1	Microbial competition reduces interaction distances to the low µm-range
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18 Abstract

19 Metabolic interactions between cells affect microbial community compositions and hence 20 their function in ecosystems. It is well-known that under competition for the exchanged 21 metabolite, concentration gradients constrain the distances over which interactions can occur. 22 However, interaction distances are typically quantified in two-dimensional systems or 23 without accounting for competition or other metabolite-removal, conditions which may not 24 very often match natural ecosystems. We here analyze the impact of cell-to-cell distance on 25 unidirectional cross-feeding in a three-dimensional system with competition for the 26 exchanged metabolite. Effective interaction distances were computed with a reaction-27 diffusion model and experimentally verified by growing a synthetic consortium of 1 µm-28 sized metabolite producer, receiver and competitor cells in different spatial structures. We 29 show that receivers cannot interact with producers ~15 µm away from them, as product 30 concentration gradients flatten close to producer cells. We developed an aggregation protocol 31 and created variants of the receiver cells' import system, to show that within producer-32 receiver aggregates even low affinity receiver cells could interact with producers. These 33 results show that competition or other metabolite-removal of a public good in a three-34 dimensional system reduces the interaction distance to the low micrometer-range, 35 highlighting the importance of concentration gradients as physical constraint for cellular 36 interactions.

37 Introduction

Microbial interactions are observed in dense biofilms (0 µm between cells) as well as in
oceans (>100 µm between cells), demonstrating that cells interact at various distances [1–4].
These interactions influence the selection pressure within an environment, and therefore
affect the structure and evolution of microbial communities [5]. As these communities play
an important role in many ecosystems, from global biogeochemical fluxes [6] to human
health [7], understanding and controlling these interactions is of high importance.

44

45 Metabolites or signaling molecules involved in interactions can be exchanged via contact-46 dependent and contact-independent transfer mechanisms. Contact-dependent mechanisms 47 require short cell-to-cell distances and use for instance direct contact between cells, vesicle 48 chains or nanotubes for exchange [5]. Contact-independent mechanisms require passive or 49 active transport of the produced compound to the extracellular space, where it subsequently 50 moves via diffusion and convection [5]. Contact-independent interactions can be local 51 (mainly between neighboring cells) or global (within the whole population), depending on the 52 profile of the concentration gradient. Saccharomyces cerevisiae for instance uses its extracellular enzyme invertase to split sucrose, resulting in a glucose and fructose gradient 53 54 around the cell. At high sucrose concentrations both aggregated and single yeast cells can 55 grow (global interactions), while at low sucrose concentrations only aggregated yeast cells 56 grow (local interactions) [8]. A similar pattern is observed for the extracellular protease of 57 Lactococcus lactis, which activity results in a peptide gradient around the cell. At high cell densities both protease positive and protease negative cells grow, while at low cell densities 58 59 mainly protease positive cells grow, since only they can benefit from their produced peptides 60 [9].

62 Whether contact-independent interactions are local or global depends on the distance between cells and the concentration gradient profile, which is affected by the metabolite source, the 63 64 metabolite-sink and the diffusion and convection rate between them. The metabolite source 65 can for instance be a producer cell [10], a nutrient pool in the environment [11] or an extracellular enzyme [8, 9]. The metabolite-sink can be a metabolite consuming cell [12, 13], 66 67 a metabolite degrading enzyme [14] or the volume of the system, as dilution reduces the metabolite concentration [8, 9]. Although the exact nature of the source and sink are often 68 69 only implicitly mentioned in these studies, their importance is well-known. Costly 70 cooperative interactions are for instance more likely to evolve when cells are close to each 71 other, because cooperators compete with wildtype non-cooperators for the excreted 72 metabolite [5, 15, 16]. Selection for interactions is therefore often done by co-culturing cells 73 on agar plates [17–19], and it is also described that interacting cells evolved aggregating 74 phenotypes [20].

75

76 These examples show that in the presence of a metabolite-removing sink, concentration 77 gradients constrain the distances over which interactions can occur. It is however not clear at what distances such interactions occur. Previous studies either quantified these distances in 78 79 two-dimensional systems [11–13] or without a metabolite-removing sink [13, 14], while 80 natural microbial communities reside in three-dimensional environments in which competing 81 metabolite consumers and other types of metabolite-removing sinks are very likely to be 82 present. We therefore combined computational and experimental analyses to provide a more 83 systematic and quantitative perspective on the impact of cell-to-cell distance on metabolic 84 interactions in three dimensions and in the presence of metabolite-removing sinks. The 85 reaction-diffusion model and experimental results show that in these conditions receiver cells fixed at ~15 µm from glucose producing cells cannot interact with the producer cells, while 86

- 87 producer-receiver aggregation facilitates metabolic interactions even when receiver cells have
- 88 a low affinity for the product caused by genetic variation of their glucose import systems.
- 89 These results suggest that competition or other metabolite-removal in a three-dimensional
- 90 system reduces interaction distances to well below 15 μm.
- 91

92 Materials and methods

93 Strains and media

- All the strains that were used are listed in Table 1. *Lactococcus lactis* NZ9000 strains
- 95 PTSman_GFP, PTScel_GFP, and glcU_GFP were obtained by a single-crossover integration
- 96 of vector pSEUDO:: Pusp45-gfp [21] into the pseudo 10 locus on the chromosome of L. lactis
- 97 NZ9000Δ*ptcC*Δ*glcU*, NZ9000Δ*ptnABCD*Δ*glcU*, and NZ9000Δ*ptnABCD*Δ*ptcC* [22],
- 98 respectively. Integration was performed as previously described [23]. Transformants were
- 99 selected on M17-agar plates supplemented with glucose, sucrose and 5 μ g/mL erythromycin.

100

- 101 L. lactis was grown in chemically defined medium (CDM) described by Otto et al. [24], with
- 102 the following changes: 0.6 g/L NH₄-citrate, 2.5 mg/L biotin, 0.02 mg/L riboflavin and no
- 103 folic acid. L. lactis NZ9000 Glc-Lac+ was pre-cultured in CDM + 0.95 wt% lactose, L. lactis
- 104 MG5267 in CDM + 0.5 wt% lactose and *L. lactis* MG1363 [25], *L. lactis* MG1363_GFP, *L.*
- 105 lactis NZ9000_GFP_PTSman, L. lactis NZ9000_GFP_glcU and L. lactis
- 106 NZ9000_GFP_PTScel in CDM + 0.5 wt% glucose. Agarose beads contained CDM + 0.4
- 107 wt% carbon source and were incubated surrounded by oil, or by CDM + 0.2 wt% carbon
- 108 source. Mono-cultures were incubated with the same carbon source as their pre-culture, co-
- 109 cultures were incubated in presence of lactose. Incubations were done at 30°C.

