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Guided assembly of multispecies positive biofilms targeting undesirable bacteria

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1 **Guided assembly of multispecies positive biofilms**

2 **targeting undesirable bacteria**

3 Short title: Guided assembly of positive biofilms

4
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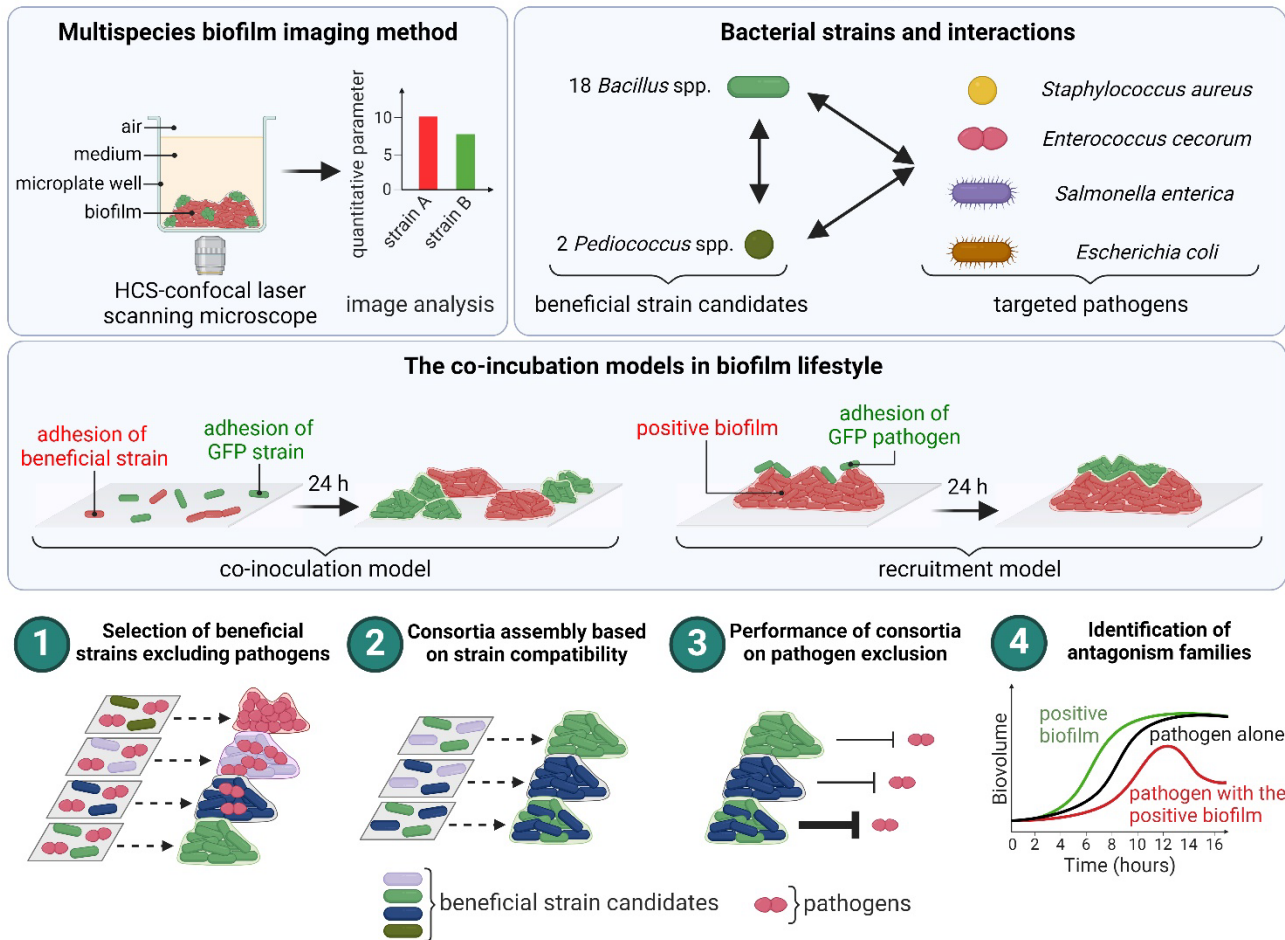
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19 Keywords: biofilms, microbial pathogens, synthetic microbial communities, *Bacillus* spp.,
20 *Pediococcus* spp., microbial growth modelling, 4D live-cell imaging

Abstract

22

23 The use of synthetic microbial communities (SynComs) engineered to form positive biofilms that
24 prevent the settlement of harmful bacteria is emerging as a promising strategy in biotechnology,
25 particularly in reducing reliance on chemical antimicrobials. Despite this potential, the rationale for
26 selecting specific strains in SynComs and the mechanisms underlying their antagonistic effects
27 remains insufficiently understood. In this study, we present a bottom-up approach integrating live-
28 cell imaging with high-throughput analysis of multi-strain biofilms across diverse scenarios. Through
29 this method, we identified beneficial strains based on their superior ability to exclude undesirable
30 bacteria and form mixed biofilms. Notably, our findings revealed that competitive strains against
31 undesirable bacteria could also exclude other beneficial strains, emphasising the need for
32 compatibility control in SynComs design. SynComs composed of *B. velezensis* and *Pediococcus* spp.
33 demonstrated enhanced pathogen exclusion compared to single strains. Temporal analysis of biofilm
34 interactions, supported by mathematical models, showed that pathogen exclusion was primarily
35 driven by nutritional competition (Jameson effect) with additional specific interference mechanisms
36 (prey-predator Lotka-Volterra model). Furthermore, pre-establishing SynComs to surfaces
37 significantly increased pathogen inhibition, indicating a distinct biofilm-associated exclusion effect.
38 These insights offer a framework for rational SynCom design and deepen our understanding of the
39 mechanisms underpinning positive biofilm applications.

Design of synthetic microbial communities targeting undesirable bacteria



40

41

Introduction

42 Microorganisms predominantly exist as biofilms, which colonise a wide variety of biotopes on Earth
 43 [1]. Biofilms are intricate communities of spatially organised microorganisms embedded in a self-
 44 produced matrix. These communities thrive at interfaces, interacting with each other and their
 45 environment [2]. The three-dimensional structures, coupled with diverse cell populations and matrix,
 46 endow biofilms with unique properties distinct from planktonic lifestyles [3–5]. Biofilms typically
 47 comprise multiple species engaged in various interactions, including resource competition,
 48 cooperation, and inhibition of other species [6,7]. Deciphering these complex interaction networks to
 49 predict biofilm behaviour and functions is a pivotal challenge in microbial ecology.

50 In line with the “One Health” concept to rationalise the use of chemical antimicrobials,
51 biotechnological solutions are being developed to regulate interactions within complex biofilm
52 communities through guided microbial ecology [8]. For instance, single strains or synthetic microbial
53 communities (SynComs) [9,10] capable of forming positive biofilms with antagonistic activity
54 against undesirable microorganisms can be intentionally introduced in the foods chain [11], directly
55 onto hosts [12,13] but also more recently on surfaces such as livestock buildings [14].

56 A competitive ecological process can be established between bacterial species, resulting in the
57 dominance of one and the exclusion of the other [15]. Pathogen exclusion can be attributed to
58 nutritional [16] and spatial competition [17], as well as the secretion of interfering molecules through
59 quorum sensing modulation [18], bacteriostatic [19], or bactericidal effects [20]. Each exclusion
60 mechanism can be represented using mathematical modelling tools based on temporal analyses [21].
61 For instance, the Jameson-effect model can be described as competition between species over the use
62 of environmental resources in order to maximise their growth and population. This model accounts
63 for a nutritional competition which results in the deceleration of the population growth when the
64 common resource(s) are exhausted [16]. Another model, Lotka-Volterra prey-predator model,
65 implicates the secretion of interfering molecules, leading to the decline of the prey population. [22].
66 These models provide quantitative parameters describing the evolution of the partners and their
67 mutual influence. However, quantifying antagonism typically relies on assessing planktonic
68 interactions, which do not accurately reflect real conditions under which microbial communities
69 reside in biofilms [23]. Furthermore, the inclusion of multiple strains in SynComs lacks clear
70 justification and assurance of compatibility. In addition, the enhanced effects of SynComs compared
71 to their constituent strains are often unclear.

72 To enhance the design of SynComs for positive biofilm applications, we developed a systematic
73 pipeline to select beneficial strain candidates that specifically antagonise pathogens through their
74 biofilm formation. SynComs were designed based on the ability of strains to coexist in the same
75 positive biofilms without excluding partners. The objective was to identify compatible beneficial

76 strains able to form a mixed biofilm with enhanced pathogen exclusion and biofilm formation
77 capability compared to individual strains. This bottom-up approach relies on non-destructive
78 observation of multispecies biofilm phenotypes using high-content screening confocal laser scanning
79 microscopy (HCS-CLSM) combined with genetically engineered fluorescent strains and dedicated
80 image analysis [24]. The study includes over 23 000 meticulously analysed z-stack images,
81 predominantly utilising two colour channels to extract quantitative data on positive biofilm efficiency.
82 The collection of tested beneficial strain candidates comprised 18 *Bacillus* and 2 *Pediococcus* strains.
83 These genera are renowned for their biofilm-forming abilities [25,26], exclusion capabilities [20,27],
84 and versatile applications in biotechnology, biocontrol [28] and biopreservation [11], making them
85 attractive candidates for investigating their potential to create positive biofilms. The 20 candidate
86 strains were screened for their impact on the growth and establishment of several pathogenic bacteria
87 affecting people and/or animals, including *Staphylococcus aureus*, *Enterococcus cecorum*,
88 *Escherichia coli*, and *Salmonella enterica* serovar Enteritidis (*S. enterica*), in two submerged mixed-
89 species biofilm models developed for this study.

