Making yogurt with the ant holobiont uncovers bacteria, acids, and enzymes for food fermentation

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Abstract

Milk fermentation has a rich history in which food culture, the environment, and microbes intersect. However, the biocultural origins of fermentation practices and microbes have largely been replaced by industrial processes. Here, we consider a historical fermentation originating from Turkey and Bulgaria – ant yogurt. We revisit the traditional practices and modern gastronomic applications that use red wood ants (*Formica rufa* group) to initiate milk fermentation. Subsequently, we characterize the ants and experimental ant-derived yogurts. We uncover that the ant holobiont, which consists of the ants and their microbes, contributes key acids and enzymes to fermentation. Metabarcoding and culturing revealed that lactic and acetic acid bacteria, including species related to those in conventional yogurt and sourdough, originate from the live ants and proliferate in the milk. The ants and bacteria consequently introduce formic, lactic, and acetic acid, advantageous for yogurt acidification and coagulation. Last, proteases with the potential to act on casein and alter yogurt texture are produced by the ants and bacteria. The ant holobiont thus facilitates fermentation akin to the microbial consortia in other ferments. Our findings highlight the value of integrating traditional, gastronomic, and biological frameworks to uncover the origins and applications of microbes for fermented foods.

Introduction

The fermentation of milk into products such as yogurt, cheese, and kefir originate from ancient practices and has dramatically shaped food cultures. The oldest evidence for dairying dates to 9,000 years ago in Anatolia (modern-day Turkey) (Evershed et al., 2008). Prehistoric dairy fermentation potentially occurred as early as 7,000 years ago, based on fat and protein residues isolated from ceramics resembling cheese strainers (Evans et al., 2023; Salque et al., 2013). In the millennia to follow, diverse dairy practices transformed milk into a preservable, widespread, and nutritious resource (Fisberg & Machado, 2015; Scott et al., 2022; Warinner et al., 2014). Following suit, dairy fermentation became indispensable to regional cuisines and languages (Multu Sirakova, 2023; Öney Tan, 2010). Yogurt, a tangy fermented milk product, was thus a functional cultural adaptation dependent upon interactions between people, dairy animals, the environment, and most importantly, microbes. It is microbes that enter the milk, and through their enzymatic processes, catalyse the fermentation to acidic, viscous yogurt (Nagaoka, 2019). These interspecies relationships are reflected in the Turkish word for a fermentation starter, *maya*, that ultimately “comes from relations within the broader web of life”, including microbes, animals, plants, and human culture (Multu Sirakova, 2023).

In the early 1900s, microbiologists characterized the first yogurt culture, laying the foundation for a pivotal shift from the diversity inherent in traditional yogurt to a simplified industrialized yogurt. Stamen Grigorov and Ilya Metchnikoff isolated and popularized a species of bacteria from Bulgarian *maya*, *Lactobacillus delbrueckii* subsp. *bulgaricus* (Grigoroff, 1905; Metchnikoff, 1908). Following Metchnikoff, the industrialization of yogurt focused on a small number of bacterial taxa, predominantly *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (FAO & WHO, 2018). Both species are lactic acid bacteria, which play an important role in industrial and wild fermentations through food preservation, flavour generation, and potential health benefits (Hu et al., 2022; Wang et al., 2021; Zapasnik et al., 2022). However, the focus on a few bacterial species overlooks the biodiversity embodied in traditional yogurts, which can include multiple species and strains (Demirci et al., 2022; Velikova et al., 2018). Revisiting biocultural origins of yogurt fermentation offers opportunities to explore ecological niches of food microbes for novel starter cultures (Sedó Melina et al., 2022), to understand the interconnectedness of food systems, and to illuminate the history of yogurt and its cultural praxis.

The bacteria in yogurt stem from the multispecies dimensions of *maya*, which we aim to elucidate for a traditional Turkish and Bulgarian yogurt-making practice - ant yogurt. Generally, the bacterial

community that first colonizes the milk and establishes the fermentation ecosystem may stem from the dairy animal (Wouters et al., 2002), the person making the ferment (Kothe et al., 2024; Reese et al., 2020), or the environment, such as vegetation (Michaylova et al., 2007). Yogurt cultures are then propagated by adding a small amount from a primary or old yogurt to new milk, a process referred to as “backslopping.” A historic practice for starting the first or primary yogurt in Turkey (Öney Tan, 2010) has been documented by the ethnographer Ali Rıza Yalman, “If the nomads want to make yogurt and cannot find enough starter culture to make yogurt, they crush the tiny eggs of the ants sheltering under the stones in their palms. When you put this into the milk […], that milk becomes yogurt.” (Yalman, 1977). In neighbouring Bulgaria, a traditional spring practice involves fermenting yogurt within a red wood ant colony (Multu Sirakova, 2023). Further oral histories from Sharri mountains of Albania and North Macedonia also recall the use of ants in traditional yogurt fermentation (F. Demiraj, personal communication, May 2023). These countries are connected by cultural threads preceding the establishment of national boundaries, contributing to general continuity between their culinary practices, both today and across millennia. The ethnographic evidence of ant yogurt across regions suggests ants may play an overlooked functional role in fermentation.

Here, we test the hypothesis that yogurt fermentation can be initiated by the ant “holobiont”, which includes both the ant and the microbial communities inherent to it (Bordenstein & Theis, 2015; Margulis & Fester, 1991). The ant holobiont may contribute acids and enzymes key to the fermentation (Figure 1). First, we explored the potential of red wood ants (*Formica rufa* group) (Robinson & Stockan, 2020) as starter cultures for yogurt based on ethnographic accounts and as culinary ingredients for modern gastronomy. Then, we characterized the microbial community of the ants *F. rufa* and *F. polyctena* and yogurts derived from *F. polyctena*, quantified organic acids in the yogurts, and assessed the proteases and peptidases originating from ants and bacteria (Figure 1 - figure supplement 1). The study overall examines an overlooked ecological niche of bacteria in the context of traditional practices to identify microbes key to past and future fermented foods.

Results

The ant holobiont serves as a catalysing agent for food applications

We worked towards a holistic understanding of traditional uses of ants as a yogurt starter by conducting fieldwork in Bulgaria in a community that retained an oral history of the practice (Multu Sirakova, 2023; Sinotte et al., 2023). This community also is the ancestral home of one of the

authors (S. Mutlu Sirakova). We used an ant colony selected by members of the community, which was the species *F. rufa*. This is one of four species in the *F. rufa* group that maintain species ranges within Bulgaria (Antonova & Marinov, 2021). To make the yogurt, four live ants were added to the jar of warmed raw milk (Figure 2A), a cheese cloth was placed over the top of the glass jar, and the jar was left to ferment within the colony overnight (Figure 2B). The jar was retrieved after a day of incubation. The acidity, texture, and flavour were indicative of early stages of yogurt fermentation. We observed that the milk had acidified to pH 5, coagulated at the bottom of the container (Figure 2C), and “had a slight tangy taste with mild herbaceousness and pronounced flavours of grass-fed fat” (D. Zilber).

Culinary applications of the *F. rufa* ants were created by the research and development team at Restaurant Alchemist, ranked number eight in the world (50 Best, 2024), holding two Michelin stars (Guide, 2024), and known for science-centred innovation. Co-authors based these applications on previous experiments where the addition of ants to milk caused coagulation. Presumably, this coagulation was due to the *F. rufa* ants' formic acid. Discussions on the traditional uses of ants to coagulate milk (T. Tan, personal communication, April 2022) further directed the development of culinary applications. Thus, the innovations leverage the potential of ant-derived formic acid and microbe-derived organic acids to coagulate milk.

Three culinary applications were developed using live ants and ants that were frozen and subsequently dehydrated. First, the “ant-wich” contained ice cream derived from sheep yogurt made with live ants as starter culture (Figure 2D). The ants provided a distinct, pungent acidity that contrasts with the fat of the milk, and a serving temperature of -11ºC balances the desert. Second, a goat milk “mascarpone” was developed using dehydrated ants to catalyse the milk coagulation (Figure 2E). The texture was similar to commercial mascarpone, yet the flavour was pungent and aromatic, like a mature pecorino cheese. Third, a milk wash cocktail was created, which is a dairy-based cocktail dating to the early 1700s (Wondrich, 2010). Classically, the milk is curdled with acid from citrus and subsequently filtered to remove the dairy solids, resulting in a clear beverage with more richness and body. In this case, dehydrated ants induced the curdling and separation of milk (Figure 2F). The cocktail had fruity notes from the apricot liqueur and the brandy (raisins, dried figs, and caramelized apples), and a silky texture from the residual milk whey. Replacing citrus with ants resulted in a milder acidity and a distinct flavour with additional fruity notes. Despite the potential of these culinary innovations, we note that ants are not included among the four insects authorized in the European Union according to Regulation 2015/2283 on Novel Foods.

130 Given that ants have diverse culinary applications with potential to initiate fermentation, we aimed
131 to further elucidate the role of the ant holobiont. We hypothesized that the ant holobiont, consisting
132 of the ant and its microbial partners, contributes to fermentation with bacteria, acids, and
133 enzymes. To address this hypothesis, we created three elaborations of fermented milk with live,
134 frozen, or dehydrated ants, per the modern gastronomic applications. The ants were collected in
135 late spring and early autumn to further determine the impact of the season on the microbiome
136 and the fermentation.

137 *Live ants provide small, stable, and controlled yogurt microbiomes*

138 We first characterized the bacterial microbiome of red wood ant sister species *F. rufa* and *F.*
139 *polyctena* (Borowiec et al., 2021) with 16S *rRNA* metabarcoding. Our aim was to examine how
140 the ant yogurt practice may be translated across species and geographies. These ants have
141 ranges spanning Denmark, where experimental yogurts were made, and Bulgaria or Turkey
142 (Guénard et al., 2017; Janicki et al., 2016), where ethnographic histories originated (Figure 3A).
143 Both species are found along the edges of pine forests, have thatched pine needle mounds, and
144 near-identical morphological characteristics (Seifert, 2021). These similarities make them all but
145 indistinguishable in the field to ant experts (Seifert, 2021), suggesting both may have been used
146 in traditional and modern culinary applications.

147 The microbiomes of the two *Formica* species were dominated by lactic acid bacteria
148 (Lactobacillaceae), acetic acid bacteria (Acetobacteraceae), and obligate intracellular bacteria
149 (Anaplasmataceae) (Figure 3A). The bacterial families and genera (Figure 3 – figure supplement
150 1A) align with *Formica*-specific microbiomes associated with species across the Northern
151 Hemisphere (Jackson et al., 2023; Jackson et al., 2022; Kaczmarczyk-Ziemba et al., 2020; Zheng
152 et al., 2022). *Formica* ant microbiomes were seasonal. We observed an increase in relative
153 abundance of lactic and acetic acid bacteria (Figure 3A) and overall microbial biomass (i.e. load)
154 in the autumn, compared to spring (Figure 3 – figure supplement 1B). Seasonal microbiomes
155 have not been previously documented in *Formica* ants and are germane to the use of these ants
156 in fermentation. Overall, the consistent presence of lactic and acetic acid bacteria in the *F. rufa*
157 group indicates the ant microbiome may be pertinent to food fermentation.

