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

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Summary

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 However, the biocultural origins of fermentation practices and microbes have largely been replaced by industrial processes.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 conventional 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 may alter yogurt texture and are produced by the ants and bacteria. The ant holobiont thus catalyses 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 originates from ancient practices and has dramatically shaped food cultures. The oldest archaeological 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 this biodiversity 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), air, or containers used. 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 S1). 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.

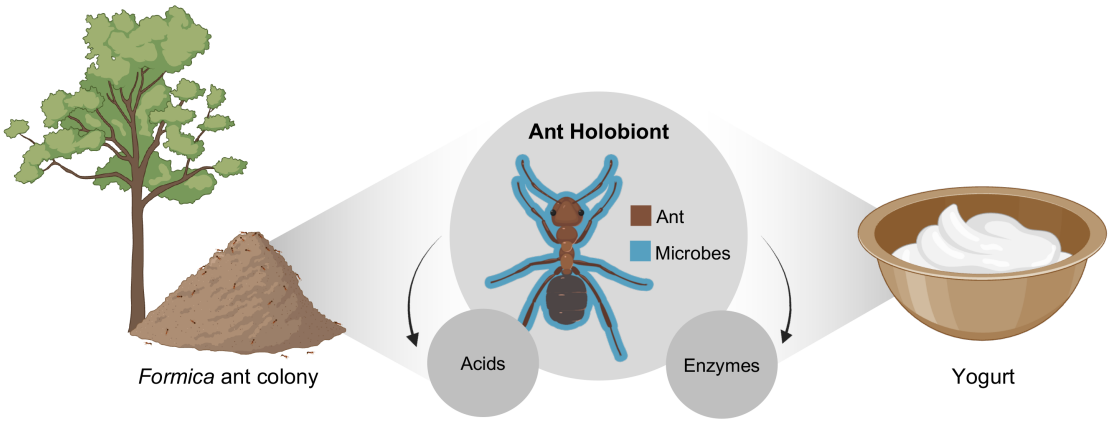

Figure 1. Hypothesized contributions of the ant holobiont to yogurt fermentation. *Formica* red wood ants create characteristic thatched mounds (left), which may have been used in traditional yogurt fermentation practices (Mutlu Sirakova 2023). The traits of the ant holobiont, the composite of both ant 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.

Results

The ant holobiont serves as a catalysing agent for dairy 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). Here, the ant could act as an inoculum, and the colony could serve as an incubator since the nest itself is known to produce heat (Coenen-Stass et al., 1980). 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).

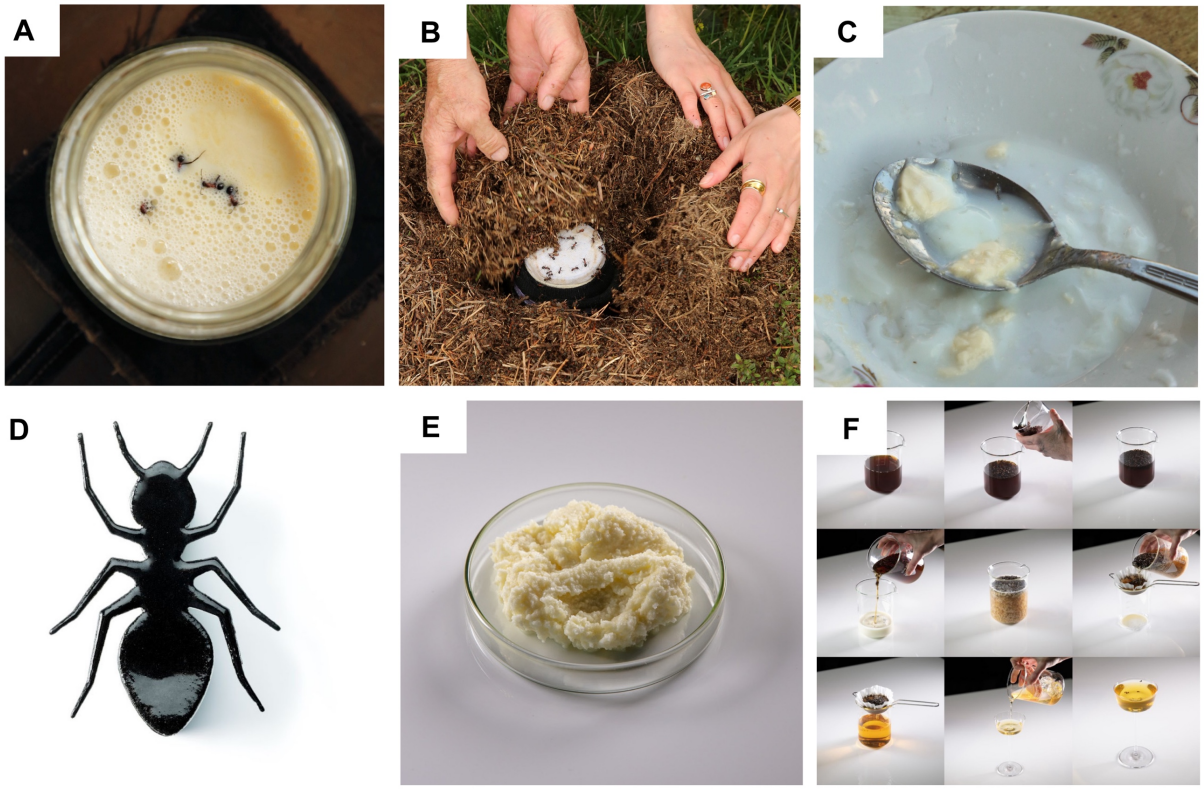

Figure 2. The ant holobiont serves as a catalysing agent for dairy applications. (**A-C**) Photographs of traditional yogurt fermentation initiated by *Formica* ants taken during field work in Bulgaria. (**A**) Ants were added to warmed milk that was then (**B**) buried within the ant colony and left 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).

