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Rapid phenotypic and metabolomic domestication of wild *Penicillium* molds on cheese

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ABSTRACT Fermented foods provide novel ecological opportunities for natural populations of microbes to evolve through successive recolonization of resource-rich substrates. Comparative genomic data have reconstructed the evolutionary histories of microbes adapted to food environments, but experimental studies directly demonstrating the process of domestication are lacking for most fermented food microbes. Here we show that during the repeated colonization of cheese, phenotypic and metabolomic traits of wild *Penicillium* molds rapidly change to produce mutants with properties similar to industrial cultures used to make Camembert and other bloomy rind cheeses. Over a period of just a few weeks, populations of wild *Penicillium* strains serially passaged on cheese resulted in the reduction or complete loss of pigment, spore, and mycotoxin production. Mutants also had a striking change in volatile metabolite production, shifting from production of earthy or musty volatile compounds (e.g. geosmin) to fatty and cheesy volatiles (e.g. 2-nonanone, 2-undecanone). RNA-sequencing demonstrated a significant decrease in expression of 356 genes in domesticated mutants, with an enrichment of many secondary metabolite production pathways in these downregulated genes. By manipulating the presence of neighboring microbial species and overall resource availability, we demonstrate that the limited competition and high nutrient availability of the cheese environment promote rapid trait evolution of *Penicillium* molds.

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32 **IMPORTANCE** Industrial cultures of filamentous fungi are used to add unique aesthetics and
33 flavors to cheeses and other microbial foods. How these microbes adapted to live in food
34 environments is generally unknown as most microbial domestication is unintentional. Our work
35 demonstrates that wild molds closely related to the starter culture *Penicillium camemberti* can
36 readily lose undesirable traits and quickly shift toward producing desirable aroma compounds.
37 In addition to experimentally demonstrating a putative domestication pathway for *P.*
38 *camemberti*, our work suggests that wild *Penicillium* isolates could be rapidly domesticated to
39 produce new flavors and aesthetics in fermented foods.

40

41

42 **INTRODUCTION**

43 Fermented foods such as cheese, miso, sourdough, and sauerkraut are hybrid
44 microbiomes where wild microbial species from the environment mix with domesticated
45 microbes that are added as starter cultures. Microbes from natural ecosystems have the
46 potential adapt to these resource-rich environments where they may rapidly evolve new traits
47 and/or lose traits that are not maintained by selection in these novel environments (1). Previous
48 evidence for microbial domestication in fermented foods comes from comparative genomic
49 studies of food isolates and closely-related wild strains (1–4). For example, in the fungus
50 *Aspergillus oryzae*, which is used in the production of soy sauce, miso, and sake, both structural
51 and regulatory genomic changes are correlated with the evolution of non-toxic and flavorful
52 *Aspergillus oryzae* strains from a highly toxic ancestor (*Aspergillus flavus*) (2). The evolutionary
53 origins of most domesticated microbes remain enigmatic in large part because domestication of
54 microbes is usually unintentional and the processes driving microbial domestication have not
55 been experimentally recreated (1).

56 *Penicillium* species colonize the surfaces of aged cheeses around the world either as
57 starter cultures that are intentionally added during the cheese making process (5, 6) or as non-

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58 starter *Penicillium* species that enter cheese production facilities from natural fungal populations
59 (7–9). The white surface of Camembert and Brie cheeses is created by a variety of strains of the
60 starter culture *P. camemberti.* This domesticated fungus is white, makes fewer conidia (asexual
61 spores) than most wild *Penicillium* species, does not make detectable levels of mycotoxins, and
62 produces desirable mushroomy and fatty volatiles during cheese ripening (10–12) (**Fig. 1A**). In
63 contrast, the putative ancestor *P. commune* and other closely related *Penicillium* species are
64 generally greenish-blue, make large numbers of conidia, and produce mycotoxins and other
65 undesirable volatiles that negatively impact cheese quality (**Fig. 1B-C)**. Historical accounts
66 suggest that white mutant strains of *Penicillium* species were either directly isolated from French
67 cheeses or produced in laboratories in France (13), but the potential domestication processes
68 that generated these iconic cheese mold species have not been identified.

69 Here we used experimental evolution to determine how wild *Penicillium* molds may be
70 unintentionally domesticated in the cheese aging environment. We specifically determined how
71 quickly *Penicillium* could evolve new phenotypes on cheese, how *Penicillium* traits change
72 during domestication on cheese, and what properties of the cheese environment promote
73 domestication of *Penicillium*. Using our cheese rind model (14, 15), we serially passaged
74 populations of wild *Penicillium* and tracked the emergence of phenotypic mutants. We found
75 that mutants with substantially reduced mycotoxin levels, reproductive output, and pigment
76 production rapidly emerge in these experimental populations. Volatile profiling and RNA-
77 sequencing also demonstrate a substantial remodelling of metabolism in domesticated mutants.
78 These findings illustrate the potential for rapid domestication of filamentous fungi in cheese
79 caves around the world.

80

81 **RESULTS and DISCUSSION**

82 **Non-starter *Penicillium* species rapidly evolve novel phenotypes on cheese**

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83 To experimentally evolve *Penicillium* on cheese, two non-starter *Penicillium* strains
84 (*Penicillium commune* strain 162_3FA and *Penicillium* sp. strain 12) isolated from a cheese
85 cave in Vermont in the United States were serially passaged on cheese curd medium. These
86 molds were isolated from a cheese aging facility that was colonized by the molds within the past
87 five years. These cheese cave isolates have wild-type phenotypes (pigmented, high spore
88 production, musty odors, and mycotoxin production), and are closely related to *P. camemberti*
89 strains used in cheese production (**Fig. 1D, Fig. S1**). At each passage, replicate populations
90 were sampled to determine population size and mutant frequency. Colonies were considered
91 phenotypic mutants if they had altered surface color or texture indicating changes in pigment or
92 spore production. To determine how competition from neighboring cheese microbes impacts the
93 rate of phenotypic diversification, we serially passaged replicate populations alone ("*Penicillium*
94 alone") or in the presence of a mix of three competitors ("*Penicillium* + community", including the
95 yeast *Debaryomyces hansenii*, and the bacteria *Brachybacterium alimentarium* and
96 *Staphylococcus xylosus*) that commonly co-occur with *Penicillium* species in cheese rinds (14,
97 16, 17).

98 Within four weeks of serial passage on cheese, mutant phenotypes began to emerge in
99 our experimental *Penicillium* populations, reaching 71.5% of the population in the *Penicillium*
100 alone treatments by the end of the experiment (**Fig. 2A**). The presence of neighbors strongly
101 inhibited mutant phenotype frequency in the *Penicillium* + community treatments (mean of
102 26.2%, repeated-measures ANOVA $F_{1,6}$= 86.5, p < 0.001, Fig. 2A). Neighbors also decreased
103 total population size with an average of 42% decrease in total CFUs across the experiment
104 (repeated-measures ANOVA $F_{1,6}$= 10.3, p = 0.02, **Fig. S1**). Similar patterns of phenotypic
105 diversification alone and inhibition with neighbors were observed with *Penicillium* sp. strain 12
106 (**Fig. S2A-B, Table S2A-S2B**). These results suggest that cheese can promote the rapid
107 phenotypic diversification of *Penicillium* molds and that biotic interactions in cheese rinds can
108 inhibit this diversification.

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109 Cheese is a high-resource environment compared to environments where *Penicillium*
110 molds naturally occur (soils, leaves, etc.), with an abundance of carbon, nitrogen, and other
111 resources stored in the protein casein (18). To determine if the high resource availability of
112 cheese promotes the rapid domestication of *Penicillium* molds, we repeated the short-term
113 experimental evolution above with the standard cheese curd ("normal cheese"), with a "low
114 cheese" treatment with 1/10th the normal amount of cheese curd, and with an "alternating
115 cheese" treatment with alternating normal and 1/10th cheese curd agar at every other passage.
116 The low cheese treatment was designed to reduce total nutrient availability while keeping pH
117 and other environmental variables similar. The alternating treatment was designed to simulate
118 alternating colonization of a high resource environment (cheese) and a low resource
119 environment (soil, wood) which could occur in a cheese aging facility.

120 As with our first set of experiments, the normal cheese treatment resulted in the rapid
121 evolution of phenotypic mutants by the fourth week of the experiment with a mean mutant
122 frequency of 89.0% (+/- 12.2) across four replication populations at the end of the experiment
123 (**Fig. 2B**). Both the alternating cheese and low cheese treatment had significantly lower mutant
124 frequencies across the duration of the experiment (mean mutant frequency at end of
125 experiment: alternating cheese = 73.0 +/- 14.6%, low cheese= 0.16 +/- 0.19%; repeated-
126 measures ANOVA $F_{2,9}$= 149.6, $p < 0.0001$). As with the competition treatment above, population
127 sizes were significantly lower in the low cheese treatment (repeated-measures ANOVA $F_{2,9}$=
128 105.1, $p < 0.0001$) and may explain the substantially suppressed rate of diversification (**Fig. S4**).
129 These results suggest that the high resource environment of cheese promotes the rapid trait
130 evolution of *Penicillium*.