158 We then tested the hypothesis that lactic and acetic acid bacteria in the ants may transfer to the
159 yogurt. Based on 16S *rRNA* metabarcoding of experimental yogurts made with *F. polyctena* under
160 aseptic conditions, we confirmed our hypothesis that bacteria from the ants contribute to the
161 yogurt microbiome. The preparation of the ants, namely whether they were live, dehydrated, or

162 frozen, distinctly impacted the bacteria communities in the resulting yogurts (Figure 3B). Like the
163 ants themselves, the live ant yogurts were consistently dominated by the lactic and acetic acid
164 bacteria, which displayed seasonal differences in composition. Here, the most abundant bacterial
165 genus was *Fructilactobacillus* (Figure 3 – figure supplement 1C). We note that these true
166 abundances may have been higher, considering our metabarcoding positive control indicates we
167 slight underestimation of the abundance of lactic acid bacteria in samples (Figure 3 – figure
168 supplement 2). In contrast, dehydrated ant yogurts exhibited variable microbiomes. Frozen ant
169 yogurts contained two bacteria groups: Bacillaceae, which can proliferate in the yogurt, and
170 Anaplasmataceae, which obligately lives in ant cells (Buchner, 1965; Jackson et al., 2022) and
171 thus cannot grow in the yogurt. Here, dehydration and freezing likely reduced bacterial viability
172 (Santivarangkna et al., 2011) and consequently favoured stochastic community assembly or
173 freeze-resistant Bacillaceae (Nicholson et al., 2000). The persistence of lactic and acetic acid
174 bacteria in live ant yogurt suggests it is the best starter for fermentation and provides biological
175 support for the traditional, live ant-based yogurts.

176 We determined the consistency of the bacterial microbiome composition across the yogurts.
177 Alpha and beta diversity metrics were used, where alpha diversity is the number of bacterial
178 strains per sample (i.e. amplicon sequence variants) and beta diversity is the difference in the
179 composition of strains from one sample to the next, while weighting relative abundance. The ant
180 preparations significantly affected the alpha and beta diversity of the yogurts (LM: $F_{2,27}$=5.387,
181 p=0.0108, based on Shannon index; Adonis: $F_{2,27}$= 8.4402, p<0.0001, based on Bray Curtis
182 distances). The alpha diversity of the live ant yogurts was intermediate (Figure 3C), but the beta
183 diversity was very low (Figure 3D). Therefore, the live ant yogurts tended to have the same
184 species and composition from one sample/preparation to the next, as one would hope to see in
185 predictable and controlled fermentations.

186 *Bacteria from the ants proliferate in the milk*

187 We hypothesized that bacteria from the ant holobiont grew in the milk, facilitating fermentation.
188 To assess growth, we determined the bacterial load in ants and yogurts with quantitative PCR
189 (qPCR) and identified viable bacteria with culturomics using 16S *rRNA* Sanger sequencing. Live
190 ants introduced lactic acid bacteria that proliferated in the yogurt. In the spring, yogurts contained
191 a higher loads of lactic acid bacteria than the ants introduced as a starter (Figure 4A; t(2, 10) = -
192 2.975, p=0.0139). However, in autumn, we observed the opposite pattern (Figure 4A; t(2, 10) =
193 3.269, p=0.0084), suggesting that not all lactic acid bacteria from the ants enter the milk, or that

194 the bacteria associated with the autumn ants are less prolific. While lactic acid bacteria dominated
195 live ant yogurts, it made up a marginal amount the bacterial load in frozen and dehydrate yogurts
196 (Figure 4 – figure supplement 1A).

197 Yogurt made with live ants contained several species of culturable lactic acid bacteria (Figure
198 4B). Aligned with the dominance of *Fructilactobacillus* in the community analysis, we isolated *F.*
199 *sanfranciscensis*, a bacterium not only associated with ants (Zheng et al., 2022) but also
200 sourdough bread fermentation (Gänzle et al., 2023; Kline & Sugihara, 1971). We further cultured
201 *Lactobacillus delbrueckii* subsp*. bulgaricus* and *Lacticaseibacillus casei*, typically found in
202 fermented dairy products (FAO & WHO, 2018; Zheng et al., 2020). Last, we isolated the acetic
203 acid bacteria, *Oecophyllibacter saccharovorans* (Figure 4 – figure supplement 2), previously
204 characterised in association with ant genera closely related to *Formica* (Blaimer et al., 2015;
205 Brown & Wernegreen, 2016; Chua et al., 2018). The diversity of culturable lactic acid bacteria
206 thus indicates a niche overlap between ants and fermented foods.

207 In the yogurts made with dehydrated and frozen ants, the bacterial load disproportionately came
208 from spore-forming Bacillaceae. Notably, there is little to no Bacillaceae biomass in the ants,
209 especially when compared to the yogurts (Figure 4C; $t_{frozen}(2, 10) = -2.307$, $p=0.0437$), and this
210 Bacillaceae did not proliferate in live ant yogurts (Figure 4 – figure supplement 1B). We cultured
211 several species of Bacillaceae, stemming largely from dehydrated or frozen ants or yogurts
212 (Figure 4D). This included the food contaminant, *Bacillus cereus* (Dietrich et al., 2021). Although
213 we cannot determine if the *Bacillus* load was sufficient to be problematic for consumption, it still
214 indicates a potential risk. Therefore, we conclude that the microbiome of dehydrated and frozen
215 ants and their corresponding yogurts are undesirable for food fermentations.

216 *Ants and bacteria contribute to the acidification of yogurt*

217 We hypothesized that the ant holobiont, composed of both the ant and its microbes, may
218 contribute acids key to fermentation. In yogurt, bacteria metabolize lactose and consequently
219 produce high amounts of lactic acid and often low amounts of acetic and formic acid (Sieuwerts
220 et al., 2010). These acids are essential to the tangy flavour, thick texture, and preservation of
221 yogurt (Fernandez-Garcia & McGregor, 1994). Moreover, *Formica* ants (though, notably, not all
222 ants) have a venom gland that contains largely formic acid that may be up to 10% its body weight
223 (O'Rourke, 1950), although the whole ant contains a more complex mélange of chemicals
224 (Löfqvist, 1976). Thus, the acid from the ant holobiont may have three effects. First, it may
225 engender yogurt tastes and textures, even without the effects of microbes. Second, it can create

226 acidic conditions that favour acid-producing microbes. Third, the acid-producing microbes can
227 themselves alter the acidity and flavour of the ferment. To investigate if the components of the
228 ant holobiont, body and microbes, contribute organic acids to the yogurt, we quantified formic,
229 lactic, and acetic acid in yogurts and controls by HPLC and measured pH before and after
230 fermentation (Figure 5).

231 The ant holobiont contributed formic acid, which was the most abundant acid found in the yogurts
232 (Figure 5A). The amount of formic acid was in a similar range to that in the control, in which formic
233 acid alone was added to pH 4.6 to induce coagulation. The season, but not the type of yogurt
234 (live, dehydrated, frozen) further dictated the formic acid levels (LM results). The higher levels in
235 autumn (t-test: $t_{live}(2, 10) = -3.500$, $p = 0.0057$; $t_{dehydrated}(2, 10) = -3.297$, p=0.0081) may be due to
236 the seasonality of formic acid production (O'Rourke 1950). Therefore, the acid from the ants
237 entered the milk and likely shaped the unique acidic flavour and texture of the culinary
238 applications, contrasting the rounded flavour of lactic acid typically predominant in fermented
239 dairy.

240 Lactic and acetic acid were also detected in the ant yogurts, validating that ant-associated
241 bacteria likely contributed acids during fermentation (Figure 5A). Acid profiles align with the
242 microbiome data. Lactic and acetic acid bacteria were the most abundant genera in live ant
243 yogurts (Figure 3A). Similarly, these acids are highest in live ant yogurts compared to dehydrated
244 ($t_{lactic}(2, 10) = 4.270$, $p=0.0052$; $t_{acetic}(2, 10) = 3.065$, $p=0.0220$) and frozen yogurts ($t_{lactic}(2, 10)$
245 $=3.842$, $p=0.0060$; $t_{acetic}(2, 10) = 3.653$, $p=0.0147$). In live yogurts, we observed lactic and acetic
246 acid were higher in spring ($t_{lactic}(2, 10) = 2.897$, $p=0.0231$; $t_{acetic}(2, 10) = 3.325$, $p=0.012$), the season
247 in which we isolated the greatest diversity of lactic acid bacteria (Figure 4C). This suggests
248 different strains of bacteria may inherently vary in acid production. Notably, ant yogurts had less
249 lactic acid, acetic acid (Figure 5A), and bacterial biomass (Figure 5 – figure supplement 1A) than
250 conventional or homemade yogurts. We did not establish and perform the optimal fermentation
251 conditions for the ant yogurt, and thus the fermentation was incomplete. However, we can
252 conclude that the ant microbes contribute organic acids to fermentation.

253 Ant yogurts contained on average 2.5 g/L total organic acids, while conventional yogurts
254 contained up to 12 g/L total organic acid. Thus, the ant yogurt remained in a pH range of 5.0-5.9
255 and did not reduce to a pH of 4.2 of conventional yogurt (Figure 5 – figure supplement 1B), given
256 the conditions and timing of our experiment. Initially, the pH of the milk dropped upon the addition
257 of ants, likely due to the formic acid from the ants (Figure 5 – source data 1). The pH continued
258 to decrease through the fermentation (Figure 5 – figure supplement 1B), likely because of acid-

259 producing microbes. Typically, in yogurt fermentation, a drop to pH 5.3 initiates coagulation, and
260 a further acidification to pH 4.6 completes coagulation by causing aggregation of casein micelles
261 (Robinson et al., 2006) The ant yogurt coagulated yet largely did not reach such a low pH. This
262 suggests another aspect of the fermentation denatured caseins, namely proteases.

263 *Ants and microbes contribute proteases and peptidases, potentially modifying yogurt texture*

264 Our final hypothesis was that the ant holobiont may contribute enzymes, in particular proteases,
265 that modify the yogurt texture. Proteases can differentially cleave and degrade casein, the main
266 protein in milk, and result in a firm or fluid yogurt texture (Amani et al., 2017; Gassem & Frank,
267 1991). Based on the pH and coagulation results, we predicted the ants themselves may contain
268 milk-clotting proteases, similar to those previously identified in mealworms (Yang et al., 2022).
269 Casein proteases and peptidases are also typically produced by the bacteria in yogurt (Amani et
270 al., 2017). Thus, the ant holobiont, ants and bacteria, may contribute proteases to the yogurt
271 fermentation. To test this hypothesis, we conducted an untargeted proteomics analysis based on
272 a self-curated database that contained proteases from *Formica* ants, ant-yogurt bacteria,
273 conventional yogurt bacteria, and milk proteins.

274 We confirmed our hypothesis that the ant holobiont contributes proteases and peptidases to the
275 yogurt fermentation (Figure 5B). Several ant proteases were identified in the yogurt (Figure 5B),
276 one of which, an ATP-dependent CLP protease, is known to have caseinolytic potential (Maurizi
277 et al., 1990). Ant-associated bacteria further contributed proteases and peptidases. The lactic
278 acid bacteria *F. sanfranciscensis* produced two proteases, ATP-dependent CLP protease and
279 zinc metalloprotease, both known to have caseinolytic homologues (Jia et al., 2015; Maurizi et
280 al., 1990). Proteases and peptidases typically belonging to bacteria in conventional yogurt, *L.*
281 *delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, were found, as well as ant yogurt
282 bacteria *Paenibacillus* and *Wolbachia pipentis*. Last, we identified milk proteins such as casein,
283 which made up a high percentage 96.19 ± 1.07% of the proteome of samples (Figure 5 – source
284 data 2), potentially because of the low bacterial load compared to conventional yogurt. There
285 were no significant differences in the relative abundance of all proteins in the proteome between
286 live and dehydrated ant yogurts or seasons (Figure 5 – figure supplement 2). Thus, proteases in
287 both live and dehydrated yogurts may, in part, catalyse coagulation in the experimental yogurts
288 and in the modern culinary applications. Taken together, the ant holobiont, ants and microbes,
289 contribute proteases potentially relevant to food fermentation.