90 Through 4D (xyzt) HCS-CLSM imaging of interspecies interactions in biofilms, the underlying
91 family of mechanisms driving antagonistic interactions on pathogens by the SynComs were
92 elucidated. Moreover, we showed by modelling of biofilm interaction curves that these mechanisms
93 depend on the initial quantities of each partner in the mixed biofilm and are specific to the biofilm
94 lifestyle.

95 Together, these results allow for the rationalisation of SynCom formulation for positive biofilm
96 application to limit pathogen growth and establishment on surfaces, while understanding the families
97 of interactions involved.

Materials and methods

98

99 *Bacterial strains and genetic constructs*

100 The wild-type (WT) bacterial strains and genetic constructions used in the study are listed in **Sup. 1**.
101 The phylogenetic analysis of the strains was conducted using the bacterial phylogenetic tree service
102 provided by BV-BRC [29]. The phylogenetic tree was then constructed using the default codon tree
103 method. For species assignments, the genome sequences were compared to those of the closest
104 species within the *Bacillus* or *Pediococcus* genus in the BV-BRC database, using 1,000 genes with a
105 tolerance for five deletions. Subsequently, the beneficial strains were ordered based on their
106 phylogenetic distances (**Sup. 2**). The transformation protocol using the pCM11 plasmid derivatives,
107 carrying the GFP or the mCherry-encoding genes, was adapted for each strain and is detailed in **Sup.**
108 **3**. Briefly, wild type *E. coli* and *S. enterica* were transformed using a standard heat shock protocol
109 [30]. The protocols for preparing electrocompetent cells and transforming *E. cecorum* were adapted
110 from Dunny *et al.* [31]. *B. velezensis* was transformed based on its natural competence [32] using a
111 methodology inspired by Dergham *et al.* [33]. The plasmid stability assay is presented in **Sup. 4**.

112 *Biofilm models*

113 All experiments were conducted at 30°C using Tryptic Soy Broth (TSB) medium (BioMérieux,
114 France) supplemented with antibiotics (5 µg/mL erythromycin for Gram-positive species or 100
115 µg/mL ampicillin for Gram-negative species) when appropriate. 5 mL overnight cultures, inoculated
116 from a glycerol stock at -80°C, were centrifuged at 5000 g for 5 minutes and then re-suspended in
117 fresh TSB medium prior to conducting each experiment. The biofilms were cultivated at the bottom
118 of a µclear® 96-well plate (Greiner Bio-one, France) which is compatible with high-resolution
119 fluorescence microscopy. In this study, we have developed two biofilm models to study microbial
120 interactions in multi-strain biofilms.

121 *Co-inoculation model*

122 The co-inoculation model was developed with the objective of facilitating the controlled ratios of
123 adhered strains on samples while limiting the presence of swimming planktonic cells. Multi-strain
124 biofilms were established using fluorescent genetically labelled pathogenic strains in conjunction
125 with non-labelled strains (one, two, or three strains). The initial adhesion biovolume between the
126 fluorescent strain and the non-labelled strains was standardised using the protocol described by
127 Guéneau *et al.* [24], which is based on a dual labelling by the GFP and the SYTO61, a cell-permeant
128 red dye that binds to nucleic acid (Invitrogen, Carlsbad, CA, USA.). In this system, cells expressing
129 the GFP will exhibit both green and red fluorescence, whereas untagged cells will only display the
130 red fluorescence. In our kinetic measurements, the SYTO61 has been replaced by the FM4-64, a
131 lipophilic red dye that binds to cell membranes (Invitrogen, Carlsbad, CA, USA) at a vital
132 concentration of 1 µg/mL [34]. Three initial adhesion ratios were subjected to kinetic experiments.
133 The first comprised two times more pathogens compared to the beneficial strain(s) (ratio 1). The
134 second had two times more beneficial strain(s) compared to the pathogens. The third had 10 times
135 the amount of the beneficial bacteria candidate(s) (ratio 3).

136 In practice, overnight cultures of the GFP-labelled and genetically unlabelled strains were diluted in
137 1 mL of TSB to achieve the desired adhesion ratio. 200 µL of the bacterial solution were added to the
138 µclear 96-well plates and allowed to adhere statically at 30°C for 1.5 hours. The supernatant was then
139 replaced with fresh TSB and the cultures were incubated for 24 hours at 30°C. The same protocol
140 was employed to assess the compatibility between the GFP-labelled *B. velezensis* strains and the other
141 beneficial strain candidates. The adhesion ratios were determined with the GFP-labelled *B. velezensis*
142 12048 and subsequently applied to *B. velezensis* 11285 and *B. velezensis* ILPB8. In the kin
143 consortium compatibility assay composed of these three *B. velezensis* strains, the SYTO9, a cell-
144 permeant green dye that labels nucleic acid, was employed at a final concentration of 2 µg/m. In
145 conjunction with the SYTO9, another nucleic acid labelling dye, DAPI was used (Invitrogen,
146 Carlsbad, CA, USA). In this experiment, a LSM 700 inverted Confocal Scanning Laser Microscope

147 (Carl Zeiss, Germany) equipped with a 405 nm laser was utilised to facilitate the excitation of the
148 blue nucleic acid dye DAPI.

149 *Recruitment model*

150 The recruitment model was developed for the purpose of examining pathogen adhesion and growth
151 on a pre-established positive biofilm. The overnight cultures of pathogens were subjected to
152 centrifugation at 5,000 rpm for 10 minutes, and re-suspended in fresh TSB medium in order to remove
153 any residual antibiotics. In the case of the kinetics experiment, the resulting suspension was then
154 supplemented with 1 µg/ml of FM4-64 membrane dye. A 50-microliter aliquot of a GFP-labelled
155 pathogen suspension was added to wells containing a 24-hour-old positive biofilm and allowed to
156 adhere statically at 30°C for 1.5 hours. Subsequently, the supernatants containing non-adhered cells
157 were replaced with 200 µL of fresh TSB. CLSM acquisitions were conducted either directly
158 (recruitment t=0h) or after 24 hours of growth at 30°C (recruitment t=24h). Prior to acquiring images
159 via CLSM, 50 µL of a TSB solution containing SYTO61 at 2 µg/mL or FM4-64 at 1 µg/mL (for the
160 kinetic assays) was added to the wells.

161 *Live-cell fluorescent imaging using CLSM*

162 Live-cell fluorescent imaging was conducted using a Leica SP8 AOBS inverted high-content
163 screening confocal laser scanning microscope (HCS-CLSM, LEICA Microsystems, Germany,
164 MIMA2 platform of INRAE, <https://doi.org/10.15454/1.5572348210007727E12>). Biofilm images
165 were acquired using a 63x water immersion objective with a numerical aperture of 1.2. Imaging was
166 performed at a frequency of 600 Hz, with images taken every micrometre to ensure comprehensive
167 coverage of the full height of the biofilm. The images had a resolution of 512 × 512 pixels, covering
168 a physical area of 184.52 µm x 184.52 µm with a pixel size of 0.361 µm. For fluorescence detection,
169 SYTO9 and GFP were excited with an argon laser at 488 nm, and the emitted fluorescence was
170 collected using a hybrid detector (HyD LEICA Microsystems, Germany) in the range 500-550 nm.

171 FM4-64 was excited through the use of a helium-neon laser at 561 nm, and the emitted fluorescence
172 subsequently collected with a hybrid detector in the range of 600-750 nm. Excitation of SYTO 61
173 was achieved at 633 nm and the emitted fluorescence was collected with a hybrid detector in the range
174 of 650-750 nm.

175 For 4D (xyzt) acquisitions, the temperature was maintained at 30°C and 150 µm stacks were
176 automatically acquired every hour using the HCS mode of the confocal microscope. Each experiment
177 included between 3 and 6 biological replicates, with 4 technical replicates per biological replicate,
178 thereby providing at least 12 technical values per condition.

179 *CLSM image analysis*

180 Two-dimensional projections of biofilms and movies were generated using IMARIS 9.3.1 software
181 (Bitplane, Zurich, Switzerland). Quantitative analysis of image stacks were performed using the
182 BiofilmQ software [24,35]. Each fluorescence channel was analysed separately using the OTSU
183 thresholding method and “global biofilm properties” were selected to extract the biovolume of the
184 binarized images.