111 Table 1. Bacterial strains and plasmids used in this study

L. lactis strain	Description	Reference
NZ9000 $\Delta ptcC\Delta glcU$	Derivative of NZ9000 containing a 1254 bp deletion in <i>ptcC</i> and a 864 bp deletion in <i>glcU</i> .	[22]
NZ9000 $\Delta ptnABCD\Delta glcU$	Derivative of NZ9000 containing a 1736 bp deletion in <i>ptnABCD</i> and a 864 bp deletion in <i>glcU</i> .	[22]
$NZ9000\Delta ptnABCD\Delta ptcC$	Derivative of NZ9000 containing a 1736 bp deletion in <i>ptnABCD</i> and a 1254 bp deletion in <i>ptcC</i> .	[22]
MG5267	<i>L. lactis</i> MG1363 with the lactose operon integrated into the genome.	[26]
NZ9000 Glc-Lac+	NZ9000∆ <i>glk∆ptnABCD</i> containing a 657-bp deletion in <i>ptcBA</i> , carrying pMG8020 (lactose mini-plasmid of 23.7 kb, containing <i>lacFEGABCD</i> , derivative of pLP712).	[27]
MG1363_GFP	MG1363 carrying pSEUDO:: <i>P</i> _{usp45} -gfp.	[21]
NZ9000_PTSman_GFP	Ery ^r , NZ9000 $\Delta ptcC\Delta glcU$ carrying pSEUDO:: P_{usp45} -gfp.	This work
NZ9000_PTScel_GFP	Ery ^r , NZ9000 $\Delta ptnABCD\Delta glcU$ carrying pSEUDO:: P_{usp45} -gfp.	This work
NZ9000_glcU_GFP	Ery ^r , NZ9000 $\Delta ptnABCD\Delta ptcC$ carrying pSEUDO:: P_{usp45} -gfp.	This work
Plasmids	Description	Reference
pSEUDO:: <i>P</i> _{usp45} -gfp	Ery ^r , integration vector, pSEUDO:: P_{usp45} - sfgfp(Bs) derivative, carrying the gene coding for the green fluorescent protein (Dasher-GFP).	[21]

113 Aggregation protocol

114 Producer and receiver cell pre-cultures (10 mL) were washed three times with 0.9% sodium 115 chloride. Receiver cells were resuspended in 2 mL 0.225% sodium chloride, producer cells 116 were resuspended in 0.9% sodium chloride and diluted to an OD_{600} of 1.1. Both were 117 incubated in an ultrasonification bath (Branson 200 Ultrasonic cleaner, Branson Ultrasonics, 118 Danbury, CT, USA) at 46 kHz for 3 minutes to ensure complete resuspension to single cells. 119 The surface of (non-)producer cells was charged positively by electrostatic deposition of 120 polyethyleneimine (PEI; Mr 600 000 - 1 000 000; ~50% in H₂O; Sigma-Aldrich, Saint Louis, 121 MO, USA) as follows. Sonicated producer cells were mixed with 0.25% PEI (hydrated, pH 7) 122 in a 1:1 (v/v) ratio and incubated at room temperature for 5 minutes. After incubation cells 123 were collected by centrifugation (900 g, 3 minutes) and washed by replacing the supernatant 124 with 0.9% sodium chloride five times without resuspending the pellet. Washed cells were 125 resuspended in 200 µL 0.9% sodium chloride and sonicated as described above. The surface 126 of washed receiver cells was negatively charged and therefore not further modified [28]. Cell 127 concentrations in the prepared producer and receiver suspensions were measured with flow 128 cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA). Aggregates were formed electrostatically by mixing the positively charged producer cells 129 130 with the negatively charged receiver cells, such that the oppositely charged cells stuck to each 131 other. The suspension with negatively charged receiver cells was mixed using a T10 basic 132 ULTRA TURRAX homogenizer with an S10N-5G dispersing element (IKA, Staufen, 133 Germany) at 8000 rpm for 15-20 minutes. While mixing, the positively charged producer 134 cells were added to the negatively charged receiver cells using a 1 mL syringe (Terumo, 135 Tokyo, Japan), a Chemyx Fusion 200 syringe pump (125-400 µL/h, Chemyx Inc., Stafford, 136 TX, USA) and polyethylene tubing (inner diameter 0.38 mm, BD, Franklin Lakes, NJ, USA).

137	The mixing time (15-20 minutes) and syringe pump flow rate (125-400 μ L/h) were adjusted
138	within the mentioned ranges such that the final aggregate percentage was $\sim 3\%$.

139

140 Agarose beads formation and analysis

141 Agarose beads in oil were made by mixing a water and an oil phase. The oil phase contained

142 Novec HFE 7500 fluorinated oil (3M, Maplewood, MN, USA) and 0.2% PicoSurf 1

143 surfactant (Sphere Fluidics, Cambridge, UK). The water phase contained CDM, 1 wt%

144 melted agarose with ultra-low gelling temperature (Type IX-A, A2576, Sigma-Aldrich, Saint

145 Louis, MO, USA) and cells, and it was prepared as follows. Pre-cultures were washed with

146 phosphate buffered saline (PBS) and the OD_{600} was measured to determine the cell

147 concentration (assuming OD $1 = 10^9$ cells/mL). The total cell concentration in the aggregate

148 suspension was determined using flow cytometry (Accuri C6). The producer cell or aggregate

149 concentration in CDM with agarose was set to $2.7 \cdot 10^6$ /mL, the receiver cell concentration to

150 $8.9 \cdot 10^7$ cells/mL.

151 300 µL water phase and 700 µL oil phase were mixed using a T10 basic ULTRA TURRAX

152 homogenizer with an S10N-5G dispersing element at 8000 rpm for 5 minutes. Emulsions

153 were subsequently placed on ice for at least 20 minutes, to solidify the agarose beads. After

154 solidification cells could not move and growth therefore resulted in micro-colony formation

155 within the agarose bead. Formed agarose beads had an average diameter of $37 \,\mu m$

156 (supplementary information, section 1). Based on this average diameter, each bead contained

157 on average 8 receiver cells. In addition to the receivers, $\sim 2\%$ of the beads contained two or

158 more producer cells/aggregates and ~19% contained one producer cell/aggregate (~79%

159 contained no producer/aggregate).

