1 **Maintenance of microbial cooperation mediated by public goods in**

2 **single and multiple traits scenarios**

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9 **ABSTRACT**

10 Microbes often form densely populated communities, which favor competitive

11 and cooperative interactions. Cooperation among bacteria often occurs through

12 the production of metabolically costly molecules produced by certain individuals

13 that become available to other neighboring individuals, called public goods. This

14 type of cooperation is susceptible to exploitation, since non-producers of a

15 public good can benefit from it while saving the cost of its production (cheating),

16 gaining a fitness advantage over producers (cooperators). Thus, in mixed

17 cultures, cheaters can increase in frequency in the population, relative to

18 cooperators. Sometimes, and as predicted by simple game-theoretic

19 arguments, such increase in the frequency of cheaters causes loss of the

20 cooperative traits by exhaustion of the public goods, eventually leading to a

21 collapse of the entire population. In other cases, however, both cooperators and

22 cheaters remain in coexistence. This raises the question of how cooperation is

23 maintained in microbial populations. Several strategies to prevent cheating have

24 been described involving a single trait and a unique environmental constraint. In

25 this review, we describe current knowledge on the evolutionary stability of

26 microbial cooperation, discussing recent discoveries describing the

27 mechanisms operating in multiple traits and multiple constraints settings. We

28 conclude with a consideration of the consequences of these complex

29 interactions, and we briefly discuss the potential role of social interactions

30 involving multiple traits and multiple environmental constraints in the evolution

31 of specialization and division of labor in microbes.

INTRODUCTION

33 Microbes often live in highly dense communities where interactions among

34 individuals are both inevitable and essential (1). In uni-directional interactions

35 between two individuals, the one which engages in an action can be defined as

36 the 'actor' and the one influenced by the effects of the action as the 'recipient'. If

37 a social behavior influences positively the fitness of the recipient regardless of

38 its fitness effect on the actor, it is considered a cooperative behavior (2, 3).

39 Cooperation in microbes often involves the production of public goods by the

40 actor (cooperator) which can benefit both the actor and the recipient. The nature

41 of these public goods and the mechanisms by which they affect the fitness are

42 diverse: materials to generate protective structures (e. g. exopolysaccharides to

43 generate biofilms), toxins to kill competitors, enzymes to digest food,

44 biosurfactants for group motility, proteins to detoxify the environment, molecules

45 to scavenge nutrients, etc. (4, 5).

46 Cooperation, however, can be vulnerable to exploitation, as individuals that are

47 not contributing to the cooperative behavior (cheaters) by not producing public

48 goods can benefit from them. Given that cheaters are not paying the cost but

49 benefit from the cooperative behavior, they gain a relative fitness advantage

50 over cooperators (2, 3). Thus, in mixed populations, cheaters can increase in

51 frequency, sometimes causing loss of cooperation and a decrease in population

52 size. In other cases, however, there is maintenance of cooperation, even if

53 there is a shift in the proportions of cooperators and cheaters in the population.

54 The study of how cooperation is maintained in populations led to the

55 identification of different mechanisms for protection against possible

56 exploitations by cheaters (recently reviewed in 6).

57 **MECHANISMS TO PREVENT CHEATING ON SINGLE TRAITS**

58 *Quorum sensing.* Bacteria can produce, secrete and detect signaling

59 molecules, called autoinducers, whose concentration in the medium increases

60 as cells divide. When this concentration reaches a certain threshold, bacteria

61 engage synchronously in a coordinated response. This process is called

62 quorum sensing, since it enables bacteria to make coordinated 'decisions'

63 depending on their '*quorum*' (7, 8, 9).

64 The expression of genes involved in the production of public goods is often

65 controlled by quorum sensing (10, 11, 12, 13), which causes the public goods to

66 be produced only when cooperative individuals are able to reach the quorum

67 threshold. Thus, in this scenario, public goods production only occurs when

68 cooperators are in greater numbers, precisely when cooperative behavior

69 provides the greatest benefit: at high bacterial density, there is more efficient

70 usage of public goods than at low bacterial density, where accessibility is lower

71 (14). Therefore, via quorum sensing regulation, production of public goods is

72   restricted to the conditions where it is more productive for the producer cells,

73   thus decreasing the chances for cheaters to outcompete cooperators and

74   contributing to preventing the complete loss of the cooperative behavior (15)

75   (**Fig. 1A**).

76   Protection of cooperation mediated by traits regulated by quorum sensing has

77   been both inferred theoretically (16, 17) and observed experimentally (18, 19,

78   20, 21, 22). However, quorum sensing cheaters still arise. For example, it has

79   been shown that mutants of *Pseudomonas aeruginosa* blind to the quorum-

80   sensing signal can avoid the cost of producing the public good regulated by

81   quorum sensing (23, 24, 25).