185 To assess the activity of *Bacillus* species strains against pathogens, the GFP biovolume ($\mu\text{m}^3/\mu\text{m}^2$) of
186 submerged mixed biofilms was quantified and normalised to the biovolume of biofilms of a specific
187 pathogen labelled with GFP. To standardise a score reporting biofilm anti-pathogenic activity, values
188 were centre-reduced to fall between 0 (indicating the lowest activity) and 1 (representing the highest
189 activity). This scoring method facilitated comparison of the anti-pathogenic activity of different
190 *Bacillus* strains, allowing the identification of the most promising potential.

191 *Modelling microbial growth and competition*

192 GFP-measured biovolumes served as model inputs for both pathogens. For *Bacillus* in monoculture,
193 biovolumes corresponding to the FM4-464 marker were used. In co-culture, beneficial strain(s)

194 biovolumes were determined by subtracting the GFP-biovolume of the co-inoculated pathogen, from
195 the total biovolume.

196 For monoculture experiments, biovolume increase was described using a generic primary growth
197 model [36] :

$$\frac{1}{N(t)} \frac{dN(t)}{dt} = \mu_{max} \cdot \alpha(t) \cdot f(t)$$

198
199 This model defines μ_{max} as the exponential growth rate, $\alpha(t)$ as the adjustment function, and $f(t)$ as the
200 inhibition function. The nlsMicrobio package was employed to fit the Baranyi & Roberts primary
201 growth model, providing estimates for μ_{max} , lag phase (derived from $\alpha(t)$), maximum population size
202 (N_{max} , derived from $f(t)$), and initial population size (N_0).

203 Two types of models were fitted to the competition data between pathogens and bioprotective flora.

204 The first type of model is the Jameson-type model [37]. The Jameson effect can be described as
205 competition between species to use environmental resources in order to maximise their growth and
206 population. When the common resource(s) are exhausted, the competition is over and the growth of
207 each species in the population stops.

208 The growth stops simultaneously by both populations. Both competitors share the inhibition function
209 ($f(t)$) of the exponential growth.

$$\frac{1}{N_A(t)} \frac{dN_A(t)}{dt} = \mu_{max A} \cdot f_A(t)$$

$$\frac{1}{N_B(t)} \frac{dN_B(t)}{dt} = \mu_{max B} \cdot f_B(t)$$

210

211 The inhibition function being

$$f_A(t) = f_B(t) = \begin{cases} 1 & \text{if } t < t_{max} \\ 0 & \text{if } t \geq t_{max} \end{cases}$$

212

213 where t_{max} is the time at which the stationary phase begins for populations A and B.

214 The second type of model is the Lotka-Volterra model. In this model, the inhibition functions can be
215 described as follow:

$$\begin{cases} f_A(t) = \left(1 - \frac{N_A(t) + \alpha_{AB}N_B(t)}{N_{maxA}} \right) \\ f_B(t) = \left(1 - \frac{N_B(t) + \alpha_{BA}N_A(t)}{N_{maxB}} \right) \end{cases}$$

216

217 where the parameters α_{AB} and α_{BA} are the coefficients of interaction measuring the effects of one
218 species on the other.

219 It makes no specific assumptions about the mechanisms underlying species interactions; they can be
220 parameterised in ways that approximate any combination of underlying mechanisms. For example,
221 in a system with two species, if both α_{AB} and α_{BA} are less than zero, species suppress each other's
222 growth, indicating competition.

223 *Statistical analysis*

224 Results related to the description of biofilm phenotypes were presented as mean and standard
225 deviation (SD). Statistical analysis was performed using two-way ANOVA followed by Fisher's least
226 significant difference without correction, utilising PRISM software (GraphPad, USA, California).
227 Statistical significance was determined at a p -value less than 0.05. The significance levels are denoted
228 as follows: * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, **** for $P < 0.0001$.
229 The nlsMicrobio [38] and gauseR [39] packages were respectively used to fit the Barnayi&Roberts
230 growth model, Jameson-type and Lotka-Volterra models to biovolumes.

231

Results

232 *Selection of beneficial strains for pathogen exclusion*

233 A collection of 20 beneficial strains from various isolation origins was first characterised for different
234 surface-biofilm and motility phenotypes. These phenotypes included the formation of macro-
235 colonies, the development of submerged biofilm, and the ability of strains to swarm at the surface of
236 semi-solid agar. This phenotypic characterization revealed a wide range of phenotypic diversity (**Sup.**
237 **5**). While differentially affected by the ability to spread on solid (macrocolony) or semi solid
238 (swarming) surfaces, all strains were observed to develop submerged biofilms of varying thickness
239 and structures. This observation led us to use the submerged biofilm as a working model in our study.
240 A screening process was conducted to identify beneficial strains capable of restricting the growth and
241 establishment of four pathogens, *E. cecorum*, *S. aureus*, *E. coli* and *S. enteritica* (**Fig. 1A, B**). To this
242 end, we developed two co-incubation biofilm assays. These assays were based on the formation of
243 mixed biofilms after the co-inoculation of strains at different ratios and on the potential recruitment
244 of a pathogenic strain by a preformed biofilm composed of potentially beneficial bacilli strain(s). To
245 ensure a balanced initial co-inoculation of both the beneficial and pathogen strains, the adhesion ratio
246 was first validated by CLSM (**Sup. 6**). After co-inoculation and 24 hours of growth, the raw
247 biovolume of GFP-labelled pathogens from z-stacks were extracted by image analysis (**Sup. 7**).
248 Regarding the recruitment assay, the raw biovolumes of the pathogen were quantified to investigate
249 its adhesion (recruitment t=0 h, **Sup. 8**) and growth (recruitment t=24 hours, **Sup. 9**) on an established
250 positive biofilm. The data were used to calculate antagonistic scores for each beneficial strain against
251 pathogens (**Fig. 1C**). Our results revealed different modes of interactions between the beneficial
252 bacilli and the pathogen. For instance, in a co-inoculation assay, *E. coli* and *S. enterica* were not
253 excluded. Conversely, in the recruitment assay, the *B. velezensis* strains as well as *Paenibacillus* spp.
254 1167 exhibited a strong capacity to exclude *S. enterica*. Pathogen adhesion to pre-established positive

255 biofilms (*i.e.*, recruitment at T=0 h) was reduced with most beneficial strains, except for *S. enterica*.
256 Overall, *B. velezensis* consistently demonstrated superior pathogen exclusion capacity across the two
257 interaction models in comparison to other beneficial strains. Remarkably, *B. velezensis* strains 11285,
258 12048, and ILPB8 consistently achieved the highest scores and illustrated superior performance
259 against *E. cecorum* and *S. aureus*.

260 Cell counts were performed using *B. velezensis* ILPB8 strain, which exhibits a high antagonistic
261 score, thereby confirming that the reduction in the GFP signal in our experiments was a direct result
262 of a decline in cell numbers (**Sup. 10**).

263 *Engineering a mixed biofilm from compatible beneficial bacterial strains*

264 We selected *B. velezensis* strains 11285, 12048, and ILPB8 based on the highest sum of antagonist
265 scores against pathogens. We employed a co-inoculation model to identify compatible mixed biofilms
266 resulting from interactions between the three genetically GFP-tagged *B. velezensis* strains and other
267 beneficial candidate strains. Initially, the adhesion ratios between *B. velezensis* 12048 GFP and 20
268 beneficial strains were determined and applied subsequently to *B. velezensis* 11285 GFP and *B.*
269 *velezensis* ILPB8 GFP (**Sup. 11**). After 24 hours of growth, the biovolumes were extracted from the
270 z-stacks of images. The GFP/SYTO61 ratio was calculated to assess the strains' ability to coexist or
271 exclude each other (see **Fig. 2A, B**). Our results suggest that beneficial strains exhibit mutual
272 exclusion tendencies. Mixed biofilm formation occurred only when the three GFP-labelled *B.*
273 *velezensis* strains were combined with their genetically unlabelled counterparts or with the two
274 phylogenetically more distant *Pediococcus* spp. Notably, none of the combinations exhibited a total
275 biovolume (SYTO61) significantly different from that of either strain when grown individually (**Sup.**
276 **12**).

277 *A three-strain B. velezensis kin-compatible consortium covers more surface*
278 *area but do not exhibit greater antagonism than single strains*

279 Further studies were carried out to assess the potential of the selected *B. velezensis* strains 11285,
280 12048 and ILPB8, to form a three-strain mixed biofilm. Co-inoculation experiments were performed,
281 incorporating two of the *B. velezensis* strains, genetically tagged with GFP or mCherry, together with
282 the wild-type of the third *B. velezensis* strain (**Fig. 3A**). After staining the consortium with the DNA
283 binding fluorescent dye DAPI, the observations and proportion quantifications of the three strains
284 revealed a uniformly mixed biofilm (**Fig. 3B**). The strains within the kin consortium showed no
285 significant differences in their biovolume (**Fig. 3C**). However, the kin consortium was found
286 to exhibit a significantly higher surface area coverage than the one- and two-strains combinations
287 (**Fig. 3D**).