161 To incubate agarose beads in CDM, 1 mL CDM and 1 mL perfluorooctanol (PFO, Alfa 162 Aesar, Ward Hill, MA, USA) were added to the emulsion after solidification. This leads to 163 the breaking of the emulsion and separation of the water and oil phase upon gently mixing. 164 Subsequently the water phase, containing agarose beads in CDM, was separated from the oil 165 phase and incubated while rotating. For incubation in presence of competing glucoseconsumers 10⁹ L. lactis MG1363 cells per mL were added to the CDM surrounding the 166 agarose beads (supplementary information, section 2). 167 168 169 Growth in agarose beads was analyzed with flow cytometry (Accuri C6). Agarose beads in 170 CDM were measured directly. For agarose beads in oil the emulsion was first broken by 171 adding 240 µL PBS and 300 µL PFO to 60 µL emulsion, followed by gently mixing. The 172 water phase, containing agarose beads in PBS, was separated from the oil phase and 173 measured using flow cytometry. Details about the flow cytometry gating strategy and data

analysis are shown in supplementary information section 3.

175

176 Three-dimensional reaction-diffusion model

177 A three-dimensional, numerical reaction-diffusion model was implemented in COMSOL 178 Multiphysics (COMSOL 5.0, Comsol Inc., Burlington, MA, USA). Two spherical agarose 179 beads were placed in a cubic computational domain. One bead contained a producer cell that 180 secreted glucose with a constant rate, and both beads contained eight receiver cells that 181 consumed glucose based on Monod (saturation) kinetics. The concentration at the agarose 182 bead surface resulted from a partition coefficient which was set to 0 to model incubation in 183 oil, and to 1 to model incubation in CDM. The diffusion coefficient of glucose was set to 6.7.10⁻¹⁰ m²/s [29] both inside and outside agarose beads [30], and 10 times lower in micro-184 185 colonies [29, 31]. A time-dependent study yielded the spatial distribution of glucose. See

186 supplementary information section 4 for more details about the geometry and used

187 parameters.

188 **Results**

189 Reaction-diffusion modelling predicts short interaction distances in three-dimensional 190 systems

191 To compare concentration gradients in two- and three-dimensional systems we made 192 reaction-diffusion models in COMSOL Multiphysics (supplementary information, section 193 4.2). The concentration gradients around a producer cell were calculated either in cube to 194 mimic a three-dimensional system, or in a thin plate to mimic a two-dimensional system 195 (plate thickness of 1.1 μ m, roughly matching the producer cell diameter of 1 μ m). In both cases the total volume was 1 nL (10^6 cells/mL). The model predicted that in the thin plate the 196 197 maximal concentration is halved at 24 µm from the producer cell, while in the cube this 198 distance is 0.6 µm (Figure S5). This indicated that in three-dimensional systems the distances 199 at which cells can interact are significantly shorter than in two-dimensional systems.

200

201 Design of a synthetic consortium and three-dimensional spatial structure for growth

202 To study how concentration gradients constrain interactions between micro-organisms in a 203 three-dimensional environment, we extended the cubic model to contain producer and 204 receiver cells, and analyzed the impact of cell-to-cell distance on the interaction 205 (supplementary information, section 4.3). To experimentally validate the model results we 206 constructed synthetic consortia using four L. lactis strains. 1) A "producer" that takes up 207 lactose and hydrolyzes it intracellularly to glucose and galactose. It was engineered to not 208 metabolize glucose, which was therefore secreted while the cells grew on galactose. 2) A 209 GFP-expressing "receiver" that can take up and grow on glucose, but not lactose. 3) A "non-210 producer" that takes up lactose. It uses both the glucose and galactose moiety for growth, and 211 therefore does not secrete glucose. 4) A "competing glucose-consumer" (Figure 1A). To co-212 culture these cells in a three-dimensional system, glucose-producers and -receivers (the

213 unidirectional cross-feeders) were encapsulated in solidified agarose beads with an average

- 214 diameter of ~40 µm. For negative controls, glucose-producers were replaced by glucose-
- 215 "non-producers". Cells were embedded in the beads either as separate cells (~15 µm between
- 216 cells) or as aggregates (0 µm between cells) (Figure 1B). During incubation agarose beads
- 217 were separated either by oil or by CDM (Figure 1C). Separation by oil prevented diffusion of
- 218 glucose from beads, enabling us to validate that cells can grow and interact in agarose beads.
- 219 Separation by CDM resulted in glucose diffusion from beads, enabling us to study
- 220 unidirectional cross-feeding in presence of a concentration gradient in a three-dimensional
- system. To investigate the effect of metabolite-removal on the interaction distances,
- 222 interactions were analyzed in presence and absence of competing glucose-consumers outside
- the beads (Figure 1C).



agarose beads surrounded by oil

no diffusion from beads



no competing glucose-consumers



agarose beads surrounded by CDM diffusion from beads



226 Figure 1. Metabolic interactions in three-dimensional spatially structured environments. (A)

227 The four L. lactis strains that were used to make synthetic consortia: 1) "producers" which take up 228 lactose and secrete glucose, 2) "receivers" which take up glucose and express GFP, 3) "non-229 producers" which take up lactose but do not secrete glucose and 4) "competing glucose-consumers" 230 which take up glucose (supplementary information, section 2). (B) The three-dimensional spatial 231 structure within agarose beads. A distance of 15 µm between cells is comparable to a homogeneous 232 distribution of 3.108 bacteria/mL. For visibility reasons the microscope picture shows a GFP-233 expressing cell surrounded by non-fluorescent cells. Aggregates used in experiments were formed 234 oppositely: a (non-)producer cell was surrounded by GFP-expressing receivers. (C) The three-235 dimensional spatial structure between agarose beads. Aggregates were only incubated in presence of

competing glucose-consumers.

237

238 To analyze if we could detect growth in agarose beads we cultured producers and receivers in 239 beads surrounded by oil (no glucose diffusion from beads) and analyzed the beads with flow 240 cytometry before and after incubation. Beads inoculated with mono-cultures of producers 241 (lactose as carbon source) or receivers (glucose as carbon source) showed an increased 242 forward scatter after incubation, indicating that cells could grow inside beads. In agreement 243 with our expectation the fluorescence/scatter ratio of beads with growth was low for beads 244 with producers and high for beads with GFP-expressing receivers (Figure 2B). 245 To validate that cells could also interact within beads, we made beads such that $\sim 21\%$ of 246 them contained both a producer and receivers and ~79% contained receivers only. Because 247 the metabolic interaction is unidirectional, we expected that in presence of lactose the 248 producers would always grow, while the receivers would only grow when glucose, secreted 249 by producers, was available to them (Figure 2A). After incubation 14±1% of the agarose beads showed an increased forward scatter, and these beads had a high fluorescence/scatter 250

ratio (Figure 2B, Table 2). This is close to the expected 21% of beads with producer and

252 receivers, indicating that receivers could only grow in beads with producers.