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

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

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

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

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

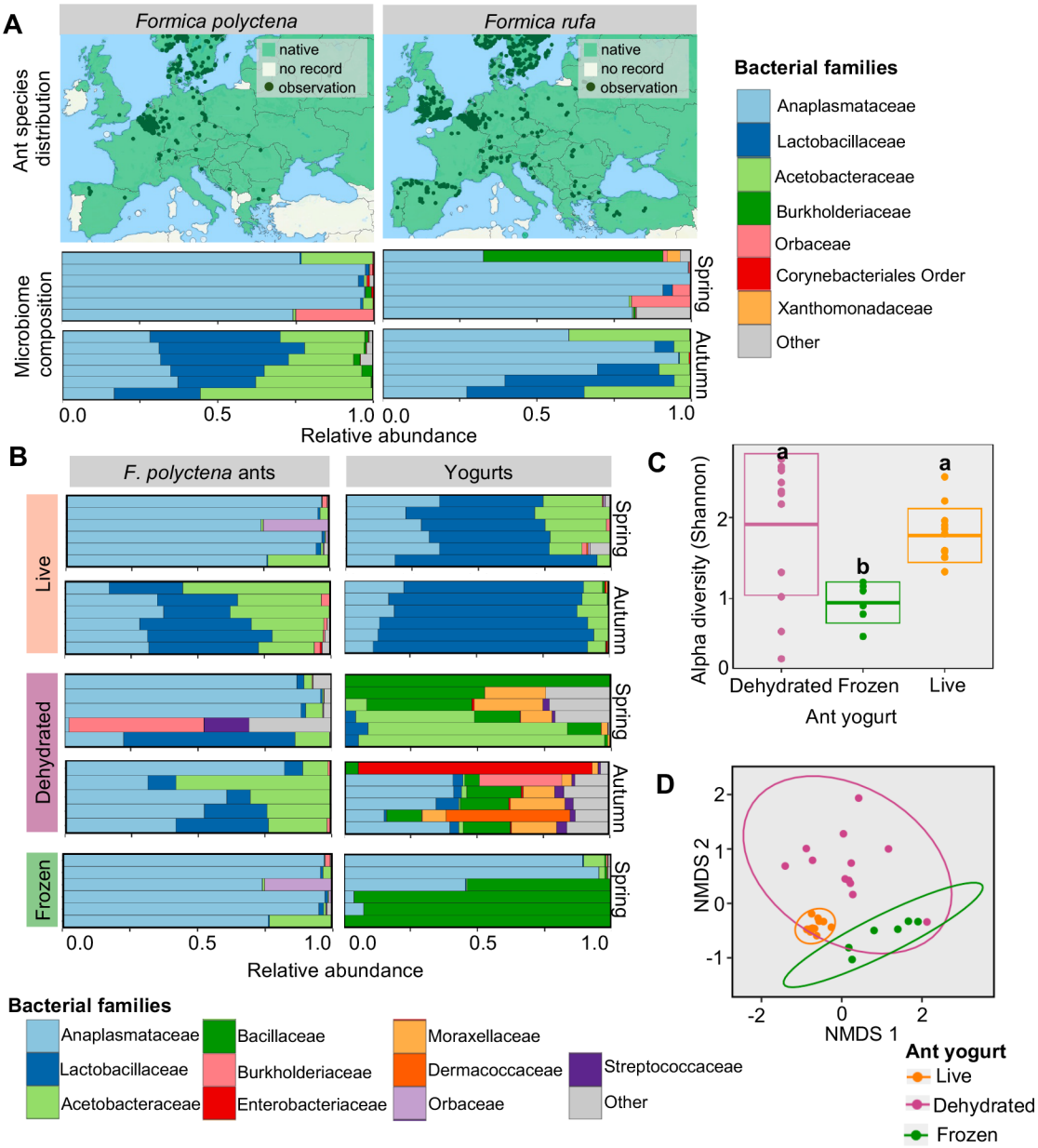

Figure 3. Live ants provide small, stable, and controlled yogurt microbiomes. (**A**) European species ranges of *F. polyctena* and *F. rufa* (top), both within the *F. rufa* group of red wood ants (maps modified from antmaps.org). The microbiome composition of ant species across seasons (bottom). Bar represents four pooled ants from a colony of each respective species, collected in Denmark. The top 8 bacterial families among the samples are illustrated. (**B**) The bacterial microbiome of the three ant preparations and corresponding ant yogurts made in spring and autumn. Bars represent replicates, and the top ten bacterial families are shown. Live ant microbiomes are also represented in panel (**A**), and live and frozen ants are identical, as they live ants that were frozen prior to extraction are also representative of the frozen ants themselves. 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

177 standard deviations. (**D**) The beta diversity of ant yogurts based on Bray-Curtis distances and
178 Non-Metric Multidimensional Scaling (NMDS) analysis. The grouping is supported by a
179 PERMANOVA.

180 The microbiomes of the two *Formica* species were dominated by lactic acid bacteria
181 (Lactobacillaceae), acetic acid bacteria (Acetobacteraceae), and obligate intracellular bacteria
182 (Anaplasmataceae) (Figure 3A). The bacterial families and genera (Figure S2A) align with
183 *Formica*-specific microbiomes associated with species across the Northern Hemisphere (Jackson
184 et al., 2023; Jackson et al., 2022; Kaczmarczyk-Ziemba et al., 2020; Zheng et al., 2022). *Formica*
185 ant microbiomes were seasonal. We observed an increase in relative abundance of lactic and
186 acetic acid bacteria (Figure 3A) and overall microbial biomass (i.e. load) in the autumn, compared
187 to spring (Figure S2B). Seasonal microbiomes have not been previously documented in *Formica*
188 ants and are germane to the use of these ants in fermentation. Overall, the consistent presence
189 of lactic and acetic acid bacteria in the *F. rufa* group indicates the ant microbiome may be pertinent
190 to food fermentation.

191 We then tested the hypothesis that lactic and acetic acid bacteria in the ants may transfer to the
192 yogurt. Based on 16S *rRNA* metabarcoding of experimental yogurts made with *F. polyctena* under
193 aseptic conditions, we confirmed our hypothesis that bacteria from the ants contribute to the
194 yogurt microbiome. The preparation of the ants, namely whether they were live, dehydrated, or
195 frozen, distinctly impacted the bacteria communities in the resulting yogurts (Figure 3B). Like the
196 ants themselves, the live ant yogurts were consistently dominated by the lactic and acetic acid
197 bacteria, which displayed seasonal differences in composition. Here, the most abundant bacterial
198 genus was *Fructilactobacillus* (Figure S2C). We note that these true abundances may have been
199 higher, considering our metabarcoding positive control indicates a slight underestimation of the
200 abundance of lactic acid bacteria in samples (Figure S3). In contrast, dehydrated ant yogurts
201 exhibited variable microbiomes. Frozen ant yogurts contained two bacteria groups: Bacillaceae,
202 which can proliferate in the yogurt, and Anaplasmataceae, which obligately lives in ant cells
203 (Buchner, 1965; Jackson et al., 2022) and thus cannot grow in the yogurt. Here, dehydration and
204 freezing likely reduced bacterial viability (Santivarangkna et al., 2011) and consequently favoured
205 stochastic community assembly or freeze-resistant Bacillaceae (Nicholson et al., 2000). The
206 persistence of lactic and acetic acid bacteria in live ant yogurt suggests it is the best starter for
207 fermentation and provides biological support for the traditional, live ant-based yogurts.