131

132 **Domestication of *Penicillium* on cheese leads to stable reductions in reproductive**
133 **output, pigmentation, and mycotoxin production**

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134 In our work above, all strains that emerged with altered colony morphotypes were
135 grouped together to obtain overall rates of phenotypic evolution in different biotic and abiotic
136 environments. To provide a finer-scale analysis of how reproductive and metabolic traits shifted
137 during adaptation to cheese, we measured reproductive output and mycotoxin production of
138 representative strains of *P. commune* 162_3FA that spanned the spectrum of mutant colony
139 phenotypes (Tables S1-S2). Reproductive output was measured as the number of CFUs
140 produced per unit area of a fungal colony and measured both spore and hyphal propagules. We
141 also measured production of the mycotoxin cyclopiazonic acid (CPA) by ancestral and mutant
142 strains when grown on cheese curd. Cyclopiazonic acid is commonly produced by *Penicillium*
143 *commune* and other closely related *Penicillium* species that colonize cheese surfaces, but it is
144 generally not produced or only produced in small quantities by *P. camemberti* strains used in
145 cheese production (7, 12). Pigment production was also qualitatively described by
146 photographing colonies of each strain grown on cheese curd agar. Spore, mycotoxin, and
147 pigment production are traits that are often co-regulated by global regulators in *Aspergillus* and
148 *Penicillium* species (19, 20). Mycotoxin and pigment production are thought to be important
149 traits for filamentous fungi to compete with other microbes or tolerate oxidative stress (21, 22);
150 however, these traits are frequently lost within fungal populations, suggesting that they are
151 costly (23). We predicted that adaptation to cheese might lead to the loss of some of these
152 costly traits in the high-resource and reduced competition environment of cheese rinds.

153 Trait analysis of the ancestor and seven mutant strains found a general pattern of
154 reduced reproductive output, mycotoxin, and pigment production. Reproductive output was
155 significantly lower in all mutant phenotypes compared to the ancestral strain (ANOVA with
156 Dunnett's test: $F_{8,18}$=178.6, p < 0.001), with some strains having approximately 4-log reductions
157 in spore production (**Fig. 2D**). We did not separate spores from hyphae in our analysis of
158 reproductive output. However, we suspect the drop in reproductive output is largely due to loss
159 of spore production because colonies of white mutants lost the characteristic dusty appearance

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160 created by spore-bearing conidiophores (**Fig. 2C**). We also detected a substantial loss of CPA
161 production across all mutants of *P. commune* 162_3FA (ANOVA with Dunnett's test: $F_{8,18}$=38.2,
162 $p < 0.001$), with three strains (M6, M7, and M8) having no detectable levels of CPA. Pigment
163 production followed the pattern of loss of reproductive output and CPA production, with
164 intermediate light blue mutants that had intermediate levels of reproductive output and CPA
165 production (M2, M9, and M10) and completely white mutants having the lowest levels of
166 reproductive output and CPA production (M3, M5, M6, M7, and M8). The observed phenotypic
167 changes were not transient; repeated transfer of mutants on cheese curd agar did not lead to
168 reversions to wild-type morphologies (**Fig. S5**). These trait analyses suggest co-regulated loss
169 of reproductive output, mycotoxin production, and pigmentation during adaptation of *P.*
170 *commune* to the cheese environment.

171 The fitness of mutants may differ from the ancestral strain when growing on the rich
172 cheese medium because mutants may shift resource allocation from costly traits (e.g.
173 secondary metabolite production) to growth. To test whether mutants had a higher fitness
174 compared to the ancestor, we used competition experiments where equal amounts of a mutant
175 strain (M9) were co-inoculated with the ancestor (WT). We were only able to conduct these
176 competition experiments with mutants that still had some level of spore production because it is
177 difficult to standardize inputs of wild-type spore producers and white mutant strains that had
178 almost no spore production. As predicted, the mutant outcompeted the ancestor after ten days
179 (**Fig. 2E**), suggesting that the coordinated loss of traits during domestication leads to a higher
180 fitness in the cheese environment.

181

182 **Domestication shifts the *Penicillium* volatilome from musty to cheesy aromas**

183 While working with the mutant strains, we noticed that they had strikingly different
184 aromas compared to the wild-type ancestral strain. The ancestral strain smelled musty and
185 earthy while the mutants smelled fatty, green, and surprisingly reminiscent of aged cheese. The

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186 aromas of cheese are volatile organic compounds (VOCs) that are produced by filamentous
187 fungi and other microbes during proteolysis, lipolysis, and other processes that decompose the
188 cheese substrate (11, 24, 25). These VOCs are important determinants of how consumers
189 perceive the quality of a cheese and can create variation in aroma profiles across surface-
190 ripened cheese varieties (26, 27). To quantitatively assess whether domestication of *Penicillium*
191 on cheese alters volatile aroma production, we captured volatiles produced by wild-type and
192 mutant *Penicillium* using headspace sorptive extraction followed by analysis with gas
193 chromatography-mass spectrometry (GC-MS) (28, 29). We compared the ancestor *P. commune*
194 162_3FA with three mutants - M2, M5, and M6 - that spanned the continuum of reproductive
195 output, mycotoxin, and pigment traits (**Fig. 2D**).

196 As suggested by our preliminary olfactory observations, the composition of volatiles
197 produced by mutants shifted substantially from the ancestor (**Fig. 3**, ANOSIM $R = 1.0$, $p <$
198 0.001). Geosmin was the only VOC that was produced by the ancestor and was absent in all
199 three of the mutant strains (6.722% contribution in SIMPER analysis of ancestor vs. all mutants
200 pooled together; **Fig. 3, Table S3**). Geosmin is widely recognized for contributing a musty
201 aroma to environmental and food samples and is produced by both bacteria and fungi including
202 *Penicillium* (30–32). It has a very low odor threshold meaning that even small amounts of
203 geosmin produced by *Penicillium* can be perceived as strong aromas (33). The loss of geosmin
204 production could be the major driver of the perceived loss of musty aromas in domesticated
205 *Penicillium* mutants.

206 In addition to a loss of musty aromas, mutant strains produced higher amounts of methyl
207 ketones and other VOCs associated with molds used in cheese production. Typical Camembert
208 flavor has been defined in patents as containing 2-heptanone, 2-heptanol, 8-nonen-2-one, 1-
209 octen-3-ol, 2-noanol, phenol, butanoic acid, and methyl cinnamate (11). All of these VOCs,
210 except butanoic acid and methyl cinnamate, were detected in our GC-MS profiling and several
211 were major drivers of differences in VOC profiles of mutants compared to the ancestor (**Fig. 3;**

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212 2-heptanone = 12.1% SIMPER contribution, 8-nonen-2-one = 8%, 1-octen-3-ol = 7%
213 contribution). Other methyl ketones that have been detected in *P. camemberti* (34), including 2-
214 nonanone (13.8% contribution) and 2-undecanone (12.3%), were also detected in higher
215 concentrations in mutants compared to the ancestor and contributed strongly to differences in
216 VOC profiles (Fig. 3). These methyl ketones are perceived as cheesy, fatty, fruity, and green
217 aromas that are typically associated with ripened cheeses (35, 36). Collectively, these VOC
218 data demonstrate a dramatic remodelling of the volatilome of *P. commune* as a result of rapid
219 domestication on cheese.
220

221 **Comparative transcriptomics demonstrates global down-regulation of secondary**
222 **metabolite production in domesticated *Penicillium* mutants**

223 To explore additional shifts in metabolic processes in cheese-adapted *Penicillium* not
224 captured by our targeted metabolomics above, we used RNA-sequencing (RNA-seq) to
225 compare global expression patterns of the wild-type *P. commune* to one mutant strain (M5).
226 This mutant was selected because it had an intermediate reduction of reproductive output, CPA
227 production, and pigment production. We predicted that in addition to shifts in gene expression
228 related to spore and pigment production, genes associated with other secondary metabolites
229 not measured would also be downregulated.

230 The transcriptome of mutant M5 had 356 genes with significantly lower expression
231 compared to the ancestor, or about 3.2% of all protein-coding genes (**Fig. 4A**). Only 86 genes
232 had higher expression levels in the mutant compared to the ancestor. An enrichment analysis of
233 GO terms associated with these differentially expressed genes highlights the substantial
234 downregulation of genes associated with secondary metabolite production (**Fig. 4B**). Many
235 pathways that were significantly enriched in the list of downregulated genes were associated
236 with pigment production (melanin biosynthesis) and production of a range of secondary
237 metabolites including chanoclavine-I, austinol, and dehydroaustinol (**Fig. 4B, Table S4**). One

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238 striking example is the ergot alkaloid synthesis (*eas*) gene cluster. Ergot alkaloids can be toxic
239 to mammals and recent work demonstrated that genomes of *P. camemberti* from cheese
240 contain some genes in the ergot alkaloid biosynthesis pathway and can produce some early
241 precursors of ergot alkaloids (37). The *eas* gene cluster is also present in *P. commune* 162_3FA
242 and is strongly downregulated (a mean of -42 fold-change across *dmwA*, *easE*, *easF*, and *easC*
243 genes) in the mutant compared to the ancestor (**Fig 4C**). In addition to the dramatic decrease in
244 the expression of genes associated with secondary metabolite production, the observed
245 reduction in conidia production by mutants is supported by strong downregulation of *abaA*,
246 which regulates condia development in *Aspergillus* (38) (**Table S4**).
247

248 **Domesticated mutants of *Penicillium* are found at low frequencies in cheese caves**

249 Our work above provides experimental evidence that *Penicillium* molds can rapidly
250 domesticate in the cheese environment. But does this domestication produce phenotypic
251 mutants in the more realistic conditions of a cheese cave? To answer this question, we deeply
252 sampled a population of *Penicillium* sp. strain 12 from a cheese cave in Vermont, USA.
253 Sampling of *Penicillium* sp. strain 12 occurred four years after the initial isolation of this strain.
254 We removed patches of the fungus from the surface of 43 different wheels of cheese and plated
255 out each of the patch samples to determine the frequency of wild-type vs. mutant colonies.
256 Much of our experimental work focused on *P. commune* 162_3FA and ideally we would have
257 sampled a population of this fungus. However, we were unable to find a large enough
258 population of *P. commune* 162_3FA in the cave where it was originally isolated.