253 Together this setup forms a synthetic consortium where spatial interactions can be

254 manipulated in a three-dimensional environment, and which allows the detection of growth

- and interactions using flow cytometry.
- 256

257 Under glucose competition receivers cannot interact with producers ~15 µm away

In the example above glucose could not diffuse from beads and each agarose bead acted as an individual compartment. In contrast, when glucose can diffuse from agarose beads the model predicted that the glucose concentration flattens close to the producer. In that case receivers at a distance of 15 µm from a producer in the same bead are exposed to similar glucose

262 concentrations as receivers in beads without a producer (Figure 2C). If this prediction was

263 correct, we expected that most receivers can grow when the global glucose concentration

builds up, while in case of glucose competition the glucose concentration stays low and even

265 $\,$ receivers 15 μm away from a producer in the same bead should not be able to grow. We

therefore incubated agarose beads in CDM, which allows glucose diffusion from beads.

267 Without competing glucose-consumers in the CDM outside the beads 75±7% of the beads

showed growth and these beads had a high fluorescence/scatter ratio (Figure 2D, Table 2).

269 This indicates growth of both receivers with and receivers without a producer in their bead.

270 When we took the same beads but added competing glucose-consumers outside the beads,

271 only $15\pm3\%$ of the beads contained growth. In the beads where growth was observed the

272 fluorescence/scatter ratio was low, indicating that only producers grew (Figure 2D, Table 2).

273 These results are consistent with the model predictions and show that under glucose

274 competition receivers cannot interact with producers even if they are only ~15 μ m away.

275

276 Without competing glucose-consumers still 25±7% of the beads were gated as "no growth", 277 although the model predicted that all receivers could grow (Figure 2C and 2D). These beads 278 could be false negatives caused by our conservative gating strategy, or by empty beads with 279 single fluorescent cells attached to their outside. Conversely, for the beads gated as "growth", 280 we observed an increased fluorescence/scatter ratio compared to the single receiver controls 281 (Figure 2D). It is known that fluorescence of individual cells increases with decreasing 282 growth rate [32, 33], suggesting that in co-cultures the higher fluorescence/scatter ratio could 283 be caused by glucose limited and therefore slower growth of the receivers in the beads. 284 Together this data shows that competition for glucose in a three-dimensional environment 285 prevents interactions at ~15 µm distance, because the presence of competing public good-286 consumers leads to steep concentration gradients.

287

288 Aggregated producers and receivers interact even under glucose competition

289 In the presence of steep concentration gradients microbial interactions might be facilitated by bringing producers and receivers in close proximity. Consistently, the model predicted that 290 291 cell aggregation would allow receivers to grow under glucose competition (Figure 2E). We 292 developed a protocol to make producer-receiver aggregates. Defined aggregates were formed 293 by adding positively charged producers to an excess of negatively charged receivers, ensuring 294 that producers were directly surrounded by receivers. In this way we obtained a mixture of 295 single receivers and aggregates of one producer and approximately eight receivers (Figure 296 1B). We aimed to add an aggregate to $\sim 21\%$ of the beads, but we could only roughly estimate 297 the aggregate concentration in the mixture based on the added amount of positively charged 298 cells. However, underestimating this percentage would not affect the results, as we only 299 analyze agarose beads with growth after incubation (supplementary information, section 3).

300	We incubated the forme	d agarose beads in CDM	with competing glucose-consumers	and

- 301 after incubation we saw an increased scatter in $3\pm 2\%$ of the beads (Figure 2F, Table 2),
- 302 indicating only growth in beads with both producers and receivers. The fluorescence/scatter
- 303 ratio of beads with growth was increased compared to the producer mono-culture (Figure 2F),
- 304 indicating growth of both producers and receivers. Beads with grown aggregates of non-
- 305 producers and receivers showed a fluorescence/scatter ratio similar to the producer mono-
- 306 culture (Figure 2F), indicating that the aggregation protocol or the data analysis procedure did
- 307 not influence the readout.
- 308 Therefore, the results show that cell aggregation facilitates microbial interactions, even in a
- 309 three-dimensional system with competition for the public good.
- 310

311 Table 2. Consortium response in different spatial structures. Summary of the experimental results
312 of Figure 2.

in a bation and dition	cell-to-cell distance	producer + receivers (~21%) receivers only (~79%)		non-producer + receivers (~21%) receivers only (~79%)	
		beads with growth	fluorescence/ scatter ratio	beads with growth	fluorescence/ scatter ratio
no diffusion from beads	~15 µm	$14 \pm 1\%$	High	$16 \pm 0\%$	Low
diffusion from beads, no competing glucose consumers	~15 µm	$75\pm7\%$	High	$6 \pm 1\%$	Low
diffusion from beads,	~15 µm	$15 \pm 3\%$	Low	$9\pm2\%$	Low
consumers	0 µm	$3 \pm 2\%$	Medium	$4\pm2\%$	Low



315 Figure 2. Consortium response in different spatial structures. Panels (A), (C), and (E) show the 316 predicted concentration gradient at the diagonal of the cube, for the following spatial structure: (A) 317 No diffusion from beads, (C) Diffusion from beads, $\sim 15 \,\mu m$ between cells within a bead and (E) 318 diffusion from beads, aggregated cells within a bead. In panels (B), (D) and (F) the experimental 319 results are shown for these different spatial structures. The forward scatter histograms show the 320 populations that were gated as "growth" in the producer-receiver co-cultures (n=3). From these 321 populations the fluorescence/scatter signal was determined. Next to the producer-receiver co-culture 322 control samples were included: receivers only, producers only and co-cultures of non-producers and 323 receivers (n=3 for each of them). The non-producers and receivers, and the producers only controls 324 are overlapping in all plots. The schematic drawing at the right is based on the experimental data and 325 shows which cells could grow in producer-receiver co-cultures.