290 **Discussion**

291 In this study, we explored the traditional practice of using ants as a starter for yogurt fermentation.
292 We hypothesized that the ant holobiont, both the ant and its microbes, catalysed the fermentation.
293 First, we uncovered that bacteria from the ants shaped the yogurt microbiome. Using live ants as
294 a starter, in contrast to frozen or dehydrated ants, resulted in a consistent microbiome containing
295 lactic and acetic acid bacteria, desirable for fermentation. These ant-bacteria included some
296 species thought of as canonical fermentation microbes, such as *L. delbrueckii* subsp. *bulgaricus*
297 and *F. sanfranciscensis*. Second, we discovered that the addition of the ant holobiont to milk
298 introduced acids and proteases. Formic acid originated from the ant, while lactic and acetic acid
299 were likely produced by bacteria. The ants and bacteria also contributed proteases with
300 caseinolytic potential. Our results revealed that the ant holobiont can contribute to fermentation
301 and thus prompted reconsideration of the microbial consortia in our ferments, their origins, and
302 applications to fermented food.

303 We uncovered how ants and their bacteria synergistically shape the fermentation by applying the
304 holobiont framework. Holobiont theory considers a host and its associated microbes collectively,
305 where the interplay between animal and microbes shapes the resulting ecology and evolution of
306 the whole, host plus microbes (Bordenstein & Theis, 2015; Margulis & Fester, 1991; Zilber-
307 Rosenberg & Rosenberg, 2008). When animals or plants are used in fermentation, their
308 holobionts may structure that fermentation in ways that reach beyond the effect of the individual
309 parts. *Formica* ants produce formic acid for defence (O'Rourke, 1950; Tragust et al., 2020) and
310 feed on the sugar-rich honeydew (Ivens & Kronauer, 2022) and protein-rich insects (Robinson &
311 Stockan, 2020). In response, the lactic and acetic acid bacteria are hypothesized to metabolize
312 the honeydew (Zheng et al., 2022), are highly acid tolerant (Tragust et al., 2020) and are potential
313 contributors to proteolysis. Once ants are introduced into milk, the properties of the ant holobiont
314 extend to the yogurt ecosystem. Formic acid initially acidifies the milk, ant-bacteria break down
315 milk sugars producing additional acid, and proteases liberate peptides and amino acids for
316 bacterial growth. By looking through the lens of the holobiont, we could clarify why *Formica* ants
317 and bacteria are pre-adapted for the fermentation of yogurt and potentially other foods.

318 The effect of the ant holobiont on yogurt fermentation parallels the role of microbes in other
319 fermented foods. In most ferments, multiple species of microbes offer complementary
320 contributions to the fermentation process (Canon et al., 2020; Sieuwerts et al., 2008). In
321 conventional yogurt, the bacteria *S. thermophilus* produces formic acid that is then metabolized
322 by *L. delbrueckii* subsp. *bulgaricus*, and in return, *L. delbrueckii* subsp. *bulgaricus* produces
323 proteases. Collectively, these bacteria then metabolize lactose and produce lactic acid to

transform milk into tangy yogurt (Sieuwerts, 2016; Sieuwerts et al., 2010). In sourdough, yeast and lactic acid bacteria can secrete factors that promote one another's growth (Sieuwerts et al., 2018), and overall have complementary roles in the fermentation of sugars and protein derivatives (Gänzle, 2014; Gänzle et al., 2023). Together, sourdough microbes create leavened bread with acidic, kokumi, and umami flavours (Gänzle et al., 2023). In the ant yogurt, our results suggest the separate elements of the ant holobiont, the ant body and microbes, act synergistically. Consequently, the ant holobiont introduces acids, proteases, and potentially other compounds that contribute to the organoleptic characteristics such as distinct acidity, coagulation, and flavour.

Intriguingly, we isolated two species of lactic acid bacteria from the ant yogurt that are commonly found in fermented foods, *L. delbrueckii* subsp. *bulgaricus* and *F. sanfranciscensis*. *L. delbrueckii* subsp. *bulgaricus* was first scientifically isolated from Bulgarian yogurt (Grigoroff, 1905; Metchnikoff, 1908) and is presently used in conventional yogurt around the world (FAO & WHO, 2018). The phylogeny indicates that related species are associated with bees and vertebrates such as pigs and hamsters (Duar et al., 2017). It has also been identified on the leaves of the snowdrop flower, *Galanthus nivalis*, endemic to Bulgaria (Michaylova et al., 2007). We now add the ant holobiont to the list of potential natural habitats of these bacteria. The second bacterial species, *F. sanfranciscensis,* is one of the most prevalent bacteria in sourdough bread globally (Gänzle & Zheng, 2019; Landis et al., 2021). The *Fructilactobacillus* clade appears to have diversified with ants (Jackson et al., 2023) and related social insects (Duar et al., 2017). We suspect that *F. sanfranciscensis* has evolved in ants over millions of years and was only introduced into fermented foods with the advent of bread making in the last several thousand years (Arranz-Otaegui et al., 2018; Carretero et al., 2017). These results lay the groundwork for unveiling the diversity of fermentative bacteria residing in ants and for elucidating the bacterial evolution from ant holobionts to fermented foods.

Our results bring forth new questions we believe are central to understanding the ecological and ancient origins of fermentative microbes. The ecological origins and assembly of ferment microbiomes, which colonize fermentations in lieu of backslopping, are only being to be characterized (Louw et al., 2023). Recently, it has been convincingly argued that the wine yeasts can originate from social wasps, where the symbiosis between wasps and yeasts is essential for the microbial transfer to wine ecosystems (Di Paola et al., 2023; Madden et al., 2018; Stefanini et al., 2012). Similarly, our results are reconcilable with the idea that bacteria for yogurt fermentation may originate from ants. This poses the question whether the ant holobiont was influential to the historic origins of yogurt or bread fermentation. Beyond traditional practices using ants in yogurt

(Multu Sirakova, 2023; Öney Tan, 2010; Yalman, 1977), ant colony materials may be integrated in sourdough fermentation (Mutlu Sirakova 2023), providing further avenues for microbial transfer. Conceivably, the ant bacteria then could be backslopped in traditional fermentations, successively propagating and evolving in yogurts. Although it is not clear when these traditions arose, it is noteworthy that the earliest evidence of yogurt and architectural depictions of ants and bees were found in prehistoric remains from our study region (Hendy et al., 2018; Walter, 2021). While these fragmentary anecdotes do not reconcile or resolve the origins of fermentative microbes, they highlight cultural threads that may connect ants and ferments.

As a group of co-authors, including anthropologists, culinary innovators, and food scientists, we differentially perceive the implications of the ant holobiont on yogurt-making. From an anthropological viewpoint, it challenges the ethos of conventional yogurt fermentation. Unlike traditional yogurts, large-scale industrial yogurt production relies on a limited number of bacterial strains. Conversely, the lactic acid bacteria found in ant yogurt are maintained in the ants, yogurt, and environment, and are cultured through traditional dairy practices passed down over generations (Multu Sirakova, 2023). To preserve this microbial diversity, we must uphold its cultural heritage, along with the conservation of red wood ants (Balzani et al., 2022). In relation to modern gastronomy, elaborations on the use of ants in milk, along with their microbes and enzymes, may hold wider purchase for fermented foods. It opens new culinary possibilities for other dairy products and fermented beverages that are sour or effervescent. Beyond the possibility of imagining new foods, exposing the public to familiar foods made with microbes and insects could conceivably help change consumer perceptions of entomophagy. From the food science perspective, microbes or enzymes from ant yogurts can be integrated into the food system to explore novel applications and flavours. For example, these ant microbes hold potential promise for new plant-based foods, such as dairy-free yogurts. While we differ in what the future of ant holobiont-facilitated fermentation may be, we agree it provides a powerful example that can broaden our thinking, as a society, about what is possible.

Materials and Methods

Traditional and modern culinary applications of ants

We travelled to Nova Mahala, Bulgaria to recreate ant yogurt with communities that retained memories and oral histories of the ant yogurt tradition. We based the yogurt fermentation on freshly acquired raw cow milk and *F. rufa* ants, from a colony found just outside of the village. The milk was warmed until nearly scalding, such that it could “bite your pinky finger” (Multu Sirakova,

2023). Four live *F. rufa* ants were added to 400 mL of milk. A cheesecloth was secured over the milk, and fabric was wrapped around the glass container for insulation. The milk jar was then buried inside the ant colony, covering it completely with the mound material. The milk was left within the colony and retrieved the next day, 26 hours later. The yogurt was then tasted, checked with pH strips, and stirred to observe coagulation.

Three culinary applications of the *Formica rufa* ants were developed by the research and development team at Restaurant Alchemist.

Ant yogurt ice cream sandwich: The “ant-wich” consisted of ant yogurt ice cream, ant gel filling, and ant tuile. To make the ant yogurt ice cream, organic 7% fat sheep’s milk (Hårbølle Mejeri, Møn, Denmark) was fermented with 1.6% live ants. Live ants were crushed and mixed in an aliquot of milk, which was passed through a strainer with pore size 100 μmM (Sigma Aldrich, Denmark) to remove ant body parts and potential parasites that may occur in low prevalence (Jensen et al., 2017). The strained aliquot was mixed with the rest of the milk, followed by an incubation period of 8 h at 40°C and subsequent storage at 4°C overnight. To sweeten and thicken the yogurt into ice cream, 300 g of sheep’s cream (Hårbølle Mejeri Møn, Denmark), and 22 g of sugar (Nordic Sugar, Denmark) was added to 380 g of ant yogurt, followed by 3 g of gelatine sheets (Condi, bloom 220, Denmark) and 5 g of ice cream stabilizer. The ant gel filling, based on an ant infusion, was made for the ice cream sandwich. For the infusion, dehydrated ants (*Formica rufa*) and water (filtered, pH 7) were mixed at the ratio of 10/100 (v/w), vacuum infused at 85°C for 8 h, strained through a fine mesh, and kept at 4°C. Subsequently, the ant gel was made with 100 g of ant infusion, 7.5 g of powdered glucose 33DE (Sosa, Spain), 70 g of inverted sugar (Trimoline, Cristalco, France), and 5 g of NH pectin (Sosa, Denmark). These were mixed, cooked for 5 min, and set to rest at 4°C for 24 h. Finally, an ant tuile was made to sandwich the ant ice cream and filling. The tuile was made by mixing 100 g of sweetened condensed milk (Tørsleffs, Denmark), 100 g of 00 wheat flour (Condi, Denmark), 100 g of egg whites, 3 g of baking powder (Condi, Denmark), 2 g of salt (Maldon), and 8 g of charcoal-black colorant (Sosa, Spain). The mixture was blended for 5 min, spread to measure 2 mm in height, and baked in the oven at 155 °C for 15 min. The final “ant-wich” was assembled to take the shape of an ant using a laser-cut stencil and served at -11 °C.

Ant mascarpone-like cheese: A mascarpone-like cheese was developed using ants as the coagulant. In common practice, lemon juice and milk or cream is added to make mascarpone, where the citric acid coagulates the dairy. Here, the acid from the ants likely contributed to the coagulation. Pasteurized sheep's milk (7% fat) and cream (64% fat) (Hårbølle Mejeri, Møn,

422 Denmark) were mixed with the proportions being 30% and 70%, respectively. The dairy mixture
423 was homogenized and heated to 95°C. Then, 10 mL ant water infusion (described above) was
424 added for each kilogram of this dairy mixture, stirring until complete coagulation (Munieweg et al.,
425 2021). The curd was cooled to 4°C for 6 h and strained through a cheesecloth. The mascarpone-
426 like cheese was stored at 4°C.