288 Further exploration of the mechanisms underlying the exclusion of *E. cecorum* and *S. enterica* by *B.*
289 *velezensis* was conducted using HCS-CLSM kinetic experiments applied to the two co-incubation
290 models. (**Sup. 13**). To achieve this, the vital membrane-dye FM4-64 was used to visualise the entire
291 biofilm without altering the growth of the pathogen (**Sup. 14**). Biovolume curves over time were
292 extracted from the images and processes using the Jameson or Lotka-Volterra mathematical models
293 to determine the growth rate and the growth potential (**Sup. 15**). Additionally, to enhance our
294 understanding of interaction mechanisms, distinct initial adhesion ratios of *B. velezensis* and
295 pathogens were applied. The first ratio (ratio 1) comprised twice the number of pathogens in
296 comparison to the beneficial strain(s). The second ratio (ratio 2) contains twice more beneficial
297 strain(s) compared to the pathogens. Finally, the third ratio (ratio 3) has 10 times the amount of the
298 beneficial bacteria candidate(s). We observed that the growth rate of *E. cecorum* was reduced in the
299 presence of *B. velezensis* under all conditions except when the initial ratio started with a higher
300 proportion of the pathogen (ratio 1) (**Fig. 4A**). For all ratios, the growth potential was found to
301 decrease and even to become negative with the ratio 1, indicating a decline of *E. cecorum* biovolume

302 at the end of the kinetics compared to the initial situation (**Fig. 4B**). In this specific case, the
303 interactions were found to adhere to a Lotka-Volterra model. The growth rate and growth potential
304 of *S. enterica* in the presence of *B. velezensis* were only observed to be altered in the context of
305 recruitment (**Fig. 4C, D**). With the exception of the ratio 1 in co-inoculation with *E. cecorum*, the
306 interaction profiles were found to adhere to a Jameson model for all the strains of *B. velezensis* and
307 their associated consortia against pathogens. However, significant differences in exclusion capacity
308 between the *B. velezensis* strains against the two pathogens could be observed (**Sup. 16-17**).

309 *Additive anti-pathogenic effects of B. velezensis and Pediococcus spp.* 310 *compatible consortia*

311 We demonstrated that the Kin consortium could form compatible and homogeneous mixed biofilms
312 with the two *Pediococcus* strains *P. acidilactici* R1001 or *P. pentosaceus* R1094 (**Fig. 5 A, B**). The
313 four-strain consortia covered over 80% of the surface, mainly due to the influence of *Pediococcus*
314 spp. biofilm, which developed between the clusters formed by the Kin consortium (**Fig. 5 A, C**). This
315 biofilm exhibited a greater biovolume compared to controls (**Fig. 5 D**).

316 The most effective exclusion activity against pathogens at 24h is achieved by mixed biofilms
317 composed of *Pediococcus* spp. and *B. velezensis* strains from the Kin consortium (**Sup. 18 A**). Indeed,
318 the consortia composed of *B. velezensis* and *Pediococcus* spp. demonstrated a consistently enhanced
319 ability to exclude *S. enterica* at 24 hours compared to that of individual beneficial strains in the
320 recruitment model ($P < 0.0001$). In addition, the consortia performed at least as well as the best
321 individual strains (**Sup. 18 B-G**). Therefore, we investigated the mechanisms of *S. enterica* exclusion
322 in the first 12h of recruitment using HCS-CLSM coupled with modelling (**Fig. 6 A**). The growth rate
323 of *S. enterica* was found consistently reduced in the presence of beneficial strains, with no
324 improvement observed in the Kin consortium when combined with *Pediococcus* spp. (**Fig. 6 B**).
325 However, a significant ($P < 0.01$) decrease in *S. enterica* growth potential was observed when *P.*

326 *pentosaceus* R1094 was added to the Kin consortium, compared to the performance of either the Kin
327 consortium or *P. pentosaceus* R1094 alone (**Fig. 6 C**).

328 We investigated whether the acidification of the environment by *Pediococcus* spp. was involved in
329 enhancing the effect of *B. velezensis* on the exclusion of *S. enterica* in the recruitment t=24h model
330 (**Sup. 19**). We demonstrated that acidification proportionally reduced the biovolume of *S. enterica*
331 biofilms but did not affect the exclusion by *B. velezensis* ILPB8 (**Sup. 20**).

332 Interestingly, the biofilm lifestyle influences interactions differently compared to the planktonic
333 lifestyle. Indeed, in the planktonic mode, *Pediococcus* spp. are excluded by *B. velezensis* (**Sup. 21**).
334 *S. enterica* systematically excludes *B. velezensis* or *Pediococcus* spp. when starting with the same
335 ratio of the two partners in planktonic cultures. Furthermore, starting with the same initial inoculum
336 as for recruitment, but agitating the cultures under planktonic conditions, a culture containing *B.*
337 *velezensis* is able to completely inhibit the growth of *S. enterica*, whereas *Pediococcus* spp. alone was
338 excluded (**Sup. 22**).

339 Discussion

340 This study presents a rational and systematic approach for selecting and combining strains within
341 SynComs to form stable biofilms with robust antagonistic activity against bacterial pathogens. By
342 employing a non-destructive high-throughput imaging pipeline, we demonstrated that compatible
343 strains, such as *B. velezensis* and *Pediococcus* spp., can form stable mixed biofilms with enhanced
344 pathogen exclusion capabilities. These findings underscore the potential application of these
345 communities in positive biofilm strategies for surface microbial management.

346 Common methods for studying microbial interactions in multi-species communities, such as
347 metabarcoding [40], optical density measurements [41], or colony-forming unit (CFU) counting [42],
348 often disrupt intricate three-dimensional structures crucial in biofilm studies [43]. In contrast, our
349 approach utilised non-destructive imaging techniques, allowing precise quantification of individual

350 community members and observation of their spatial arrangement over time. This approach addresses
351 significant gaps in existing methodologies, which often neglect critical factors such as adhesion rates
352 and the formation of filamentous structures or aggregates [2,25]. Our co-inoculation model, which
353 used image-based post-adhesion biovolume calibration, ensured accurate initial quantification of both
354 partners on the substrate - a crucial step in understanding biofilm dynamics, where spatial
355 arrangements strongly influence microbial interactions [44].

356 Despite the advantages of microscopy techniques, including their capacity for high throughput and
357 detail analysis, they are inherently time-consuming and generate large volumes of data. Furthermore,
358 distinguishing the contributions of each strain within biofilms composed of more than three species
359 presents significant challenges. To overcome these hurdles, genetic manipulation of strains for the
360 expression of distinct fluorophores or the use of specific dyes is necessary. The selection of
361 fluorophores with distinct absorption and emission spectra is critical to prevent overlap within the
362 same sample. These technical limitations restricted our ability to explore all potential strain
363 combinations, such as co-culturing *P. acidilactici* and *P. pentosaceus*, due to the absence of
364 corresponding fluorescent strains. Future investigations should consider the microbiota context of the
365 studied ecosystem, as it may influence observed interaction phenomena [45].

366 Through temporal imaging and mathematical modelling, we identified key mechanisms driving
367 pathogen exclusion within biofilms, primarily involving spatial and nutritional competition, along
368 with the potential synthesis of antimicrobial compounds. Our findings suggest that pathogen
369 exclusion is largely governed by the Jameson effect, coupled with specific interference mechanisms,
370 where both the growth potential (nutritional competition) and the growth rate (secretion of
371 bacteriostatic molecules) of the pathogen are reduced [16]. While this image-based approach provides
372 a general framework for understanding these dynamics, it does not pinpoint specific molecular
373 effectors responsible for pathogen exclusion. Future studies using omics approaches in
374 combination with genetic analysis may help to elucidate these molecular underpinnings of pathogen
375 exclusion [46].

376 The inhibitory effect of *B. velezensis* on a broad range of bacteria is well documented, often attributed
377 to the secretion of interfering molecules [47], some of which are specifically expressed in biofilms
378 [48]. In interactions that align with the Lotka-Volterra prey-predator model, mutual influence
379 between microbial partners is observed [22]. Interestingly, at certain concentrations, the positive
380 biofilm would secrete a molecule capable of killing *E. cecorum*, although the specific nature of this
381 molecule remains unknown. These findings emphasise the need to consider the biofilm lifestyle, as
382 their related kinetics differ significantly from those in planktonic cultures, even when starting with
383 the same initial ratios of beneficial strains and the evaluated pathogen. This distinction is crucial, as
384 metabolic processes vary widely between biofilm and planktonic states [5]. Therefore, the biofilm
385 lifestyle should be a key consideration in designing SynComs intended for environments where
386 bacteria are likely to form biofilms. Moreover, the experiments were conducted under controlled
387 laboratory conditions with a maximum of 5 partners. A future perspective of this work would be to
388 consider the microbiota of the specific surfaces to be treated in order to study the interactions, which
389 is important to take into account, as it can potentially exclude both pathogens and intentionally
390 applied beneficial SynComs [49].

391 Before evaluating the antagonistic effects of SynComs on pathogen growth, we systematically
392 assessed the compatibility of strains to establish stable mixed biofilms without excluding other
393 partners. Our results revealed that *B. velezensis* strains, selected for their ability to exclude pathogens,
394 also tend to exclude other *Bacillus* spp. However, the compatibility between the three *B. velezensis*
395 strains is consistent with the principle of kin discrimination, whereby organisms
396 differentiate between genetically related (kin) and unrelated (non-kin) individuals [50,51]. This
397 discrimination is likely based on the detection of small signalling molecules or specific flagellin
398 motifs in *B. velezensis* [52].