326

Aggregation results in dense micro-colonies, facilitating growth of receivers with low affinity and low V_{max} glucose transporters

329 To study the effect of glucose uptake efficiency on the receiver response, we modelled 330 producer-receiver aggregates with receivers that have glucose-transporters with different 331 affinities (K_m) and maximal uptake rates (V_{max}). Specifically, we modelled receivers that had one of the three different glucose transporters of L. lactis [22] (supplementary information, 332 333 section 4.5). Within aggregates the effective diffusion coefficient ($D_{eff,s}$) is described to be 334 10-70% of the diffusion coefficient in water (D_s), depending on the aggregates' density [29, 335 31]. When we set D_{eff,s} to10% of D_s, the model predicts that receivers with the low K_m and high V_{max} transporter PTSman (K_m = 0.013 mM, V_{max} = 0.22 µmol/min/mg protein) consume 336 337 about 90 times more glucose than receivers with a high K_m or low V_{max} transporter (PTScel: 338 $K_m = 8.7 \text{ mM}, V_{max} = 0.25 \mu \text{mol/min/mg}$ protein, glcU: $K_m = 2.4 \text{ mM}, V_{max} = 0.08$ 339 μ mol/min/mg protein) (supplementary information, section 4.5). When D_{eff.s} is 70% of D_s, this difference is almost 350 fold. 340

341 As the model predicted less glucose consumption by receivers with low glucose affinities and 342 low maximal glucose uptake rates, we constitutively expressed GFP in three engineered L. 343 *lactis* NZ9000 strains which each contain only one of the three glucose transporters [27]. We 344 subsequently analyzed if their uptake was high enough to interact with producers. As we saw 345 before, the fluorescence/scatter ratio in mono-culture controls decreased with an increasing 346 growth rate (Figure 3 and supplementary information section 5). The data further show that in producer-receiver aggregates even the low affinity receivers could grow and the differences 347 348 in fluorescence/scatter ratio between the mutants were small (2-3 fold). Based on the model 349 this data suggests that dense micro-colonies with a low D_{eff.s} were formed. Aggregates with 350 receivers containing the low K_m and high V_{max} transporter PTSman showed the highest 351 fluorescence/scatter ratio. Consistent with the model predictions this result suggests that 352 PTSman containing receivers have the highest glucose uptake rate. 353 Altogether the data show that in a three-dimensional system with a metabolite consuming 354 sink a steep concentration gradient is obtained, and cells only $\sim 15 \,\mu m$ away from each other 355 cannot interact through glucose cross-feeding. This physical constraint can be overcome by 356 bringing cells together in the low micrometer range, as achieved through cell aggregation -357 physical contact.





381 Discussion

382 Contact-independent interactions can be local or global, depending on the profile of the 383 concentration gradient. Previous studies quantified interaction distances either in monolayers 384 of cells (two-dimensional system) or in absence of a competing metabolite-sink [13, 14]. 385 While these studies give valuable insight, they have a limited resemblance to natural 386 ecosystems, which are typically three-dimensional and harbor competing organisms and other 387 metabolite-removing sinks. A reaction-diffusion model predicted that in three-dimensional 388 systems the concentration gradient drops much faster than in two-dimensional systems (the 389 maximal concentration is halved at 0.6 µm instead of 24 µm), suggesting that in natural 390 ecosystems interaction ranges might be much shorter than the *in vitro* two-dimensional 391 systems predict. To better understand how concentration gradient constraints affect the 392 composition and fitness of natural communities, we analyzed the impact of cell-to-cell 393 distance on unidirectional cross-feeding in three dimensions in the presence and absence of a 394 competing metabolite consumer as a public good-removing sink.

395

396 Without competing glucose-consumers we observed a global response: receivers grew at all 397 distances from producers (Figure 2D, Table 2). When we added competing glucose-398 consumers only receivers aggregated with producers could grow (Figure 2D and 2F, Table 2). 399 Diener et al. observed a similar pattern of local and global interactions during S. cerevisiae 400 mating [14]. Haploid cells secrete a peptide, which is sensed and degraded by haploid cells of 401 the opposite mating type. This results in a local high peptide concentration and local 402 interactions: cells from opposite mating types initiate mating specifically in each other's 403 direction. However, incubation of mutants that could not degrade the peptide resulted in a 404 global high peptide concentration, and independent of their location cells initiated mating in 405 different directions. For wildtype cells Diener *et al.* predicted that the maximum information

406 content of the peptide distribution is similar for cells ~ 17 and $\sim 2 \,\mu m$ away from each other 407 [14], suggesting that yeast cells interact efficiently when they are 17 µm away from each 408 other. Similar interaction distances (3.2-12.1 µm) were found by Dal Co et al. when they 409 grew bidirectionally cross-feeding E. coli cells in a microfluidic chamber [13]. Our data 410 however show that in a three-dimensional environment with a metabolite-sink the interaction 411 distances are shorter, as producers and receivers ~15 µm away from each other could not 412 cross-feed (Figure 2D). Within aggregates cross-feeding was possible, but it was still less 413 efficient than what was achieved in presence of high concentrations of the secreted 414 metabolite (Figure 2B and 2F). 415 These results match the model prediction that interaction distances in three-dimensional 416 systems are shorter than in two-dimensional systems. It furthermore indicates that the 417 presence of a metabolite-sink also affects the interaction distance. In the set-ups of Dalco et 418 al. and Diener et al. the metabolite is degraded/consumed only by the receiver itself, so the 419 metabolite concentration will only decrease close to receiver cells. When receivers compete 420 with other metabolite-sinks, such as competing metabolite consumers or a dilute system, the 421 overall metabolite concentration will be lowered, resulting in shorter interaction distances. 422 Indeed, Koschwanez et al. showed that at low cell densities and low sucrose concentrations, 423 where the volume acts as the main metabolite-sink, S. cerevisiae cannot grow, even though 424 invertase splits sucrose into glucose and fructose in the periplasmic space, so very close to the 425 receiver cell [8].

426

In the presence of a competing metabolite-sink the concentration of the exchanged metabolite
is low, and we therefore expected that variation in the receivers' import affinity would
influence the interaction efficiency. However, within aggregates we observed only small
differences in growth of high and low affinity receivers (Figure 3), suggesting that dense

micro-colonies with a low diffusion rate were formed (supplementary information, section
4.1). Aggregating cells therefore seem to kill two birds with one stone: they decrease both the
cell-to-cell distance and the diffusion rate, two factors which were previously reported to
promote interaction [15, 34]. The resulting increase of the local concentration might also
explain why Koschwanez *et al.* saw that yeast cells which could not grow on low sucrose
concentrations due to their low cell density, could grow when the same amount of cells was
aggregated [8].

In our set-up the local glucose concentration increases with time, because the producers grew
independently from the receivers. Future research could focus on bidirectional cross-feeding
systems, where producer growth is limited by receiver growth. In that case the initial
production rate will be lower and receiver affinity might play a bigger role.