427 *Cocktail clarified with ant milk-wash*: Milk-wash is a technique commonly used in cocktails to
428 clarify a liquid. Milk Punch, the common drink prepared with this technique, is a dairy-based drink
429 dating to the late 1600s and early 1700s. The earliest written recipe of clarified milk punch comes
430 from a 1711 cookbook by Mary Rockett (Wondrich, 2010). To do so, the milk is curdled by lowering
431 the pH, which makes it possible to strain out the dairy solids. The result is a clear beverage with
432 more richness and body. It is also a popular modern technique at cocktail bars, normally produced
433 in large amounts (Wondrich, 2010). Using this technique, a cocktail was developed, but by
434 inducing the milk coagulation with dehydrated ants. The formic acid replaced other products
435 commonly used to lower the pH, such as lemon, lime, or any other acids like citric or ascorbic
436 acid. For the cocktail, an alcoholic base was made using 181 g of brandy (Ximenez Spinola
437 Battonage Brandy, Spain), 101 g of genepi liqueur (Genepi Grand Tetras, France), and 118 g of
438 apricot liqueur (Bitter Truth Apricot Liqueur, Germany) mixed in a glass jar. 10 g of dehydrated
439 ants (*Formica rufa*) were added and infused for 2 h at RT. The alcoholic mix plus dehydrated ants
440 were poured into a glass jar with 200 g of milk (5% fat), stirred, and kept at 2 °C for 6 h to curdle
441 the milk. Subsequently, the mixture was filtered through a coffee filter and kept at 2 °C until
442 serving. The cocktail was garnished with 4 frozen ants. The cocktail is in the "aperitif" style of
443 cocktails.

444 *Ant collection and processing for experimental yogurt*

445 Experimental yogurts were made in spring, according to the tradition (Multu Sirakova, 2023), and
446 in autumn, to test the seasonality of the fermentation. We used the red wood ant *Formica*
447 *polyctena* for microbiome characterization and experimental yogurt fermentation, and *F. rufa* for
448 microbiome characterization. *F. polyctena* is the sister species of *F. rufa*, where the later was
449 used in the ethnographic account and at the Alchemist R&D. *F. polyctena* worker ants were
450 collected at the end of May and the start of October 2022, from a colony in Rude Skov, Denmark
451 (55.8334720, 12.474938410). Similarly, worker ants from a colony of *F. rufa*, found in
452 Bøllemosen, Denmark (55.827676, 12.534667), were collected at the end of May and late
453 September 2023. Ants were taken without disturbing the mound structure, considering several

454 species within the group have experienced species declines within Europe (Balzani et al., 2022).
455 Ants were gathered with sterilized equipment and kept on ice while transported to the lab.

456 *F. polyctena* worker ants were then processed to make the 3 elaborations of ant yogurt, live,
457 frozen, and dehydrated. Live ants were provided sterile 15% sucrose as food and used to make
458 ant yogurt within the week of collection. Prior to freezing, ants were aseptically removed from the
459 nest material, then snap-frozen and stored at -20°C. Dehydrated ants were prepared by placing
460 frozen ants in a vented Petri dish with sterile filter paper and incubated at 40°C for 16 h and were
461 used within a week. Live and dehydrated ants were frozen at -20°C for later microbiome
462 characterization.

463 The ant species were identified by barcoding of the *cytochrome c oxidase I gene (COXI)*. DNA
464 was extracted with Chelex (Sigma Aldrich, Denmark), amplified, cleaned, and sequenced (Sinotte
465 et al., 2021). PCRs were performed with primers LCO1490 5´-
466 GGTCAACAAATCATAAAGATATTGG-3´and HCO2198 5´-
467 TAAACTTCAGGRTGACCAAAAAATCA-3´ (Zuccon et al., 2012). PCR products were cleaned
468 with PureIT ExoZap (Amplicon, Denmark) and sequenced at Macrogen (the Netherlands).
469 Sequences were trimmed and aligned using default settings in Geneious (Biomatters Ltd., New
470 Zealand), and the consensus sequence was compared to NCBI's nucleotide database with
471 BLASTn.

472 *Live, dehydrated, and frozen elaborations of experimental ant-yogurt*

473 Ant yogurts were made under sterile conditions within a laminar flow hood, to assure that the ants
474 were the sole source of microbes. The yogurt was made with 10% w/v reconstituted milk powder
475 in distilled water (Sigma Aldrich, Denmark) and sterilized at 121 °C for 5 min to avoid
476 caramelization. Yogurts were made in Falcon tubes with 30 mL of sterile milk and 0.5 g live ants,
477 crushed with a sterile pestle, 0.5 g frozen ants, or 0.4 g dehydrated ants. Yogurts and negative
478 controls were incubated at 42°C for 8 h and then stored at 4°C overnight, according to standard
479 yogurt-making practices (Nagaoka, 2019; Robinson et al., 2006). The following day yogurts were
480 subsampled for microbial culturomics, acid quantification, DNA extraction, and proteomics.
481 Subsamples were stored at -20 °C.

482 In the spring, six samples of each of the live, frozen, and dehydrated ant-yogurts were made as
483 well as 2 negative controls of milk alone, one incubated and one not incubated. In the autumn,

484 the same design was used, but frozen ant-yogurt was not made as it was found to contain
485 pathogens.

486 *DNA extraction of yogurt and ants*

487 DNA was extracted from the yogurts and ants to assess the microbial community composition. All
488 extractions were performed under sterile conditions in a laminar air flow. The yogurt samples were
489 first homogenized by vortexing, then passed through a Corning cell strainer with pore size 100
490 µm (Sigma Aldrich, Denmark) to remove ant body parts. The yogurt underwent three washes prior
491 to lysis to remove excess fats that may act as inhibitors and to pellet microbial cells. First, 3 g of
492 yogurt and 10 mL of sterile 2% sodium citrate solution pH 7.4 were incubated at room temperature
493 for 10 min, then centrifuged at 5,000 g for 10 min at 4°C. Second, the pellet was retained and
494 washed in 15 mL TES buffer (50 mM Tris, 1 mM EDTA, 20% sucrose) and centrifuged at 5,000 g
495 for 10 min at 4°C. A final wash was done in 1 mL TES, which was pelleted, and supernatant
496 removed. For the ants, two ants were used per DNA extraction. Prior to extraction, 200 µL
497 molecular water was added to the ants and they were crushed with a sterile pestle.

498 DNA extraction was performed with the DNeasy Blood and Tissue Kit (Qiagen, Germany)
499 according to manufacturer's instructions for gram positive bacteria with the following
500 modifications. Physical lysis of ants and yogurts was performed via bead-beating with 0.1 mm G2
501 DNA/RNA Enhancer beads (VWR, Denmark) in a TissueLyser (Qiagen, Denmark) at 30 Hz for 3
502 min. Samples then underwent chemical lysis, including 30 min lysis in lysozyme with an addition
503 of 2 µL of mutanolysin (25 U/µL) followed by 1 h proteinase K lysis. The final elution of the DNA
504 extract was 200 µL in AE buffer, passed twice through the column to maximize yield. Due to
505 exceptionally low DNA concentrations in some experimental yogurts, three extractions were
506 performed per sample, and thus 9 g of yogurt in total was washed, extracted, and pooled per
507 sample. Similarly, two ant extractions were pooled for a final sample representing four ants. The
508 elutes were pooled and concentrated with AMP Pure XP Reagent (Beckman Coulter, Denmark)
509 according to manufacturer's instructions, then underwent a clean-up for inhibitors with the DNeasy
510 PowerClean Pro kit (Qiagen, Denmark) according to manufacturer's instructions.

511 The final samples for sequencing included yogurts, ants, and several controls. Yogurt samples
512 thus included 20 samples from spring and 13 from autumn, as previously described. From the
513 ants, there were six final samples for live and for dehydrated *F. polyctena* each from spring and
514 autumn, and six samples of live *F. rufa* in spring and autumn. In addition to the experimental

515 yogurts and ants, a mock community of standard bacteria (Zymobiomics, Nordic Biosite,
516 Denmark) was included to determine any community bias generated by extraction and later
517 metabarcoding. Further, three conventional plain yogurts were included as positive controls. Last,
518 five blank DNA extractions were included as negative controls for DNA extraction.

519 *Quantification of bacterial load*

520 The bacterial microbial loads of the yogurt, ant, and control samples were assessed with real-
521 time PCR (qPCR). To quantify the bacteria in samples, the Femto Bacterial DNA Quantification
522 Kit (Zymobiomics, Denmark) was used according to the manufacturer's instructions. The Femto
523 Bacterial Kit targets the 16S *rRNA* gene of bacteria and estimates the amount of bacterial DNA
524 (ng/μL) in a sample based on a series of standards and non-template control. qPCR of samples
525 was run in duplicate on Mx3005p (Agilent, Germany). The technical replicates demonstrated
526 minimal variation and were thus averaged. Subsequently, the amount of DNA/mL of yogurt, in the
527 total yogurt, or ant starter, was back calculated based on the standards and experimental
528 parameters.

529 *Bacterial community metabarcoding and analysis*

530 The bacterial community of the ants, yogurts, and controls was characterized with bacterial
531 metabarcoding. Library preparation and targeted sequencing of the 16S *rRNA* V3-V4 region was
532 performed with the primers 341F (5' -CCTAYGGGRBGCASCAG- 3') and 806R (5' -
533 GGACTACNNGGGTATCTAAT- 3') at Novogene Europe (Cambridge, United Kingdom). In brief,
534 amplicons for the targeted region underwent size selection, then equivalent amounts of PCR
535 product were end-repaired, A-tailed, and ligated with Illumina adapters. Library concentrations
536 were checked with Qubit and qPCR, and size distribution was checked with BioAnalyzer. Libraries
537 were pooled and sequenced on Illumina for pair-ended 250 bp at a depth of 30k per sample. After
538 sequencing, primers and adaptors were removed by Novogene, and raw reads were provided for
539 analysis.

540 The analysis of sequences was carried out in R (v. 4.3.1) (Team, 2023), which included
541 determination of amplicon sequence variants (ASVs), taxonomic assignment, visualization, and
542 statistical analyses. The dada2 pipeline (v. 1.30.0) (Callahan et al., 2016) was used for ASV and
543 taxonomic assignment. Reads were not trimmed due to the overall high quality of sequences, but
544 were filtered with the default parameters, with *maxEE* adjusted to (2,4). Reads were then paired
545 with *mergePairs*, *minOverlap* set to 16, and chimeras were removed. Taxonomy was assigned to

546 the species level where possible with the SILVA database (v. 138.1) (Glöckner et al., 2017).
547 Potential contaminants were then removed using SCRuB (v.0.0.1) (Austin et al., 2023) based on
548 the blank negative controls that were successfully sequenced. Mitochondria, chloroplasts, and
549 Eukarya were removed, resulting in 63.87% of ASVs with genus-level classification and 82.23%
550 with family-level. The mock community was then assessed to determine any potential bias created
551 by DNA extraction and sequencing. All eight bacterial genera present in the mock community
552 were validated.

553 The metabarcoding data was then analysed and visualized using the packages phyloseq
554 (v.1.46.0) (McMurdie & Holmes, 2013), microviz (v.0.12.0) (Barnett et al., 2021), and ggplot2
555 (v.3.4.4) (Wickham, 2011). The composition of the microbiome of ants and yogurts was visualized
556 with microviz. The beta diversity of the yogurts was visualized with phyloseq through a NMDS
557 ordination of their Bray-Curtis distances, resulting in a plot ordinated by yogurt treatments. The
558 alpha diversity of the yogurts was further determined with phyloseq. Last, the microbial loads of
559 Lactobacillaceae and Bacillaceae in the ant starters and yogurts were estimated by multiplying
560 the relative abundance of each genus, based on the metabarcoding data, by the quantified
561 bacterial load, based on 16S qPCR. This was visualized using ggplot2.