82   Bacteria, however, have mechanisms to avoid the spread of these types of

83   mutants. An interesting example involves *Bacillus subtilis* (26). Like other

84   bacterial species, wild populations of *B. subtillis* display a high degree of

85   diversity in functional quorum-sensing alleles, called pherotypes. Each

86   pherotype responds to an autoinducer similar to its own but not to autoinducers

87   produced by other pherotypes. As such, a minority pherotype exploits the public

88   goods produced by the majority of pherotypes (hence having the same fitness

89   as obligatory cheaters) but resumes the production of the public good on its

90   own when its density reaches a high value (26). Moreover, if cheaters take over

91   a population by exploiting a public good regulated by quorum sensing, the

92   decrease in the overall population density can go below the quorum threshold

93   and that would cause the cooperators to seize the production of this public good

94   due to the quorum sensing regulation. In absence of the public good and with

95   the cooperators saving the cost of its production, the fitness advantage of the

96   cheaters would decrease and the population would stay in a polymorphic state.

97   We recently provided experimental evidences of this; we showed that when

98   cooperators are forced to produce constitutively the quorum sensing-regulated

99   good (by adding exogenous quorum sensing signal), cheaters can invade the

100   population to fixation, thereby causing full loss of cooperators and the

101   subsequent loss of the public good trait (27). Thus, it can be said that the

102   quorum sensing regulation of a public good trait can prevent the extinction of

103   the cooperator phenotype in the population and preserve the genetic

104   architecture of quorum sensing (21, 27).

105

106   **Partial privatization**. In contrast to public goods, private goods are only

107   accessible to producers, making cheating practically impossible. Privatization of

108   public goods is well documented in yeast (28, 29, 30). Invertase is an

109   extracellular enzyme produced by *Saccharomyces cerevisiae* that hydrolyzes

110   sucrose into glucose and fructose, which then can be used by the entire

111   population. Thus, the products of invertase activity mostly function as public

112   goods. However, invertase producers have a certain degree of priority to access

113   the monosaccharides generated by the invertase activity, preventing cheating

114   on this trait (**Fig. 1B**). Although 99% of the monosaccharides are accessible to

115   the entire population and therefore can be used by cheaters, the 1% of

116 privatized monosaccharides accessible only to cooperators is enough to avoid

117 cheaters to outcompete them. Even though a mutant strain that does not

118 produce invertase is able to invade a population of invertase producers, the

119 latter are also able to increase in frequency if initially at very low frequencies.

120 This leads to a steady state coexistence of invertase producers and cheaters

121 (29). Interestingly, there is also density dependence fitness. The fitness of

122 mutants is lower when the total population density is low because there are not

123 enough cells producing invertase (31). In other words, some goods are public if

124 population density is high, but can only be used privately if density is low. The

125 dependence of the fitness of cheaters on population density seems to simply

126 result from the fact that, at higher densities, cheaters are closer to producers of

127 the public good. This was demonstrated through mathematical models and

128 experimental data obtained from *P. aeruginosa* producing iron-scavenging

129 siderophore molecules as public goods (32). Other systems for which it has

130 been shown that the fitness of cheaters depends on total population density

131 include: the production of siderophore molecule enterochelin by *Escherichia coli*

132 (33), the production of adhesive exopolysaccharides (EPS) in *B. subtilis* biofilms

133 (34) and the synthesis of the enzyme β-lactamase, which degrades β-lactam

134 antibiotics such as ampicillin, by *E. coli* (35). In the latter example, non-

135 producers of β-lactamase may still survive in mixed populations at high cell

136 density (when sensitive bacteria grow close to producers), showing that

137 antibiotic resistance can also display positive density-dependence (35, 36, for a

138 review about degradation and modification of antibiotics, see 37). These

139 examples, taken from very different systems, show that privatization of

140 extracellular public goods is efficient at low, but not necessarily at high density.

141 As a consequence, in these systems, usually neither producers nor cheaters

142 reach fixation.

143 Although perhaps not perfect, in nature one finds examples of paths towards

144 privatization. A very interestingly example is the molecular structure of iron-

145 scavenging siderophores (38). Using data from 189 secreted siderophores

146 (considering three different parameters for each enzyme) from 124 bacterial

147 species, Kummerli *et al.* showed that their diffusivity inversely correlate with the

148 structure level of the habitat they occupy. That is, highly diffusible siderophores

149 are preferentially present in species living in structured habitats, such as soil

150 and hosts, while poorly diffusible siderophores are mostly secreted by species

151 from seawaters and other unstructured habitats.