208 We determined the consistency of the bacterial microbiome composition across the yogurts.
209 Alpha and beta diversity metrics were used, where alpha diversity is the number of bacterial

210 strains per sample (i.e. amplicon sequence variants) and beta diversity is the difference in the
211 composition of strains from one sample to the next, while weighting relative abundance. The ant
212 preparations significantly affected the alpha and beta diversity of the yogurts (LM: $F_{2,27}$=5.387,
213 p=0.0108, based on Shannon index; Adonis: $F_{2,27}$= 8.4402, p<0.0001, based on Bray Curtis
214 distances). The alpha diversity of the live ant yogurts was intermediate (Figure 3C), but the beta
215 diversity was very low (Figure 3D). Therefore, the live ant yogurts tended to have the same
216 species and composition from one sample/preparation to the next, as one would hope to see in
217 predictable and controlled fermentations.

218 *Bacteria from the ants proliferate in the milk*

219 We hypothesized that bacteria from the ant holobiont grew in the milk, facilitating fermentation.
220 To assess growth, we determined the bacterial load in ants and yogurts with quantitative PCR
221 (qPCR) and identified viable bacteria with culturomics using 16S *rRNA* Sanger sequencing. Live
222 ants introduced lactic acid bacteria that proliferated in the yogurt. In the spring, yogurts contained
223 a higher loads of lactic acid bacteria than the ants introduced as a starter (Figure 4A; t (2, 10) = -
224 2.975, p=0.0139). However, in autumn, we observed the opposite pattern (Figure 4A; t (2, 10) =
225 3.269, p=0.0084), suggesting that not all lactic acid bacteria from the ants enter the milk, or that
226 the bacteria associated with the autumn ants are less prolific. While lactic acid bacteria dominated
227 live ant yogurts, it made up a marginal amount of the bacterial load in frozen and dehydrate
228 yogurts (Figure S4A).

229 Yogurt made with live ants contained several species of culturable lactic acid bacteria (Figure
230 4B). Aligned with the dominance of *Fructilactobacillus* in the community analysis, we isolated *F.*
231 *sanfranciscensis*, a bacterium not only associated with ants (Zheng et al., 2022) but also
232 sourdough bread fermentation (Gänzle et al., 2023; Kline & Sugihara, 1971). We further cultured
233 and isolate of *Lactobacillus delbrueckii* subsp*. bulgaricus*, typically found in fermented dairy
234 products (FAO & WHO, 2018). Last, we isolated the acetic acid bacteria, *Oecophyllibacter*
235 *saccharovorans* (Figure S5), previously characterised in association with ant genera closely
236 related to *Formica* (Blaimer et al., 2015; Brown & Wernegreen, 2016; Chua et al., 2018). The
237 diversity of culturable lactic acid bacteria thus indicates a niche overlap between ants and
238 fermented foods.

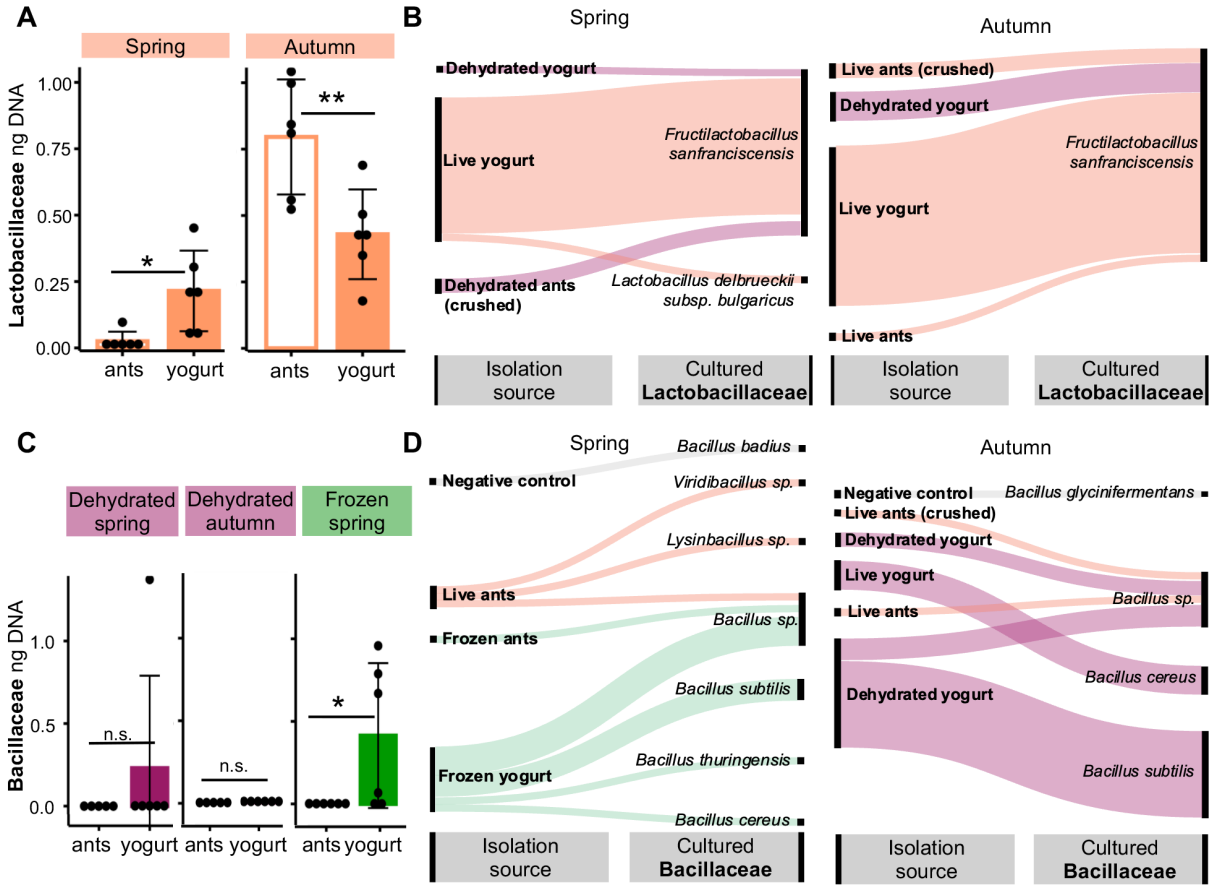

Figure 4. Bacteria from the ants proliferate in the milk. (**A**) The estimated amount of lactic acid bacteria (Lactobacillaceae) DNA, representative of lactic acid bacterial load, in the live ants and resulting yogurts across seasons. (**B**) The diversity of culturable species of lactic acid bacteria 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, yogurts, and negative control 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).