259 White mutant colonies were detected on 12 of the 43 wheels of cheese and were
260 infrequent relative to wild-type (0.36% frequency). This low abundance of mutants in these
261 multispecies rind communities aligns with the low mutant frequency observed when *Penicillium*
262 sp. strain 12 evolved in the presence of competitors (2.23%, **Fig S3B**). This survey
263 demonstrates that phenotypic mutants of wild molds can be detected in caves where cheeses

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264 are aged. These rare mutants were likely the source of the original white molds used in
265 industrial Camembert production.
266
267 **Conclusions**
268 Novel ecological opportunities are thought to promote the diversification of plants and
269 animals during adaptive radiations (39–41) and similar processes may occur when wild
270 microbial populations colonize the high-resource environments of fermented foods. Cheese is a
271 resource-rich substrate that provides microbes from natural populations with novel ecological
272 opportunities. Facilities where natural rind cheeses are aged are relatively stable environments
273 where stressors of the natural world, including resource-limitation and UV stress, are relaxed.
274 While these facilities are carefully managed to keep out pathogens, wild molds from natural
275 populations commonly colonize the surfaces of certain cheeses where a natural rind is desired
276 (6, 7, 14). Using experimental evolution, we demonstrate that cheese aging environments have
277 the potential to promote rapid trait evolution of *Penicillium* species. Changes in the cheese
278 environment that suppressed population size, including competition and decreased resource
279 availability, inhibited trait evolution during domestication. Previous comparative genomic studies
280 of *Penicillium* molds from cheese and fermented meat have identified genomic signatures of
281 evolution over longer time-scales in fermented food environments (3, 42). Our experimental
282 work demonstrates that in just a few weeks, *Penicillium* molds can adapt to the cheese
283 environment through the loss of energetically costly traits, including spore production, pigment
284 production, and mycotoxin production.
285 Previous studies in *Aspergillus* and *Penicillium* species have observed similar rapid trait
286 change when fungi are subcultured in rich lab media over many generations (43–45). This
287 phenomenon has been called degeneration because desired industrial traits or traits of interest
288 for laboratory studies are lost. These cultures experience a similar transition from a high
289 competition and low resource natural environment (plants, soil, etc.) to the low competition and

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290 high resource environment (rich lab media) as is the case when fungi colonize cheese. For
291 example, serial transfer of *A. parasiticus* caused the rapid loss of secondary metabolite
292 production, decreased sporulation, and changes in pigment production at a similar timescale
293 (several weeks) to that observed in our work on cheese (44). A similar pattern of trait
294 degeneration with serial transfer was observed with *A. flavus* (45). Together with our work on
295 *Penicillium*, these studies demonstrate the rapid and coordinated phenotypic and metabolomic
296 shifts in a range of filamentous fungi as they are stably maintained in high-resource
297 environments.

298 One of the most striking changes that we observed in our *Penicillium* mutants is a shift in
299 VOC production. Even though the domestication process in our experiments was undirected,
300 white mutants stopped producing the musty VOC geosmin that is generally considered
301 undesirable in foods (31) and increased production of ketones and other VOCs that are
302 considered desirable in Camembert and other cheeses (11, 32). The genes and pathways
303 responsible for production of the secondary metabolite geosmin and other VOCs are not well-
304 characterized in cheese *Penicillium* species (46) so we are unable to explicitly link the
305 transcriptomic data with the VOC data. Ketone production by cheese fungi is a result of lipases
306 releasing fatty acids from lipids that are then converted into ketones, alcohols, and other VOCs
307 (25, 46, 47). The observed shifts in VOC production could reflect a generalized loss of
308 secondary metabolite production and increased lipid degradation of the cheese substrate.

309 We did not identify a specific genetic mechanism controlling the observed trait evolution
310 in *Penicillium* and a genetic mechanism underlying degeneration of *Aspergillus* cultures has
311 also not been identified. It is possible that genomic evolution, including structural changes such
312 as chromosomal rearrangements or mutations in specific genes, could drive the phenotypic and
313 metabolomic shifts observed. Alternatively, the rapid trait evolution in *Penicillium* may be
314 explained not by genomic evolution, but by transgenerational epigenetic inheritance which has
315 been proposed to be important in filamentous fungi (48). Global regulators of genes involved

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316 with pigment, toxin, and spore production have been identified in *Aspergillus* and other fungi
317 and some of these regulators, including the methyltransferase LaeA, have been demonstrated
318 to epigenetically regulate transcription (49, 50). Future work further characterizing these
319 mutants will identify the specific genetic and molecular changes driving domestication.

320 A detailed record of how contemporary *P. camemberti* mutants used in cheese
321 production were derived is not available (13), so we cannot know precisely how and when *P.*
322 *commune* was domesticated to become *P. camemberti*. It is possible that the industrial starter
323 cultures used today were isolated as mutants from cheese caves in Europe. Regardless of how
324 these mutants were ultimately acquired, our work demonstrates the potential for *Penicillium*
325 molds to rapidly evolve without intentional selection for desired cheese-making traits. Because
326 we observed similar trait shifts in two different *Penicillium* species, it is possible that phenotypic
327 mutants of many different *Penicillium* species are continuously evolving in cheese caves around
328 the world. Our laboratory domestication suggests that new strains of *Penicillium* for cheese
329 production could be generated through more intentional and controlled domestication
330 processes. Most strains used in mold-ripened cheese production originate from Europe,
331 providing a limited palette of textures and flavors. The experimental domestication of *Penicillium*
332 on cheese could lead to the production of locally adapted strains for cheese production in other
333 parts of the world.
334

335 **METHODS**

336 **Isolation and manipulation of *Penicillium* cultures.** Two non-starter *Penicillium* strains,
337 *Penicillium* sp. 162_3FA and *Penicillium* sp. 12, were used in the experiments throughout this
338 study. Both fungal strains were isolated from the surface of a natural rind cheese produced and
339 aged in Vermont, USA. A third non-starter mold, *Penicillium* sp. MB was isolated from a natural
340 rind cheese production and aging facility in California. It was sequenced as part of this work and
341 included in the phylogenomic analysis, but was not used in the experimental portion of the

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342 paper because mutant scoring was not as clear as with the other two strains. To determine the
343 putative taxonomic identity of these two molds, whole genome sequences were obtained using
344 Illumina sequencing as previously described for a *Mucor* isolate (51). Genomes were
345 assembled using the *de novo* assembler in CLC Genomics Workbench and annotated using
346 GenSAS (https://www.gensas.org/).

347 **Construction of a phylogenomic tree of *Penicillium*.** To reconstruct the evolutionary
348 relationships among described *Penicillium* species and the two isolates used in this work, we
349 obtained a comprehensive set of genomes from *Penicillium* species using NCBI's Taxonomy
350 Browser. We downloaded the 33 available *Penicillium* genomes on February 5th 2018. In
351 addition to the 33 *Penicillium* genomes, we also downloaded three genomes from
352 representative species in the genus *Aspergillus* for use as outgroup taxa. Altogether, our
353 dataset contains a total of 39 taxa – three *Penicillium* genomes sequenced in the present study,
354 33 publicly available *Penicillium* genomes, and three *Aspergillus* taxa.

355 To identify orthologous genes, we used the Benchmarking Universal Single-Copy
356 Orthologs (BUSCO), version 2.0.1 (52), pipeline and the Pezizomycotina database (creation
357 date: 02-13-2016) from ORTHODB, version 9 (53). The BUSCO pipeline uses hidden Markov
358 models for 3,156 previously established Pezizomycotina orthologs (hereafter referred to as
359 BUSCO genes) to individually search each genome for their corresponding orthologs. Identified
360 orthologs are classified as "single copy" if only a single full length orthologous gene is identified,
361 "duplicated" if two or more full length predicted orthologs are identified, "fragmented" if the
362 identified orthologous gene is less than 95% of the sequence length of the gene's multiple
363 sequence alignment used for constructing the hidden Markov model, or "missing" if no
364 orthologous gene is found in the genome.

365 Examination of BUSCO genes reveals that the genomes sequenced and assembled in
366 the present study exhibited high genome completeness. For example, using 3,156 universally
367 single-copy orthologs from Pezizomycotina (or BUSCO genes), we observed that the

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368 MB_DraftGenome, *Penicillium commune* (162_2_DraftGenome), and 12_DraftGenome isolates
369 have 3,099 / 3,156 (98.2%), 3,097 / 3,156 (98.2%), 3,096 / 3,156 (98.1%) of BUSCO genes
370 present in their genomes, respectively. Notably, these values are similar to those of other
371 publically available genomes; for example, *P. citrinum* and *P. camemberti* have 3,095 / 3,156
372 (98.1%) and 3,100 / 3,156 (98.3%) of BUSCO genes present in their genomes, respectively.