399 Notably, only the three competitive *B. velezensis* strains were able to form mixed biofilms with one
400 another and with the more distantly related *Pediococcus* spp. Even so, no significant increase in
401 pathogen exclusion was observed in these kin-compatible consortia compared to individual strains,

402 likely due to the presence of shared competition mechanisms and substantial overlap in gene content.
403 By selecting more phylogenetically distant but compatible species, such as *B. velezensis* and
404 *Pediococcus* spp., we have achieved genetic diversification, enhanced exclusion mechanisms and
405 extended coverage to different physico-chemical environments [49]. Interaction experiments
406 confirmed that consortia composed of *B. velezensis* and *Pediococcus* spp. exhibited antagonistic
407 scores against pathogens at least as high as the best-performing individual strain. Additionally,
408 consortia of *B. velezensis* and *Pediococcus* spp. demonstrated a systematic additive effect against *S.*
409 *enterica* in the recruitment model, where the positive biofilm is pre-established before pathogen
410 arrival. This effect is not only attributed to pH reduction by *Pediococcus* spp., but rather to spatial
411 and nutritional competition, as supported by our modelling experiments. This finding is consistent
412 with the high surface coverage capacity and improved biofilm production observed in consortia of *B.*
413 *velezensis* and *Pediococcus* spp.

414 The use of consortia composed of a *B. velezensis* strain from the Kin consortium and a *Pediococcus*
415 spp. strain results in more intense exclusion of *S. enterica* after 24 hours in the recruitment model
416 compared to the performance of the individual strains. Kinetic experiments over 12 hours in the
417 recruitment model demonstrate that the Kin consortium, when paired with the *P. pentosaceus* R1094
418 strain, significantly reduces the growth potential of *S. enterica*. This suggests that the combination of
419 the Kin consortium with the R1094 strain allows for more effective early exclusion, which levels out
420 over time with the *P. acidilactici* R1001. Further studies should investigate potential beneficial
421 metabolic exchanges between *B. velezensis* and *Pediococcus* spp. as well as the dynamics of pathogen
422 exclusion during interference. This should be achieved through the use of omics approaches [53] and
423 genome-based modelling [54].

424 In conclusion, our study highlights the compatibility between selected *B. velezensis* and *Pediococcus*
425 spp. strains, resulting in improved pathogen elimination with a specific additive effect on *S. enterica*.
426 The compatibility and exclusion capacity of strains are intricately linked to biofilm formation,
427 emphasising the importance of pre-establishing positive biofilms on surfaces to achieve additive

428 effects. The imaging methodology presented provides a rational framework for assembling SynComs
429 in a biofilm context, with a focus on strain compatibility and antagonistic potential against pathogens.
430 Moreover, the techniques developed in our study have broad potential applications in
431 biotechnological fields aimed at pathogen control, bioremediation, biopreservation and probiotics.

432 **Data availability**

433 Raw stacks of images dataset corresponding to the figure 1 and 2 has been deposited in Data INRAE
434 (accession number <https://doi.org/10.57745/XRXQEI>). Further inquiries can be directed to the
435 corresponding author. Dataset kinetics and R codes used to assess growth parameters are available
436 on Github: https://github.com/lguillier/dataset_kin

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442 **Conflict of interest**

443 VG, CB, GJ, JP-G and MC are employees of the Lallemand SAS company. All the authors declare
444 no competing interests.

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451 (<https://www.biorender.com/>).

452 **Author contributions**

453 VG, LG, MC and RB: conceptualization and methodology. MC and RB: validation and supervision.
454 VG, LG, CB: formal analysis and data curation. VG, LG, JP-G, MC, and RB: investigation. PS et M-
455 FN-G contribute to develop the transformation protocol of the strains. GJ generates the phylogenetic
456 tree. MC and RB: resources, project administration, and funding acquisition. VG: writing the original
457 draft. M-FN-G, PS, LG, MC and RB: reviewing, and editing. All authors have read and agreed to the
458 published version of the manuscript.