442

443 Controlled metabolite exchange is a critical feature of living cells [35], and forms the basis 444 for extracellular metabolism of nutrients and interactions with other cells. Concentration 445 gradients constrain the distance over which these interactions can occur and it therefore 446 shaped the evolution of the molecular mechanisms involved in these interactions. Slow 447 diffusion of large, aggregated resources like particulate iron (>0.4 µm) can for instance cause 448 cellular iron uptake to become diffusion limited. It is therefore hypothesized that cells secrete 449 siderophores to form fast diffusing iron-siderophore complexes after iron dissolution, in this 450 way increasing their iron uptake rate [36]. It is furthermore known that many extracellular 451 substrate-degrading enzymes are attached to the cell, which places the source (enzyme) close 452 to the receiver (cell). Invertase is for instance located in the periplasmic space of S. cerevisiae 453 [37], the protease of L. lactis is attached to the cell wall [9] and in both fungi and bacteria 454 cellulosomes are also attached to the cell wall [38]. Hauert et al. argue that when a producer 455 also benefits from its own product, which is the case for extracellular enzymes, spatially

456 structured localization of cells is only advantageous when the enzyme production costs are 457 high [39]. Attachment of extracellular enzymes to the cell wall therefore suggests that these 458 enzymes are costly, and indeed, Bachmann *et al.* showed in *L. lactis* that protease negative 459 strains outcompeted protease positive strains with a cell wall bound protease, unless they 460 were >1 mm apart (cell density of <10³ cells/mL) [9].

461 In the presence of a competing public good-sink interacting cells can aggregate, for example 462 in biofilms, to reduce the diffusion distance and diffusion rate [40], which increases the 463 efficiency of their interactions [15, 34]. During evolution of cooperation in which costly 464 compounds are secreted, wildtype non-cooperators typically form such a competing public 465 good-sink, indicating that cell-to-cell distances well below 15 µm are required to evolve 466 costly cooperation. However, aggregation is not always increasing interaction efficiency, 467 because it also slows down the diffusion of inhibiting metabolic end-products from the 468 micro-colony and the diffusion of extracellular nutrients into the micro-colony. Aggregation 469 of the cross-feeding yoghurt consortium (Lactobacillus bulgaricus and Streptococcus 470 *thermophilus*) in 100-300 µm capsules reduced for instance their growth and acidification 471 rates, and proteolysis was only faster in the first hour [41], indicating that in this case the 472 aggregation costs did not outweigh the benefits. Aggregation also allows (evolution of) 473 contact-dependent transfer mechanisms, like nanotubes or vesicle chains. To our knowledge 474 L. lactis does not exchange cytosolic material using these contact-dependent transfer 475 mechanisms and the model indicates that just diffusion can explain our experimental results. 476

477 Consequences of concentration gradient constraints are not limited to bacteria and yeasts.
478 Plants, fungi and other (organisms with) large cells use intracellular concentration gradients
479 to regulate amongst others cell polarity, cell division and cell size [42–44]. Although the rate
480 of diffusion in the cytoplasm is fast, cells can use spatially structured protein modification

- 481 systems as source or sink, to create concentration gradients [45, 46]. It is therefore important
- 482 to think about the constraints and opportunities that concentration gradients may impose
- 483 on cellular interactions, how it shaped their evolution and their role in microbial consortia,
- 484 and how researchers can use these principles to understand and steer these processes.

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496

497 Author contributions

- 498 R.J.v.T., B.T. and H.B. conceived the study, designed experiments, interpreted the data and
- 499 wrote the paper. R.J.v.T., I.v.S. and E.Z. carried out the experiments. T.R. and A.L.
- 500 developed the aggregation protocol. C.P. and R.J.v.T. built the COMSOL Multiphysics
- 501 model. J.A.H. and O.P.K. constructed the strains L. lactis NZ9000_PTSman_GFP, L. lactis
- 502 NZ9000_PTScel_GFP and *L. lactis* NZ9000_glcU_GFP. All authors helped improving the503 manuscript.

504

505 **Competing interests statement**

H.B. is also employed by NIZO Food Research, a contract research organization. NIZO Food
Research had no role in the study design, data collection and analysis, decision to publish, or
preparation of the manuscript.

510 Supplementary information

511 Section 1: Agarose bead size- and volume-distributions

We prepared agarose beads surrounded by oil and made pictures with a microscope (9 per emulsion, Figure S1A shows an example). Pictures were subsequently analyzed with ImageJ to identify the beads (Figure S1B), and to measure size- and volume distributions (Figure S1C and S1D). Small droplets were not always identified, but as they contain only little volume this only marginally affects the analysis. Beads on the edge of the picture were excluded from the analysis. Formed emulsions were polydisperse but distributions of replicates were reproducible, with mean volume \pm SEM of 26 \pm 2 pL (diameter of 37 µm).

519



Figure S1. Agarose bead size and volume. (A) An example microscope picture of agarose beads.
(B) Agarose beads identified in (A) after ImageJ analysis. (C and D) Agarose bead size (C) and volume (D) distribution (n=10 emulsions, 9 pictures per emulsion).

543 Section 2: 10⁹ *L. lactis* MG1363 cells/mL as competing glucose-consumers

To establish if addition of 10⁹ L. lactis MG1363 cells per mL outside agarose beads 544 prevented cross-talk between beads, we mixed beads with producers and beads with receivers 545 546 and incubated them in presence of lactose in different spatial structures (Figure S2). After 547 incubation surrounded by oil only producers were grown, which was expected as glucose 548 could not diffuse from beads. When glucose could diffuse from beads and no L. lactis 549 MG1363 cells were added outside the beads, both producers and receivers grew. However, in presence of 10⁹ L. lactis MG1363 cells per mL outside the agarose beads only producers 550 551 grew, suggesting that glucose leaving beads with producers was mainly consumed by 552 L. lactis MG1363 cells outside the beads and did not reach receivers in neighboring beads. 553 The glucose concentration outside the beads probably did not exceed the low micro-molar 554 range, as the K_m for glucose of the highest affinity transporter in L. lactis MG1363 is 13 μ M 555 [22] and the L. lactis MG1363 cell density was high.



Figure S2: Addition of *L. lactis* MG1363 outside agarose beads prevents cross-talk between beads. Agarose beads with producers and agarose beads with receivers were mixed and incubated in three different spatial structures: surrounded by oil (no diffusion from beads, n=3), surrounded by CDM without *L. lactis* MG1363 (no competing glucose-consumers, n=3) and surrounded by CDM with 10^9 single *L. lactis* MG1363 cells per mL (competing glucose-consumers, n=3). Histograms show the fluorescence/scatter ratio of the populations that were gated as "growth".