373 To construct the phylogenomic data matrix, we first retained only those BUSCO genes
374 that were present in single copy in at least 20 taxa (i.e., > 50% taxon-occupancy). From this set
375 of 3,111 groups of BUSCO orthologous genes, we created individual gene alignments using
376 MAFFT, version 7.249b (54)(Katoh and Standley, 2013) with the BLOSUM62 matrix of
377 substitutions (Mount, 2008), the "geneafpair" parameter, a gap penalty of 1.0, and 1,000
378 maximum iterations. To thread nucleotide sequences on top of each amino acid multiple
379 sequence alignment, we used a custom PYTHON, version 3.5.2 (https://www.python.org/), script
380 using BIOPYTHON, version 1.7. Individual nucleotide alignments were trimmed using TRIMAL,
381 version 1.4 (55), with the "automated1". Thereafter, the 3,111 groups of BUSCO orthologous
382 genes were concatenated into a single phylogenomic data matrix that contained 5,498,894 sites
383 and had taxon occupancy of 97.65 ± 5.23%.

384 To reconstruct the evolutionary history of *Penicillium* species, we used the gene-based
385 maximum likelihood schemes of concatenation and coalescence (56–58). We first determined
386 the best-fitting models of sequence substitutions and conducted maximum likelihood searches
387 of single gene trees for all 3,111 genes in our data matrix. Using IQ-TREE, version 1.6.1 (59),
388 we determined the best-fitting model for each single gene or partition according to the Bayesian
389 Information Criterion. We inferred each gene's evolutionary history using the "-m TEST"
390 parameter and distinct randomly generated seeds specified using the "-seed" parameter.
391 Among best-fitting models, the most commonly observed one was "TN+F+I+G4" (60, 61), which
392 was observed in 537 / 3,111 (17.26%) genes, "TIM2+F+I+G4" (61), which was observed in 440 /

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393 3,111 (14.14%) genes, and "GTR+F+I+G4", which was observed in 400 / 3,111 (12.86%) genes
394 (61, 62).

395 To infer evolutionary history using the gene-based concatenation approach, we first
396 created a partition-file that describes the best-fitting parameters for each gene and conducted
397 five independent searches for the maximum likelihood topology. More specifically, to allow each
398 of the 3,111 genes in the phylogenomic data matrix to have its own substitution model and rate
399 heterogeneity across sites parameters, we created a nexus-format partition file that detailed
400 these parameters and gene boundaries. Using this file along with the "-spp" parameter, each
401 partition was also allowed to have its own set of evolutionary rates (63). We also increased the
402 number of candidate trees used during the search for the optimal tree from 5 to 10 using the "-
403 nbest" parameter. Using this scheme, we conducted 5 independent searches with 5 distinct
404 seeds for the maximum likelihood topology. We next evaluated support for the maximum
405 likelihood topology using the ultrafast bootstrap approximation approach (UFBoot) (64).

406 To infer evolutionary history using coalescence, we combined all single gene
407 phylogenies into a single file. Using this file, we inferred the evolutionary history of *Penicillium*
408 species using ASTRAL-II, version 4.10.12 (57) using default parameters and assessed
409 bipartition support using local posterior probability (65).

410 Examination of the resulting phylogeny revealed high concordance with previous whole-
411 genome based analyses (66) with full support at every internode using UFBoot and local
412 posterior probability except for the split of section *Exilicaulis* and *Lanata-divaricata*, which
413 received local posterior probability value of 0.97. Importantly, our analyses place the newly
414 sequenced *Penicillium commune* in section of *Fasciculata* (Fig A), as previously described (67).

415 To explore putative biological drivers of incongruence in the phylogenies, we determined
416 how many individual gene trees supported any or none of the three alternative topologies by
417 calculating their gene support frequencies (68). More specifically, we counted how many single
418 gene trees supported a sister group relationship of *Penicillium biforme* and *Penicillium*

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419 *camemberti,* how many a sister group relationship of *P. biforme* and *Penicillium commune*, and
420 how many a sister group relationship of *P. camemberti* and *P. commune*. We also counted how
421 many gene trees did not infer these three species as a monophyletic group. Finally, we also
422 kept track of how many genes could not be used due to incomplete taxon representation to
423 determine if incomplete taxon representation was contributing to incongruence. Gene support
424 frequencies were determined by examining the sister species of *Penicillium commune* using
425 NEWICK UTILITIES, version 1.6 (69).

426 **Experimental evolution of *Penicillium* on cheese.** Each of the *Penicillium* strains was grown
427 in our experimental cheese system consisting of 20 mL of cheese curd agar (CCA) in a
428 standard 100 x 15 mm Petri dish. Cheese curd agar (CCA) is composed of freeze-dried
429 unsalted cheese curd from a blue cheese produced in Vermont (100 g/L), xanthan gum (5 g/L),
430 salt (30 g/L) and agar (17 g/L). CCA allows for controlled manipulations of cheese rind
431 communities and accurately mimics the dynamics of cheese rind development (14). To start the
432 evolution experiment, each strain was initially inoculated with 500 colony-forming units (CFUs)
433 across the surface of the CCA plate. Each experimental cheese community was incubated for 7
434 days in the dark at 24ºC and 95% humidity. Experimental communities were serially transferred
435 to new CCA every week for a period of 8 weeks.

436 To manipulate the biotic environment throughout the evolution experiment, cheese rind
437 bacteria and yeasts were added to four replicate communities to create a "*Penicillium* +
438 Community" treatment. The yeast *Debaryomyces hansenii* strain 135B and the bacteria
439 *Staphylococcus xylosus* BC10 and *Brachybacterium alimentarium* strain JB7 were added at the
440 same density as *Penicillium* at the initial inoculation. We selected these three microbial species
441 for the *Penicillium* + Community treatment because they represent taxa that are common
442 members of natural rind cheese microbiomes (14, 16, 51) and were stably maintained during
443 the duration of the experiment. We acknowledge that these community members may have

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444 evolved during the experimental domestication experiment and we did not attempt to control for
445 their evolution throughout the experiment.

446 To manipulate total resource availability throughout the evolution experiment, we created
447 "low cheese" which contained the same components as CCA except 10 g/L of freeze-dried
448 unsalted cheese curd (instead of 100 g/L in "normal cheese") was used in the medium. The pH
449 of "low cheese" was identical to that of "normal cheese". In the "alternating normal/low"
450 treatment, we alternated transfers each week between full-strength and dilute CCA, starting with
451 full-strength CCA when setting up the experiment.

452 At each transfer, the cheese curd agar from each community was removed from the
453 Petri dish and homogenized inside a Whirl-Pak bag containing 30 mL of 1X phosphate buffered
454 saline (PBS). From this homogenized mixture, an aliquot of 100 µL was plated onto new CCA to
455 seed a new community. Another aliquot was serially diluted and plated onto PCAMS media with
456 50 mg/L of chloramphenicol (to inhibit bacterial neighbors in the "*Penicillium* + Community"
457 treatment) for colony counting and scoring colony phenotypes. Glycerol stocks were made at
458 each transfer so that communities could be archived and revived later if needed.

459 Phenotypic evolution was tracked by scoring wild-type and mutant colonies at each
460 transfer. Mutant colonies were considered to have differences in pigment intensity, distribution
461 of pigment around the colony, colony texture, degree of sporulation, and extent of mycelium
462 production (explained in detail in Table S1A-S1B). Phenotyping was done after 5 days of
463 incubation of PCAMS plates containing the output of each transfer. PCAMS output plates were
464 incubated at 24ºC for 5 days before phenotyping was completed. Phenotyping occured on
465 plates with at least 100 colonies.

466 **Reproductive and mycotoxin trait analysis.** Reproductive and mycotoxin traits were only
467 measured for the ancestor and select mutants of *Penicillium* sp. 162_3FA because it is most
468 closely related to *P. camemberti*. The following strains were used in these assays: Ancestor,
469 M2, M3, M5, M6, M7, M9, and M10. These strains were selected for trait profiling because they

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470 spanned the spectrum of visible colony types ranging from similar to wild-type (slightly less blue)
471 to completely white (Table S1A-S1B). To determine reproductive output, each strain was
472 inoculated on three replicate plates at a density of 50 CFUs on the surface of 20 mL of cheese
473 curd agar (CCA) in a standard 100 x 15 mm Petri dish. At this density, individual CFUs were
474 discernible. Plates were incubated for 7 days at 24ºC and 95% humidity. From three individual
475 colonies, a sterile circular cork borer with a diameter of 0.7cm was inserted into the center of the
476 colony. The excised colony plug was serially diluted and CFUs were determined on PCAMS.

477 To determine how production of mycotoxin cyclopiazonic acid changed in mutant strains
478 compared to ancestors, we measured CPA production in the following strains: Ancestor, M2,
479 M3, M5, M6, M7, M9, and M10. 40,000 CFUs of each strain was spread across the surface of
480 20 mL cheese curd in a 100 x 15 mm Petri dish. Three biological replicates of each strain were
481 used in the experiments. Plates were incubated in the dark for 3 days at 24°C and 11 days at
482 4°C. After the 14 day incubation, the medium was harvested from the plate, placed into a Whirl-
483 pak bag, and homogenized. Samples were frozen at -80°C until analysis.