152

153 ***Policing.*** Policing can be defined as an action that influences negatively the

154 fitness advantage of cheaters, by either limiting the benefit that cheaters obtain

155 from the cooperative behavior or by increasing the advantage that it provides to

156 cooperators (39) (**Fig. 1C**). Several examples of policing against cheaters have

157 been repeatedly observed in many eukaryotes (40, 41, 42, 43) and in bacteria

158 (44, 45, 46). For instance, in *P. aeruginosa*, the production of cyanide and the

159 resistance against it are coupled traits, both regulated by the RhlR system (47,

160 48), which is in turn controlled by LasR (49, 11), the master regulator of quorum

161 sensing (50, 51, 52). LasR controls the production of many public goods,

162 including elastase (50, 51), a secreted metalloprotease capable of digesting

163 proteins, such as casein, into amino acids (53, 54). Therefore, in casein

164 medium, *lasR* mutants can cheat (24). However, mutations in *lasR* cause the

165 loss of cyanide production and, importantly, the resistance to it. Thus, LasR+

166 cells (cooperators) are immune to the cyanide they produce, whereas *lasR*

167 mutants (cheaters) are not. In that scenario, the coupling of cyanide immunity

168 with a public good production polices against cheater invasions, preventing the

169 collapse of the population and therefore protecting cooperation (45). Inter-

170 species policing, involving reciprocity as a punishing/rewarding behavior, have

171 been observed in polymicrobial communities (55) and in mutualistic

172 relationships between certain bacteria and their hosts, such as *Rhizobia*-plants

173 (56, 57) and *Vibrio fischeri*-squids (58, 59).

174

175 **Spatial structure.** Spatial structures, such as biofilms, favor related cells to

176 stay in close proximity and limit the diffusion of the public goods, increasing the

177 benefit for producers and diminishing the chances for cheating (60, 61) (**Fig.**

178 **1D**). Biofilms are one of the most common ways that bacteria generate spatial

179 structure (62). Lipids, polysaccharides, and nucleic acid molecules secreted by

180 bacteria function as an extracellular matrix (63) where the cells can anchor and

181 switch from motile to static phase (62). The matrix limits the diffusion of the

182 public goods secreted by cooperators (64, 65), making them to be less

183   accessible to cheaters, reducing their fitness advantage and therefore
184   preventing cheating (66, 61, 64, 67). Interestingly, predictions based on
185   computer simulations of multi-species biofilm formation showed that, when
186   resources abound and therefore competitive effects are less severe, the
187   production of public goods may be maintained in a focal species simply due to
188   the mere presence of cells belonging to other species, which completely
189   surround producers of the exoproduct. This results in the insulation of
190   producers from non-producer cheater mutants of the same species, preventing
191   intraspecific competition between the two social actors, therefore favouring
192   within-species cooperation (68). Interestingly, biofilms often require the
193   production of a costly exoproduct, namely EPS (adhesive exopolysaccharides),
194   that can be a public good itself and, in thosee cases, is susceptible to
195   exploitation (64). In other cases, however, similar but non-costly exoproducts
196   involved in biofilms formation can be social but relatively nonexploitable (69).

197   A particular effect of spatial segregation can occur, for instance, in a population
198   divided in a set of subgroups composed by mixtures of cooperators and non-
199   cooperators in different proportions. If the cooperative behavior is costly, a
200   decrease in the proportion of cooperators within each subgroup is expected.
201   However, if the proportion of cooperators in each subgroups is sufficiently
202   different, the overall proportion of cooperators in the whole population may
203   increase. This is a statistical effect termed "Simpson's paradox" after its
204   discoverer, the statistician Edward H. Simpson (70). This effect was later shown
205   by D. S. Wilson to be relevant for the problem of maintenance of cooperation

206   (71, 72). The reason for the increase in the proportion of cooperators in the

207   overall population is that subgroups with more cooperators are also more

208   productive, therefore resulting in higher cell yields. Interestingly, this effect is in

209   fact a direct consequence of kin selection (selection of a costly trait due to

210   increased fitness of individuals genetically related to those possessing that

211   trait), as both kin selection and group selection (selection at the level of group

212   instead of at level of the individual) can be shown to be mathematically

213   equivalent (73). If, from time to time, subpopulations join and then separate

214   again, there should be a mechanism assuring a certain variance of cooperators

215   among subgroups for the proportion of cooperators to continue increasing (71).

216   This phenomenon was experimentally observed in microbial communities (74,

217   75). Griffin *et al.* showed that if competition is local, the higher productivity of

218   groups with more cooperators is (exactly) compensated by a stronger

219   competition between kin individuals (74, 76, 77, 78, 2). Therefore, this effect is

220   mostly observed if competition is global.