In the yogurts made with dehydrated and frozen ants, the bacterial load disproportionately came from spore-forming Bacillaceae. Notably, there is little to no Bacillaceae biomass in the ants, especially when compared to the yogurts (Figure 4C; t_{frozen} (2, 10) = -2.307, p=0.0437), and this Bacillaceae did not proliferate in live ant yogurts (Figure S4B). We cultured several species of Bacillaceae, stemming largely from dehydrated or frozen ants or yogurts (Figure 4D). This

258 included the food contaminant, *Bacillus cereus* (Dietrich et al., 2021). Although we cannot
259 determine if the *Bacillus* load was sufficient to be problematic for consumption, it still indicates a
260 potential risk. Therefore, we conclude that the microbiome of dehydrated and frozen ants and
261 their corresponding yogurts are undesirable for food fermentations.

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

263 We hypothesized that the ant holobiont, composed of both the ant and its microbes, may
264 contribute acids key to fermentation. In yogurt, bacteria metabolize lactose and consequently
265 produce high amounts of lactic acid and often low amounts of acetic and formic acid (Sieuwerts
266 et al., 2010). These acids are essential to the tangy flavour, thick texture, and preservation of
267 yogurt (Fernandez-Garcia & McGregor, 1994). Moreover, *Formica* ants (though, notably, not all
268 ants) have a venom gland that contains largely formic acid that may be up to 10% its body weight
269 (O'Rourke, 1950), although the whole ant contains a more complex mélange of chemicals
270 (Löfqvist, 1976). Thus, the acid from the ant holobiont may have three effects. First, it may
271 engender yogurt tastes and textures, even without the effects of microbes. Second, it can create
272 acidic conditions that favour acid-producing microbes. Third, the acid-producing microbes,
273 independent of the ant, can themselves alter the acidity and flavour of the ferment. To investigate
274 if the components of the ant holobiont, body and microbes, contribute organic acids to the yogurt,
275 we quantified formic, lactic, and acetic acid in yogurts and controls by HPLC and measured pH
276 before and after fermentation (Figure 5).

277 The ant holobiont contributed formic acid, which was the most abundant acid found in the yogurts
278 (Figure 5A). The amount of formic acid was in a similar range to that in the control, in which formic
279 acid alone was added to pH 4.6 to induce coagulation. The season, but not the type of yogurt
280 (live, dehydrated, frozen) further dictated the formic acid levels (LM results). The higher levels in
281 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
282 the seasonality of formic acid production (O'Rourke 1950). Therefore, the acid from the ants
283 entered the milk and likely shaped the unique acidic flavour and texture of the culinary
284 applications, contrasting the rounded flavour of lactic acid typically predominant in fermented
285 dairy.

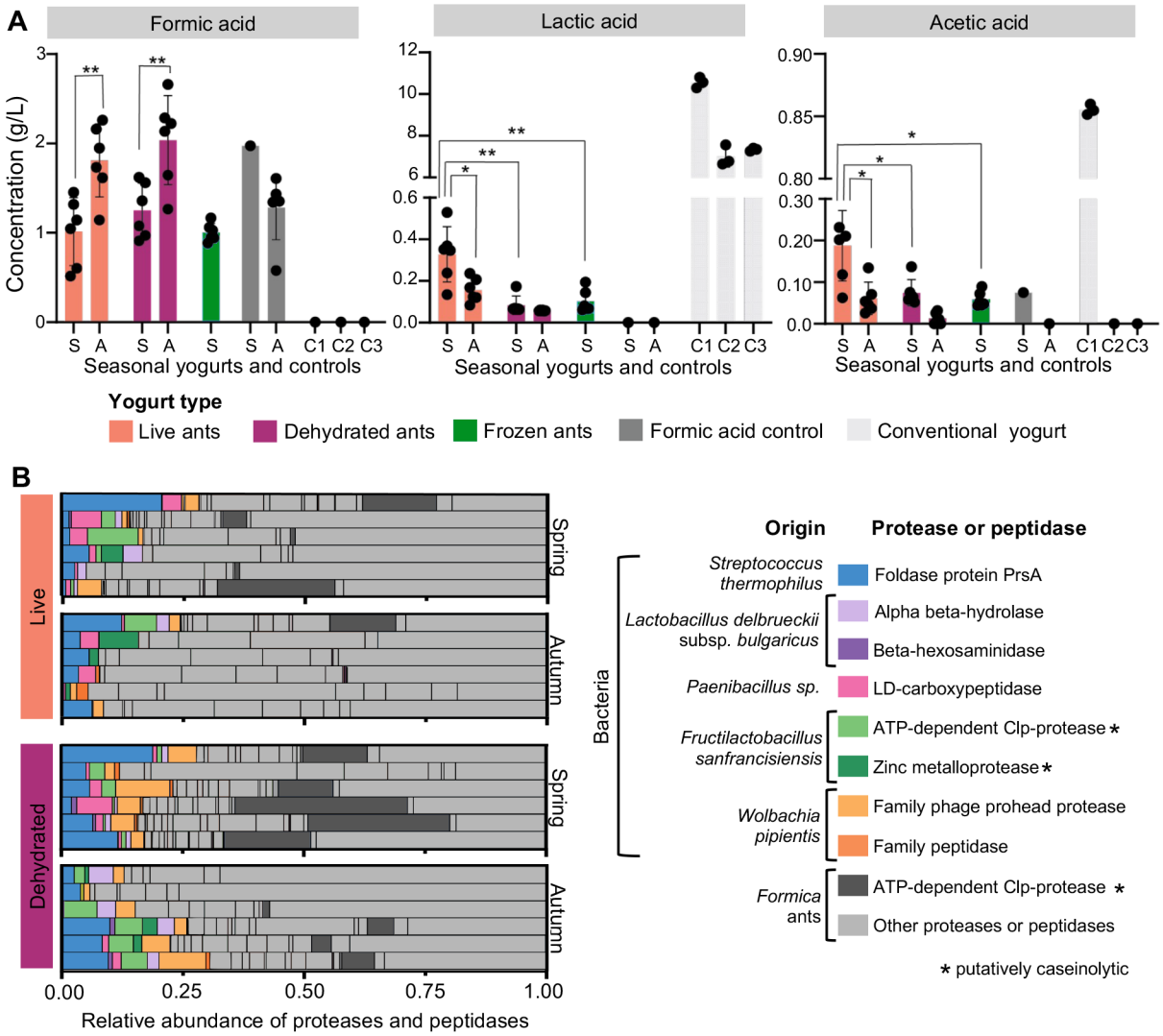

Figure 5. Organic acids, proteases, and peptidases in ant yogurts. (**A**) Concentration of metabolites formic acid, lactic acid, and acetic acid in the yogurts from spring (S) and autumn (A). The samples include yogurts made with live, dehydrated, and frozen ants, formic acid, and three conventional 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 Table S2.