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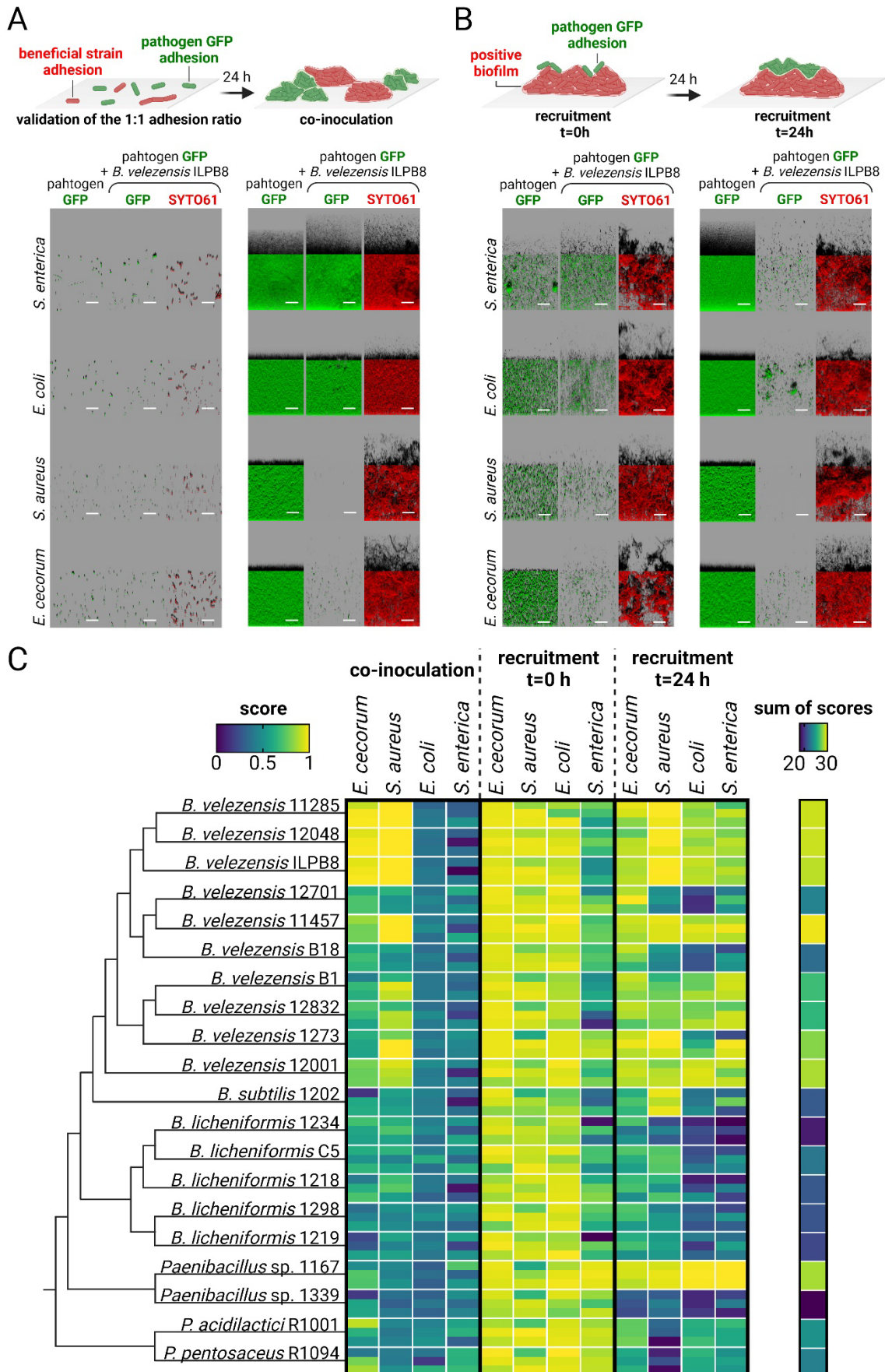
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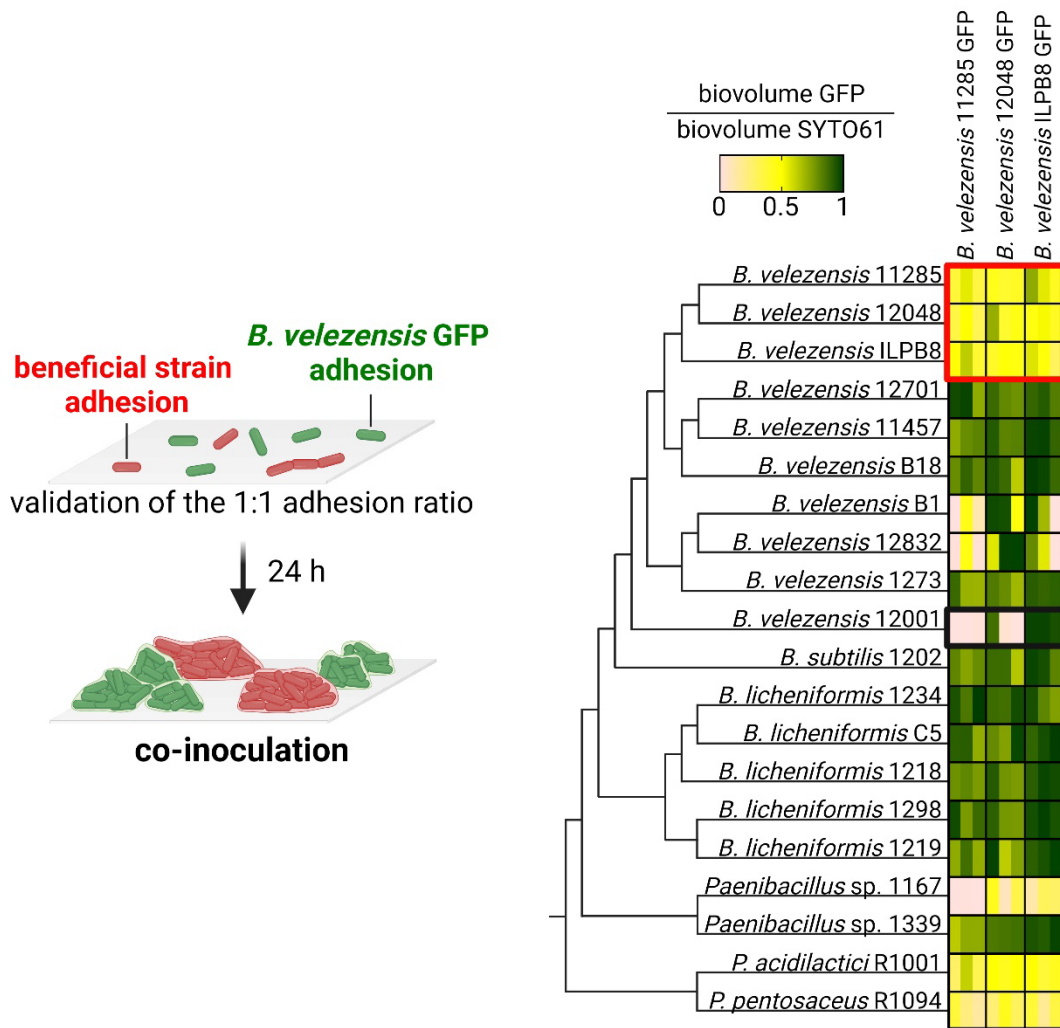
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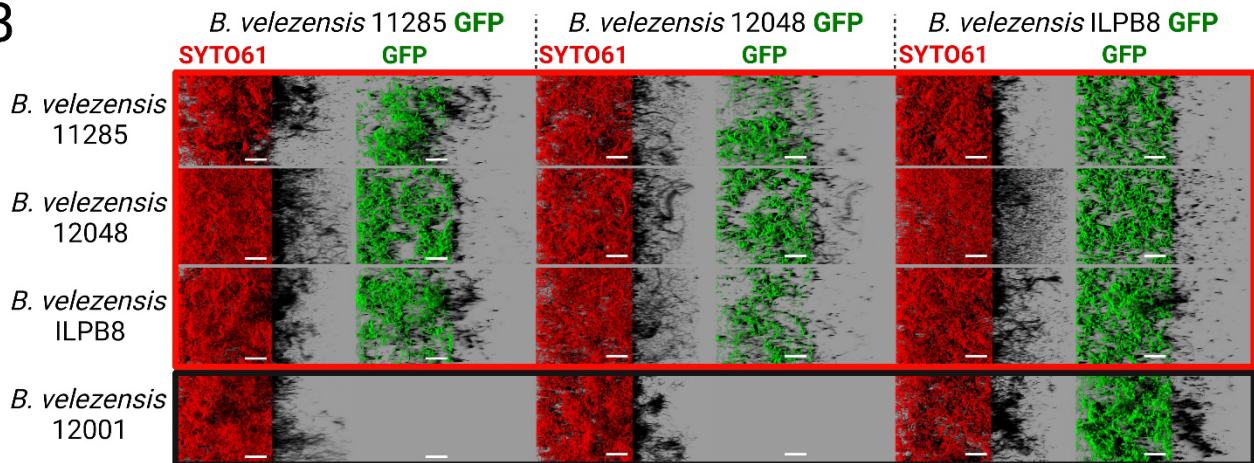
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587 **Figure 1: Antagonism scores of the candidate beneficial strains against pathogens in two co-**
588 **incubation models.** (A) In the co-inoculation model, adhesion ratios (0.50 +/- 0.25) of both
589 pathogenic and beneficial strains were determined by image analysis of CLSM observations.
590 Following adhesion with these pre-determined ratios, biofilms were incubated 24 hours before being
591 observed with CLSM. (B) In the second model, a planktonic culture of the pathogen was added to a
592 pre-established biofilm for 24 hours prior CLSM observation. After adhesion, a 24-hour incubation
593 period was followed by CLSM observation. In both models, SYTO61 was used to visualise the entire
594 population in red (i.e. pathogens and beneficial strains). Pathogen GFP biovolumes were quantified
595 to identify beneficial strains that reduced pathogen biovolume compared to pathogens growing alone
596 under the same conditions. Representative images are shown from the strain *B. velezensis* ILPB8.
597 Scale bar = 40 µm. (C) Pathogens were grouped by model and the 20 candidate beneficial strains
598 were ordered according to a phylogenetic tree reflecting their relatedness. In the heat map, a score of
599 1 represents the highest antagonist activity and 0 represents the lowest, calculated based on pathogen
600 GFP biovolume in the presence of a beneficial strain normalised to the GFP biovolume of the
601 pathogen growing alone. Each square represents one interaction, displaying the means of three
602 biological replicates, each calculated from four technical replicates. The sum of scores against each
603 pathogen across all models was calculated to determine the total antagonistic power of each beneficial
604 strain.
605

A



B

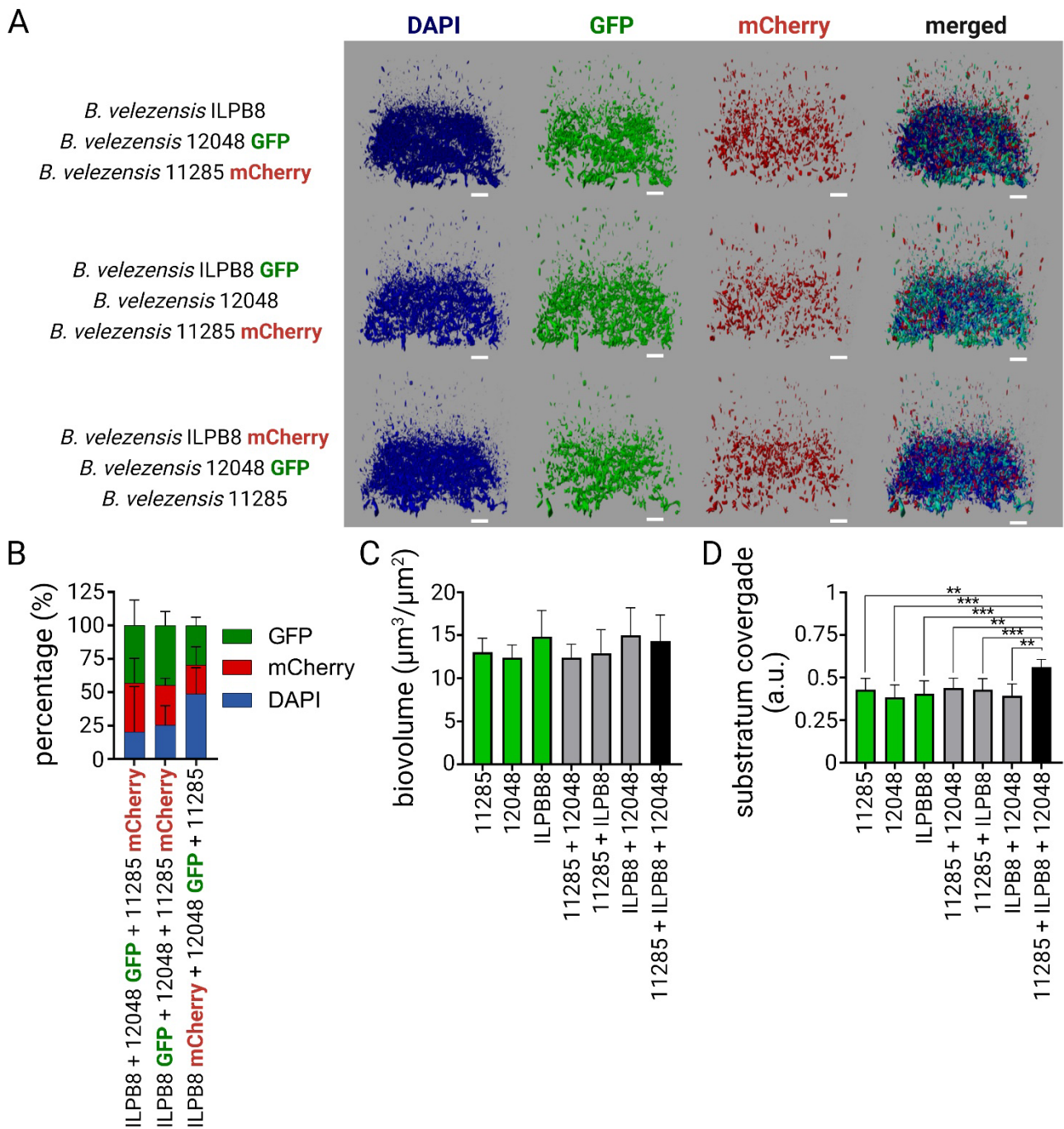


606

607 **Figure 2: Mixed biofilm compatibility experiment between candidate beneficial bacterial**
 608 **strains.** (A) The ability of three *B. velezensis* strains, expressing GFP and exhibiting high antagonistic
 609 activity against pathogens, to form mixed submerged biofilms with other beneficial strains was
 610 evaluated using the co-inoculation model. Strains were ordered according to their phylogenetic
 611 distance. The SYTO61 and GFP signals were quantified to calculate the GFP biovolume to SYTO61