562 We assessed the effect of treatment (live, dehydrated, or frozen) on the beta diversity of the
563 yogurts using Bray-Curtis distances and an adonis PERMANOVA using the package vegan
564 (v.2.6.4) (Oksanen et al., 2013) and function *adonis2*. Subsequently, we performed a pairwise-
565 comparison between the three treatments based on the PERMANOVA using the package
566 pairwiseAdonis (v.0.4.1) (Martinez, 2017), which performs *p*-value adjustment for multiple
567 comparisons. The alpha diversity of the three yogurts was based on the Shannon index. We
568 performed a general linear model to determine the effect of treatment on the alpha diversity,
569 confirming the parametric assumptions of the model. Pairwise comparisons were then performed
570 with a Tukey HSD. Pairwise comparisons of the Lactobacillaceae and Bacillaceae load in the ant
571 starters and yogurts was performed with two sample t-tests for equal or unequal variance
572 depending upon the data.

573 *Bacterial culturomics of milk ferments and ants*

574 Bacteria from the ant yogurts and ants were cultured and identified with Sanger sequencing. 100
575 µL of yogurt sample was inoculated and plated in De Man, Rogosa and Sharpe (MRS) (VWR
576 Chemicals, US), Gifu Anaerobic Medium, modified (GAM) (HyServe GmbH & Co. KG, Germany),

577 yeast peptone dextrose (YPD), and Luria-Bertani (LB) media. YPD contained 10 g/L yeast extract,
578 20 g/L peptone, and 20 g/L D-glucose. LB contained 10 g/L peptone, 5 g/L yeast extract and 0.5
579 g/L sodium chloride. A total of 20 g/L agar was added for solid cultivations. For ants, individual
580 ants were inoculated into liquid media. Tubes and plates were incubated at 30°Cover 2 days, or
581 until growth was visible. All four media were cultivated in aerobic conditions and MRS and GAM
582 were also incubated anaerobically. Subcultures were plated and incubated in the same
583 conditions. Distinct bacterial colonies from plates were identified with Sanger sequencing of the
584 conserved 16S *rRNA* gene, which included colony PCR (cPCR) with primers 5´-
585 AGAGTTTGATCCTGGCTCAG-3´ and 5´-CCGTCAATTCCTTTRAGTTT-3´ (Cebeci & Gürakan,
586 2008). All cPCR reactions consisted of 12.5 µL of Red-Taq Mastermix (VWR, Denmark), 0.5 µL
587 of forward 10 µM primer, 0.5 µL of reverse 10 µM primer, 11.5 µL of water and a bacterial colony.
588 The reactions followed the same thermocycler program that consisted of an initial 4 min at 94 °C,
589 followed by 35 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min with a final 72 °C for 10
590 min. PCR products were Sanger sequenced (Eurofins Scientific SE). Results from sequencing
591 were taxonomically classified using BLAST and presented in a Sankey diagram
592 (app.rawgraphs.io).

593 *Quantifying pH, lactic, acetic, and formic acid in yogurt*

594 The pH and coagulation of all yogurts were assessed before and after fermentation. After
595 fermentation, the pH was measured from one sample of each of the groups, and the coagulation
596 of the treatment group was assessed. Given the abundance of formic acid found in *Formica* ants
597 (O'Rourke, 1950), we also examined the effect of formic acid on coagulation. Formic acid (Sigma
598 Aldrich, Denmark) was added to the sterile milk until pH 4.6, 5.15, 5.9, and 6.6, which represented
599 the range of pHs observed in conventional yogurts, ant yogurts, and sterile milk. The pH of the
600 negative control was also measured. Additionally, pH of all the samples was measured prior to
601 DNA extraction and enzyme analysis, after they had undergone a cycle of freezing and thawing.
602 This was performed on a separate pH reader than that which provided the initial measurement
603 before and after fermentation.

604 The amount of formic, lactic, and acetic acid in the samples was quantified with high-performance
605 liquid chromatography (HPLC). Yogurt samples were centrifuged at 5000 rpm for 5 min at 4°C,
606 and their supernatant was passed through a 0.2 µm syringe filter (Sigma Aldrich, Denmark). The
607 filtrate was analysed by a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA)
608 equipped with a refractive index detector and a Bio-Rad Aminex HPx87 column (Bio-Rad, USA).

609 The column oven temperature was set at 30°C. The flow rate was set at 0.6 mL/min with 5 mM
610 H_2SO_4 (Sigma Aldrich, Denmark) as an eluent. The injection volume was 10 µL. Peaks were
611 identified by comparison to the prepared standards, and integration of the peak areas was used
612 to quantify acids from obtained standard curves using the software Chromeleon7 (Thermo Fisher
613 Scientific, USA). Plots were visualised in GraphPad Prism. T-tests were used to compare organic
614 acid levels between samples, with Benjamini-Hochberg correction for multiple testing.

615 *Proteomics of ant yogurts*

616 Sample preparation for proteomics analysis was performed directly from the supernatant of the
617 yogurt samples after centrifugation for 10 min at 14,000 g and further processed as described
618 earlier (Kozaeva et al., 2021). After centrifugation, the supernatants were subjected to the
619 bicinchoninic acid (BCA) assay to estimate protein concentrations. Trypsin and LysC digestion
620 mix (Promega) was added to 20 µg protein of each sample and incubated for 8 h. Trifluoroacetic
621 acid was added to halt digestion and the samples were desalted using C18 resin (Empore, 3M)
622 before HPLC-MS analysis.

623 HPLC-MS analysis of the samples was performed on an Orbitrap Exploris 480 instrument
624 (Thermo Fisher Scientific) preceded by an EASY-nLC 1200 HPLC system (Thermo Fisher
625 Scientific). For each sample, 1 µg of peptides was captured on a 2 cm C18 trap column (Thermo
626 Fisher 164946). Subsequently separation was executed using a 70 min gradient from 8% (v/v) to
627 48% (v/v) of acetonitrile in 0.1% (v/v) formic acid on a 15-cm C18 reverse-phase analytical column
628 (Thermo EasySpray ES904) at a flow rate of 250 nL/min. For data-independent acquisition, the
629 mass spectrometer was run with the HRMS1 method (Xuan et al., 2020) preceded by the FAIMS
630 Pro Interface (Thermo Fisher Scientific) with a compensation voltage (CV) of -45 V. Full MS1
631 spectra were collected at a resolution of 120,000 and scan range of 400-1,000 m/z, with the
632 maximum injection time set to auto. MS2 spectra were obtained at a resolution of 60,000, with
633 the maximum injection time set to auto and the collision energy set to 32. Each cycle consisted
634 of three DIA experiments each covering a range of 200 m/z with a window size of 6 m/z and a 1
635 m/z overlap, while a full MS scan was obtained in between experiments.

636 To assign the proteases to their potential organisms of origin, we created a database consisting
637 of the proteases, peptidases and proteins based on UniProt reference proteomes, the gold
638 standard in proteomics. The proteomes consisted of the ant host, bacteria from the ant yogurt,
639 and two conventional yogurt bacteria. For the ant, we used the *F. executa* proteome, since the *F.*

polyctena proteome was not publicly available, and *F. execta* is a closely related species. From the *F. execta* proteome, we selected the proteases specifically. For all the bacteria included, we refined our databased to only include proteases and peptidases in the proteome. We selected bacteria most prevalent among the bacteria isolated from the live ant yogurt bacterial metabarcoding and culturomics: *Fructilactobacillus sanfranciscensis*, *Oecophylibacter saccharovorans*, and *Paenibacillus sp.* We also included *Wolbachia pipentis*, which is closely related to the obligate intracellular bacteria found in *Formica* ants (Jackson et al., 2022) and is represented in our analysis. We included the conventional yogurt bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus. Streptococcus* was only present in low abundance in the bacteria metabarcoding data, and *L. delbrueckii* subsp. *bulgaricus* was isolated in one instance. We included them to determine if the ant yogurt contained proteases with the same or similar functionality. Last, we included common milk proteins, such as caseins, found in the *Bos taurus* proteome. For DIA data analysis, Spectronaut v18 (Biognosys, Switzerland) was used for protein identification and relative quantification of peptides. The default settings for "directDIA" were applied with an FDR cut-off of 1%, except for MS1 quantification for the peptides. Protein abundances were inferred from the peptide abundances using the MaxLFQ algorithm available within Spectronaut.

In the analysis of the results, first we determined if proteases, peptides, and milk proteins, changed in relative abundance between spring or autumn and live or dehydrated samples. We were unable to determine the abundance of a subset of proteases identified, likely due to their low abundance, and they were therefore not included in the analyses. For each protein, the average relative abundance was computed across all autumn samples and all spring samples. Similarly, the average relative abundance was computed across all live samples and all dehydrated samples.
The log2 fold change (log2FC) was calculated using the formula:
log2FC=log2(average abundance in condition 1/ average abundance in condition 2)

P-values were obtained by comparing the relative abundances of each protein across all samples and their replicates in the autumn versus spring conditions. Similarly, *p*-values were calculated by comparing the relative abundances of each protein across all samples and their replicates in the alive versus dehydrated conditions. The Benjamini-Hochberg procedure was applied to adjust the *p*-values for multiple comparisons, controlling the false discovery rate (FDR). The adjusted *p*-values were then transformed to their -log10 values for further analysis and visualization.

Second, we visualized the composition of the proteases and peptidases in GraphPad Prism. To do so, we removed the milk proteins originating from *Bos taurus*, given they made up a high proportion of the proteome. The aim was thus to clarify ant or bacterial contributions to the enzymatic profile. The relative abundance of each protease or peptidase visualized was thus based on the total abundance of proteases and peptidases in each sample, and not the proteome.

Data availability

Amplicon sequences from metabarcoding and isolated bacterial strains will be made available on the SRA archive in GenBank upon publication. Proteome data will be made available on the RCSB Protein Database Archive upon publication. Additional data frames will be made available on FigShare upon publication.

Code availability

The code for analyses had been deposited in Zenodo upon publication.

Author contributions

Conceptualization: V.M.S., L.J.J., R.R.D., V.R-V., D.P.V., S.M.S; Methodology: V.M.S., V.R.-V., D.P.V, N.R.V, S.K.T.P, E.M.V, S.B.A; Formal analysis: V.M.S., V.R.-V., S.K.T.P; Investigation: V.M.S., V.R.-V. D.P.V., S.M.S., N.R.V., A.C-M., S.K.T.P., E.M.V., D.Z.; Resources: L.J.J., S.B.A, R.M., S.M.S.; Data curation: V.M.S.; Writing – original draft preparation: V.M.S., V.R-V., L.J.J., R.R.D; Writing – review & editing: all authors; Visualisation: V.M.S., V.R.-V.; Supervision: L.J.J., R.D.D., S.B.A., R.M.; Project administration: V.M.S., L.J.J.; Funding acquisition: S.B.A., L.J.J., R.R.D., R.M.