221

222   **Facultative cooperation.** Another bacterial mechanism to overcome

223   cheating is facultative cooperation, which can operate through metabolic

224   prudence (79). *P. aeruginosa* can produce high levels of rhamnolipids (80), a

225   carbon-rich surface polymer, which enables bacteria swarming to colonize new

226   niches (81, 82). Xavier and colleagues showed that this bacterium only invests

227   in rhamnolipid production under conditions of metabolic unbalance, i. e. when

228   carbon concentration is higher than nitrogen concentrations. Bacteria require

229   both carbon and nitrogen to divide, whereas the production of rhamnolipids

230   requires carbon but not nitrogen. Thus, by investing in rhamnolipid production

231   only when the C/N ratio does not favor cell division, bacteria minimize the cost

232   of this public good under conditions of strong competition, preventing

233   cooperators to be outcompeted by non-producers (cheaters). Under nitrogen

234   deprivation, the induction of rhamnolipid production allows bacteria to swarm

235   away towards areas that might contain both nitrogen and carbon, where

236   bacteria can start dividing again. Thus, facultative induction of cooperative

237   behaviors reduces the chances of cheaters to invade bacterial populations of

238   producers (79). Xavier *et al.* showed that, if production is constitutive,

239   maintenance of cooperation would be possible only if producers of rhamnolipid

240   were mostly surrounded by other producers of rhamnolipid, a very stringent

241   situation. By facultatively producing rhamnolipid, however, producers strongly

242   decrease the cost of its production, what allows the maintenance of cooperation

243   even if producers and non-producers are mixed (83) (**Fig. 1E**). Another

244   mechanism of facultative cooperation involves the production of a public good

245   molecule (in this case, the siderophore pyoverdine in *P. aeruginosa*) during

246   exponential phase, but not at stationary phase (84). Interestingly, production of

247   this type of reusable public good molecules with long durability only when

248   necessary is also a way of diminishing their production cost (85).

249

250   ***Antagonistic pleiotropy.*** Antagonistic pleiotropy can also contribute to

251   stabilize cooperation (86). Indeed, some mutations that cause individuals to

252   become cheaters (by ceasing to cooperate with other individuals), have

253   pleiotropic effects and these mutants also become unable to perform a private

254   behavior or to accomplish a fundamental metabolic step (87). Therefore, the

255   pleiotropic effects of these mutations result in a direct fitness cost to these

256   cheaters (**Fig. 1F**). Antagonistic pleiotropy has been observed in different

257   systems, including instances in which cooperation is mediated by public goods.

258   In these cases, the production of public and private goods is coupled (88). For

259   instance, LasR regulates the production of various public and private goods in

260   *P. aeruginosa* (10, 11, 12, 13). As mentioned before, one of the LasR-regulated

261   public goods is elastase (50, 51). Thus, when casein is the sole carbon source,

262   *lasR* mutants can behave as cheaters (24). However, *lasR* also controls the

263   production of the enzyme necessary to digest adenosine (89, 90). In contrast to

264   elastase, adenosine is maintained intracellularly, thus acting as a private good.

265   When casein and adenosine are the only carbon sources available, cooperators

266   (LasR+ cells) can use both while *lasR* mutants (cheaters) can only use the

267   casamino acids generated by elastase activity, and not adenosine (90). The

268   extra carbon source accessible to the cooperators provides them with an

269   advantage over the cheaters, thus protecting the population against cheater

270   expansion (88, 91).

271

272 **Influence of phenotypic heterogeneity on cooperation.** In certain

273 cases, the different social roles involved in cooperation can be played by

274 genotypically identical strains that differentiate phenotypically into functionally

275 diverse social actors. Phenotypic differentiation in distinct cell types interacting

276 cooperatively protects the population against invasion by cheaters for any of the

277 functions exerted by the different cell types, since each cell type saves the costs

278 of producing the public goods generated by the other cell types, and this

279 reduces the competitive advantage of cheaters for either of the traits. This type

280 of interactions has been observed in biofilms of *Myxococcus xanthus*, *B.*

281 *subtilis*, and *P. aeruginosa* (92). A similar example of cooperation mediated by

282 phenotypic heterogeneity was described in *B. subtilis* sliding motility, where the

283 division of labor of two cell types (producers of either surfactin or extracellular

284 matrix) allows a special structural organization that increases the rate of colony

285 expansion, generating a strong ecological advantage and protecting the

286 bacterial population against non-producers of either surfactin or matrix (93).

287 There are also examples of cooperation mediated by phenotypic variation in

288 host-pathogen interactions. For instance, the bistable expression of virulence

289 determinants during infection of the mice gut by *Samonella* Typhimurium

290 protects the bacterial population against the invasion by avirulent mutants,

291 thereby promoting the evolutionary stability of virulence (94).