612 biovolume. Each square represents one interaction and shows the means of three biological replicates,
613 each derived from four technical replicates. Red and black rectangles highlight the examples
614 illustrated below. (B) Representative examples illustrate the interactions between the three *B.*
615 *velezensis* GFP strains capable of forming two-strain mixed biofilms. The exclusion scenario is
616 demonstrated by *B. velezensis* 12001, which excludes *B. velezensis* 11285 and *B. velezensis* 12048,
617 while it is excluded by *B. velezensis* ILPB8. Scale bar = 40 μm .

618



619

620 **Figure 3: Mixed biofilm composed of three kin-compatible strains of *B. velezensis*.** (A)

621 Representative images of mixed biofilms using *B. velezensis* ILPB8 strains wild type, or genetically

622 labelled with GFP or mCherry, along with the addition of DAPI in the biofilm to visualise the entire

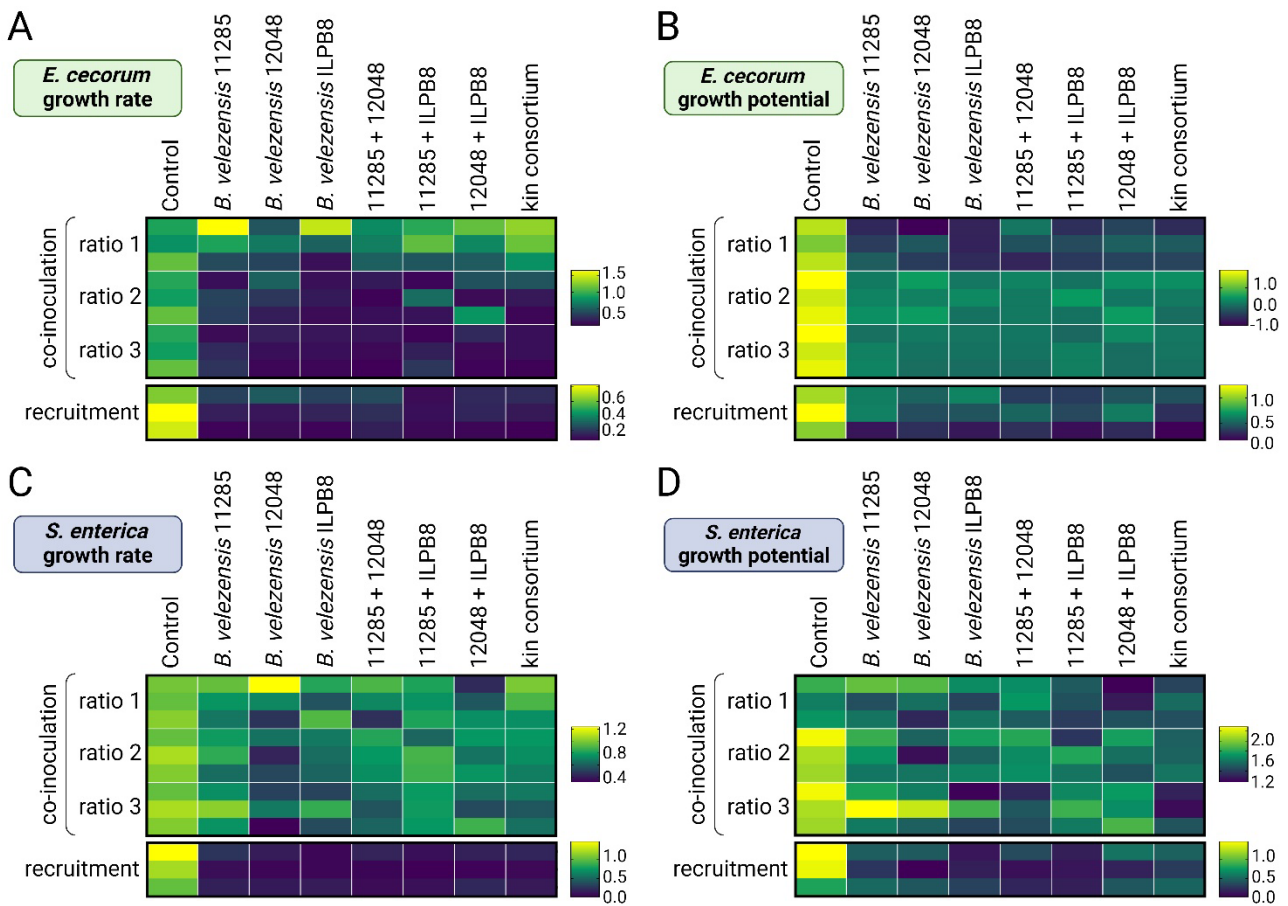
623 population in blue. Scale bar = 30 μm . (B) The percentage of signal from GFP-, mCherry- and DAPI-

624 labelled cells relative to the total biovolume of DAPI in biofilms of the three strains is presented. (C)

625 The co-inoculation model was employed using each wild-type strains, either alone or in combination,

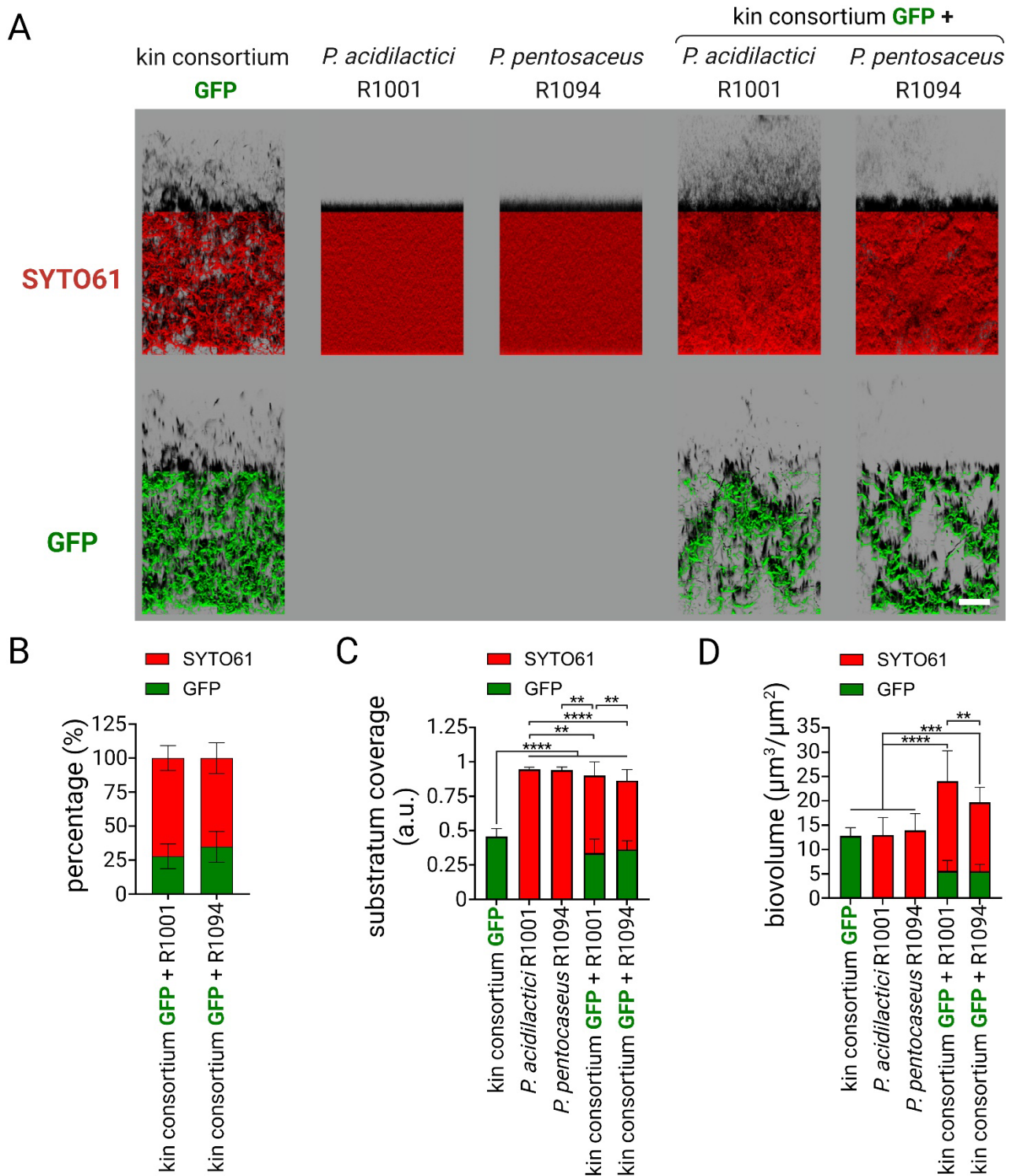
626 and the biofilms were labelled with SYTO9 and observed using the Leica SP8 HCS-CLSM to