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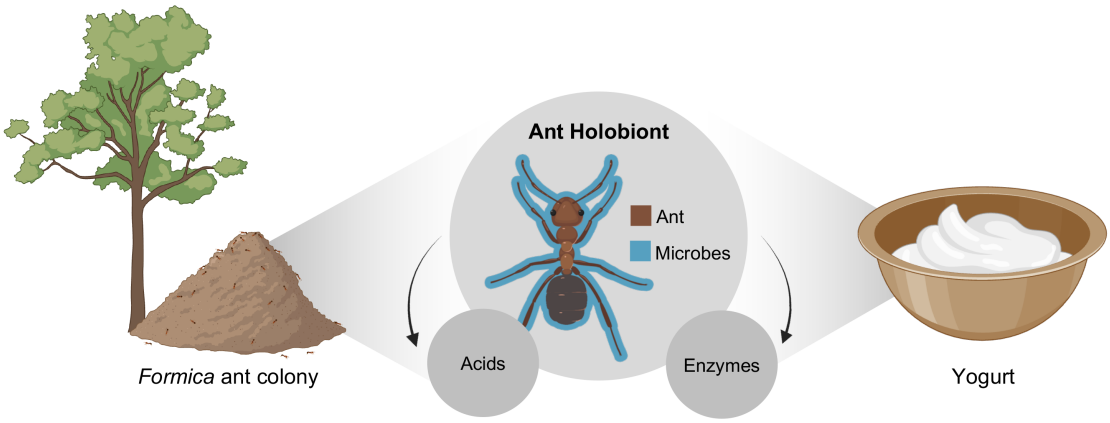

Figure 1. Hypothesized contributions of the ant holobiont to yogurt fermentation. *Formica* red wood ants create characteristic thatched mounds (left), which may have been used in traditional yogurt fermentation practices (Multu Sirakova 2023). The traits of the ant holobiont, the composite of both ant and the microbes found within it, are hypothesized to act as the starter of the yogurt fermentation (centre). These include the microbes (lactic acid and acetic acid bacteria) that are found in and on the ants, acids that are produced by the ant and the ant-associated microbes, and enzymes that have ant or microbial origins.

Figure supplement 1. Experimental workflow to characterize microbes, acids, and proteases in ant yogurt.

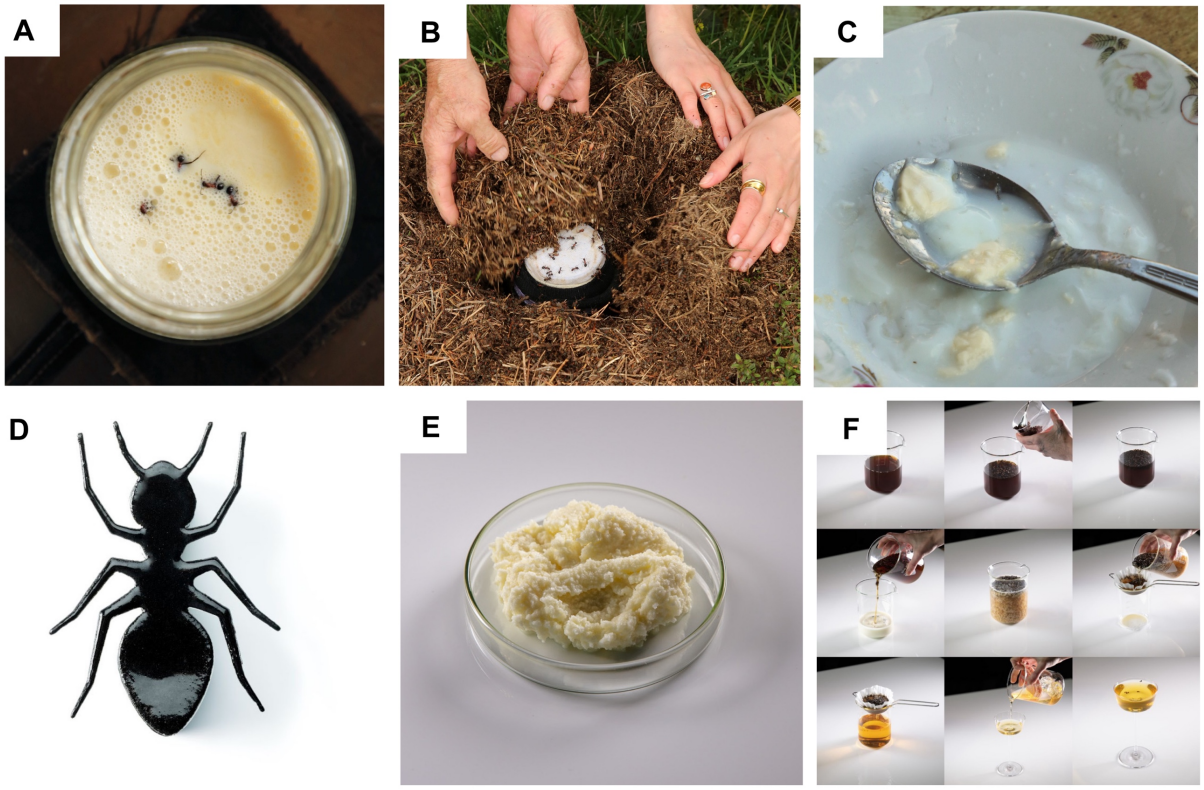

Figure 2. Ants as culinary catalysts of milk fermentation and coagulation. (**A-C**) Photographs of traditional yogurt fermentation initiated by *Formica* ants taken during field work in Bulgaria. (**A**) Ants were added to warmed milk that was then (**B**) buried within the ant colony and left to ferment overnight. (**C**) The resulting milk had started to coagulate and acidify, indicative of early stages of yogurt fermentation. (**D-F**) Culinary applications created by restaurant Alchemist's research and development team using *F. rufa* ants: (**D**) ant yogurt ice cream sandwich, (**E**) ant "mascarpone-like" cheese, and (**F**) milk-wash cocktail (Photos a-c by David Zilber, and d-f by Søren Gammelmark and Kåre Knudsen of Restaurant Alchemist).

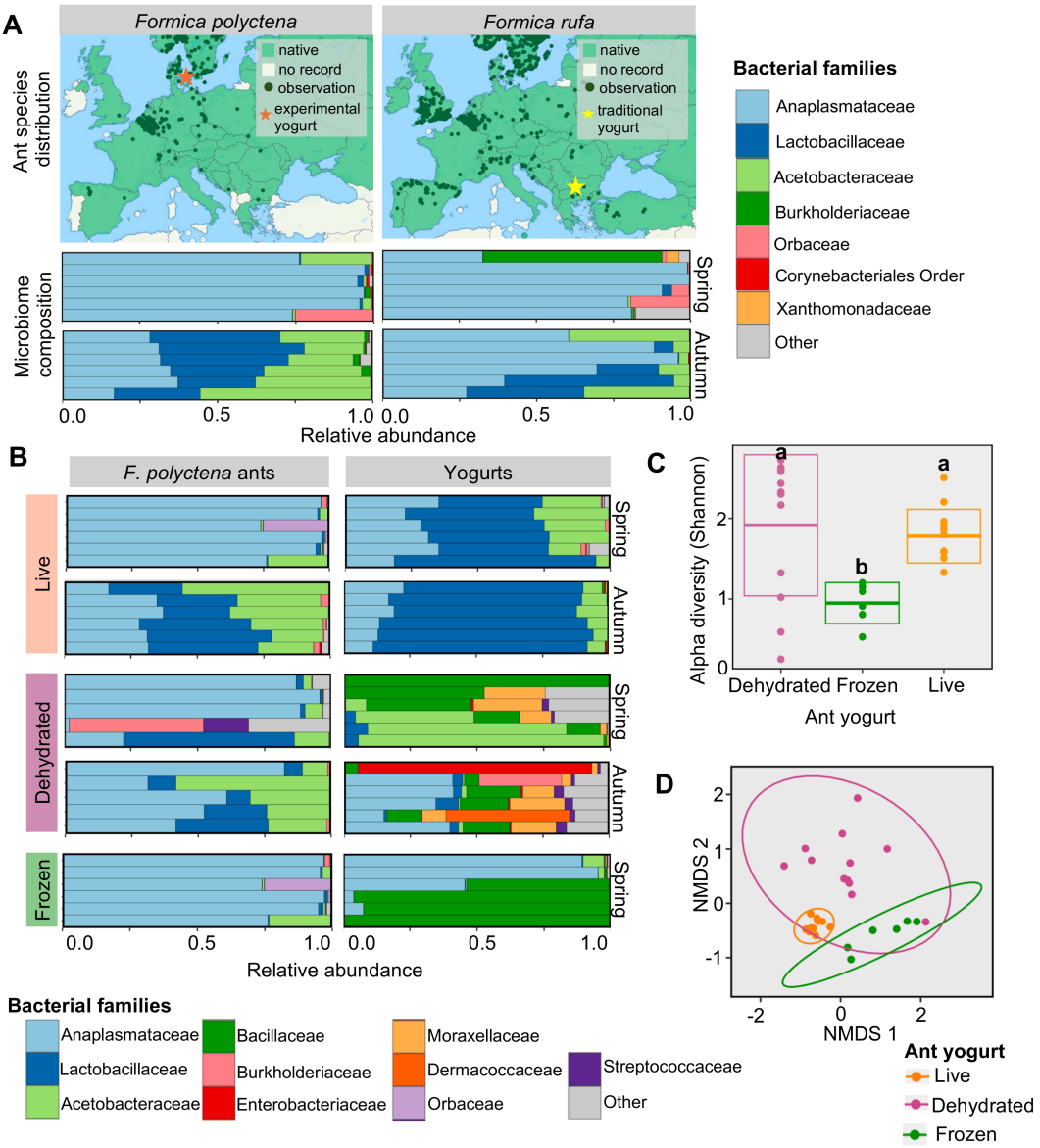

Figure 3. *Formica* ants host a stable, seasonal microbiome, and preparation of the ants affects the corresponding yogurt microbiome. (**A**) European species ranges of *F. polyctena* and *F. rufa* (top), both within the *F. rufa* group of red wood ants (maps modified from antmaps.org). The microbiome composition of ant species across seasons (bottom). Bar represents four pooled ants from a colony of each respective species, collected in Denmark. (**B**) The bacterial microbiome of the three ant preparations and corresponding ant yogurts made in spring and autumn. Bars represent replicates. Live ant microbiomes are also represented in panel (**A**), and live and frozen ants are identical. They are shown for direct comparison to the corresponding yogurts. (**C**) The alpha diversity of ant yogurts. Letters indicate pairwise statistical differences (Tukey HSD: $p<0.05$), and boxes illustrate standard deviations. (**D**) The beta diversity of ant yogurts based on

1044 Bray-Curtis distances and Non-Metric Multidimensional Scaling (NMDS) analysis. The grouping
1045 is supported by a PERMANOVA.
1046 **Figure supplement 1.** Genus-level microbiomes and seasonal bacterial loads of ants.
1047 **Figure supplement 2.** Detection bias of bacterial abundances based on mock community control.

