Droplet printing reveals the importance of micron-scale structure for bacterial ecology

Ravinash Krishna Kumar1, Thomas A. Meiller-Legrand2,3, Alessandro Alcinesio1, Diego Gonzalez3,5, Despoina A. I. Mavridou4, Oliver J. Meacock2,3,6, William P. J. Smith2,3, Linna Zhou1, Wook Kim3,7, Gökçe Su Pulcu1, Hagan Bayley1✉ & Kevin R. Foster2,3✉

Bacteria often live in diverse communities where the spatial arrangement of strains and species is considered critical for their ecology. However, a test of this hypothesis requires manipulation at the fine scales at which spatial structure naturally occurs. Here we develop a droplet-based printing method to arrange bacterial genotypes across a sub-millimetre array. We print strains of the gut bacterium Escherichia coli that naturally compete with one another using protein toxins. Our experiments reveal that toxin-producing strains largely eliminate susceptible non-producers when genotypes are well-mixed. However, printing strains side-by-side creates an ecological refuge where susceptible strains can persist in large numbers. Moving to competitions between toxin producers reveals that spatial structure can make the difference between one strain winning and mutual destruction. Finally, we print different potential barriers between competing strains to understand how ecological refuges form, which shows that cells closest to a toxin producer mop up the toxin and protect their clonemates. Our work provides a method to generate customised bacterial communities with defined spatial distributions, and reveals that micron-scale changes in these distributions can drive major shifts in ecology.
Bacteria commonly exert strong effects on the growth and survival of surrounding cells via mechanisms that include nutrient competition, quorum sensing and toxin production\(^1\)–\(^4\). Consequently, the position of different strains and species in space is thought to be critical for the ecology of bacterial communities, and indeed how they affect us\(^5\)–\(^11\). In particular, the way in which different strains of bacteria are arranged when a community first develops is expected to be a key predictor of which strains dominate and, more generally, whether the community thrives or perishes\(^12\)–\(^21\). When strains and species grow apart in distinct patches, for example, this is expected to limit the impacts of competitive mechanisms, thereby promoting both coexistence and productivity.

The importance of spatial structure is broadly supported by comparisons of liquid with solid-surface (agar) culture, and agar-based manipulations on the millimetre-to-centimetre scale (Fig. 1b)\(^22\)–\(^27\). However, natural spatial structure often occurs at much finer scales, with data from the mammalian microbiome suggesting that the micron-to-millimetre scale is particularly important\(^6\),\(^11\),\(^28\). For example, while there appears to be significant genotypic mixing in the gut microbiome\(^29\), the dental and tongue microbiome is more structured, with patches of bacteria dominated by one genotype reaching tens to hundreds of microns in scale\(^8\),\(^9\),\(^30\). Moreover, there is evidence that solute gradients within bacterial communities can limit the distance at which cells affect each other to micron scales\(^31\)–\(^33\), raising the possibility that...
differences in the arrangement of genotypes at these scales will have impacts.

While fine-scale spatial structure is hypothesised to be important for the form and function of microbial communities, direct tests are needed. A key barrier is a requirement for a technology that can manipulate the patterning of interacting bacteria at the relevant spatial scales. A range of printing technologies exists to position and pattern microbes\(^\text{34-39}\). However, the typical focus of this work has been the production of biomaterials, such as bacterial sensors (e.g., bacteria embedded in a 3D hydrogel), rather than ensuring that bacteria can grow and interact as a community. Moreover, current printing methods typically have a resolution at the millimetre scale rather than the micrometre scale at which natural communities appear often to be structured. Here, we present a high-resolution droplet printing method that allows us to position interacting microbes in specific patterns in submillimetre arrays. With this, we are able to manipulate and study the ecology of microbial communities patterned at micron scales (Fig. 1). Our experiments reveal that changing the arrangement of bacterial genotypes across these fine scales can be critical for ecological outcomes.