300 Lactic and acetic acid were also detected in the ant yogurts, validating that ant-associated
301 bacteria likely contributed acids during fermentation (Figure 5A). Acid profiles align with the
302 microbiome data. Lactic and acetic acid bacteria were the most abundant genera in live ant
303 yogurts (Figure 3A). Similarly, these acids are highest in live ant yogurts compared to dehydrated
304 (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)
305 =3.842, p=0.0060; t_{acetic} (2, 10) =3.653, p=0.0147). In live yogurts, we observed lactic and acetic
306 acid were higher in spring (t_{lactic} (2, 10) =2.897, p=0.0231; t_{acetic} (2, 10) =3.325, p=0.012), the
307 season in which we isolated the greatest diversity of lactic acid bacteria (Figure 4C). This suggests
308 different strains of bacteria may inherently vary in acid production. Notably, ant yogurts had less
309 lactic acid, acetic acid (Figure 5A), and bacterial biomass (Figure S6A) than conventional or
310 homemade yogurts. We did not establish and perform the optimal fermentation conditions for the
311 ant yogurt, and thus the fermentation was incomplete. However, we can conclude that the ant
312 microbes contribute organic acids to fermentation.

313 Ant yogurts contained on average 2.5 g/L total organic acids, while conventional yogurts
314 contained up to 12 g/L total organic acid. Thus, the ant yogurt remained in a pH range of 5.0-5.9
315 and did not reduce to a pH of 4.2 of conventional yogurt (Figure S6B), given the conditions and
316 timing of our experiment. Initially, the pH of the milk dropped upon the addition of ants, likely due
317 to the formic acid from the ants (Table S1). The pH continued to decrease through the
318 fermentation (Figure S6B), likely because of acid-producing microbes. Typically, in yogurt
319 fermentation, a drop to pH 5.3 initiates coagulation, and a further acidification to pH 4.6 completes
320 coagulation by causing aggregation of casein micelles (Robinson et al., 2006) The ant yogurt
321 coagulated yet largely did not reach such a low pH. This suggests another aspect of the
322 fermentation denatured caseins, namely proteases.

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

324 Our final hypothesis was that the ant holobiont may contribute enzymes, in particular proteases,
325 that modify the yogurt texture. Proteases can differentially cleave and degrade casein, the main
326 protein in milk, and result in a firm or fluid yogurt texture (Amani et al., 2017; Gassem & Frank,
327 1991). Based on the pH and coagulation results, we predicted the ants themselves may contain
328 milk-clotting proteases, like those previously identified in mealworms (Yang et al., 2022). Casein
329 proteases and peptidases are also typically produced by the bacteria in yogurt (Amani et al.,
330 2017). Thus, the ant holobiont, ants and bacteria, may contribute proteases to the yogurt
331 fermentation. To test this hypothesis, we conducted an untargeted proteomics analysis based on

332 a self-curated database that contained proteases from *Formica* ants, ant-yogurt bacteria,
333 conventional yogurt bacteria, and milk proteins.

334 We confirmed our hypothesis that the ant holobiont contributes proteases and peptidases to the
335 yogurt fermentation (Figure 5B). Several ant proteases were identified in the yogurt (Figure 5B),
336 one of which, an ATP-dependent CLP protease, is known to have caseinolytic potential (Maurizi
337 et al., 1990). Ant-associated bacteria further contributed proteases and peptidases. The lactic
338 acid bacteria *F. sanfranciscensis* produced two proteases, ATP-dependent CLP protease and
339 zinc metalloprotease, both known to have caseinolytic homologues (Jia et al., 2015; Maurizi et
340 al., 1990). Proteases and peptidases typically belonging to bacteria in conventional yogurt, *L.*
341 *delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, were found, as well as ant yogurt
342 bacteria *Paenibacillus* and *Wolbachia pipentis*. Last, we identified milk proteins such as casein,
343 which made up a high percentage 96.19 ± 1.07% of the proteome of samples (Table S2),
344 potentially because of the low bacterial load compared to conventional yogurt. There were no
345 significant differences in the relative abundance of all proteins in the proteome between live and
346 dehydrated ant yogurts or seasons (Figure S7). Thus, proteases in both live and dehydrated
347 yogurts may, in part, catalyse coagulation in the experimental yogurts and in the modern culinary
348 applications. Taken together, the ant holobiont, ants and microbes, contribute proteases
349 potentially relevant to food fermentation.

350 **Discussion**

351 In this study, we explored the traditional practice of using ants as a starter for yogurt fermentation.
352 We hypothesized that the ant holobiont, both the ant and its microbes, catalysed the fermentation.
353 First, we uncovered bacteria from the ants shaped the yogurt microbiome. Using live ants as a
354 starter, in contrast to frozen or dehydrated ants, resulted in a consistent microbiome containing
355 lactic and acetic acid bacteria, desirable for fermentation. These ant-bacteria included some
356 species thought of as canonical fermentation microbes, such as *F. sanfranciscensis*. Second, we
357 discovered that the addition of the ant holobiont to milk introduced acids and proteases. Formic
358 acid originated from the ant, while lactic and acetic acid were likely produced by bacteria. The
359 ants and bacteria also contributed proteases with caseinolytic potential. Our results revealed that
360 the ant holobiont can contribute to fermentation and thus prompted reconsideration of the
361 microbial consortia in our ferments, their origins, and applications to fermented food.

362 We uncovered how ants and their bacteria synergistically shape the fermentation by applying the
363 holobiont framework. Holobiont theory considers a host and its associated microbes collectively,
364 where the interplay between animal and microbes shapes the resulting ecology and evolution of
365 the whole, host plus microbes (Bordenstein & Theis, 2015; Margulis & Fester, 1991; Zilber-
366 Rosenberg & Rosenberg, 2008). When animals or plants are used in fermentation, their
367 holobionts may structure that fermentation in ways that reach beyond the effect of the individual
368 parts. In this study, it was thus essential to include the entire ant holobiont, rather than isolating
369 the bacteria alone for fermentation. *Formica* ants produce formic acid for defence (O'Rourke,
370 1950; Tragust et al., 2020) and feed on the sugar-rich honeydew (Ivens & Kronauer, 2022) and
371 protein-rich insects (Robinson & Stockan, 2020). In response, the lactic and acetic acid bacteria
372 are hypothesized to metabolize the honeydew (Zheng et al., 2022), are highly acid tolerant
373 (Tragust et al., 2020) and are potential contributors to proteolysis. Once ants are introduced into
374 milk, the properties of the ant holobiont extend to the yogurt ecosystem. Formic acid initially
375 acidifies the milk, ant-bacteria break down milk sugars producing additional acid, and proteases
376 liberate peptides and amino acids for bacterial growth. By looking through the lens of the
377 holobiont, we could clarify why *Formica* ants and bacteria are pre-adapted for the fermentation of
378 yogurt and potentially other foods.