484 The cyclopiazonic acid concentration of the cheese curd agar was measured using liquid
485 chromatography with tandem mass spectrometry (LC-MS/MS) at Romer Labs (Union, Missouri,
486 USA). The homogenized cheese curd sample was extracted in a 50/50 mixture of acetonitrile
487 and deionized water by shaking for 90 minutes. The supernatant was filtered and 10 mL was
488 mixed with 500 µL of acetic acid. 1 mL of this solution was vortexed in a MycoSpin Column
489 (Romer Labs), vortexed for 1 minute, and centrifuged for 30 seconds at 10,000 rpm. 75 µL of
490 the purified extracted was injected into a Shimadzu HPCL with a Phenomenox Gemini HPCL
491 C18 column (4.6 X 150 mm, 5 µm) with mobile phase A consisting of ESI + 5 mM ammonium
492 formate with 0.1% formic acid in deionized water and mobile phase B consisting of acetonitrile.
493 The injection volume was 40 µL, the flow rate was 1.0 mL/min, and the column temperature was
494 40°C. Internal standards of CPA were used to construct a calibration curve.

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495 Stability of traits was assessed in two mutant strains M5 and M6. Three replicate plugs
496 of each of these strains was transferred to fresh CCA weekly using heat-sterilized stainless
497 steel cork borers (6mm diameter). Colonies were photographed at each transfer as in **Fig. S5**.
498 **Competition experiments.** We competed ancestor *Penicillium* sp. 162_3FA with the mutant
499 strain M9 to determine whether evolved mutant strains have a higher fitness compared to the
500 ancestor. It is challenging to standardize input densities of the white mutants because they
501 produced many fewer spore compared to the ancestor. Mutant M9 of *Penicillium* sp. 162_3FA
502 was chosen as a competitor because it still produced significant numbers of spores making it
503 possible to make comparable initial inoculum for both the ancestor and evolved strains.
504 Experiments were conducted in 96-well plates with 150µL of 10% CCA added to each well and
505 200 CFUs of each strain added at the start of the experiment. Six replicate experimental cheese
506 communities containing the wild-type and mutant mix were incubated in the dark at 24°C for ten
507 days. To determine the abundance of WT and M9 at the end of the experiment, each replicate
508 community was removed from the 96-well plate, homogenized in 600 µL 1X PBS, serially diluted
509 onto PCAMS, and then WT and M9 colonies were counted.
510 **Volatile profiling.** Cheese volatiles were collected from fungal cultures by headspace sorptive
511 extraction (HSSE) using a glass encapsulated magnetic stir-bar coated with 0.5mm
512 polydimethylsiloxane (PDMS). Before each sample was collected, the stir-bars were heated
513 from 40°C to 300°C at 5°C/min and flushed with 50 mL/min nitrogen (Airgas) to desorb sorbed
514 organics using a Gerstel (Baltimore, MD) TC2 tube conditioner. HSSE is an equilibrium-driven,
515 enrichment technique in which 10mm long stir-bars, Twister™ from Gerstel, were suspended 1
516 cm above the sample by placing a magnet on the top side of the collection vessel cover. All
517 cultures were sampled in quadruplicate (n=4) for 4 hr. One replicate of the Control (WT) was
518 lost during sample processing. After collection, the stir-bar was removed and spiked with 10
519 ppm ethylbenzene-d_{10}, an internal standard obtained from RESTEK (Bellefonte, PA). Organics
520 were introduced into the gas chromatograph/mass spectrometer (GC/MS) by thermal

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521 desorption. In addition to Twister blanks, analysis of the agar media was made to ensure
522 background interferents were minimal. If present, these compounds were subtracted from the
523 fungal data.

524 Analyses were performed using an Agilent (Santa Clara, CA) 7890A/5975C GC/MS
525 equipped with a 30 m x 250 μm x 0.25 μm HP5-MS column. The instrument was equipped with
526 an automated multi-purpose sampler (MPS), thermal desorption unit (TDU), and a CIS4
527 programmable temperature vaporizing (PTV) inlet from Gerstel. The TDU, operating in splitless
528 mode, transferred the sample from the stir-bar to the CIS4, which was held at -100°C, by
529 ramping the temperature from 40°C to 275°C at 720°C/min, then held isothermal for 3 min
530 under 50 ml/min helium gas flow. Once transferred, the CIS4 was heated from -100°C to 280°C
531 at 12°C/min, and held for 5 min. The GC temperature was held at 40°C for 1 min, then ramped
532 to 280 °C at 5°C/min, and held for 5 min. The MS was scanned from 40 to 250 *m/z*, with the EI
533 source at 70 eV. A standard mixture of C7 to C30 n-alkanes, purchased from Sigma–Aldrich (St.
534 Louis, MO), was used to calculate the retention index (RI) of each compound in the sample.

535 The Ion Analytics (Gerstel) spectral deconvolution software was used to analyze the
536 GC/MS data. Peak identification was performed through comparison of sample and reference
537 compound spectral patterns and retention indices, NIST05, Adams Essential Oil Library, and
538 literature. Compound identity was based on the following set of conditions. First, peak scans
539 must be constant for five or more consecutive scans (differences ≤ 20%). Second, the scan-to-
540 scan variance (SSV or relative error) must be < 5. The SSV calculates relative error by
541 comparing the mass spectrum at each peak scan against another. The smaller the difference,
542 the closer the SSV is to zero, the better the MS agreement. Third, the Q-value must be ≥ 93.
543 The Q-value is an integer between 1 and 100; it measures the total ratio deviation of the
544 absolute value of the expected minus observed ion ratios divided by the expected ion ratio times
545 100 for each ion across the peak. The closer the value is to 100, the higher the certainty
546 between database and sample spectra. Finally, the Q-ratio compares the ratio of the molecular

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547 ion intensity to confirming ion intensities across the peak; it also must be ≤ 20%. When all
548 criteria are met, the software assigns a compound name or numerical identifier to the peak from
549 the database.

550 To cluster the VOC data, a UPGMA tree with 100 bootstraps was constructed using a
551 Bray-Curtis dissimilarity matrix in PAST3. Analyses of similarity (ANOSIM) was used to test
552 whether there were differences between the ancestor and mutant in VOC profiles. ANOSIM R
553 values indicate the degree to which groups separate, with 1 being complete separation and 0
554 indicating a complete lack of separation. Similarity percentage analysis (SIMPER) on Bray-
555 Curtis dissimilarity distances was used to identify the compounds that contributed most to
556 differences in VOC profiles.

557 **RNA-sequencing.** To determine global changes in gene expression in cheese-adapted
558 *Penicillium*, we compared the ancestor and mutant M5 of *Penicillium* sp. 162_3FA were
559 inoculated onto cheese curd agar. Inoculum of both strains came from streaks of the fungi
560 growing on plate count agar with milk and salt (PCAMS) that had been growing for one week. A
561 1 cm^2 plug was taken from the leading edge of mycelium and then homogenized in 500 µL of
562 phosphate buffered saline (PBS). At three evenly spaced locations on a 100cm wide Petri dish
563 containing 20 mL of cheese curd agar, 20 µL of the inoculum was spotted onto the agar surface.
564 After 72 hours of growth in the dark at 24 °C, the spots were 1.5cm in width. The wild-type
565 162_3FA had produced spores and was blue in color and the mutant M5 162_3FA was white in
566 color. The entire fungal mass from each of the three spots was cut away from the CCA and then
567 placed in RNAlater (Qiagen) and stored at -80 °C. Four biological replicates were sampled for
568 each of the two strains.

569 RNA was extracted from one of the three spots from each replicate plate using a Qiagen
570 RNeasy Plant Mini Kit after grinding the sample in liquid nitrogen with an autoclaved mortar and
571 pestle. Approximately 100 mg of ground fungal biomass was placed in 750µl of Buffer RLT with
572 10µl of β-mercaptoethanol per 1 ml added to the Buffer RLT. The manufacturer's recommended

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573 protocol was followed for RNA extraction, including an on column DNAse treatment. To isolate
574 mRNA, the NEBNext ® Poly (A) mRNA Magnetic Isolation Module (New England Biolabs) was
575 used. This mRNA was used to generate RNA-seq libraries using the NEBNext ® Ultra II RNA
576 Library Prep Kit for Illumina following the manufacturer's recommended protocol. The RNA-seq
577 libraries were sequenced using 125 base-pair length, paired-end Illumina sequencing on a
578 HiSeq at the Harvard Bauer Core.

579 After trimming low quality sequences and removing failed reads using CLC Genomics
580 Workbench, sequencing yielded 3.5 to 22 million forward reads that were used for read
581 mapping and differential expression analysis. Reads were mapped to a reference genome of *P.*
582 *commune* 162_3FA that was sequenced using pair-end 125 base-pair length Illumina
583 sequencing, assembled with CLC Genomic Workbench de novo assembler, and annotated
584 using GenSAS (https://www.gensas.org/). Read mapping was performed with the CLC
585 Genomics Workbench RNA-seq analysis pipeline with the following settings: mismatch cost of
586 2, insertion cost of 3, deletion cost of 3, length fraction of 0.8, and similarity fraction of 0.8. The
587 number of unique reads mapped (mapped to one specific gene and not additional locations in
588 the genome) was used to determine expression levels and quantile normalization was used to
589 take into account different levels of sequencing across replicates. Other methods of calculating
590 gene expression and normalization were assessed (e.g. RPKM) and did not change the main
591 findings of the differential expression analysis. Identification of genes that were differentially
592 expressed in the cheese-adapted mutant compared to the ancestor was completed by using the
593 empirical analysis of differential gene expression tool in CLC Genomics Workbench. This
594 pipeline uses the exact test for two-group comparisons (70). We considered those genes with
595 greater than 5-fold change in expression and FDR corrected p-values of < 0.05 as differentially
596 expressed genes. To identify specific biological pathways that were enriched in the sets of
597 downregulated or upregulated genes, we used a KOBAS 2.0 to conduct a hypergeometric test