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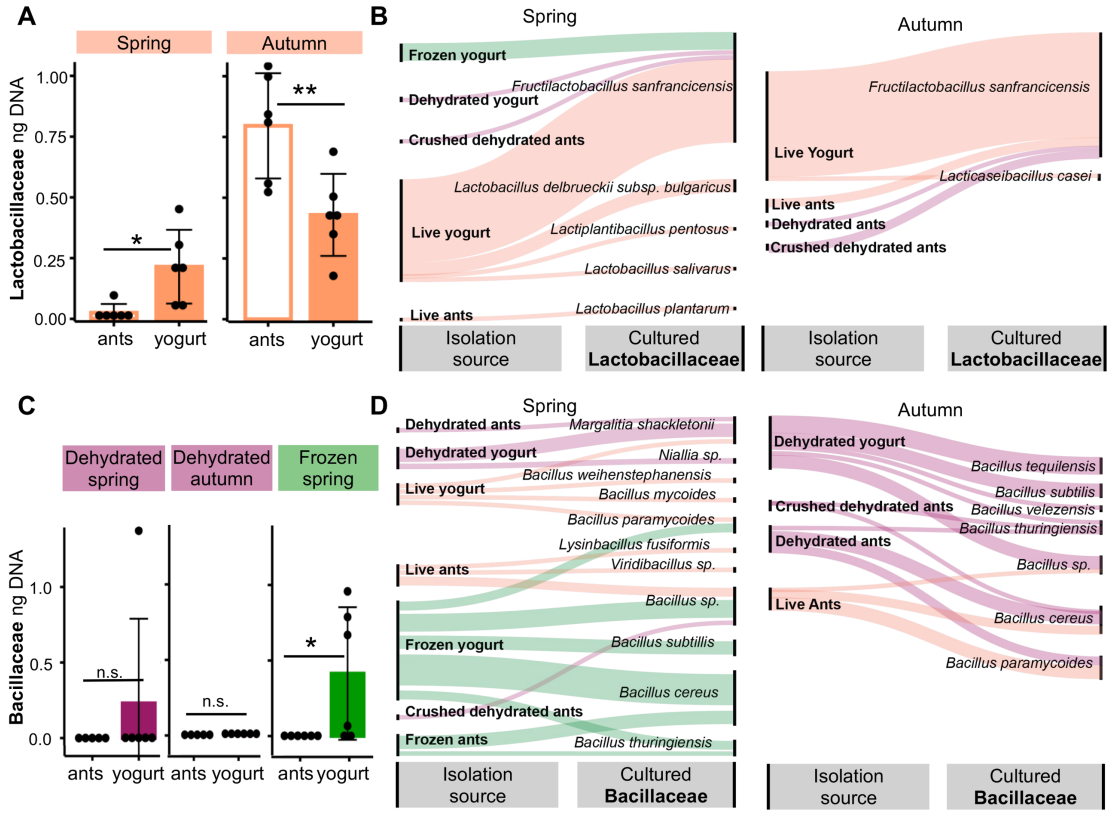


Figure 4. Bacterial biomass and culturable diversity of lactic acid and spore-forming bacteria vary based on the preparation of ant yogurts. (**A**) The estimated amount of lactic acid bacteria (Lactobacillaceae) DNA, representative of lactic acid bacterial load, in the live ants and resulting yogurts across seasons. (**B**) The diversity of culturable species of lactic acid bacteria from the ants and yogurts, where the majority of isolates stem from live ants and live ant yogurts. The thickness of each line approximates abundance of culturable bacteria by representing the number of microbial media plates where the bacteria grew compared to other samples. (**C**) The amount of DNA from spore-forming Bacillaceae, indicative of Bacillaceae bacterial load, is exceptionally low in dehydrated and frozen ants and increases in the respective yogurt fermentations. (**D**) Culturable species of Bacillaceae isolated from ants and yogurts. Frozen ant yogurts were not made in autumn. Line thickness represents an approximation of culturable bacteria as above. (* = $p \leq .05$; ** = $p \leq .01$; *** = $p \leq .001$; n.s. = p>0.05; y-axis present on a logarithmic scale).

Figure supplement 1. Bacterial load and relative abundance of Lactobacillaceae and Bacillaceae.

Figure supplement 2. All bacteria cultured from ants and ant yogurts in spring and autumn.

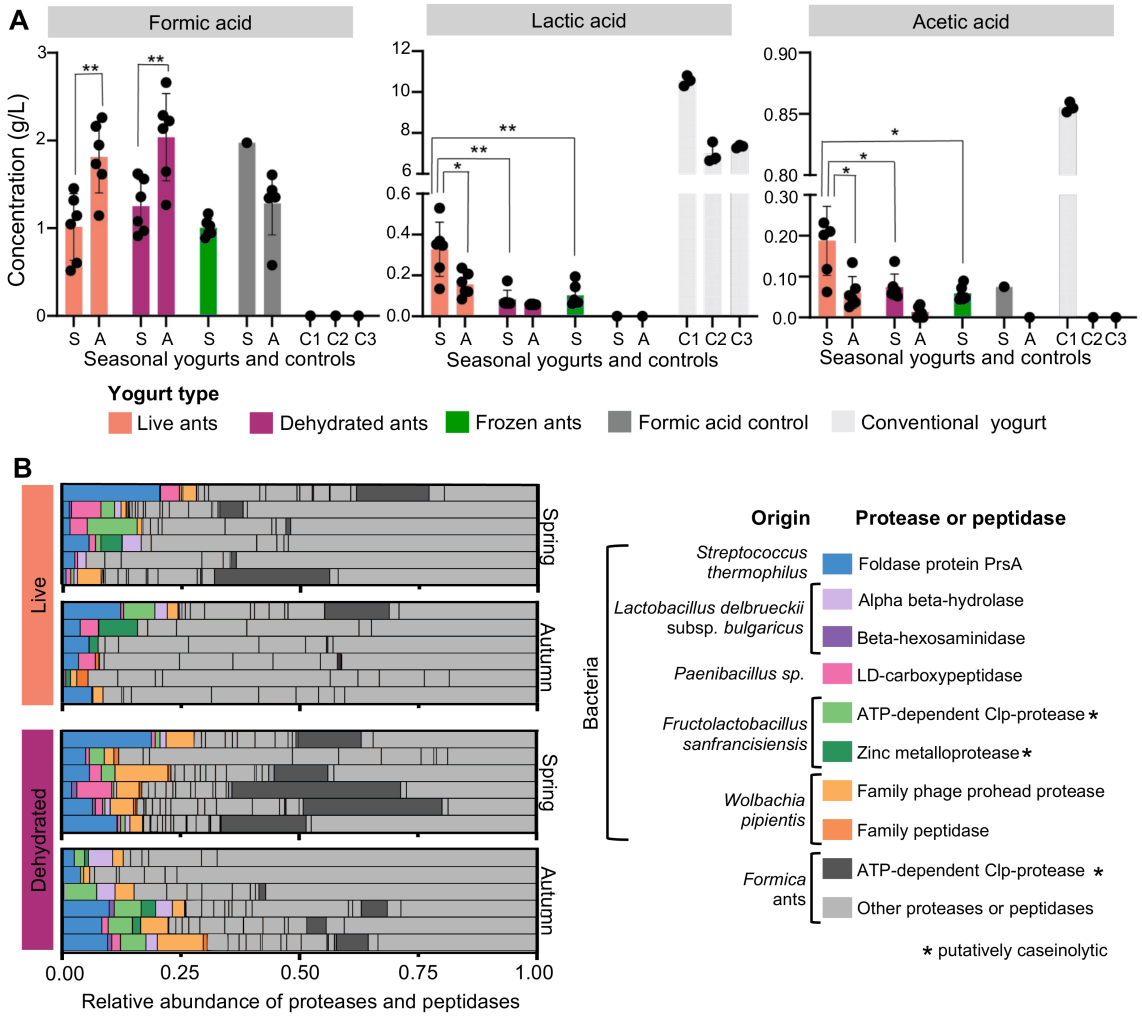
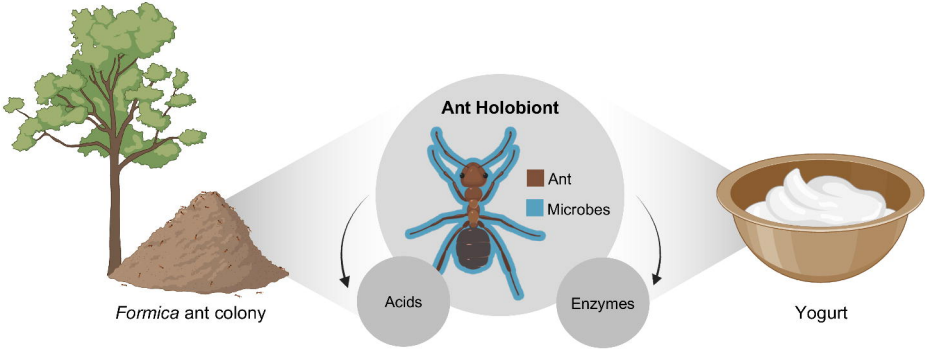
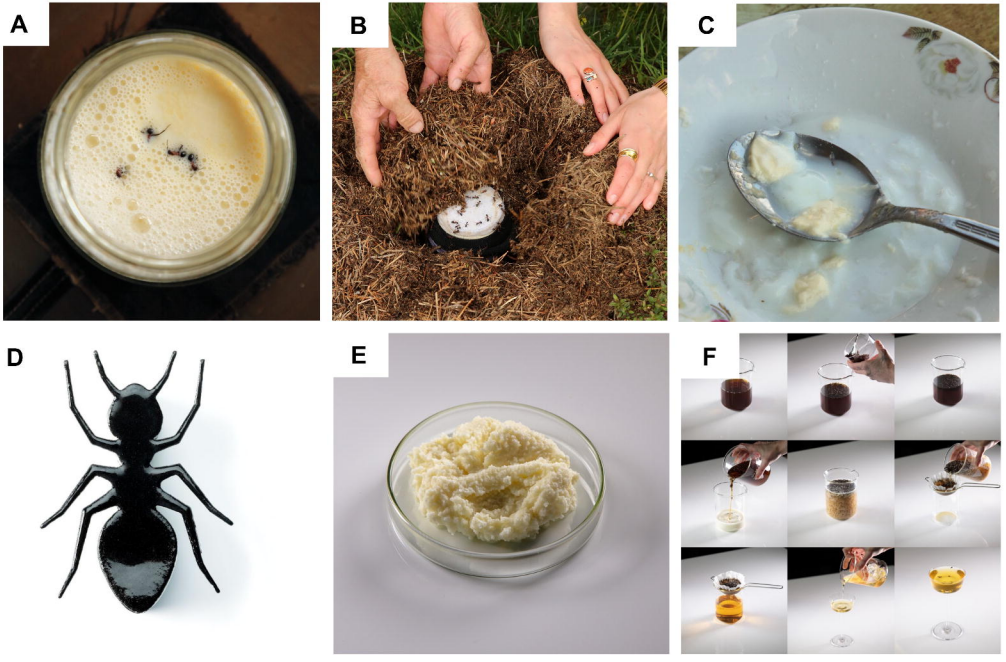


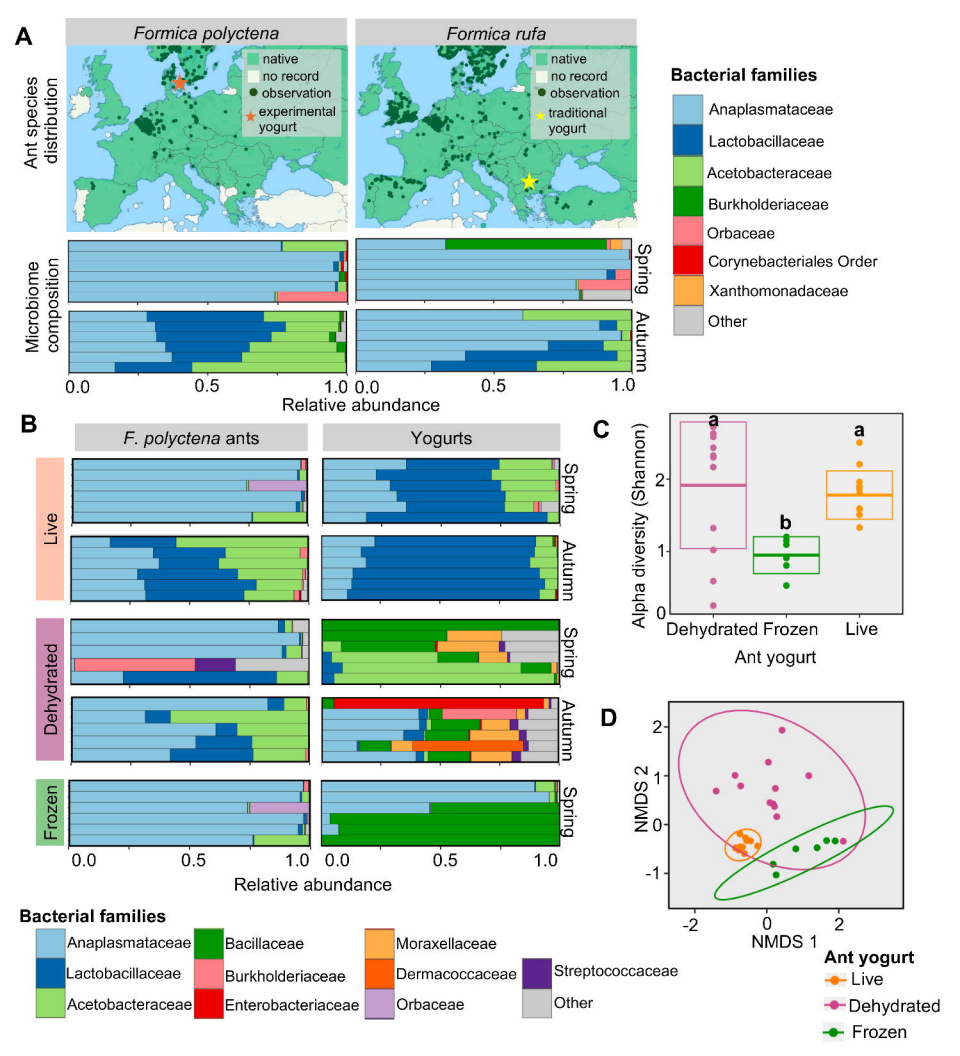
Figure 5. Organic acids, proteases, and peptidases in ant yogurts. (**A**) Concentration of metabolites formic acid, lactic acid, and acetic acid in the yogurts from spring (S) and autumn (A). The samples include yogurts made with live, dehydrated, and frozen ants, formic acid, and three conventional yogurts as controls (C1-3). Data points represent biological replicates and error bars represent the standard deviation. Statistical analysis was performed using Student's t-test (two-sample unequal variance; * = P ≤ .05; ** = P ≤ .01; *** = P ≤ .001). (**B**) Proteases and peptidases detected in live and dehydrated ant yogurt samples. Proteases and peptides originate from the ant holobiont, including the ant and its bacteria. It also includes bacteria found in conventional yogurt, that were potentially present in low abundances. The rows represent biological replicates. Milk proteins were removed to improve the visualization of the proteases and peptidases. Milk proteins made up 96.19 ± 1.07% of the samples. The absolute abundance of all proteins in the proteomes can be found Figure 5 – source data 1.