379 The effect of the ant holobiont on yogurt fermentation parallels the role of microbes in other
380 fermented foods. In most ferments, multiple species of microbes offer complementary
381 contributions to the fermentation process (Canon et al., 2020; Sieuwerts et al., 2008). In
382 conventional yogurt, the bacteria *S. thermophilus* produces formic acid that is then metabolized
383 by *L. delbrueckii* subsp. *bulgaricus*, and in return, *L. delbrueckii* subsp. *bulgaricus* produces
384 proteases. Collectively, these bacteria then metabolize lactose and produce lactic acid to
385 transform milk into tangy yogurt (Sieuwerts, 2016; Sieuwerts et al., 2010). In sourdough, yeast
386 and lactic acid bacteria can secrete factors that promote one another's growth (Sieuwerts et al.,
387 2018), and overall have complementary roles in the fermentation of sugars and protein derivatives
388 (Gänzle, 2014; Gänzle et al., 2023). Together, sourdough microbes create leavened bread with
389 acidic, kokumi, and umami flavours (Gänzle et al., 2023). In the ant yogurt, our results suggest
390 the separate elements of the ant holobiont, the ant body and microbes, act synergistically.
391 Consequently, the ant holobiont introduces acids, proteases, and potentially other compounds
392 that contribute to the organoleptic characteristics such as distinct acidity, coagulation, and flavour.

393 Intriguingly, we isolated a species of lactic acid bacteria from the ant yogurt that is commonly
394 found in fermented foods, *F. sanfranciscensis*. The bacterial species *F. sanfranciscensis,* is one

395 of the most prevalent bacteria in sourdough bread globally (Gänzle & Zheng, 2019; Landis et al.,
396 2021). The *Fructilactobacillus* clade appears to have diversified with ants (Jackson et al., 2023)
397 and related social insects (Duar et al., 2017). We suspect that *F. sanfranciscensis* has evolved in
398 ants over millions of years and was only introduced into fermented foods with the advent of bread
399 making in the last several thousand years (Arranz-Otaegui et al., 2018; Carretero et al., 2017). In
400 the future, the bacteria isolated from these ants may be assayed for key functions in ferments,
401 such as sugar metabolism and exopolysaccharide formation. These results lay the groundwork
402 for unveiling the diversity of fermentative bacteria residing in ants and for elucidating the bacterial
403 evolution from ant holobionts to fermented foods.

404 Our results bring forth new questions we believe are central to understanding the ecological and
405 ancient origins of fermentative microbes. The ecological origins and assembly of ferment
406 microbiomes, which colonize fermentations in lieu of backslopping, are only beginning to be
407 characterized (Louw et al., 2023). Recently, it has been convincingly argued that the wine yeasts
408 can originate from social wasps, where the symbiosis between wasps and yeasts is essential for
409 the microbial transfer to wine ecosystems (Di Paola et al., 2023; Madden et al., 2018; Stefanini et
410 al., 2012). Similarly, our results are reconcilable with the idea that bacteria for fermentation may
411 originate from ants. This poses the question whether the ant holobiont was influential to the
412 historic origins of yogurt or bread fermentation. Beyond traditional practices using ants in yogurt
413 (Multu Sirakova, 2023; Öney Tan, 2010; Yalman, 1977), ant colony materials may be integrated
414 in sourdough fermentation (Mutlu Sirakova 2023), providing further avenues for microbial transfer.
415 Conceivably, the ant bacteria then could be backslopped in traditional fermentations, successively
416 propagating and evolving in yogurts or sourdoughs. Although it is not clear when these traditions
417 arose, it is noteworthy that the earliest evidence of yogurt and architectural depictions of ants and
418 bees were found in prehistoric remains from our study region (Hendy et al., 2018; Walter, 2021).
419 While these fragmentary anecdotes do not reconcile or resolve the origins of fermentative
420 microbes, they highlight cultural threads that may connect ants and ferments.

421 The ant yogurt highlights the knowledge that can gained from traditional practices and ecological
422 interactions, yet large-scale production of the yogurt itself may not be advisable. *Formica* red
423 wood ants are of conservation concern due to recent declines in populations (Balzani et al., 2022).
424 The bacteria within these ants, which can be isolated and used separately for fermentation,
425 underscores the value of this biodiversity. However, public collection of ants is thus not
426 sustainable. Furthermore, preparation of ant yogurt requires specialised knowledge. In modern
427 gastronomy, *Formica* ants are typically frozen or placed in alcohol to kill a parasite the ants can

428 carry, which is infectious to humans (Jensen et al., 2017). We found that fermentation started with
429 frozen ants promote the growth of unfavourable bacterial communities, and that live ants must be
430 used. In culinary applications with live ants, we then strained the starter with a microbiology-grade
431 sieve to remove any potential parasites. In the traditional ant yogurt, knowledge holders may have
432 maintained practices to prevent the spread of this parasite, but we did not address this in the
433 scope of our research. Thus, careful consideration of both the cultural and biological aspects of
434 traditional or historical practices is paramount when exploring their re-creation.

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

453 **Materials and Methods**

454 *Traditional and modern culinary applications of ants*

455 We travelled to Nova Mahala, Bulgaria to recreate ant yogurt with communities that retained
456 memories and oral histories of the ant yogurt tradition. We based the yogurt fermentation on
457 freshly acquired raw cow milk and *F. rufa* ants, from a colony found just outside of the village. The
458 milk was warmed until nearly scalding, such that it could “bite your pinkie finger” (Multu Sirakova,
459 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 nest itself is known to produce heat (Coenen-Stass et al., 1980), and thus may act as an incubator for the yogurt fermentation. 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,

493 Denmark) were mixed with the proportions being 30% and 70%, respectively. The dairy mixture
494 was homogenized and heated to 95°C. Then, 10 mL ant water infusion (described above) was
495 added for each kilogram of this dairy mixture, stirring until complete coagulation (Munieweg et al.,
496 2021). The curd was cooled to 4°C for 6 h and strained through a cheesecloth. The mascarpone-
497 like cheese was stored at 4°C.