Results

A new method to print patterned microbial communities. Our method works by piezo injection of tailored ‘bioinks’ comprising bacterial cells (\(E.\ coli\), \(10^6\) cells mL\(^{-1}\)), molten agarose (1.5% w/v) and lysogeny broth (LB) medium into an oil/phospholipid solution at 37°C. The method generates 110-µm-diameter droplets (\(\approx 70\) cells per droplet), which are deposited into 2D patterns by line-by-line printing (Fig. 1a). The droplets are initially surrounded by monolayers of phospholipid, which form bilayers on contact with one another, thereby creating a stabilised, support-free structure\(^\text{40,41}\) (Fig. 1a). The resulting array is then gelled, and the lipid bilayers removed by the addition of an oil (silicone oil AR20, which causes the lipid to precipitate) to create a final printed community of bacteria in agarose suspended in LB medium (‘Methods’) (Fig. 1a, c–f). Within the printed community, bacteria grew from single-cell dispersions (with starting 2D-centre-to-centre distances of \(18 \pm 2\) µm) into 3D microcolonies (with median diameters of 15 (10–21) µm) over 18 h of growth (see Supplementary Fig. I and Supplementary Methods I for further characterisation of printed arrays). To validate the method, we printed two fluorescently labelled strains of \(E.\ coli\). This demonstrated the ability to print specific patterns and, with them, to vary the amount of genetic mixing (defined by a segregation index (SI))\(^\text{16}\), see ‘Methods’ and Supplementary Fig. 2) at the scale of the arrays (Fig. 1g). Importantly, the calculated SI of our printed arrays matched well with the theoretical SI of the respective printing maps (Fig. 1h), and the imposed patterns did not impact the frequencies of strains that differ only in the fluorophore that they carry (Fig. 1i) (Supplementary Fig. 2 and Supplementary Methods I.2). With this method, therefore, we can systematically alter the spatial arrangement of bacterial strains across naturally relevant scales to study how they interact and grow.

Competition between toxigenic and susceptible cells. We next sought to test whether this spatial structure is important for the ecology of interacting bacteria. To do this, we studied a common ecological interaction in the mammalian gut: interference competition between strains of \(E.\ coli\) that produce protein toxins known as colicins\(^\text{24–45}\). While colicin production can be critical for survival, there is diversity in the types of colicins carried by \(E.\ coli\) strains, and many strains carry none at all—suggesting that colicin release is not always beneficial\(^\text{44}\) (Supplementary Methods 2.1). We began by performing two-way competitions between susceptible non-colicin-producing strains (S) and a range of colicin-producing strains (P) in printed communities at different SI values (Fig. 2) (see Table 1 for strains used). Theory suggests that genetic mixing can favour colicin producers, by giving them more access to susceptible cells. Conversely, increasing spatial structure has the potential to isolate producer genotypes from susceptible genotypes, which might protect susceptible genotypes if the competition is restricted to the boundaries between genotypes (Fig. 2b, c)\(^\text{46–48}\). These predictions are borne out in experiments with bacteriocins performed at large spatial scales\(^\text{23,49}\). However, it was far from clear whether the micron-scale spatial structure would have similar effects in our experiments. Colicins are released from cells (they do not remain attached), will easily diffuse through agar\(^\text{26,49}\) and are highly toxic, killing with single-hit kinetics\(^\text{30,51}\). These features of colicin biology mean they are able to diffuse and kill across the millimetre-to-centimetre scale, which might overwhelm any structure at the micron scale.