577 Section 3: Flow cytometry gating strategy and data analysis

578 Figure S3 shows the flow cytometry gating strategy and data analysis procedure.

579



10⁻⁴ 10⁻² 10⁰ 10² Fluorescence/Side scatter

606 Figure S3. Flow cytometry gating strategy and data analysis.

607 Overview. Each agarose bead sample was measured twice. We first acquired an overview of the 608 complete sample, containing both single cells and agarose beads. Drawn gates were based on 609 measurements of empty agarose beads and single cells. Thereafter we used an increased forward and 610 side scatter threshold to leave out most of the single cells, this data was used for gating and data 611 analysis. Step 1. Beads containing cells ("filled beads") were gated by including the largest agarose 612 beads and excluding the noise and the empty agarose beads. For the control samples with only 613 producers empty beads were not excluded, because before incubation agarose beads with GFP-614 producers could not be separated from empty beads. Step 2. Beads with and without growth were 615 separated with a forward scatter threshold. This threshold was set for each sample individually, based 616 on the forward scatter before incubation. This stringent gating might underestimate the amount of 617 beads with growth, but it ensures that beads without growth are excluded from analysis. Step 3. The 618 side scatter and fluorescence were background-corrected based on their values before incubation. The 619 distribution of fluorescence/scatter ratios of background-corrected data is plotted to identify which 620 cell-types were grown inside the agarose beads.

622 Section 4: Reaction-diffusion model in COMSOL Multiphysics

623 **4.1 Geometry of the agarose bead model**

624 Figure S4 shows the geometry of agarose bead model.



Figure S4: Model geometry implemented in COMSOL Multiphysics. (A) An agarose bead (sphere 644 645 with diameter of 40 µm) contains 8 receiver cells and 0 or 1 producer cells. Each cell is a sphere with 646 a diameter of 1 µm. Within the agarose bead receivers are placed at the virtual corners of a cube, and 647 the producer in the middle. The distance between the surface of producers and receivers is either 0 or 648 15 µm. (B and C) An agarose bead with one producer (bead 1) and an agarose bead without a producer (bead 2) are placed in a cube of 100 µm (corresponding to 2.10⁶ beads/mL). In (B) the 649 650 distance between the producer and receiver is 15 μ m, in (C) they are in contact (0 μ m). Producers and 651 receivers in contact are placed in a micro-colony (sphere with a diameter of 4 µm) with a reduced 652 diffusion coefficient (D_{eff,s}).

654 **4.2 Reaction-diffusion model and parameter values**

655 *Material balance*. The spatial distribution (x,y,z) and change in time (t) of the concentration 656 $C_s \text{ (mol/m}^3)$ of glucose in the bead and surrounding liquid resulted from solving the partial 657 differential equation which balances the diffusion rate with a reaction rate r_s :

$$\frac{\partial C_s}{\partial t} = D_s \left(\frac{\partial^2 C_s}{\partial x^2} + \frac{\partial^2 C_s}{\partial y^2} + \frac{\partial^2 C_s}{\partial z^2} \right) + r_s$$

658

Diffusion. The same diffusion coefficient of glucose, D_s (m²/s), was used outside and inside the agarose beads. It was set to the value of D_s in water [29], because D_s in agarose gels is similar to that in water [30]. The effective diffusion coefficient in micro-colonies depends on the void fraction, i.e. volume not occupied by cells per total micro-colony volume, and the tortuosity [13, 40]. Cells growing in an agarose matrix form dense colonies, therefore the effective diffusion coefficient within the colonies ($D_{eff,s}$) was set to 10% of the diffusion coefficient in water (D_s) [29, 31].

666

Reaction. The net glucose rate r_s (mol/m³/s) results as the difference between production and 667 consumption at a certain position in space, $r_s = q_p C_x - q_s C_x$. The specific glucose production 668 rate (qp) of L. lactis NZ9000 Glc-Lac+ is the same as its specific lactose uptake rate, as each 669 lactose molecule contains one glucose molecule. The q_p was therefore set to a constant value 670 671 of 1 molP/CmolX/h [47] and applied within the producer cells. Simulations which did include 672 the lactose concentration and Monod kinetics for lactose consumption yielded similar results 673 as simulations with a constant q_p , therefore we adopted the simpler constant rate. For 674 receivers the glucose uptake was assumed with a saturation (Monod) kinetics, $q_s = q_s^{max} \cdot C_s/(K_s + C_s)$. We used the K_s of the highest affinity glucose transporter of L. lactis 675 MG1363 [22], and qs^{max} was set to 1 molS/CmolX/h [48]. To calculate the biomass 676 concentration C_x (CmolX/m³) we assumed a molecular weight of biomass of 24.6 grams per 677

678	Cmol dry biomass ($CH_{1.8}O_{0.5}N_{0.2}$) [49, 50], a cellular water content of 70 wt% [51] and a
679	cellular density of 1000 g/L [51]. These values lead to a glucose production or maximal
680	glucose consumption rate of 38 mol/m ³ /s. A competing glucose-consumer was modelled by
681	adding glucose consumption outside agarose beads with the same Monod kinetics as that of
682	receivers. Cell growth was not incorporated in the model.

683

Boundaries. The concentration at the agarose bead surface was based on a partition
coefficient which was set to 0 when incubation in oil was modelled, and to 1 for incubation in
CDM. The liquid domain (cube) boundaries were insulated (no-flux boundary condition).

687

Parameters. Table S1 lists the default parameters used in the COMSOL Multiphysics modelwith sources for their values.

690

691 Table S1: Default parameter values of the COMSOL Multiphysics model

Parameter	Symbol	Value	Source
Maximum specific uptake rate of glucose	q _s ^{max}	$38 \text{ mol/m}^3/\text{s}$	[48]
Specific production rate of glucose	q _p	$38 \text{ mol/m}^3/\text{s}$	[47]
Half-saturation (Monod) coefficient	Ks	0.01 mM	[22]
Diffusion coefficient in water and agarose beads	D _s	$6.7 \cdot 10^{-10} \text{ m}^2/\text{s}$	[29]
Effective diffusion coefficient in micro-colonies	D _{eff,s}	$0.1 \cdot D_s$	[29, 31]

692

4.3 Predicted concentration gradients in two- and three-dimensional reaction-diffusion systems

To analyze the difference in concentration gradients in two- and three-dimensional systems
we modelled production by one producer cell in two different geometries, as represented in
Figure S5. In the two-dimensional system the model predicts that the product concentration is



halved at 24 μ m from the producer, whereas in the three-dimensional system this happens at