627 investigate their overall structure. Biovolume data were extracted and are shown in the graph. (D)
628 Substratum coverage quantification of wild-type strains alone or in consortia is presented. Error bars
629 correspond to standard deviation.
630



631

632 **Figure 4: Pathogen growth parameters in mixed biofilms with *B. velezensis*.** (A) Growth rate
 633 (h⁻¹) determination of *E. cecorum* by modelling GFP biovolume curves and (B) calculation of growth
 634 potential for *E. cecorum* determined by subtracting the initial biovolume N₀ from the final biovolume
 635 N_f (calculated in log₁₀ (biovolume μm³/μm³)). The initial biovolume ratios of *E. cecorum* GFP to *B.*
 636 *velezensis* were determined at the start of the experiment (ratio 1 = 1.4 (+/- 0.2), ratio 2 = 0.3 (+/-
 637 0.06), ratio 3 = 0.03 (+/- 0.02), recruitment = 0.2 (+/- 0.04)). (C) Growth rate determination of *S.*
 638 *enterica* by modelling GFP biovolume curves and (D) calculation of growth potential for *S. enterica*
 639 determined by subtracting the initial biovolume N₀ from the final biovolume N_f (calculated in log₁₀
 640 (biovolume μm³/μm²)). The initial biovolume ratios of *S. enterica* GFP to *B. velezensis* were
 641 determined at the start of the experiment (ratio 1 = 3.2 (+/- 0.8), ratio 2 = 0.4 (+/- 0.1), ratio 3 = 0.1
 642 (+/- 0.05), recruitment = 4.8 (+/- 0.8)). The kin consortium corresponds to the SynCom of *B.*
 643 *velezensis* strains 11285, 12048 and ILPB8. Each square represents one interaction performed using
 644 the HCS mode of the CLSM and shows the means of three biological replicates, each calculated from
 645 four technical replicates.



646

647 **Figure 5: Compatible mixed biofilm composed of the kin consortium of *B. velezensis* with**

648 ***Pediococcus* spp.** (A) Representative images showing three kin-compatible strains of *B. velezensis*

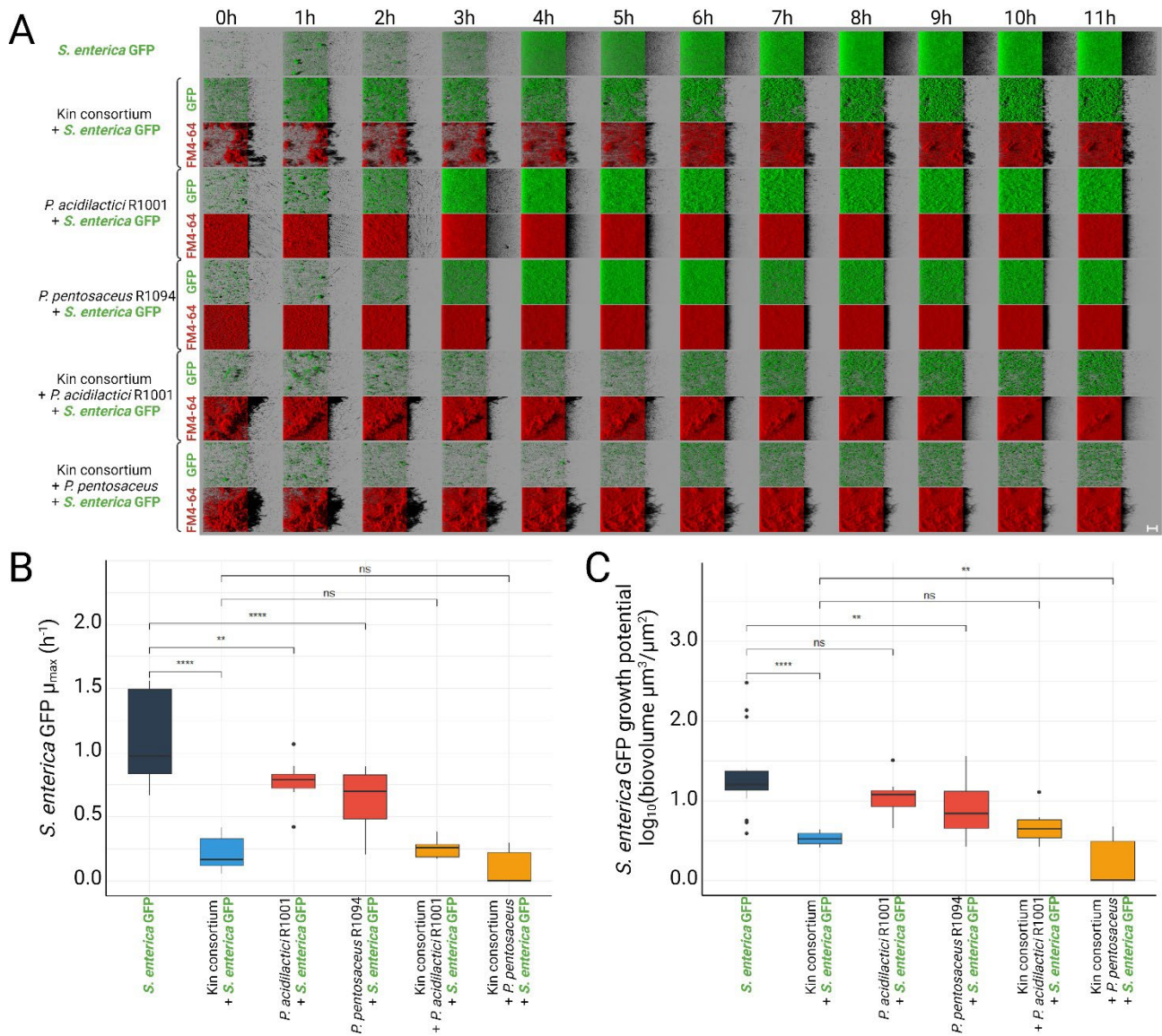
649 GFP (kin consortium: strains 11285 GFP, 12048 GFP and ILPB8 GFP) co-cultured together with

650 *Pediococcus* spp. SYTO61 was used to label all the population in red, while *B. velezensis* were

651 marked with GFP. Scale bar = 50 µm. (B) Percentage of signal from GFP and SYTO61, indicating

652 the proportion of each strain within the biofilm (C) The co-inoculation model was used to investigate
653 the overall structure of the biofilm. Biovolume data were extracted and are presented in the graph.
654 (D) Substratum coverage quantification of strains alone or in consortia, illustrating the extent of
655 surface coverage by each configuration. Error bars represent standard deviation.

656



657

658 **Figure 6: Characterisation of antagonistic activity against pathogens by consortia of *B.***
 659 ***velezensis* and *Pediococcus* spp. in the recruitment model. (A)** Representative CLSM images
 660 showing biofilm growth over time for each condition. The green colour corresponds to GFP labelling
 661 of *S. enterica*, while the red colour represents total population labelling by FM4-64. The SynCom of
 662 *B. velezensis* strains 11285, 12048 and ILPB8 (kin consortium) was co-cultured with *P. acidilactici*
 663 R1001 or with *P. pentosaceus* R1094 before the adhesion of *S. enterica* GFP in the recruitment model.
 664 The initial biovolume ratios of *S. enterica* GFP to the beneficial strains were determined at the start
 665 of the experiment (Kin consortium = 6.3 (+/- 2.2), R1001 = 0.14 (+/- 0.05), R1094 = 0.44 (+/- 0.20),
 666 Kin consortium + R1001 = 0.20 (+/- 0.03), Kin consortium + R1094 = 0.40 (+/- 0.09)). Scale bar =
 667 40 μm . (B) Box plot modelling of μ_{max} of *S. enterica* GFP in the recruitment model, showing statistical

668 analysis of the additive effects of *B. velezensis* and *Pediococcus* spp. consortia (x + y) on pathogen
669 growth inhibition. Error bars represent standard deviation. (C) Box plot modelling of growth potential
670 of *S. enterica* GFP in the recruitment model, showing statistical analysis of the additive effects of *B.*
671 *velezensis* and *Pediococcus* spp. consortia (x + y) on pathogen growth inhibition. Error bars represent
672 standard deviation.
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