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598 on functional assignments from the gene ontology (GO) database (using the *Aspergillus flavus*
599 genome as a reference for GO ID assignment) with Benjamini and Hochberg FDR correction.
600 **Cheese cave population population sampling.** Sterile toothpicks were used to sample rinds
601 of 43 wheels of a natural rind blue cheese in the same caves where *Penicillium* sp. Strain 12
602 had been previously isolated. Samples were placed in 1X PBS, stored at 4°C for 24 hours, and
603 then each sample from a wheel of cheese was plated onto PCAMS with chloramphenicol to
604 inhibit bacterial growth. Plates were incubated at 24°C for seven days before assessing plates
605 for the presence of white mutants. Camembert-style cheeses inoculated with *P. camemberti* are
606 aged in different caves at the same facility. To confirm that white mutant phenotypes were
607 derivatives of the wild-type *Penicillium* sp. strain 12 and not contamination from starter cultures,
608 we used whole genome sequencing as described above to sequence a white mutant
609 morphotype isolate. Read mapping using BowTie2 revealed that the genome had a 99.9%
610 pairwise identity to the reference genome of *Penicillium* sp. strain 12.
611 **Data availability.** Whole-genome sequences of *Penicillium commune* strain 162_3FA and
612 *Penicillium* sp. strain 12 have been submitted to NCBI as MUGJ00000000 and MUGI00000000,
613 respectively. Raw data from RNA-sequencing of *Penicillium* sp. 162_3FA strain Ancestor and
614 *Penicillium* sp. 162_3FA strain M5 and resequencing the cave isolate of *Penicillium* #12 sp.
615 have been deposited in the NCBI Sequence Read Archive as PRJNA510622.
616

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620

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801 # Figures and Figure Legends:

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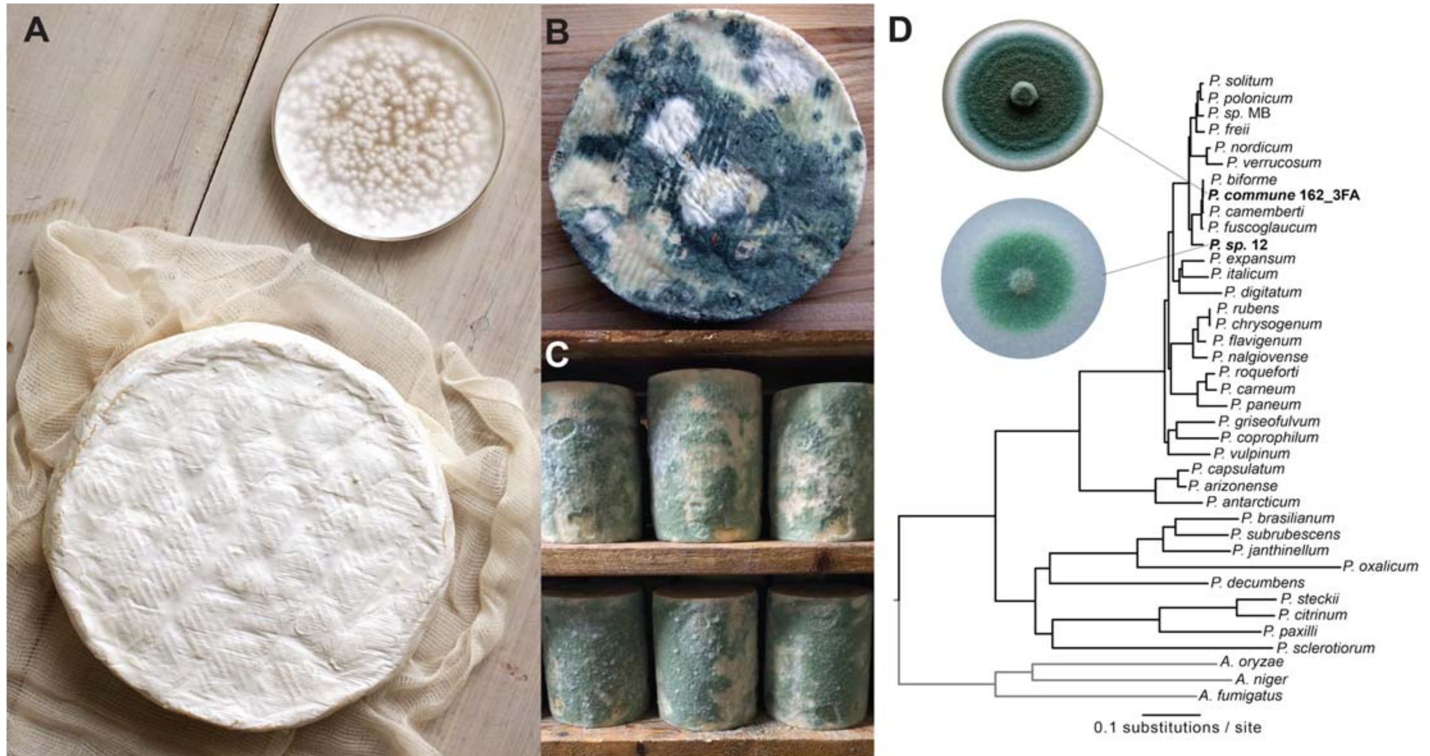

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806 **Figure 1: *Penicillium* molds in the cheese environment. (A)** The white mold known as *Penicillium*
807 *camemberti* (show in pure culture in the Petri dish) is used to make Camembert (shown), Brie, and other
808 bloomy rind cheeses. **(B)** Wild *Penicillium* molds (also known as non-starter molds) can contaminate
809 cheeses during production. **(C)** Some natural rind cheeses are intentionally colonized by wild *Penicillium*
810 molds. Shown here is *Penicillium* sp. strain 12, a strain used in the experiments in this paper, colonizing
811 wheels of a blue cheese in a cave in the United States. **(D)** A phylogenomic tree of *Penicillium*. Strains
812 used in this work are highlighted in bold. *Penicillium* sp. MB was also isolated from a natural rind cheese
813 and sequenced as part of this work, but was not used in the experiments described.

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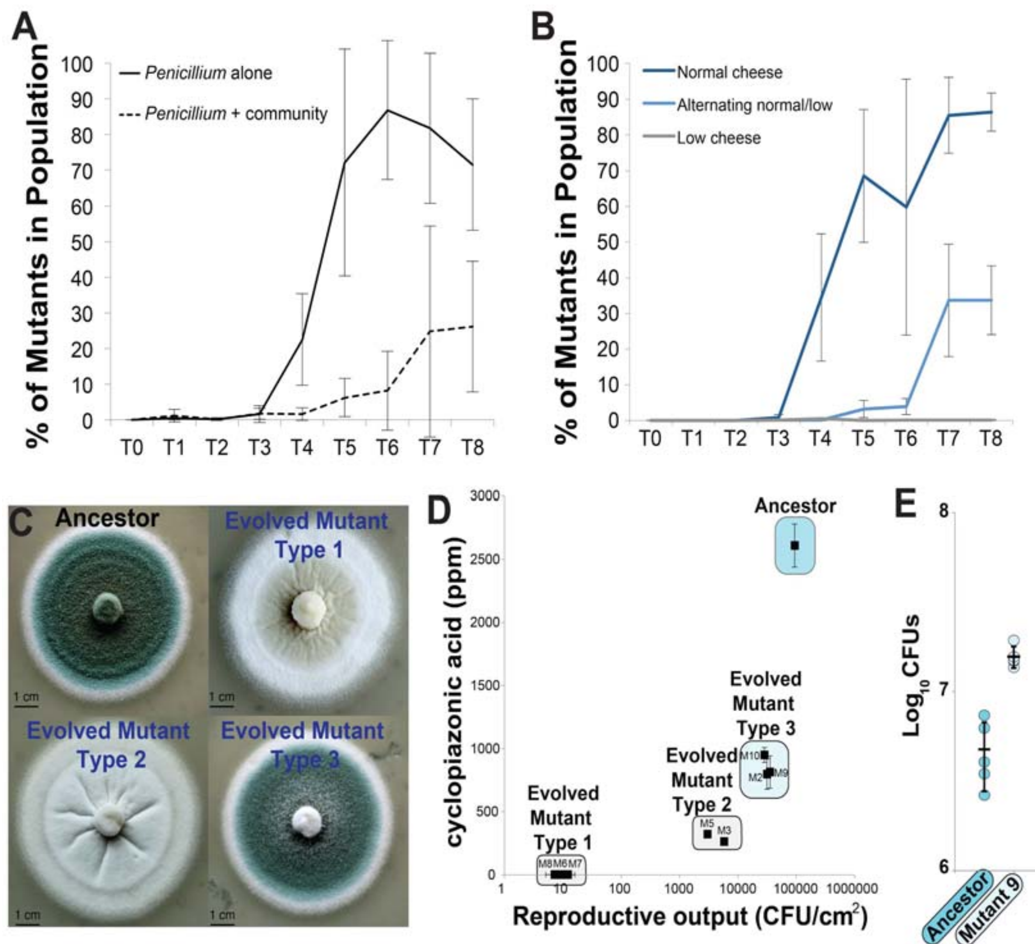

