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1 # Title Page

2 ## Full Title

3 Kombucha as a model system for multispecies microbial cooperation: theoretical
4 promise, methodological challenges and new solutions 'in solution'

5

6 ## Short Title

7 Kombucha model system solution-based methodology

8

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23

24
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41

42
Abstract

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Kombucha is a sweetened tea fermented by bacteria and yeast into a carbonated, acidic drink, producing a surface biofilm pellicle (colloquially called a SCOBY) during the process. Typically, liquid and a biofilm pellicle from a previously fermented culture is used as a starter for new cultures; however, there is no standard protocol for growing kombucha in the laboratory. In order to establish a standard protocol with low variability between replicates, we tested whether we could begin a kombucha culture with only well-mixed liquid stock. We found that viable kombucha cultures can be grown from low percentages of initial inoculum stock liquid, that new pellicles can form from liquid alone (with no 'starter' pellicle), and that the variation in the pellicle characteristics is lower when only a liquid starter is used ($p = 0.0004$). We also found that blending the pellicle before including it significantly reduces the variation among replicates, though the final pellicle was abnormal. We conclude that growing kombucha from only liquid stock is viable and provides a greater degree of experimental control and reproducibility compared to alternatives. Standardizing methodologies for studying kombucha in the lab can facilitate the use of this system for exploring questions about the evolutionary, ecological and cooperative/competitive dynamics within this multi-species system including resource transfers, functional dependence, genetic divergence, collective defense, and ecological succession. A better understanding of kombucha and other fermented foods may eventually allow us to leverage their pathogen inhibitory properties to develop novel antibiotics and bacteriocins.

Keywords

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65 Kombucha, fermentation, microbial ecology, cooperation, model system, symbiosis

66

67 Introduction

68 Kombucha is a sweetened tea that is fermented by bacteria and yeast into a
69 carbonated, acidic drink, and it produces a cellulose biofilm pellicle (colloquially known
70 as a SCOBY, or Symbiotic Community Of Bacteria and Yeast) on the surface of the
71 kombucha liquid. It is purported to have originated in the Tsin Dynasty of Ancient China
72 and then spread to Asia, Eastern Europe and Russia at the turn of the last century [1].
73 Kombucha is known by many names, including tea fungus, *Cainiigrib, Cainii kvass,*
74 *Japonskigrib, Kambucha, Jsakvasska, Heldenpilz, Kombuchaschwamm,* and
75 *Funkochinese* [2]. Kombucha is an excellent candidate model system for studying multi-
76 species cooperation because it has a long history of being artificially selected by
77 humans, it is non-toxic, it is easy to grow and grows quickly. In addition, its popularity
78 makes an effective tool for both teaching and for engaging citizen scientists.

79

80 Kombucha has several properties that make it useful as a model system for social
81 interactions in microbial ecosystems: its microbial composition has been characterized,
82 the kinetics of its fermentation have been established, it has innate antimicrobial
83 properties, and its culture conditions are simple. However, there are currently several
84 methodological challenges to using kombucha as a model system, including the need to
85 control initial conditions. Here we describe the promise of kombucha as a model
86 system, the nature of those methodological challenges, and describe results of

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87 experiments showing that starting replicates 'in solution' without a pellicle produces
88 more consistent resulting pellicle sizes compared to existing protocols that include a
89 piece of pellicle in the starting conditions. We also tested the effects of blending the
90 pellicle in the inoculum.
91
92 Multispecies ecological systems abound in the natural environment, and are also an
93 important part of many human societies and cultures in the form of fermented foods and
94 beverages. The microbial communities in these fermented foods are typically well-
95 characterized, easy to maintain, and highly reproducible; they represent a bridge
96 between fully-artificial *in vitro* systems and far more complex natural ecosystems.
97 Natural multispecies communities are enormously rich in diversity, but untangling the
98 multitudes of relationships and interactions is complex, even with modern molecular
99 tools. Additionally, many wild species are difficult or impossible to isolate or culture,
100 restricting the ability to manipulate and test hypotheses about their functional hierarchy
101 *in vitro* [3]. On the other hand, artificial microbial systems in the laboratory provide
102 powerful microcosms to test fundamental ecological concepts within tightly controlled
103 parameters, usually using well-characterized species. The downside of artificial
104 microbial model systems is that they may not be as applicable to natural microbial
105 systems. Artificial microbial models systems often have other limitations including
106 restricted scalability, unique microbial life histories that may or may not be shared with
107 natural systems, and a rapid speed of evolutionary change that can introduce
108 challenges for characterizing the dynamics among microbes within these systems [4].
109