In spite of the potentially long-range activity of colicins, we found that altering spatial structure across the micron scale had significant effects on the outcome of competition between the bacteria. For all but one of the colicin types (E8), the introduction of fine-scale spatial structure enabled a distinct patch of the susceptible strains to persist (Fig. 2d–h). The strength of this effect reflects the relative ability of each colicin strain to kill susceptible strains\(^\text{49,52}\) (Fig. 2a and Supplementary Fig. 3d–f). As might be expected, the more potent killers were less affected by
spatial structure, with the colicin E8 producer killing off the susceptible cells irrespective of whether there was structure (Fig. 2g). E8 is known to be both a potent colicin and one that is produced at high levels due to autoinduction, whereby producing cells promote toxin production in clonemates (Supplementary Fig. 3e). For all the other strains, we found that spatial structure creates an ecological refuge\textsuperscript{53} for susceptible cells. Even though the refuge is only a few hundred microns across, it allowed susceptible cells to better survive competition with toxin-producing strains for two of the combinations. For E7, this effect was so strong that the susceptible strain was able to outgrow the colicin producer when printed side by side (Fig. 2d, $f_S = 0.65 \pm\ldots$).
Fig. 2 Micron-scale structure shapes competition between susceptible and colicin-producing strains by creating ecological refuges. a The characteristics of each colicin-producing strain used in this study. The ‘killing rank’ ranks the ability of each of the colicin-producing strain to kill colicin-susceptible strains, see refs. 49,52 and data therein. b, c A schematic hypothesis, of how susceptible and colicin-producer frequencies change over time, in a genetically mixed spatial structure (SI = 0.50) (b), and in a genetically segregated spatial structure (SI = 0.94) (c), if susceptible growth rates outweigh the cost of toxin production. d, Segmented fluorescence images of five cells (white) and E7 cells (yellow) in communities printed at an initial SI of 0.50 (n = 6), 0.67 (n = 3), 0.88 (n = 3) and 0.94 (n = 6) with accompanying printing maps. A Kruskal Wallis test showed a statistically significant relationship between the change in frequencies of S with SI (Kruskal-Wallis statistic: 15.63, P < 0.001, see Supplementary Table 2 for the statistical test). e-g Segmented fluorescence images of five cells (white) and A cells (vermilion) (n = 3 and n = 4 for SI = 0.50 and 0.94, respectively) (e), and E8 cells (blue-green) (n = 7 and n = 7 for SI = 0.50 and 0.94, respectively) (g), in communities printed at an initial SI of 0.50 and 0.94. h Segmented fluorescence images of E2 cells (blue) and R1 cells (red/purple) in communities printed at an initial SI of 0.50 (n = 9) and 0.94 (n = 6). In d-h, each fluorescently segmented image was taken after 18 h of competition. Below each fluorescently segmented image is a stacked bar chart of the frequencies of strains in the array. Each data point represents a biologically independent replicate; the mean frequencies of each strain are where the bars meet. In e-h, unpaired Mann-Whitney tests were performed to assess the statistical significance of the changes in frequencies of competing strains at different SI values. For e, P = 0.6286; for f, P = 0.0020; for g, P = 0.4462; for h, P = 0.0336 (see Supplementary Tables 3 for statistical tests). In h, one-sample t and Wilcoxon tests showed a statistically significant (P = 0.0039) and non-significant (P > 0.6250) difference between the median frequencies of E2 and R1 at SI = 0.50 and 0.94, respectively (see Supplementary Tables 3 for statistical tests).

Table 1 Strains used in this study.

| Strain and genotype | Abbreviation | Toxin | Resistance to colicins | Colour code |
|---------------------|--------------|-------|------------------------|-------------|
| BZB1011             | S            | None  | No                     |             |
| BZB1011 ΔbtuB       | R1           | None  | Yes—colicin A, E7, E2, E8 |             |
| BZB1011 immE2       | R2           | None  | Yes—colicin E2         |             |
| BZB1011 pColA       | A            | Colicin A | No                  |             |
| BZB1011 pColE7      | E7           | Colicin E7 | No                   |             |
| BZB1011 pColE2      | E2           | Colicin E2 | No                   |             |
| BZB1011 immE2 pColE2| E2*          | Colicin E2 | No                   |             |
| BZB1011 pColE8      | E8           | Colicin E8 | No                   |             |

Each colicin-producing strain is immune to its cognate toxin. For BZB1011 (S), the orange and sky blue colour codes are used to represent S cells expressing sfGFP and mRFP1, respectively, in Figs. 1 and 4. The white colour code represents S cells in Figs. 2 and 3.

0.02 at SI = 0.94). Counts of colony-forming units confirmed that the imaged susceptible cells in the printed arrays were viable, despite the presence of the toxin producer (Supplementary Fig. 3i). Exploring the case of E7 in more detail revealed a graded response to spatial structure, where progressively decreasing spatial structure (decreasing SI) led to a progressive increase in the dominance of the E7 colicin producer (Fig. 2d). At high genotypic mixing, the outcome of the competition was reversed, where now the toxin producer outcompeted the susceptible strain (Fig. 2d, fS = 0.24 ± 0.05 at SI = 0.50).

We also found an effect of spatial structure when a colicin producer strain competes with a colicin-resistant strain (R1) rather than a susceptible strain (Fig. 2h). The colicin producer has a lower growth rate due to the costs of colicin production (Supplementary Fig. 3g–h). However, this growth disadvantage only translates to an ecological disadvantage in the absence of spatial structure, i.e., when the two strains are well mixed (fE2 = 0.46 ± 0.02 at SI = 0.50). When growing side-by-side in a structured community, the colicin producer reaches the same abundance as the resistant strain, despite its slower growth rate. We saw the same effect of spatial structure for another strain combination (S and R1) that also lacks any toxin-mediated competition (Supplementary Fig. 4c). In these experiments, the micron-scale segregation of the two strains shifts competition to occur more within strains than between strains. As a result, the negative effects of inter-strain competition on a slower-growing strain are weakened. While our focus is interference competition (via toxins), we find that exploitative competition (mediated by differences in growth rate) is also affected by the introduction of spatial structure in the printed arrays.