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

515 *Ant collection and processing for experimental yogurt*

516 Experimental yogurts were made in spring, according to the tradition (Multu Sirakova, 2023), and
517 in autumn, to test the seasonality of the fermentation. We used the red wood ant *Formica*
518 *polyctena* for microbiome characterization and experimental yogurt fermentation, and *F. rufa* for
519 microbiome characterization. *F. polyctena* is the sister species of *F. rufa*, where the later was
520 used in the ethnographic account and at the Alchemist R&D. *F. polyctena* worker ants were
521 collected at the end of May and the start of October 2022, from a colony in Rude Skov, Denmark
522 (55.8334720, 12.474938410). Similarly, worker ants from a colony of *F. rufa*, found in
523 Bøllemosen, Denmark (55.827676, 12.534667), were collected at the end of May and late
524 September 2023. Ants were taken without disturbing the mound structure, considering several

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

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

534 The ant species were identified by barcoding of the *cytochrome c oxidase I gene (COXI)*. DNA
535 was extracted with Chelex (Sigma Aldrich, Denmark), amplified, cleaned, and sequenced (Sinotte
536 et al., 2021). PCRs were performed with primers LCO1490 5´-
537 GGTCAACAAATCATAAAGATATTGG-3´and HCO2198 5´-
538 TAAACTTCAGGRTGACCAAAAAATCA-3´ (Zuccon et al., 2012). PCR products were cleaned
539 with PureIT ExoZap (Amplicon, Denmark) and sequenced at Macrogen (the Netherlands).
540 Sequences were trimmed and aligned using default settings in Geneious (Biomatters Ltd., New
541 Zealand), and the consensus sequence was compared to NCBI's nucleotide database with
542 BLASTn. The genetic identities of the consensus sequences were determined to be *F. polyctena*
543 and *F. rufa*. This also matched the respective polydomous and monodomous nest architecture
544 observed at the field sites and characteristic of the different species.

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

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

555 In the spring, six samples of each of the live, frozen, and dehydrated ant-yogurts were made as
556 well as 2 negative controls of milk alone, one incubated and one not incubated. In the autumn,
557 the same design was used, but frozen ant-yogurt was not made as it was found to contain
558 pathogens. Additionally, six samples of live and dehydrated at yogurt were made, where the milk
559 with ants was passed through Whatman Filter Paper Grade 4 (Sigma Aldrich, Denmark), which
560 retains particles up to 25 μm, and thus would theoretically remove any potential parasites while
561 allow the bacteria to pass through. These filtered samples were only used for bacterial
562 culturomics.

563 *DNA extraction of yogurt and ants*

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

575 DNA extraction was performed with the DNeasy Blood and Tissue Kit (Qiagen, Germany)
576 according to manufacturer's instructions for gram positive bacteria with the following
577 modifications. Physical lysis of ants and yogurts was performed via bead-beating with 0.1 mm G2
578 DNA/RNA Enhancer beads (VWR, Denmark) in a TissueLyser (Qiagen, Denmark) at 30 Hz for 3
579 min. Samples then underwent chemical lysis, including 30 min lysis in lysozyme with an addition
580 of 2 µL of mutanolysin (25 U/µL) followed by 1 h proteinase K lysis. The final elution of the DNA
581 extract was 200 µL in AE buffer, passed twice through the column to maximize yield. Due to
582 exceptionally low DNA concentrations in some experimental yogurts, three extractions were
583 performed per sample, and thus 9 g of yogurt in total was washed, extracted, and pooled per
584 sample. Similarly, two ant extractions were pooled for a final sample representing four ants. The
585 elutes were pooled and concentrated with AMP Pure XP Reagent (Beckman Coulter, Denmark)

586 according to manufacturer's instructions, then underwent a clean-up for inhibitors with the DNeasy
587 PowerClean Pro kit (Qiagen, Denmark) according to manufacturer's instructions.

588 The final samples for sequencing included yogurts, ants, and several controls. Yogurt samples
589 thus included 20 samples from spring and 13 from autumn, as previously described. From the
590 ants, there were six final samples for live and for dehydrated *F. polyctena* each from spring and
591 autumn, and six samples of live *F. rufa* in spring and autumn. In addition to the experimental
592 yogurts and ants, a mock community of standard bacteria (Zymobiomics, Nordic Biosite,
593 Denmark) was included to determine any community bias generated by extraction and later
594 metabarcoding. Further, three conventional plain yogurts were included as positive controls. Last,
595 five blank DNA extractions were included as negative controls for DNA extraction.

596 *Quantification of bacterial load*

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

606 *Bacterial community metabarcoding and analysis*

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

617 The analysis of sequences was carried out in R (v. 4.3.1) (Team, 2023), which included
618 determination of amplicon sequence variants (ASVs), taxonomic assignment, visualization, and
619 statistical analyses. The dada2 pipeline (v. 1.30.0) (Callahan et al., 2016) was used for ASV and
620 taxonomic assignment. Reads were not trimmed due to the overall high quality of sequences, but
621 were filtered with the default parameters, with *maxEE* adjusted to (2,4). Reads were then paired
622 with *mergePairs*, *minOverlap* set to 16, and chimeras were removed. Taxonomy was assigned to
623 the species level where possible with the SILVA database (v. 138.1) (Glöckner et al., 2017).
624 Potential contaminants were then removed using SCRuB (v.0.0.1) (Austin et al., 2023) based on
625 the blank negative controls that were successfully sequenced. Mitochondria, chloroplasts, and
626 Eukarya were removed, resulting in 63.87% of ASVs with genus-level classification and 82.23%
627 with family-level. The mock community was then assessed to determine any potential bias created
628 by DNA extraction and sequencing. All eight bacterial genera present in the mock community
629 were validated.

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

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

650 *Bacterial culturomics of milk ferments and ants*

651 Bacteria from the ant yogurts and ants were cultured and identified with Sanger sequencing. 100
652 µL of yogurt sample was inoculated and plated in De Man, Rogosa and Sharpe (MRS) (VWR
653 Chemicals, US), Gifu Anaerobic Medium, modified (GAM) (HyServe GmbH & Co. KG, Germany),
654 yeast peptone dextrose (YPD), and Luria-Bertani (LB) media. YPD contained 10 g/L yeast extract,
655 20 g/L peptone, and 20 g/L D-glucose. LB contained 10 g/L peptone, 5 g/L yeast extract and 0.5
656 g/L sodium chloride. A total of 20 g/L agar was added for solid cultivations. For ants, individual
657 ants were inoculated into liquid media. Tubes and plates were incubated at 30°Cover 2 days, or
658 until growth was visible. All four media were cultivated in aerobic conditions and MRS and GAM
659 were also incubated anaerobically. Subcultures were plated and incubated in the same
660 conditions. Distinct bacterial colonies from plates were identified with Sanger sequencing of the
661 conserved 16S *rRNA* gene, which included colony PCR (cPCR) with primers 5´-
662 AGAGTTTGATCCTGGCTCAG-3´ and 5´-CCGTCAATTCCTTTRAGTTT-3´ (Cebeci & Gürakan,
663 2008). All cPCR reactions consisted of 12.5 µL of Red-Taq Mastermix (VWR, Denmark), 0.5 µL
664 of forward 10 µM primer, 0.5 µL of reverse 10 µM primer, 11.5 µL of water and a bacterial colony.
665 The reactions followed the same thermocycler program that consisted of an initial 4 min at 94 °C,
666 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
667 min. PCR products were Sanger sequenced (Eurofins Scientific SE). Sequences were trimmed
668 and aligned using default settings in Geneious (Biomatters Ltd., New Zealand), and the
669 consensus sequence was compared to NCBI's nucleotide database with BLASTn. Results were
670 presented in a Sankey diagram (app.rawgraphs.io).