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110 Kombucha has many of the advantages of natural microbial systems without the
111 disadvantages of many artificial laboratory systems. Kombucha is a natural (albeit
112 artificially selected) multispecies microbial system that can be easily cultured in
113 controlled laboratory conditions. With its unique bacterial-fungal symbiosis, biofilm-
114 forming properties, and simple propagation, Kombucha may provide opportunities for
115 answering questions that cannot be answered with artificial microbial systems or with
116 completely wild microbial systems. Fermented foods in general have been domesticated
117 by humans to grow in culture conditions, originally for human consumption. Many of the
118 characteristics that make fermented foods easy to cultivate in homes and kitchens also
119 make them excellent candidates for growing in the laboratory. For these and other
120 reasons, fermented foods are now beginning to be used more broadly as alternative
121 model systems for microbial ecosystems [3].
122
123 Typically, kombucha is cultured over a 7–14 day period at room temperature. Acetic
124 acid bacteria in this community produce a thick cellulose biofilm pellicle (colloquially
125 known as a SCOBY, or Symbiotic Community Of Bacteria and Yeast) on the surface of
126 the solution, in which bacteria and yeast are embedded [5]. Fermentation begins with
127 invertase production by yeast, which may act as a public good by cleaving sucrose into
128 glucose and fructose (both of which are utilized by both yeast and bacteria, Fig 1). Then
129 the bacteria produce an encapsulating surface biofilm via cellulose polymerization of
130 glucose monomers. Each successive round of fermentation typically adds a new layer
131 of pellicle to the surface of the solution, creating a multi-layered pellicle over time (Fig
132 2). This biofilm may also act as a public good through the creation of a barrier to

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133 microbial invasion, among other putative benefits, such as: retaining buoyancy,
134 providing a substrate for colonization, competitor exclusion, water retention, and
135 resistance to UV rays [6]. The bacterial-yeast symbiosis appears to be characterized by
136 a reciprocal benefit with yeast liberating resources and bacteria protecting the system
137 from invasive microbial species. Despite kombucha's inherent ease of replication and
138 culturing, there is no existing standard protocol for generating new cultures from pre-
139 existing stocks or collected samples. Typically, liquid and a biofilm pellicle from a
140 previously fermented culture is used as a starter for new cultures; however, there is
141 often significant variation in the amount of liquid and biofilm used in these studies [2; 7].
142 Additionally, the biofilm itself is a highly spatially-structured community with uncertain
143 consistency and unknown population densities. Therefore the use of a biofilm pellicle in
144 starting new cultures may introduce unnecessary variation into the stock, resulting in
145 low experimental control and low reproducibility.
146

147 # Results

148 To address these reproducibility issues and develop standard protocols for growing
149 kombucha, we ran several experiments to determine whether a well-mixed liquid
150 inoculum from a kombucha culture can be used alone (without an initial pellicle) to
151 recreate the kombucha culture. We examined this under three levels of initial liquid
152 inoculum amounts (5%, 10% and 15%), testing whether this initial inoculum mixed with
153 tea could give rise to a new pellicle (from only the solution without initial pellicle) and
154 also whether cultures that are started with only the inoculum solution had lower variance

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155 in the weight of the pellicle that formed on the surface after 18 days, compared to those
156 cultures that were started with both inoculum and an initial pellicle.
157
158 We discovered that, contrary to documented practice, it is possible to start kombucha
159 from stock alone, without any pellicle. The size of the resulting pellicle depends on the
160 proportion of stock in the inoculum (Experiment 1; Fig 3 and Table S1). There was no
161 statistically significant difference in the resulting pellicle weight when comparing
162 kombucha started from stock alone versus stock with a pellicle fragment (T-Test $p>0.05$
163 for all comparisons; Table S2). However, using a fragment of pellicle in the starter led to
164 much higher variance in the resulting pellicles, compared to stock alone (Levene's test
165 for unequal variances $p=0.0004$; 1-way ANOVA: $F(1,16)=21.85$, $p<0.00025$).
166
167 Next, we collected 0.5 grams of pellicle per treatment by randomly sampling locations in
168 a pellicle with a 10mm punch biopsy. This collection of biopsies was either blended
169 (homogenized) or left as a collection of intact pellicle fragments (fragmented) and then
170 added to the inoculum. We predicted that starting with a blended (homogenized) pellicle
171 would lead to lower variance in the ending dry weights because the spatial variation in
172 the pellicle structure would be eliminated (compared to fragments which would maintain
173 some variability at the microstructure level). As predicted, we found that the blended
174 treatment had low variance compared to the fragmented condition (Fig 4 and Table 1)
175 and that the variance in the ending weights of the blended and liquid-only condition
176 were equal (Levene's test $p=0.06583$). When analyzed overall, the groups had unequal
177 variances (Levene's test $p=5.284\times10^{-05}$), and that all other pairwise comparisons (other

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178 than the liquid-only vs. blended treatment) had unequal variance (Table 1; column 1).
179 Natural-log transformation of these data did not abrogate the variance inequality
180 between treatments (Table 1; column 2).
181