292 **COOPERATION AND CHEATING IN MULTIPLE TRAITS**

293 **SCENARIOS**

294 The mechanisms described above allow maintenance of cooperation and

295 prevent cheating in uni-directional interactions, involving a single trait where the

296 actors are either full cooperators or full cheaters for that trait. However,

297 populations are often polymorphic and rely on more than one trait and, typically,

298 diverse environmental constraints simultaneously affect social interactions

299 among individuals. Thus, in natural settings, the most prevalent scenario is

300 possibly that of multi-directional interactions under multiple environmental

301 constraints. In these 'multiple-traits / multiple-constraints' settings, an individual

302 can be "actor" for some traits and "recipient" for others, thus behaving

303 simultaneously as a cooperator and as a cheater for different traits (95). Thus,

304 in nature, interactions among different players can affect their social roles,

305 leading to complex dynamics where the notions of "cooperation" and "cheating"

306 are context dependent. An analogy to illustrate this concept could be a

307 hypothetical study of a hunter/gatherer society by an anthropologist. In such

308 populations, both hunting and gathering vegetables can be considered

309 cooperative behaviors. Hunters are "actors" for meat obtention and "recipients"

310 for vegetable collection, whereas gatherers are "actors" for vegetable obtention

311 and "recipients" for meat acquisition. Thus the population, which requires both

312 meat and vegetables, relies on this mutualistic interaction for its sustainment. If

313 the anthropologist would study exclusively hunting as a cooperative trait, the

314 consequence would be considering hunters as cooperators and gatherers as

315 cheaters, since the latter do not hunt but eat the meat obtained by the hunters.

316 And *vice versa*, if the anthropologist would focus only on gathering, the

317 consequence would be classifying hunters as cheaters, because they are not

318 gathering but still benefit from it. To understand the social roles of both hunters

319 and gatherers, the anthropologist would need to consider simultaneously the

320 two players and the two traits involved.

321 Similarly, in order to understand how cheating affects the dynamics of other

322 biological systems involving multiple traits, the complex web of inter-species

323 and inter-strain interactions must be addressed. There has been a growing

324 literature focusing on the inter-strain and inter-species interactions in these

325 communities. Brown and Taylor (95) established a theoretical framework for the

326 evolution of joint social traits. They showed that, if two social traits interact,

327 newly arising behavior not anticipated just by considering each trait alone, can

328 occur. For example, policing mechanisms to refrain rivalry (within-groups

329 competitions) (96) or excludability (through privatization) (97, 98) are mostly

330 selected when relatedness is low. Relatedness is low when the probability that

331 a cheater interacts with a cooperator is almost as high as the probability that a

332 cooperator interacts with a cooperator. If a policing mechanism is effectively

333 decreasing rivalry within groups of individuals, conditions may be favorable

334 even when relatedness is low (95).

335

336 Cross-feeding is a relationship wherein one genotype consumes products of

337 another (unidirectional cross-feeding). However, in some cases, cross-feeding

338 can also involve interactions where more than one trait is involved. This can

339   occur when the consumer genotype also produces a metabolite useful for the

340   producer (bidirectional cross-feeding) (99). Therefore, bidirectional cross-

341   feeding is an example of multiple-traits / multiple-constraints interaction and,

342   under certain circumstances, it can influence cooperation and diversity. For

343   instance, a set of mathematical models, computer simulations and experiments

344   involving either a community formed by genetically engineered *Saccharomyces*

345   *cerevisiae* cooperating through metabolite-exchange or biofilms in which the

346   methane-producing archaeon *Methanococcus maripaludis* cooperates with the

347   bacterium *Desulfovibrio vulgaris* showed that, if the interaction between two

348   species confers a strong benefit to both species, they intermix and there is

349   cooperation between them. The intermix occurs by forming alternative layers.

350   This pattern was confirmed using two different synthetic systems composed by

351   two microbial species, but simulations predict the same intermixed pattern if

352   several species strongly interact (100). Interestingly, with such spatial structure,

353   there is maintenance of cooperation despite the appearance of cheater

354   mutants. The reason is that, the less cheater cells contribute to the other

355   partner, the more it were disfavored and eliminated (101). In another work,

356   computer simulations showed that the stronger is the interdependence between

357   interacting species, the higher is the number of mutualisms and community

358   productivity. If, in contrast, interdependence is low, conflict takes over and

359   interacting species tend to separate (102).

360   In these cases of strong interdependence upon the settlement of mutual

361   interchange of metabolites, one of the partners may lose biosynthetic functions,

362 thereby threatening cooperation. Waite and Shou used engineered *S.*
363 *cerevisiae* to recreate such scenario and observed that an adaptive race of
364 cooperators and cheaters may give the opportunity to cooperators to defeat
365 cheaters (103). However, using a similar system in *E. coli*, D'Souza and Kost
366 showed that the loss of metabolic functions is strongly selected for when the
367 corresponding metabolites can be extracted from the environment (104, 105,
368 106), in particular from cross-feeding among coexisting strains (104). Thus, the
369 development of strong interdependences can destabilize cooperation in
370 bidirectional cross-feeding scenarios. However, cooperation mediated by
371 bidirectional cross-feeding can be protected through different mechanisms, as it
372 is well illustrated in two recent papers. Germerodt *et al.* used a cellular
373 automaton to simulate a structured habitat where there were interactions
374 between six experimentally characterized genotypes. These genotypes differ in
375 their ability and propensity to produce amino-acids. Upon varying several
376 ecological parameters, the authors concluded that, in most parameters sets,
377 obligate cross-feeders arise. Moreover, the presence of cross-feeders helped to
378 stabilize genotypic diversity, while supplementing the system with required
379 metabolites significantly reduced it (107). In a second recent study, experiments
380 performed with two bacterial species, *Acinetobacter baylyi* and *E. coli,* able to
381 exchange amino-acids showed that, in a liquid environment, non-cooperating
382 strains were favored. However, in a structured environment, the authors
383 observed that auxotrophs separate from cross-feeders, hence allowing for the
384 stabilization of cooperative cross-feeding (108).