818 **Figure 2: Experimental evolution of *Penicillium* on cheese. (A)** Evolution of *Penicillium commune*
819 strain 162_3FA on cheese curd alone ("*Penicillium* alone") and in the presence of three competing
820 cheese rind microbes ("*Penicillium* + community"; *Staphylococcus xylosus, Brachybacterium*
821 *alimentarium,* and *Debaryomyces hansenii*). Lines connect points representing mean mutant phenotype
822 frequencies of four replicate populations and error bars represent one standard deviation of the mean.
823 "*Penicillium* + community" had a significantly lower mutant frequency (repeated-measures ANOVA, see
824 text for stats). **(B)** Experimental evolution of *P. commune* strain 162_3FA in different cheese nutrient
825 environments. "Normal cheese" = 10% cheese curd in agar medium. "Low cheese" = 1% cheese curd in
826 agar medium. "Alternating normal/low" = alternating 10% and 1% cheese curd at each transfer. Both "Low
827 cheese" and "Alternating normal/low" had significantly lower mutant frequencies (repeated-measures
828 ANOVA with Tukey's HSD post-hoc tests, see text for stats). Lines connect points representing mean
829 mutant phenotype frequencies of four replicate populations and error bars represent one standard
830 deviation of the mean. The "low cheese" line is difficult to see because it is at 0%. **(C)** Morphology of four
831 representative colony types. The Ancestor wild-type phenotype which is dark blue-green and dusty,
832 Evolved Mutant Type 1 which was white and flat, Evolved Mutant Type 2 which was white and
833 fuzzy/dusty, and Evolved Mutant Type 3 which was blue-green, but had less intense coloration than wild-
834 type and less fuzzy appearance. **(D)** Reproductive output and cyclopiazonic acid production of a range of
835 mutants isolated across the experimental evolution populations. Points are mean values and error bars
836 are one standard deviation of the mean. M5, M6, M7, and M8 had reduced reproductive compared to the
837 Ancestor (Dunnett's test, $p < 0.05$). All mutants had significantly reduced CPA production compared to the
838 Ancestor (Dunnett's test, $p < 0.05$). **(E)** Competition between the wild-type Ancestor *P. commune* strain
839 162_3FA and M9. M9 outcompeted the Ancestor after 10 days of growth on cheese curd agar. Points
840 represent individual replicate competition communities and horizontal line indicates mean values. Error
841 bars are one standard deviation.

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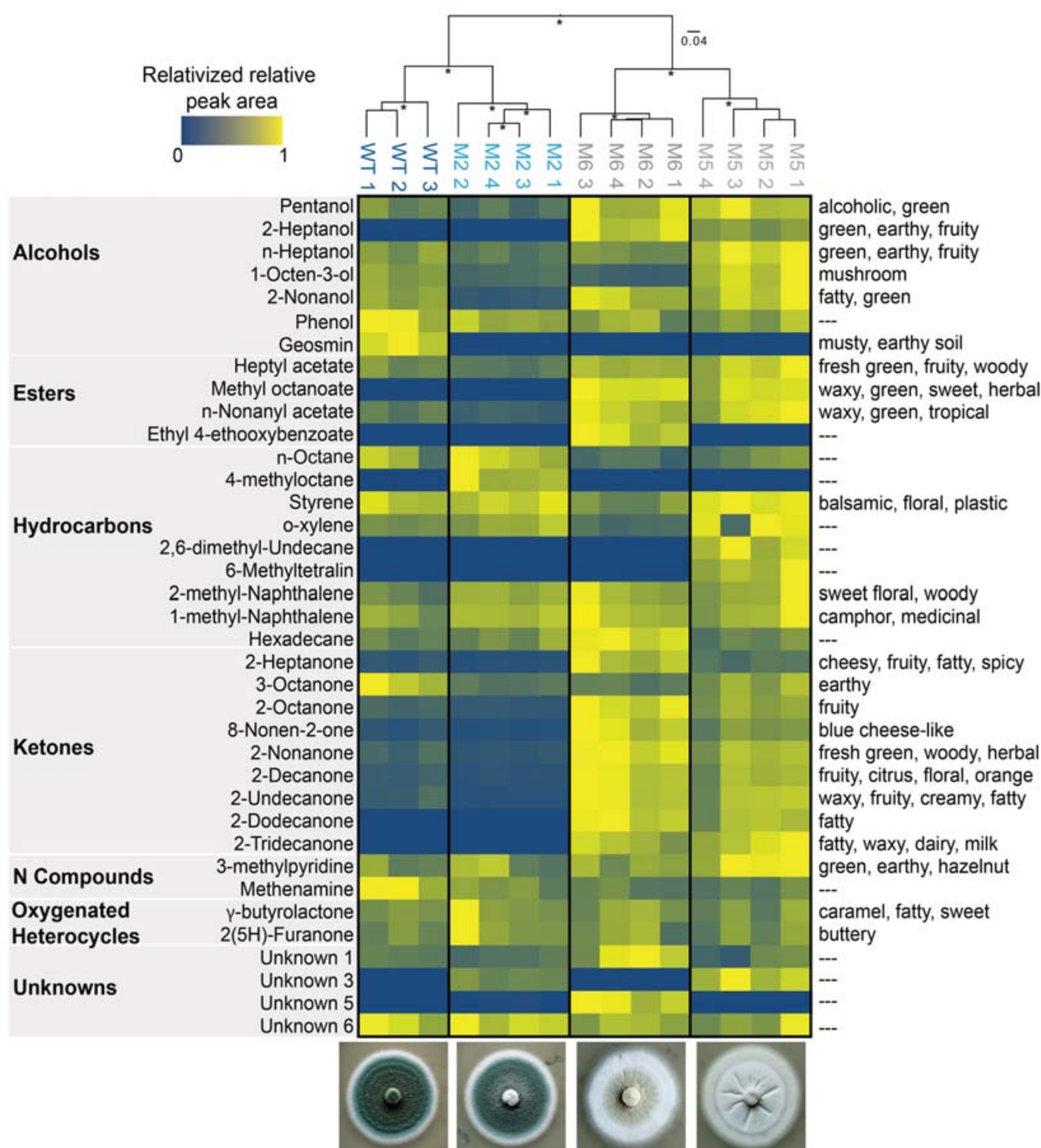

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843 **Figure 3: Volatile organic compound (VOC) production of wild-type and domesticated *Penicillium*.**
844 Because total concentrations of VOCs are highly variable across different compounds, visualization was
845 simplified by relativizing the relative peak area from GC-MS chromatograms within each VOC to the
846 highest concentration detected for that VOC. Only VOCs that were detected across all replicates are
847 shown. See Table S3 for all VOCs and their relative peak area values. The UPGMA tree is clustering the
848 VOC profiles for each replicate based on Bray-Curtis dissimilarity. Asterisks indicate clusters with > 70%
849 bootstrap support. WT = ancestor wild-type. M2, M5, and M6 are all mutants. Numbers after strains
850 (1,2,3,4) indicate biological replicates. Because of accidental sample loss during processing, only three
851 biological replicates were collected from WT. Descriptors on the right are known aroma qualities of
852 detected VOCs.

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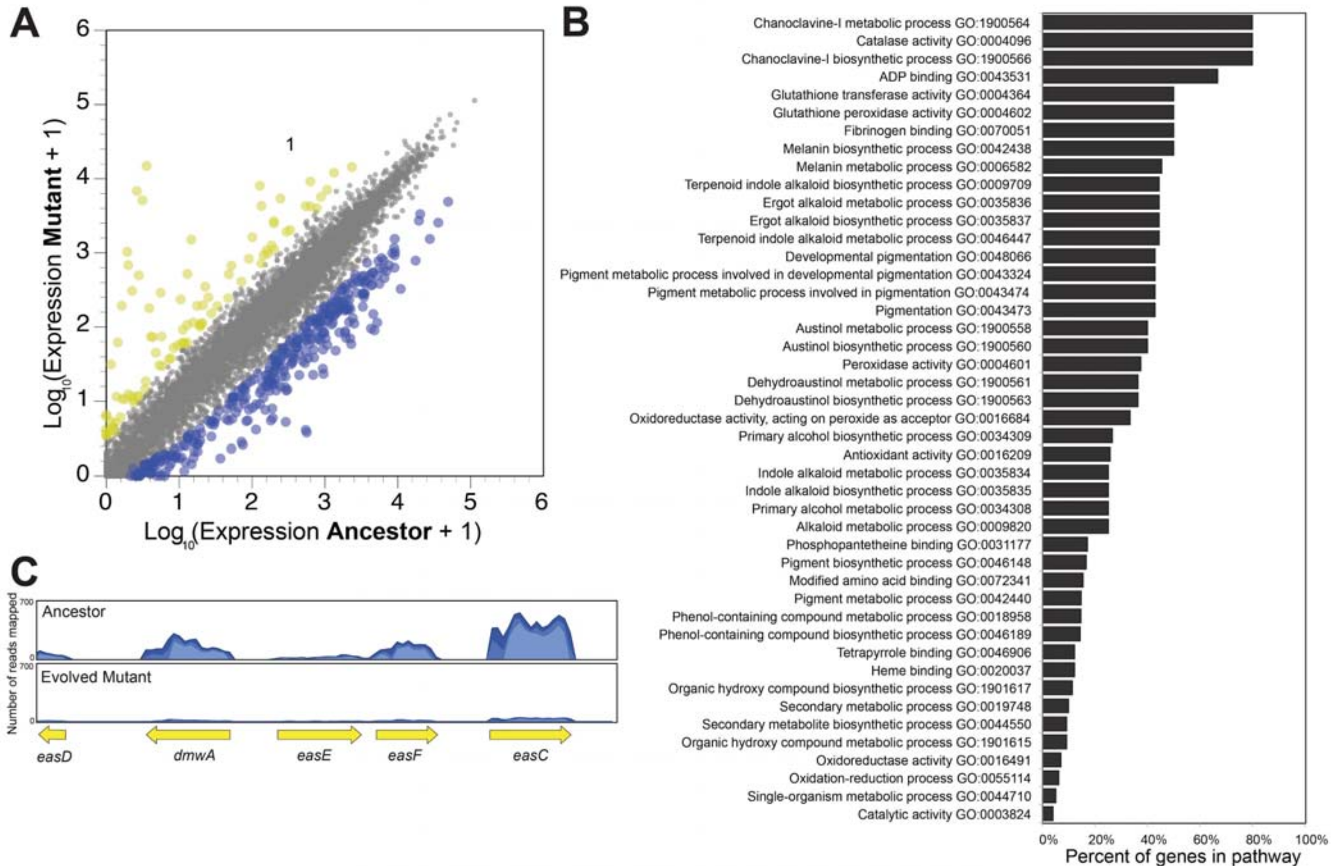