182 We found that either blending or fragmenting the starter pellicle fragment had a
183 significant effect on the resulting dry pellicle weight in experiment 2 (1-way ANOVA:
184 $F(2,9)=13.68$, $p=0.00187$, Fig 4). Blending the starter pellicle fragments led to a lighter
185 pellicle than starting from stock alone (post-hoc Tukey test, p=0.0166), and when
186 starting from intact pellicle fragments (post-hoc Tukey p=0.0014). There was no
187 significant difference in pellicle weight between conditions that were started with stock
188 alone versus stock and intact pellicle fragments (post-hoc Tukey p=0.2875), in
189 agreement with experiment 1. The blended pellicle treatments produced lighter pellicles
190 with a translucent appearance that quickly fell apart when handled. The structure of
191 these blender-derived pellicles was gelatinous and runny, unlike the firm pellicles
192 produced by the liquid-only and liquid-plus-fragments treatments. This abnormal
193 appearance and structure is a concern for using this blended protocol for future work
194 (see discussion) despite the fact that blending led to lower variance among replicates
195 than other methods involving including the pellicle.
196

197 Discussion

198 The inherent diversity of species, sources, and conditions used to cultivate kombucha
199 pose challenges for its development as a model system for multispecies interactions.

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200 We have shown that an ‘in solution’ approach using only liquid inoculum produces
201 kombucha pellicles with significantly less variance in the dry weights than those
202 produced with liquid and a supplemental pellicle inoculum. This suggests that the use of
203 a pellicle in current kombucha culturing practices may introduce unnecessary variation
204 in outcomes between conditions. Our results also suggest that the physical structure of
205 the pellicle may contribute to variance among replicates: when we used a blended
206 pellicle (SCOBY), this resulted in less variance among replicates. When we used large
207 fragments of the pellicle, we found high variance among replicates, suggesting that the
208 microstructure (smaller than the 0.5 gram fragments) may contribute to the variation
209 among the replicates.
210
211 We also showed that the variance in final pellicle weight increases with the percent of
212 stock in the inoculum. Based on these results, we suggest using a 10% stock with no
213 pellicle for the inocula for follow-up studies because this amount of stock offers a
214 reasonable growth rate without excessive variance.
215

216 Historical culturing of kombucha and problems of origin

217 Owing to its unstandardized historical transmission, the details of how to propagate
218 kombucha cultures are mostly anecdotal; there is no universal methodology for growth.
219 Nevertheless, the traditional practice is to use a whole pellicle and a portion of liquid
220 from a previously fermented batch, placing both into freshly brewed tea supplemented
221 with sucrose (table sugar). The amount and type of each ingredient and the precise

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222 timing of brewing and fermentation vary with locality [2], which may contribute to the
223 diverse microbiological communities found therein [5; 8; 9]. While this diversity is
224 intriguing from an ecological standpoint, it represents a problem for reproducibility:
225 every potential origin of kombucha is composed of different founding genera, species,
226 and strains, and has evolved under different environmental selection pressures. These
227 characteristics of kombucha create challenges for generalizing from results from
228 specific studies of kombucha. Some of these challenges can be addressed by
229 standardizing growth conditions and the initial stock.
230

231 Spatial structuring and diversity of the community

232 Kombucha has additional complexities arising from the fact that there are at least 2
233 spatially distinct regions within each culture: the pellicle and the liquid (and possibly
234 even a third distinct region: the interface between the pellicle and liquid, which has
235 tentacle-like strands descending from the pellicle to several inches in the liquid, Fig. 2).
236 These regions are known to differ in composition of their community members [5; 9] - an
237 unsurprising result, considering the microenvironmental structural differences.
238 Intriguingly, Teoh et al. [10] found that the populations of certain yeast species declined
239 in the liquid solution after 8-10 days of fermentation (as pH fell and nutrients were
240 depleted), yet were able to persist at stable levels within the pellicle beyond this
241 timeframe. The biofilm matrix may thus act as an encapsulating barrier, protecting the
242 entire community against the increasingly harsh environmental changes, and allow
243 otherwise intolerant species to remain as members of the system. This may be one of

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244 the constraints of using a liquid-only starter: by starting from kombucha liquid alone, we
245 may miss certain species that can survive in the biofilm matrix but not the liquid.
246
247 Current kombucha culturing practice utilizes both liquid and pellicle regions as inoculum
248 for new kombucha cultures. Our results suggest that this method may contribute to
249 unpredictability of growth. The amount of the pellicle and its age likely contribute to the
250 starting population size and diversity, both of which will have an effect on future
251 development and community structuring. Unless the amount of pellicle inocula is
252 carefully controlled and the composition is well-characterized, it is likely that the
253 heterogeneity present in the microstructure will bias the development of the culture. In
254 our study, including pellicle fragments did indeed generate larger variance in pellicle dry
255 weight than was the case with their stock-only treatment counterparts (Table 1).
256 Previous work suggests that the liquid medium may have lower yeast population
257 number per unit volume [10]. Despite this, we found that using well-mixed kombucha
258 liquid without pellicle resulted in less variance in dry weight. Reports are inconsistent
259 regarding whether the liquid has less diversity in yeast composition than the pellicle [5]
260 or more diversity in yeast composition than the pellicle [9], which makes it difficult to
261 contextualize our results in terms of the potential influence of yeast diversity on the
262 outcomes we observed.
263