385

386   Recently, there has been an increasing number of studies addressing bacterial

387   interactions in multiple-traits / multiple-constraints scenarios other than

388   bidirectional cross feeding. Ross-Gillespie and colleagues (109) generated

389   general predictions for different settings involving two traits, and tested these

390   predictions by analyzing the evolutionary trajectories of production of two public

391   goods genetically and functionally interlinked (the siderophores pyoverdine and

392   pyochelin) by wild-type *P. aeruginosa* during experimental evolution under

393   different levels of iron limitation (110, 111, 112). Under strong iron deprivation,

394   both non-producers of pyoverdine and pyochelin can act as cheaters (74, 109).

395   Because, under these conditions, pyoverdine-defective mutants have a larger

396   relative fitness, a gradual decrease in pyoverdine production was observed,

397   concomitant with an increase in pyochelin production. This indicates the

398   appearance of pyoverdin non-producing cheaters. In contrast, although

399   production of the two siderophores decreases over time under moderate iron

400   limitation (where only pyochelin mutants are able to cheat), the drop is stronger

401   in the case of pyochelin, revealing the rise of mutants on its production (109).

402   Thus, the work by Ross-Gillespie *et al.* suggests that regulatory cross-link

403   between two traits may help to stabilize cooperation as pyoverdine-defective

404   cheaters become pyochelin-producing cooperators, which reduces their relative

405   advantage (Fig. 2A).

406 Inglis and colleagues (113) used mathematical models and experimental

407 communities of *P. aeruginosa* to demonstrate that the presence of a loner strain

408 (non-producing the main public good but producing a functionally linked one

409 which is less efficient) can lead to rock-paper-scissor dynamics in which

410 cooperators outcompete loners, cheaters outcompete cooperators and loners

411 outcompete cheaters (**Fig. 2B**). In this scenario, the presence of the loner

412 protects cooperation in environments that would otherwise favor cheating and

413 reduction in diversity, such as well-mixed communities (113).

414 Recently, Popat and colleagues (114) explored the interaction between two

415 traits not linked at the regulatory level but connected functionally: production of

416 the quorum sensing molecule *Pseudomonas* Quinolone Signal (PQS) (115) and

417 production of siderophores in *P. aeruginosa*. PQS is a powerful iron chelator

418 that can act as an iron trap (116), decreasing iron availability and therefore

419 increasing the relative fitness of siderophore-defective cheaters. Thus, Popat *et*

420 *al.* showed that production of one social trait may indirectly affect the costs and

421 benefits of another social trait, influencing the social behavior of cheaters for the

422 latter (**Fig. 2C**). The authors speculate that similar mechanisms might have the

423 potential to contribute to preserving cooperation (114).

424 Using a novel tri-partite co-culture system involving three social actors (wild-

425 type *P. aeruginosa* as full cooperator, and *lasR* and *pvdS* mutants, unable to

426 produce   elastase   and   the   siderophore   pyoverdine,   respectively,   as

427 cheaters/partial cooperators) in single- or double-constraint environments

428   (presence of casein as sole carbon source and/or iron limitation), we studied the

429   consequences of the interactions between two regulatorily and functionally

430   independent social traits (27). When the two constraints are imposed (presence

431   of casein as sole carbon source and iron limitation), the *lasR* mutant is a

432   cheater for elastase but a cooperator for pyoverdine, whereas the *pvdS* mutant

433   cooperates for elastase production and is a cheater for pyoverdine production.

434   We observed that, under these conditions, the advantage of the *pvdS* mutant

435   for not producing pyoverdine is higher than that of *lasR* for not producing

436   elastase. As a consequence of the different costs associated with the different

437   traits, in 3-way competitions, *pvdS* causes the cessation of the cheating

438   advantage by *lasR* mutants and dominates the population. This domination of

439   *pvdS* prevents the drastic population collapse otherwise caused by the invasion

440   of *lasR* mutants, which occurs if *pvdS* is not present or in a single-constraint

441   (casein as a sole carbon source) environment. Thus, this two-traits / two-

442   constraints system allowed us to unveil the existence of strong context

443   dependent ecological interactions between cheaters for orthogonal (i. e.