713 Figure S5: Predicted concentration gradients in two- and three-dimensional reaction-diffusion 714 systems. A producer cell with a diameter of 1 µm was placed in the middle of a very thin rectangular 715 block (1.1 µm thickness) to represent a quasi-two-dimensional system. For the three-dimensional 716 system it was placed in the middle of a cube. For the cube, concentrations at the cube boundaries were 717 set to zero. For the thin plate the concentrations at the four lateral faces were set to zero, and the 718 top and bottom boundaries were insulated (no-flux boundary condition). The total volume of both systems was 1 nL (1·10⁶ cells/mL). A time dependent study in COMSOL Multiphysics 719 720 vielded concentration gradients at several moments. The figure shows the concentrations along the diagonal after 5 hours. 721

722

724 **4.4 Sensitivity analysis for** q_p **and** q_s^{max}

Figure S6 shows the predicted glucose concentration (Figure S6A) and glucose production

- rate (Figure S6B) over a plane crossing the producer cell and four of the eight receiver cells.
- 727 Profiles for aggregated cells and for cells ~15 µm away from each other are shown. Because
- 728 it is difficult to know the actual q_p and q_s^{max} inside agarose beads, we also performed a
- 729 sensitivity analysis (Figure S7). A 5-fold change in q_p and q_s^{max} resulted in similar
- 730 concentration gradients and it did not affect our hypotheses.

A. Glucose concentration



beads surrounded by CDM. The glucose concentration (**A**) and the glucose production rate (**B**) are plotted over a plane crossing the producer cell and four of the eight receiver cells. Profiles for aggregated cells and for cells ~15 μ m away from each other are shown.



Figure S7: Predicted concentration gradients after varying q_p and q_s^{max} . q_p and q_s^{max} were increased and decreased five-fold and the effects on the predicted concentration gradients in different spatial structures are shown.

782 **4.5 Model predictions for the glucose uptake of receivers with different affinities**

783 We analyzed the effect of the glucose affinity of receivers on their ability to utilize the 784 glucose made by the producer. To model the individual glucose transporters of L. lactis 785 MG1363 in COMSOL the K_s values as reported by Castro et al. were used [22]. For L. lactis NZ9000_GFP_glcU the qs^{max} was reduced with a factor four, which reflects the differences in 786 787 V_{max} of the transporters [22]. We calculated the glucose uptake for the different mutants after 5 hours in presence of competing glucose-consumers, without considering growth of the cells 788 789 (Figure S8). The effective diffusion coefficient ($D_{eff,s}$) varies from 10-70%, depending on the 790 density of the micro-colony [29, 31]. Figure S7 shows the glucose uptake when D_{eff,s} is 10%, 30% and 70%. We included a sensitivity analysis for five-fold changes in q_p and q_s^{max} values, 791 which all showed similar trends as the reference $(1x q_p \text{ and } 1x q_s^{\text{max}})$. 792



811 **Figure S8: Predicted glucose uptake.** We modelled receiver cells with different glucose affinities 812 and calculated the predicted glucose uptake. A sensitivity analysis for five-fold changes in q_p and q_s^{max} 813 values was incorporated. Each simulation contained eight receiver cells aggregated with a producer 814 (Figure S5), this figure shows the combined glucose uptake of these receivers.

816 Section 5: Growth rate determination

Strains were incubated in CDM + 0.2 wt% glucose in a 96-well plate. The OD₆₀₀ was measured every six minutes for 24 hours using a SPECTRAmax 384 plus plate reader (Molecular Devices, San Jose, CA, USA). OD₆₀₀ measurements were background corrected, ln-transformed and the slope of the region with exponential growth was calculated as the growth rate (Table S2).

822

823 Table S2. Growth rates of receivers with different glucose transporters (n=22).

Strain	Growth rate ± SD (h ⁻¹)
L. lactis MG1363_GFP	1.25 ± 0.04
L. lactis NZ9000_GFP_glcU	0.79 ± 0.04
L. lactis NZ9000_GFP_PTSman	0.74 ± 0.11
L. lactis NZ9000_GFP_PTScel	0.57 ± 0.03

825 Section 6: Flow cytometry data for receivers glucose transporters with different 826 affinities and V_{max}

We constitutively expressed GFP in three previously constructed L. lactis NZ9000 mutants 827 828 with a single glucose transporter [27], and analyzed their growth in different spatial 829 structures. This experiment focused on beads incubated in CDM (allowing glucose diffusion 830 from beads), as we expected that under these conditions the transporter characteristics of 831 receivers would be important. Figure S9A shows the experimental results when receivers 832 were ~15 µm from a producer within the same bead and incubated in CDM, whereas in Figure S9B the beads were incubated in medium with 10^9 glucose-consumers per mL. Figure 833 834 S9C shows the experimental results of producer-receiver aggregates, incubated in CDM with 10⁹ glucose-consumers per mL. Without a competing glucose-consumers we observed 835 836 growth of both receivers with and receivers without a producer in their bead (Figure S9A), 837 while with competing glucose-consumers only producers could grow (Figure S9B). In 838 producer-receiver aggregates receivers were able to grow, despite the presence of competing 839 glucose-consumers (Figure S9C). The results were similar for all glucose transporters, and 840 were consistent with the results of the wild-type (Figure 2, Table 2).



Figure S9A. Glucose accumulates, allowing receivers to grow independent of the available glucose
transporter. See complete caption on page 24.



893 Figure S9B. In presence of competing glucose-consumers receivers cannot grow, independent of the





919 Figure S9C. Despite the presence of competing glucose-consumers receivers within producer-920 receiver aggregates can grow, independently of the available glucose transporter. See complete 921 caption on page 24.

922 Figure S9: Response of consortia containing receivers with different glucose affinities. The 923 forward scatter histograms show the percentage of beads that were gated as "growth" in the co-culture 924 of producer and receivers. The fluorescence/scatter histograms show the fluorescence/scatter ratio of 925 beads that were gated as "growth" (n=3). Next to the co-culture of producer and receivers several 926 control samples are included: receivers only, producers only and co-cultures of non-producers and 927 receivers (n=3 for each of them). The non-producers and receivers, and the producers only controls 928 are overlapping in all plots. The schematic drawing at the right shows the situation after growth, based 929 on data from the two histograms. Different panels contain different spatial structures: (A) 930 Receivers $\sim 15 \,\mu m$ from a producer within the same bead, incubated in CDM. (B) Receivers $\sim 15 \,\mu m$ from a producer within the same bead, incubated in CDM with 10^9 glucose-consumers per mL. (C) 931 932 Aggregates of producers and receivers, incubated in beads surrounded by CDM with 10⁹ glucose-933 consumers per mL.

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