264 Addition of pellicle as inoculum can add variability to future pellicles

265 Despite these advantages of using well-mixed liquid without pellicle, it is important to

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266 note that liquid-only culture did result in a lower total weight of the pellicle compared to
267 liquid-and-pellicle cultures. This means that, if the goal is producing a large pellicle,
268 starting with an initial pellicle may be a good practice. Our results do not suggest that
269 use of initial pellicles is misguided in general, only that they may add variability in
270 laboratory conditions that could be better controlled by starting only with well-mixed
271 initial stock. Further, our results do not suggest that culturing practices of kombucha in
272 the home or in commercial situations should be changed so as to not include an initial
273 pellicle. Though they do suggest that including an initial pellicle may introduce more
274 variability in the outcome of the resulting pellicle that grows on the surface of the liquid.
275

276 Impact of blended homogenization of pellicle inoculum on new 277 cultures

278 One of our conditions was to fully homogenize a pellicle in a blender and add 0.5 grams
279 of the resulting material. We were curious as to whether including homogenized pellicle
280 could reduce the variance and perhaps provide an alternative to liquid-only conditions if
281 experimenters wanted to include pellicle in the initial conditions. Variance was indeed
282 lower when a blended pellicle was used as inoculum as opposed to a fragment of
283 pellicle. In fact, the variance when using a blended pellicle was no different than the
284 liquid-only condition, suggesting that a blended pellicle may be a reasonable alternative
285 to liquid. However, we also discovered that this fully blended pellicle produces a new
286 pellicle that lacks structural integrity and has reduced dry weight compared to a non-
287 blended fragment or a liquid-only condition. Whereas a typical pellicle resembles fruit

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288 leather in texture and consistency, the pellicle that resulted from the blended pellicle has
289 a mucousy and flimsy structure that comes apart easily. Future work could help to
290 distinguish between several possible explanations for this phenomenon. One possibility
291 is that, when using a normal pellicle, the cellulose fibers in the pellicle act as a nucleus
292 for the growth and organization of new fibers, like the seeding of a crystal. It may be the
293 case that disrupting that organization by blending may generate many micro-seeds that
294 do not connect well with each other as they grow, perhaps as a result of uncoordinated
295 timing of the growth of bacterial cellulose and yeast hyphae that may contribute to the
296 structural integrity. It is also possible that the blending protocol could have damaged the
297 organisms responsible for biofilm formation, or caused the release of toxins into the
298 liquid via cell lysis.
299

300 Future directions for kombucha as a model system

301 While our experiments demonstrate repeatability in pellicle formation, much remains
302 unanswered about culturing this system. Some researchers have investigated
303 alternative carbon sources and substrates for the cultivation of kombucha (summarized
304 by [2]), but little work has been done on the long-term adaptations that may occur from
305 using non-canonical sugars and nitrogen sources. From an evolutionary perspective,
306 this may provide clues as to the functional roles of the ecological partners in carbon
307 acquisition, and could reveal unexpected evolutionary trajectories for future studies. As
308 there is no universally recognized species composition for the fermentation process, it
309 would be interesting to see whether convergent evolution has occurred across cultures

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310 due to the similarities in growth conditions. Likewise, the ability of kombucha to respond
311 to environmental fluctuations could represent a powerful proxy for studying the behavior
312 of natural systems. The introduction of antibiotics, antifungals, enzymes, inhibitors,
313 neutralizing agents, toxins, or pollutants may all have unforeseen effects on the system
314 that could be extrapolated to larger scale networks.
315
316 In addition to basic scientific inquiries, kombucha holds promise for more applied
317 research. Its ability to inhibit pathogens has been studied i*n vitro* and is largely (though
318 not exclusively) attributed to its acidic character. Greenwalt et al. [11] saturated
319 cellulosic discs with solutions of fermented and unfermented black and green teas and
320 found that kombucha cultures containing 7g/L of acetic acid (33g/L total acid) had an
321 inhibitory benefit against an array of common human pathogens, though not against
322 *Candida albicans*. Upon neutralization to a pH of 7, these benefits were abrogated even
323 with increasing tea concentrations. In contrast, Sreeramulu et al. [12] demonstrated that
324 a kombucha black tea solution retained inhibitory activity against *Escherichia coli,*
325 *Shigella sonnei, Salmonella typhimurium, Salmonella enteritidus,* and *Campylobacter*
326 *jejuni* even after neutralization to a pH of 7 or thermal denaturation at 80°C for 30
327 minutes. The exact nature of these inhibitory properties are unknown, but could include
328 novel antibiotics or bacteriocins adapted to function under various environment
329 conditions.
330
331 Unlike rationally designed therapeutics that can be thwarted by the evolution of
332 resistance, the kombucha community itself can evolve and generate diverse molecules

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333 to kill competitors. Challenges to the system using invasive, human-associated
334 pathogens could provide a platform for discovering new anti-microbial compounds.
335 Additionally, the kombucha microbial community itself, while untested in human health,
336 could provide an easy way to generate diversity within the gut microbiome. Tolerance to
337 low pH would enhance survival of the microbes through the digestive tract, while the
338 biofilm itself could be used as a source of nutrients for cellulose-degrading organisms
339 during transit.
340