444   independent) social traits, which can induce or prevent a drastic collapse in

445   population fitness (helping to stabilize/destabilize cooperation) (**Fig. 2D**). We

446   also developed a mathematical model to determine the universal factors

447   governing these social interactions. The model showed that social dynamics in

448   multiple-traits / multiple-constraints systems are determined mainly by the

449   differences between the costs of the social traits involved, whereas their

450   benefits only affect population yields. Therefore, the degree of the population

451 collapse induced upon cheaters invasion depends on the benefit provided by

452 the most costly trait. This work highlights the importance of the relative costs

453 and benefits of the different cooperative traits in predicting the outcome for

454 populations in complex environments where multiple traits are required (27).

455

456 **CHEATING ON CHEATERS: A STEP TOWARDS THE DIVISION**

457 **OF LABOR?**

458 Cooperative interactions in multiple-traits / multiple-constraints scenarios tend to

459 generate polymorphism and potentially lead to the evolution of functional

460 dependency, as formulated in the Black Queen Hypothesis (117, 118).

461 Moreover, the simultaneous role of different social actors as cooperators and

462 cheaters, which generate the establishment of these inter-dependent

463 interactions, could be considered as a germinal stage in the path towards

464 functional specialization (119). Thus, social interactions in multiple-traits /

465 multiple-constraints microbial communities can function as an intermediate

466 phase towards the division of labor (120, 121, 122). Interestingly,

467 Hammerschmidt *et al.* showed that, in spatially structured microcosms inhabited

468 by "wrinkly spreader" cooperators and "smooth" cheaters of *Pseudomonas*

469 *fluorescens,* the latter can function as a germ line that facilitates the

470 reproduction of bacterial populations as collectives (123), describing a

471 mechanism for the role of cheaters in the evolution of multicellularity.

472 However, Oliveira and colleagues (124) proposed the existence of certain

473 limitations for cooperation for multiple traits. Through mathematical modeling,

474 they inferred that this type of cooperation evolves under conditions of

475 intermediate genetic mixing. They inferred that the evolution of inter-dependent

476 cooperative exchanges of multiple public goods reduces the overall productivity

477 of the community with respect to genotypes able to produce all the public goods

478 autonomously (124). Thus, there seem to be constraints to the evolution of

479 inter-dependent cooperation for multiple traits in bacterial communities.

480 Interestingly, this model does not consider situations where production of public

481 goods vary in the cost and benefits and, as discussed above, these factors can

482 change dramatically the outcome of social interactions involving multiple traits

483 (27). Additional experimental and theoretical research will help to clarify the

484 extension and limits of social interactions involving multiple traits and multiple

485 constraints in microbial communities.

486 The exploration of these thrilling research avenues will significantly contribute to

487 revealing the complex eco-evolutionary implications of social relationships in the

488 bacterial world. However, the information obtained from the study of these

489 interactions can be considered in a broader perspective. For instance, certain

490 theories in economics, such as "comparative advantage", which describes how

491 individuals, companies, or countries can save time and energy by specializing in

492 the production of one trait while obtaining others by exchanging with different

493 partners (125), are currently being tested in microbial systems (126, 127). Thus,

494 sociomicrobiology not only might help to address biomedical threats (128) but,

495 through these interdisciplinary approaches, might also contribute to further

496 understand socioeconomic challenges.

497 **ACKNOWLEDGMENTS**

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FIGURE LEGENDS

Figure 1. Mechanisms that prevent cheater invasions in single-trait scenarios.

A. Quorum sensing. Bacteria minimize the exploitation by cheaters via collectively producing public goods only when cells reach a given cell density (quorum), which is determined by sensing the concentration of signaling molecules **B. Partial privatization.** Producers of a given public good having prioritized access (represented by thicker green arrows) to it can prevent exploitation by cheaters. **C. I. Policing via direct harm.** By coupling production of a public good with production of a deleterious factor and its corresponding resistance system, cooperators harm cheaters while being immune. **C. II. Policing via reciprocity.** If, in a mutualistic public good exchange relationship, one of the actors stops their production, the other party can downregulate their own production to reciprocate the non-producer. **D. Facultative cooperation.**

545 In the case of metabolic prudence, bacteria produce a public good only when

546 the cell division is not possible; the public good can alter the environment or

547 allow bacteria to move to a new environment where growth is possible, and

548 then the public good production stops and bacteria start dividing. **E. Spatial**

549 **structure.** When sister cells can stick together, producer cells share the public

550 good mostly among other producers, minimizing the exploitation by cheaters. **F.**

551 **Antagonistic pleiotropy.** By coupling production of a public and a private

552 good, cooperators can access both, whereas the cheaters can only have

553 access to the public good. This process diminishes the relative advantage of the

554 cheaters and minimizes exploitation by cheaters.