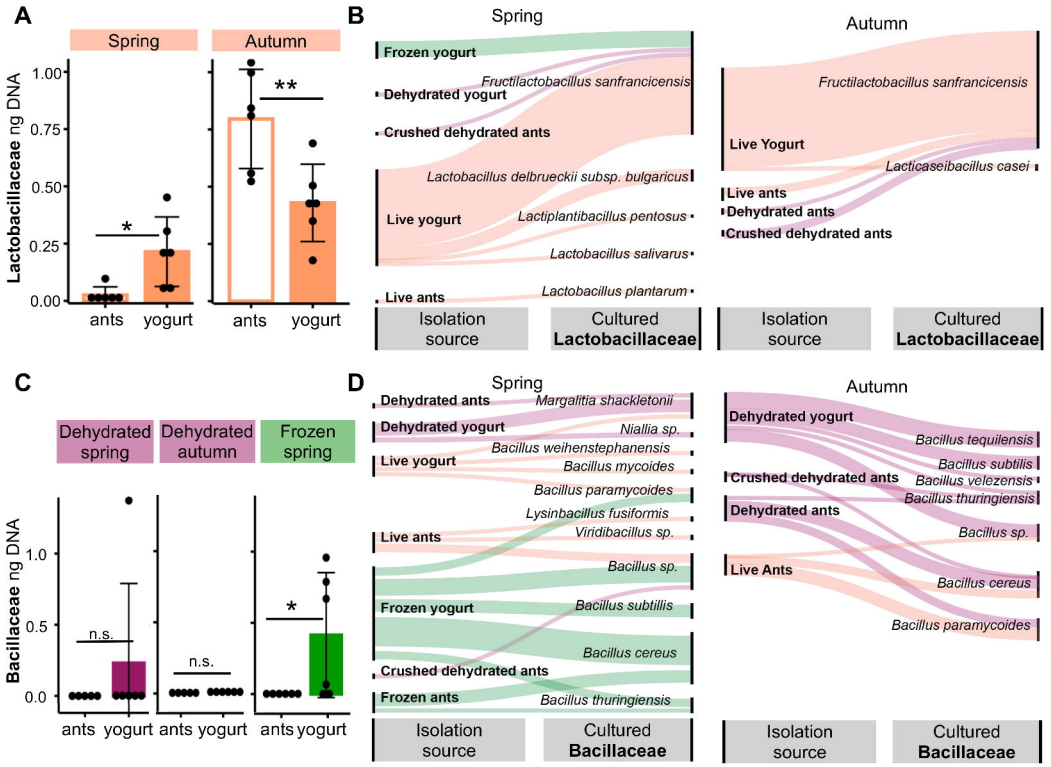
Figure supplement 1. Bacterial load and pH of ant yogurts, conventional yogurts, and controls.

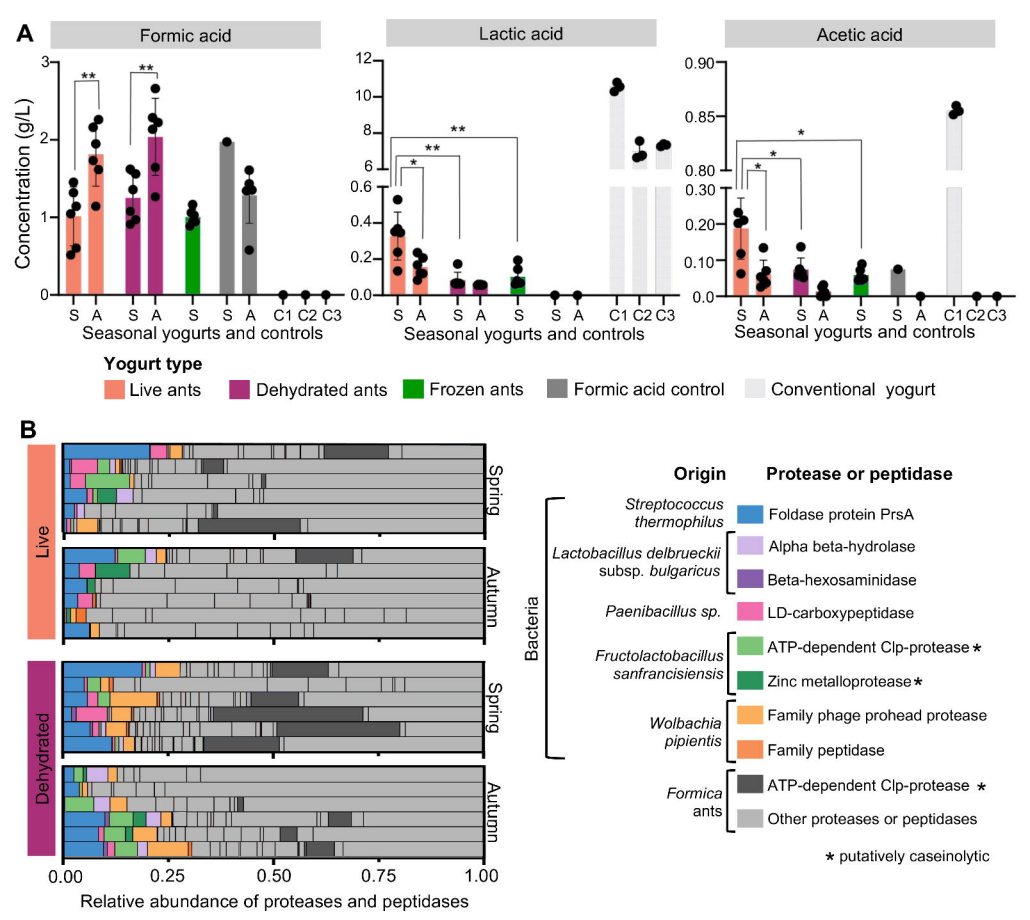
1080 **Figure supplement 2.** Differential abundance of ant yogurt proteases across seasons and
1081 elaborations.
1082 **Source data 1.** pH of yogurts and formic acid controls before and after incubation.
1083 **Source data 2.** Absolute abundance of milk proteins, proteases, and peptidases in proteomes.


Ant Holobiont
Ant
Microbes
Acids
Enzymes
Formica ant colony
Yogurt


A
B
C
D
E
F


A
Formica polyctena
Formica rufa
Ant species distribution
native
no record
observation
experimental yogurt
traditional yogurt
Microbiome composition
Spring
Autumn
0.0
0.5
1.0
Relative abundance
Bacterial families
Anaplasmataceae
Lactobacillaceae
Acetobacteraceae
Burkholderiaceae
Orbaceae
Corynebacteriales Order
Xanthomonadaceae
Other
B
F. polyctena ants
Yogurts
Live
Dehydrated
Frozen
Spring
Autumn
Relative abundance
Bacillaceae
Burkholderiaceae
Enterobacteriaceae
Moraxellaceae
Dermacoccaceae
Orbaceae
Streptococcaceae
C
Alpha diversity (Shannon)
a
b
a
Dehydrated
Frozen
Live
Ant yogurt
D
NMDS 2
NMDS 1
Ant yogurt
Live
Dehydrated
Frozen


A
Spring
Autumn
Lactobacillaceae ng DNA
1.00
0.75
0.50
0.25
0.00
*
**
ants
yogurt
ants
yogurt
B
Spring
Frozen yogurt
Dehydrated yogurt
Crushed dehydrated ants
Live yogurt
Live ants
Fructilactobacillus sanfrancicensis
Lactobacillus delbrueckii subsp. bulgaricus
Lactiplantibacillus pentosus
Lactobacillus salivarus
Lactobacillus plantarum
Isolation source
Cultured Lactobacillaceae
Autumn
Fructilactobacillus sanfrancicensis
Live Yogurt
Lacticaseibacillus casei
Live ants
Dehydrated ants
Crushed dehydrated ants
Isolation source
Cultured Lactobacillaceae
C
Dehydrated spring
Dehydrated autumn
Frozen spring
Bacillaceae ng DNA
1.0
0.5
0.0
n.s.
n.s.
*
ants
yogurt
ants
yogurt
ants
yogurt
D
Spring
Dehydrated ants
Dehydrated yogurt
Live yogurt
Live ants
Frozen yogurt
Crushed dehydrated ants
Frozen ants
Margalitia shackletonii
Niallia sp.
Bacillus weihenstephanensis
Bacillus mycoides
Bacillus paramycoides
Lysinbacillus fusiformis
Viridibacillus sp.
Bacillus sp.
Bacillus subtilis
Bacillus cereus
Bacillus thuringiensis
Isolation source
Cultured Bacillaceae
Autumn
Dehydrated yogurt
Crushed dehydrated ants
Dehydrated ants
Live Ants
Bacillus tequilensis
Bacillus subtilis
Bacillus yelezensis
Bacillus thuringiensis
Bacillus sp.
Bacillus cereus
Bacillus paramycoides
Isolation source
Cultured Bacillaceae


A
Formic acid
Lactic acid
Acetic acid
Concentration (g/L)
Seasonal yogurts and controls
S A S A S S A C1 C2 C3
Yogurt type
Live ants
Dehydrated ants
Frozen ants
Formic acid control
Conventional yogurt
B
Live
Dehydrated
Spring
Autumn
Relative abundance of proteases and peptidases
Origin
Protease or peptidase
Bacteria
Streptococcus thermophilus
Foldase protein PrsA
Lactobacillus delbrueckii subsp. bulgaricus
Alpha beta-hydrolase
Beta-hexosaminidase
Paenibacillus sp.
LD-carboxypeptidase
Fructolactobacillus sanfrancisiensis
ATP-dependent Clp-protease *
Zinc metalloprotease *
Wolbachia pipientis
Family phage prohead protease
Family peptidase
Formica ants
ATP-dependent Clp-protease *
Other proteases or peptidases
* putatively caseinolytic