341 Concluding remarks

342 There is a need for a thorough characterization of kombucha genetic and phenotypic
343 diversity. The most comprehensive metagenomics study of kombucha to date included
344 only 5 biofilm samples [5], and no studies have searched for viruses, despite viruses
345 being the most abundant entity on the planet (10^{31} viral particles at any given moment)
346 [13]. Further, resilience of the kombucha symbiotic community has not been
347 systematically investigated: the minimal starting conditions, the ranges of growing
348 conditions and the multispecies interactions required to prevent collapse are all
349 currently unknown. Our experiments are a first step towards standardizing
350 methodologies for kombucha that can facilitate the use of this system for exploring more
351 evolutionary questions, such as the nature of resource exchange, functional
352 dependence, genetic divergence, collective defense, and ecological succession.
353

354 Methods

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355 ## Kombucha Media Preparation

356 In order to produce the sweetened tea media (STM), a recipe was developed based on
357 the following protocol: 1L of deionized water (LabChem, PA) was brought to a boil
358 (approximately 90°C) in a 1.7L stainless steel kettle (Hamilton Beach, NC), and then
359 poured into a 1L Pyrex beaker containing 5g (0.5% w/v) of loose leaf black tea (Lipton,
360 Unilever, UK) with 50g (5% w/v) of sucrose (Biobasic, NY). The solution was stirred until
361 the sucrose was fully dissolved, and then allowed to steep for 10 minutes while the
362 beaker was covered with aluminum foil. At this point, the solution was strained into a
363 new Pyrex beaker through BrewRite coffee paper filter (Rockline Industries, WI) to
364 remove tea leaf debris. To ensure uniformity across all replicates, all strained tea was
365 pooled into a single graduated pitcher prior to distribution to replicate beakers. The tea
366 was allowed to equilibrate to room temperature (21°C), but further sterilization steps
367 were not performed in order to more accurately mimic the traditional processes used to
368 prepare kombucha.
369

370 ## Culturing Kombucha

371 To establish a laboratory-acclimated kombucha stock, 3 starter pellicles totaling
372 approximately 1kg (fresh weight) were donated from Dr. Aktipis's personal supply, along
373 with approximately 500mL of kombucha liquid that had been propagated for an
374 unknown length of time. This stock supply was placed into a 4 gallon (7.5L) glass jar
375 (Nantucket Glassware, MA) along with 3.5L of freshly-prepared STM, bringing the total
376 volume to 4L of liquid, plus 1kg of pellicle material. Two layers of approximately 150um-

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377 diameter pore cheesecloth (Carolina Biological Supply Company, NC) were placed over
378 the top of the jar and secured with a rubber band. This stock solution was allowed to
379 ferment continuously at room temperature, with removal and replenishment with
380 approximately 1L of STM every four to six weeks. Additionally, whenever liquid was
381 removed for experiments, an equal amount of STM was replenished into the stock
382 solution. At the time of the experiment, the stock solution had been fermenting for 6
383 months at room temperature.
384

385 Stock-Percentage Experiment

386 The goal of this experiment was to determine whether using liquid inoculum at different
387 proportions with STM and in combination with biofilm fragments could produce
388 replicable results during the generation of new kombucha cultures (Fig 5). To produce
389 replicate kombucha vessels, liquid from the Kombucha stock solution was gently
390 agitated for 15 seconds, and then combined with STM in 600mL Pyrex beakers in
391 various proportions based on the treatment. These treatments consisted of 3 replicate
392 beakers of 5% stock mix (380mL STM, 20mL stock), 10% stock mix (360mL STM,
393 40mL stock), and 15% stock mix (340mL STM, 60mL stock).
394

395 Additionally, another set of 3 replicates were prepared that contained these same
396 proportions of liquid (5% stock mix, 10% stock mix, and 15% stock mix) but also
397 included fragments of pellicle from the stock vessel totaling 3g. These fragments were
398 prepared by aseptically removing the topmost pellicle from the stock vessel (six weeks

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399 from last STM replenishment) by hand using 70% ethanol-sterilized gloves and placing
400 it onto a plastic cutting board that had been sterilized with a 10% (v/v) bleach-DI water
401 solution (after the board had dried). The pellicle was thoroughly rinsed 3 times with DI
402 water, and then dabbed with a paper towel on both sides. Average pellicle thickness
403 was measured with digital calipers (Fowler Company Inc., MA) at 4 equidistant points
404 1cm from the edge of the pellicle in addition to 1 point at the center. The average
405 thickness of the pellicle fragment added to each replicate was 3.31mm (SD: 0.51mm).
406 2cm by 2cm square fragments were cut from the pellicle using an autoclave-sterilized
407 straight razor and pooled together. Each beaker in these treatments received a random
408 assortment of these fragments weighing 3g in total. All 6 treatments consisting of 3
409 replicates each were then placed into a 30°C incubator (Model 12E, Quincy Lab
410 Corporation, IL) for 18 days, at which point they were harvested.
411