555 **Figure 2. Cheating and cooperation in multiple traits scenarios**

556 **A. Interlinked public good traits.** Regulatory and functional linkage of public

557 good production affects the course of adaptation depending on the

558 environment. Expression of pyoverdine and pyochelin differ in high and

559 moderate iron limitation environments (**I**); uni-directional inhibition of pyochelin

560 by pyoverdine (**II**) affects the evolution of these traits under different iron

561 limitation (**III**) (109) (the direction of the arrows in (**III**) indicate the changes in

562 siderophore production during propagations of cultures along the experimental

563 evolution). **B. Rock-paper-scissors.** Cheaters can outcompete cooperators,

564 loners can outcompete cheaters, and cooperators can outcompete loners. In

565 triple mixtures, loners reduce the relative advantage of cheaters and thus allow

566 the maintenance of cooperation (113). **C. Effects of one public good trait on**

567 **another via environmental modification.** Changes in the environment caused

568 by a public good trait can affect the production of another, unrelated public

569 good, and eventually affecting the relative fitness of its cheaters (114). **D.**

570 **Cheating on cheaters.** A drastic decrease in the population size due to the

571 invasion of a cheater (elastase-deficient *P. aeruginosa*) occurs in mixed

572 populations in the following scenarios: (**I**) in environments where only that public

573 good (elastase) is necessary for growth; (**II**) when a mutant of a second public

574 good trait (pyoverdine-deficient *P. aeruginosa*) is introduced but only the first

575 public good (elastase) is produced; (**III**) when both public goods (elastase and

576 pyoverdine) are necessary but the population lacks the mutant of the second

577 public good. Invasion of the first cheater and the drastic population collapse due

578 to its invasion is only avoided in the following scenario: (**IV**) when the both

579 public goods are necessary and both cheaters are present, the cheater that

580 avoids producing the most costly public good (pyoverdine-deficient *P.*

581 *aeruginosa*) outcompetes the other (elastase-deficient *P. aeruginosa*) (27).

582 The thicknesses of the arrows indicate relative differences in cost (orange

583 arrows) and benefits (green arrows). Symbols that are the same as in Figure 1

584 are described in the legend of Figure 1 to avoid repetition.

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A

- LOW CELL DENSITY
- QS OFF
- LOW PG PRODUCTION

- HIGH CELL DENSITY
- QS ON
- HIGH PG PRODUCTION

B

C

I. DIRECT HARM

II. RECIPROCITY

D

IF GROWTH IS
NOT POSSIBLE

COMPETITION

IF GROWTH IS
POSSIBLE

E

STRUCTURED
GROWTH

COMPETITION

UNSTRUCTURED
GROWTH

F

COMPETITION

◆ : QS signal

★ : Public good

↷ : Production (cost)

↷ : Coupled production (cost)

↶ : Consumption (benefit)

▲ : Private good

☠ : Toxin

🛡 : Toxin resistence

⚡ : Direct harm

★ : Exchanged public good

⬭ : Cooperator strain

⬭ : Cheater strain

⬭ : Second cooperator strain

A

I

STRONG Fe LIMITATION

PVD

PCH⁻

PVD⁻PCH⁻

CHEATING

MODERATE Fe LIMITATION

PVD

NO CHEATING

STRONG Fe LIMITATION

PCH

PVD⁻

PVD⁻PCH⁻

CHEATING

MODERATE Fe LIMITATION

PCH

CHEATING

II

PVD

PCH

III

PYOVERDINE (PVD) PRODUCTION

MODERATE Fe LIMITATION

STRONG Fe LIMITATION

PYOCHELIN (PCH) PRODUCTION

B

COOPERATOR

CHEATER

OUTCOMPETES

OUTCOMPETES

LONER

OUTCOMPETES

C

PQS

Fe LIMITATION

WT

PVD

PCH

PQS   Fe AVAILABILITY   PVD   PCH   CHEATING

PQS

WT

PVD

PVD⁻PCH⁻

OUTCOMPETES

PCH

D

I

WT

ELASTASE⁻

ELAS

II

WT

ELASTASE⁻

ELAS

PVD⁻

HIGH Fe + CASEIN

III

WT

ELASTASE⁻

PVD

ELAS

IV

WT

ELASTASE⁻

PVD

ELAS

PVD⁻

LOW Fe + CASEIN

Legend

PVD : Pyoverdine

PCH : Pyochelin

WT : Wild-type strain

PCH⁻ : Pyochelin mutant strain

PVD⁻ : Pyoverdine mutant strain

PVD⁻PCH⁻ : Pyoverdine-pyochelin double mutant strain

: High iron limitation

: Moderate iron limitation

LONER : Loner strain

: Alternative public good that the loner strain produces

OUTCOMPETES : Indicates that one strain (back of the arrow) outcompetes another (tip of the arrow)

PQS : Pseudomonas Quinolone Signal

ELAS : Elastase

: Iron-supplemented casein media

: Iron-depleted casein media