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

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

682 The amount of formic, lactic, and acetic acid in the samples was quantified with high-performance
683 liquid chromatography (HPLC). Yogurt samples were centrifuged at 5000 rpm for 5 min at 4°C,
684 and their supernatant was passed through a 0.2 μm syringe filter (Sigma Aldrich, Denmark). The
685 filtrate was analysed by a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA)
686 equipped with a refractive index detector and a Bio-Rad Aminex HPx87 column (Bio-Rad, USA).
687 The column oven temperature was set at 30°C. The flow rate was set at 0.6 mL/min with 5 mM
688 H_2SO_4 (Sigma Aldrich, Denmark) as an eluent. The injection volume was 10 μL. Peaks were
689 identified by comparison to the prepared standards, and integration of the peak areas was used
690 to quantify acids from obtained standard curves using the software Chromeleon7 (Thermo Fisher
691 Scientific, USA). Plots were visualised in GraphPad Prism. T-tests were used to compare organic
692 acid levels between samples, with Benjamini-Hochberg correction for multiple testing.

693 *Proteomics of ant yogurts*

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

701 HPLC-MS analysis of the samples was performed on an Orbitrap Exploris 480 instrument
702 (Thermo Fisher Scientific) preceded by an EASY-nLC 1200 HPLC system (Thermo Fisher
703 Scientific). For each sample, 1 µg of peptides was captured on a 2 cm C18 trap column (Thermo
704 Fisher 164946). Subsequently separation was executed using a 70 min gradient from 8% (v/v) to
705 48% (v/v) of acetonitrile in 0.1% (v/v) formic acid on a 15-cm C18 reverse-phase analytical column
706 (Thermo EasySpray ES904) at a flow rate of 250 nL/min. For data-independent acquisition, the
707 mass spectrometer was run with the HRMS1 method (Xuan et al., 2020) preceded by the FAIMS
708 Pro Interface (Thermo Fisher Scientific) with a compensation voltage (CV) of -45 V. Full MS1
709 spectra were collected at a resolution of 120,000 and scan range of 400-1,000 m/z, with the
710 maximum injection time set to auto. MS2 spectra were obtained at a resolution of 60,000, with
711 the maximum injection time set to auto and the collision energy set to 32. Each cycle consisted
712 of three DIA experiments each covering a range of 200 m/z with a window size of 6 m/z and a 1
713 m/z overlap, while a full MS scan was obtained in between experiments.

714 To assign the proteases to their potential organisms of origin, we created a database consisting
715 of the proteases, peptidases and proteins based on UniProt reference proteomes, the gold
716 standard in proteomics. The proteomes consisted of the ant host, bacteria from the ant yogurt,
717 and two conventional yogurt bacteria. For the ant, we used the *F. execta* proteome, since the *F.*
718 *polyctena* proteome was not publicly available, and *F. execta* is a closely related species. From
719 the *F. execta* proteome, we selected the proteases specifically. For all the bacteria included, we
720 refined our databased to only include proteases and peptidases in the proteome. We selected
721 bacteria most prevalent among the bacteria isolated from the live ant yogurt bacterial
722 metabarcoding and culturomics: *Fructilactobacillus sanfranciscensis*, *Oecophyllibacter*
723 *saccharovorans*, and *Paenibacillus sp.* We also included *Wolbachia pipentis*, which is closely
724 related to the obligate intracellular bacteria found in *Formica* ants (Jackson et al., 2022) and is
725 represented in our analysis. We included the conventional yogurt bacteria *Streptococcus*
726 *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus. Streptococcus* was only present in
727 low abundance in the bacteria metabarcoding data, and *L. delbrueckii* subsp. *bulgaricus* was
728 isolated in one instance. We included them to determine if the ant yogurt contained proteases
729 with the same or similar functionality. Last, we included common milk proteins, such as caseins,
730 found in the *Bos taurus* proteome. For DIA data analysis, Spectronaut v18 (Biognosys,
731 Switzerland) was used for protein identification and relative quantification of peptides. The default
732 settings for "directDIA" were applied with an FDR cut-off of 1%, except for MS1 quantification for
733 the peptides. Protein abundances were inferred from the peptide abundances using the MaxLFQ
734 algorithm available within Spectronaut.

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

744 *P*-values were obtained by comparing the relative abundances of each protein across all samples
745 and their replicates in the autumn versus spring conditions. Similarly, *p*-values were calculated
746 by comparing the relative abundances of each protein across all samples and their replicates in

747 the alive versus dehydrated conditions. The Benjamini-Hochberg procedure was applied to adjust
748 the *p*-values for multiple comparisons, controlling the false discovery rate (FDR). The adjusted *p*-
749 values were then transformed to their -log10 values for further analysis and visualization.

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

755 **Data availability**

756 Amplicon sequences from metabarcoding of ants and yogurts are available on SRA (BioProject
757 PRJNA1162421) and Sanger sequences of isolated bacterial strains (PQ535580-PQ535692 and
758 PQ651799-PQ651879) and ants (PQ453570-PQ453574) are available on Genbank. Proteome
759 data will be made available on the ProteomeXchange database under identifier identifier
760 PXD057843. Additional data frames have been deposited on FigShare and will be made public
761 prior to publication (https://figshare.com/s/a570abbd84d0fb09aa83).

762 **Code availability**

763 The code for analyses is available on GitHub and will be published on Zenodo once accepted
764 (https://github.com/vsin632/ant_yogurt.git).

765 **Author contributions**

766 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.,
767 D.P.V, N.R.V, S.K.T.P, J.G., E.M.V, S.B.A; Formal analysis: V.M.S., V.R.-V., S.K.T.P;
768 Investigation: V.M.S., V.R.-V. D.P.V., S.M.S., N.R.V., A.C-M., S.K.T.P., J.G., E.M.V., D.Z.;
769 Resources: L.J.J., S.B.A, R.M., S.M.S.; Data curation: V.M.S.; Writing – original draft preparation:
770 V.M.S., V.R-V., L.J.J., R.R.D; Writing – review & editing: all authors; Visualisation: V.M.S., V.R.-
771 V.; Supervision: L.J.J., R.D.D., S.B.A., R.M.; Project administration: V.M.S., L.J.J.; Funding
772 acquisition: S.B.A., L.J.J., R.R.D., R.M.

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