412 For the harvest, the entire mass of pellicle was removed from each replicate beaker
413 using tweezers sterilized by a 70% ethanol solution. They were rinsed with DI water and
414 dried as described in the experiments above, placed onto a large polystyrene weighing
415 boat (Cole-Parmer, IL), and then weighed on an Accuris Instruments precision balance
416 (Chemglass Life Science, NJ) to obtain a measure of fresh biomass weight. To keep a
417 representative sample for future studies, a small fragment of each pellicle was removed
418 with a razor, weighed separately, and then placed into a -20°C freezer. The remaining
419 large pellicle fragments were then placed into a 30°C incubator and allowed to dry for
420 24 hours, at which point they were weighed again. The ratio of wet to dry weight for
421 each large pellicle fragment was used to calculate the dry weight of the associated

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422 small frozen fragment. Both the recorded large fragment weight and calculated small
423 fragment weight were summed to produce a total dry weight measure for each pellicle.
424

425 Pellicle Consistency Experiment

426 The goal of this experiment was to determine whether homogenizing the pellicle could
427 lead to more consistent results in the generation of new kombucha cultures (Fig 6). 3
428 treatments of 4 replicate beakers were prepared, all containing the same proportion of
429 stock solution to STM (10%, as prepared in experiment 1, totalling 400mL), with 1
430 treatment as a liquid-only condition, 1 treatment supplemented with 0.5g of fragments of
431 pellicle and the third treatment supplemented with 0.5g of homogenized pellicle. In order
432 to standardize the extraction of pellicle fragments from the stock pellicle, the pellicle was
433 divided into a grid of 2cm by 2cm squares. After washing and drying as in experiment 1,
434 a randomly-generated number was used to select squares to extract fragments using a
435 10mm biopsy punch (AcuPunch, Ft. Lauderdale, FL). These pellicle fragments were
436 collected until their total fresh weight was 0.5g, at which point they were added to the
437 replicate beakers in this treatment. The final treatment consisted of collecting 2g of
438 biopsy punched fragments from randomly assigned squares of the same grid-marked
439 pellicle, as in second treatment. These fragments were added to a NJ600 Blender
440 (SharkNinja Operating LLC, Champlain, NY) along with 1600mL of 10% stock-STM
441 solution, and blended at ‘1’ power for 10 seconds and then ‘3’ power for 10 seconds.
442 400mL of the pellicle slurry was then distributed equally to 4 replicate beakers.
443 Incubation and harvesting proceeded as in experiment 1.

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444

445 ## Data Analysis

446 R statistical software (version 3.1, 2014) was used for all data analysis. We explored the
447 data graphically using program-generated boxplots and scatterplots, and proceeded to
448 examine linear correlations using the Spearman rank-order correlation coefficient. Wet
449 and dry pellicle weights were highly correlated (ρ=0.9071), thus dry weight was used as
450 primary dependent variable for analysis. Levene's test was performed to determine the
451 nature of variance between treatments, and data were natural-log transformed to
452 ensure homoscedasticity. For experiment 1, treatments were analyzed using a paired-
453 samples t-test using dry weight as the variable of interest. A 1-way ANOVA was then
454 performed on these data with the presence of pellicle as the factor. For experiment 2,
455 variance was analyzed using Levene's test and the data was natural-log transformed as
456 in experiment 1. A 1-way ANOVA was performed on these data with treatment as the
457 factor.

458

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494

495 Figure Captions

496 Figure 1 (made with Adobe Illustrator). Kombucha is the result of a symbiotic
497 association between acetic acid bacteria and ethanol-fermenting yeast, where the yeast
498 cleave sucrose into glucose and fructose via invertase in the cell wall, which then acts
499 as public goods for both yeast and bacteria. Bacteria produce acid, ethanol, carbon
500 dioxide as well as a cellulose biofilm that forms a barrier on the top of the solution.
501

502 Figure 2 (photograph). A typical kombucha ‘stock’ solution as it appears during growth
503 at room temperature. If allowed to ferment continuously, new pellicle layers will form at
504 the liquid-air interface upon addition of new media, forcing the older layers to submerge
505 beneath. Strand-like material is often seen dangling from the upper levels of pellicle into
506 the liquid phase.
507

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508 Figure 3 (made with R software). Average of dried pellicle weights across each
509 treatment (treatments with light blue bars contain only liquid, while treatments with dark
510 blue bars contain liquid plus pellicle fragments; the thin capped black bars represent
511 standard error of each average and individual replicate data points are represented as
512 black dots over the treatment bars). The treatments are: 5% starting stock with
513 sweetened tea (5% Stock), 5% starting stock with sweetened tea and 3g pellicle
514 fragment (5% Stock + Pellicle), 10% starting stock with sweetened tea (10% Stock),
515 10% starting stock with sweetened tea and 3g pellicle fragment (10% Stock + Pellicle),
516 15% starting stock with sweetened tea (15% Stock), and 15% starting stock with
517 sweetened tea and 3g pellicle fragment (15% Stock + Pellicle).
518

519 Figure 4 (made with R software). Average of dried pellicle weights comparing physical
520 treatment of the pellicle fragment in the inoculum (the large light blue bars are the
521 averages of each treatment, the thin capped black bars represent standard error of
522 those averages, and individual replicate data points are represented as black dots over
523 the treatment bars). The treatments are 10% starting stock with sweetened tea
524 (LiquidOnly), 10% starting stock with sweetened tea and 0.5g of pellicle fragments
525 (Fragmented), and 10% starting stock with sweetened tea and 0.5g blended pellicle
526 fragments (Blended).
527

528 Figure 5 (made with Google Slides). Schematic of the experimental design for
529 Experiment 1, showing the 2 arms of treatments: Liquid-only (A) and Liquid-and-pellicle
530 (B) (see Methods).