860 **Figure 4: Experimental domestication shifts global gene expression of Penicillium on cheese. (A)**
861 Differences in gene expression between ancestor (wild-type) and mutant strain M5 *Penicillium commune*
862 162_3FA. Each dot represents a transcript from across the genome. Yellow dots represent those
863 transcripts that had higher expression and blue dots represent those transcripts that had lower expression
864 (5-fold change in expression, FDR corrected p-value < 0.05). **(B)** Pathway enrichment analysis showing
865 distribution of GO terms that were significantly enriched in genes with decreased expression in Mutant
866 (strain M5). **(C)** Representative mapping of reads to the ergot alkaloid synthesis (*eas*) gene cluster.

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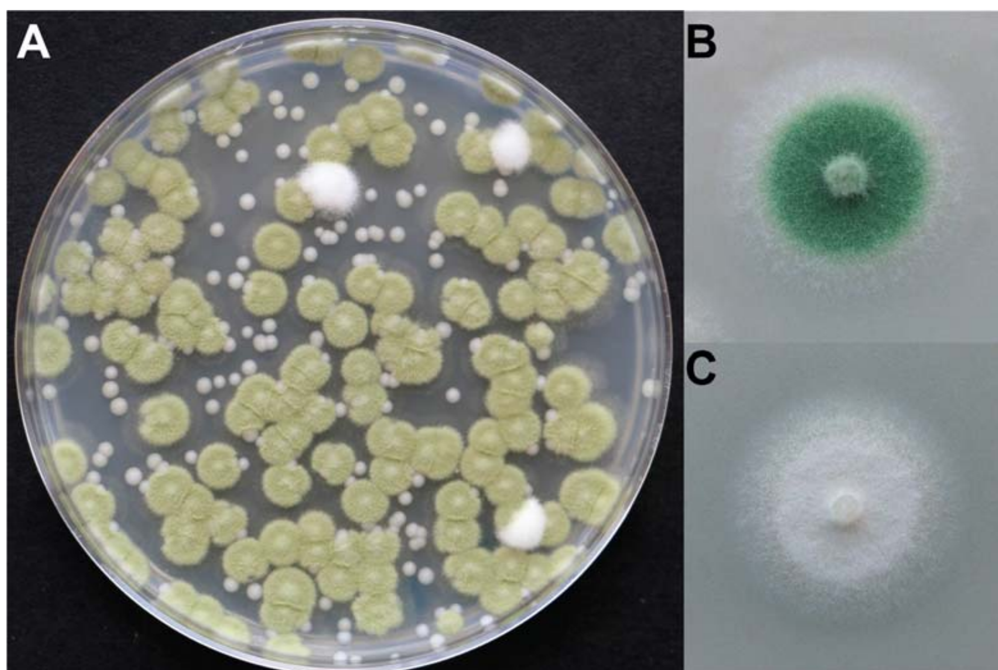

913 **Figure 5: Phenotypic mutants of *Penicillium* are present in cheese caves. (A)**
914 A plate showing *Penicillium* sp. strain 12 isolated from from a cheese cave in Vermont, USA. The fuzzy
915 white colonies are white mutants that exist at low frequencies in this fungal population. The small smooth
916 beige colonies are the yeast *Debaryomyces hansenii*. **(B)** Wild-type phenotype of the mold isolated from
917 the population and grown on cheese curd agar. Green pigmentation is different between A and B
918 because A is grown on plate count agar and B is grown on cheese. **(C)** A white mutant isolated from the
919 same population as the wild-type.
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931 # SUPPLEMENTAL FIGURE and TABLE LEGENDS

932 **Figure S1: The genome-scale phylogeny of the genus *Penicillium***
933 (A) Concatenation phylogeny with section denominations. The two strains used in the
934 experiments described in the text (shown in bold) are placed within section *Fasciculata*.
935 Furthermore, *Penicillium commune* strain 162_3FA is closely related to *Penicillium biforme* and
936 *Penicillium camemberti*. Inset depicts phylogeny with branch lengths representing substitutions
937 per site. *Penicillium* sp. MB was isolated from a natural rind cheese at the same time as the
938 other two strains and was sequenced as part of this work, but it was not used in the experiments
939 described. (B) Comparison of concatenation-based (left) and coalescence-based (right)
940 phylogenies reveals only one instance of incongruence. Specifically, whereas *P. biforme* is
941 placed sister to *P. commune* 162_3FA in the concatenation analysis, coalescence supports *P.*
942 *camemberti* as sister to *P. commune* 162_3FA. All internal branches received full support
943 except the coalescence-inferred internal branch where *Exilicaulis* and *Lanata-divaricata* split,
944 which received a local posterior probability value of 0.97. Branch lengths reflect substitutions /
945 site for concatenation and coalescence units for the coalescence inferred phylogeny.
946
947 **Figure S2: Population size of *Penicillium commune* 162_3FA when evolved alone and**
948 **with a community of cheese microbes.** Lines connect points representing mean colony
949 forming units (CFUs) of four replicate populations and error bars represent one standard
950 deviation of the mean. Total CFUs in the *Penicillium* + community treatment was significantly
951 different from *Penicillium* alone (repeated-measures ANOVA $F_{1,6}$= 10.3, p = 0.02).
952
953 **Figure S3: Experimental evolution of *Penicillium* sp. 12 alone and with a community of**
954 **cheese rind microbes. (A)** Population size of *Penicillium* sp. 12 when evolved alone and with a
955 community of cheese microbes. Lines connect points representing mean mutant phenotype
956 frequencies of four replicate populations and error bars represent one standard deviation of the
957 mean. Total CFUs in the *Penicillium* + community treatment was significantly different from
958 *Penicillium* alone (repeated-measures ANOVA $F_{1,6}$= 16.8, p =0.006). Lines connect points
959 representing mean colony forming units (CFUs) of four replicate populations and error bars
960 represent one standard deviation of the mean. **(B)** Phenotypic mutant frequency of *Penicillium*
961 sp. 12 when evolved alone and with a community of cheese microbes. Lines connect points
962 representing mean mutant phenotype frequencies of four replicate populations and error bars
963 represent one standard deviation of the mean. Mutant frequency in the *Penicillium* + community
964 treatment was significantly different from *Penicillium* alone (repeated-measures ANOVA $F_{1,6}$=
965 20.1, p <0.005).
966
967 **Figure S4: Population size of *Penicillium commune* 162_3FA when evolved in different**
968 **cheese nutrient environments.** "Normal cheese" = 10% cheese curd in agar medium. "Low
969 cheese" = 1% cheese curd in agar medium. "Alternating normal/low" = alternating 10% and 1%
970 cheese curd at each transfer. The "Low cheese" treatment suppressed population size
971 (repeated-measures ANOVA $F_{2,9}$= 105.1, p <0.0001, with Tukey's HSD post-hoc tests). Lines
972 connect points representing mean colony forming units (CFUs) of four replicate populations and
973 error bars represent one standard deviation of the mean.
974
975 **Figure S5: Stability of *Penicillium commune* 162_3FA mutant phenotypes.** Mutants were
976 transferred weekly to new cheese curd agar and colony morphology was photographed. The
977 white mutant morphology was stable over time.
978

979 **Table S1A:** Description of mutant classes and specific mutant isolates identified in the evolution

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980 of *Penicillium commune* strain 162_3FA.

981 **Table S1B:** Distribution of mutant types across replicate populations and transfers in the
982 experimental evolution of *Penicillium* commune 162_3FA. Data below show the number of
983 colonies of Ancestor or mutants counted from 10-4 dilution plates of experimental populations.
984 T1, T2, etc. = transfer number.
985
986 **Table S2A:** Description of specific mutant isolates identified in the evolution of *Penicillium* sp.
987 strain #12
988
989 **Table S2B:** Distribution of mutant types across replicate populations and transfers in the
990 experimental evolution of *Penicillium* sp. #12. Data below show the number of colonies of
991 Ancestor or mutants counted from 10-4 dilution plates of experimental populations. T1, T2, etc.
992 = transfer number.
993
994 **Table S3:** Overview of all volatile organic compounds detected in the ancestor (ANC), and
995 mutants M2, M5, and M6 of *Penicillium commune* strain 162_3FA.
996
997 **Table S4:** Overview of genes that were differentially expressed bewteen the ancestor and
998 mutant M5 of *Penicillium commune* strain 162_3FA
999