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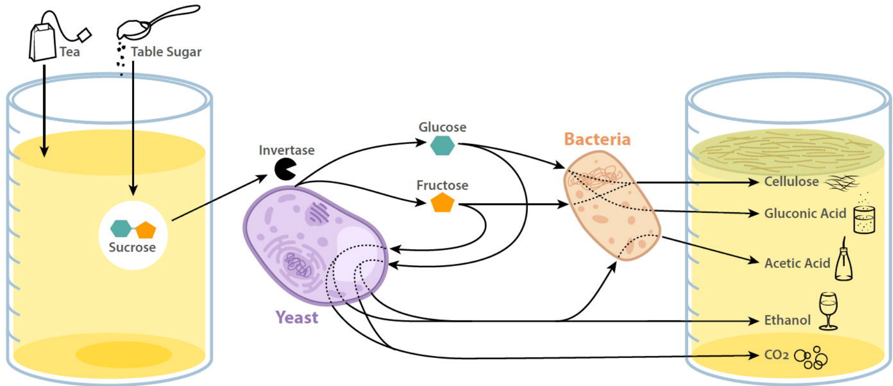
531

532 Figure 6 (made with Google Slides). Schematic of the experimental design for

533 Experiment 2 (see Methods).


Tea
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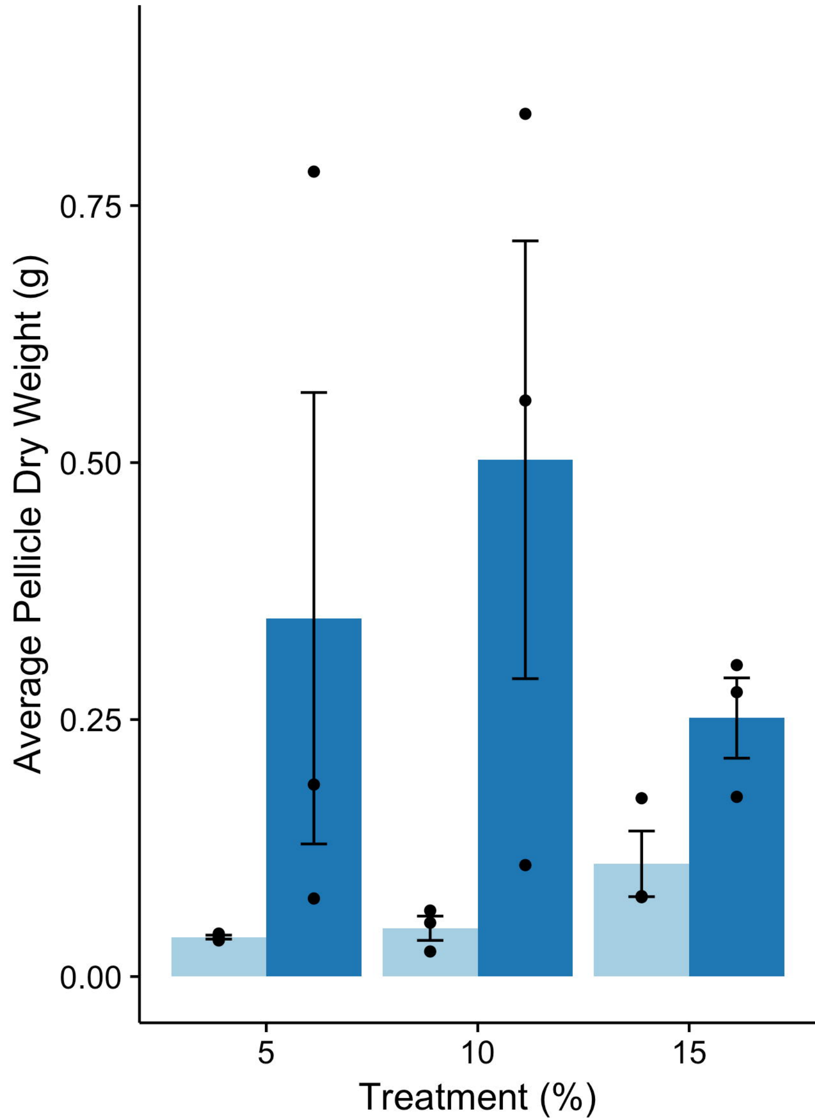
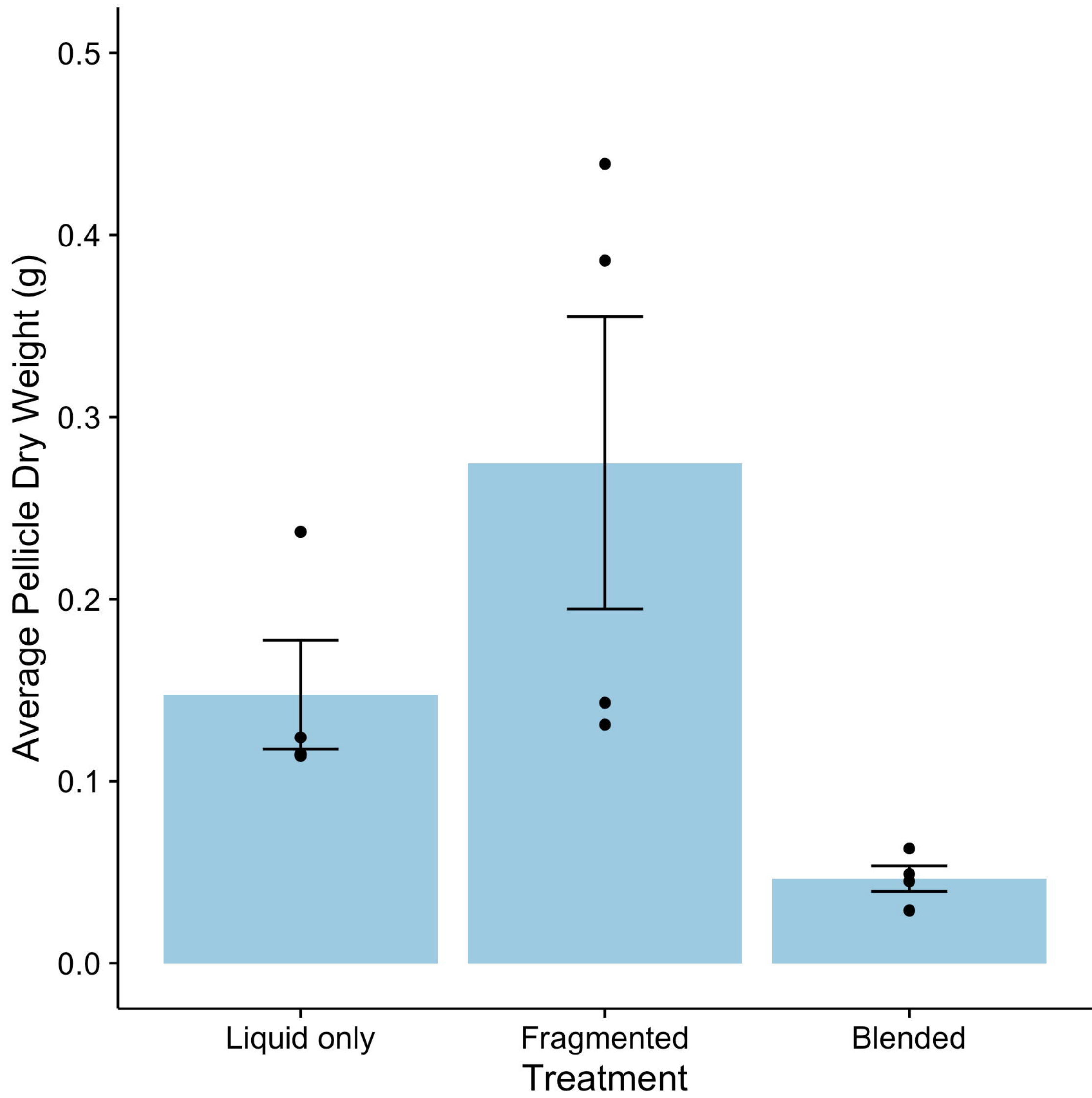
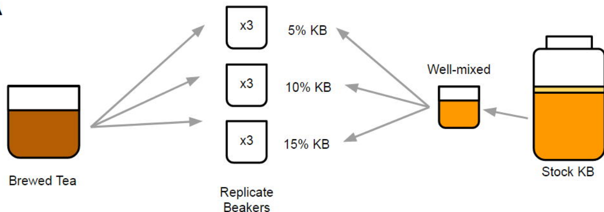
Sucrose
Invertase
Glucose
Fructose
Bacteria
Yeast
Cellulose
Gluconic Acid
Acetic Acid
Ethanol
CO2



Average Pellicle Dry Weight (g)
0.75
0.50
0.25
0.00
5
10
15
Treatment (%)



Average Pellicle Dry Weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
Liquid only
Fragmented
Blended
Treatment



B

Add 3g of pellicle to each treatment

Fragments

Pellicle

x3

x3

x3

5% KB

10% KB

15% KB

Well-mixed

Brewed Tea

Replicate Beakers

Stock KB

Brewed Tea

Liquid-Only

3g Blended

3g Fragmented

Pellicle

X4

X4

X4

Well-mixed

Stock KB

90% Tea : 10% KB