Competition between toxigenic strains. We have shown that the arrangement of bacteria at the submillimetre scale is important for the outcome of both interference and exploitative competition. Moreover, as predicted by both ecological and evolutionary theory, our results suggest that the key effect of printing strains side by side—as opposed to well mixed—is to weaken interactions (Fig. 2b, c). When exploring the importance of positioning for competition between pairs of toxin-producing strains, which are each immune to their own toxin but able to kill the other (Fig. 3). At first, these appeared to follow the same pattern as the previous (producer vs susceptible) experiments. In one (E2 and E7), we saw the same refuge effect with spatial structure, where the strain (E7) that is eliminated under mixed conditions can persist when printed side by side with the more dominant strain (E2) (Fig. 3a). The dominance of E2 is expected because a higher proportion of E2 cells make colicins than E7 cells due to autoinduction56,57. In another case, one strain (A) eliminated the other (E2) irrespective of spatial structure (Fig. 3b). Here, the potency of colicin A cells is expected because E2 is a DNase that activates the SOS response, which activates colicin A expression. By contrast, A does not activate E2 expression because colicin A targets membrane integrity rather than DNA (Fig. 2a and Supplementary Fig. 3f)26,52,58. However, a third combination (E2 and E8) had a surprising outcome (Fig. 3c). Here, without spatial structure, the dominant strain E8 eliminated E2 as though E2 were simply a susceptible strain. By contrast, with spatial structure (SI = 0.94), both strains were almost completely eliminated, something not seen when these strains grow side-by-side at larger distances on agar plates49.
We next sought to understand how spatial structure could enhance competition, and moreover do it to the extent that both populations collapse. We hypothesised that the answer lay in the fact that both of these strains exhibit autoinduction of colicin production. Similar to the effects of quorum sensing, this density-dependent regulation occurs because the import of a clonemate’s DNA-damaging colicin increases a cell’s probability of colicin release (Supplementary Fig. 3e). Autoinduction can have strong effects on competition between colicin strains. In our experiments, we hypothesised that spatial structure provided E2 cells with enough refuge to reach a sufficient cell density to activate collective colicin production through autoinduction, thereby allowing them to inhibit E8 cells more strongly. To explore this, we studied competitions between E8 and an E2 strain engineered to lack autoinduction in colicin production (E2*, see ‘Methods’) (Fig. 3d). As predicted, this greatly reduced the impact of E2 on E8 in the presence of spatial structure. Moreover, the effects of the spatial structure were reversed, with mutual destruction of the two competitors now occurring under mixed conditions without spatial structure. This ability of the engineered E2* strain to eliminate E8 under mixed conditions is likely to stem from the relatively high growth rate of E2* (Supplementary Fig. 3g, h), which allows it to rapidly establish itself in mixed arrays. In sum, the impact of the spatial structure depends on whether E2 does, or does not, perform autoinduction. Moreover, in both cases, we see a strong effect where the printed pattern determines whether one strain thrives or both are largely eliminated.
Three-genotype printing explains how ‘microrefuges’ form. The impacts of autoinduction demonstrate that fine-scale structure can have surprising and complex effects on bacterial interactions. Nevertheless, one effect that is commonly seen is that spatial structure creates a refuge, with subordinate strains better able to persist with spatial structure than without it. We sought, therefore, to understand the mechanistic basis of this refuge effect, using prints of three different types of bioinks to explore the interface between a colicin E2 producer and a susceptible strain (Fig. 4a). Printing cell-free agarose between an E2 strain and a susceptible strain removed the refuge effect, suggesting that the distribution of colicins was not limited by diffusion across the printed array (Fig. 4e). This result implied that the susceptible cells closest to the E2 producer were somehow interrupting the diffusion of colicins across the array. We hypothesised that this could occur if susceptible cells bind and thereby ‘mop up’ colicin molecules as they pass\(^5\). To explore this, we engineered a colicin-resistant strain that lacks the BtuB receptor for colicin E2 (Table 1, Fig. 4b and Supplementary Fig. 4a) (see Supplementary Methods 2.2). As predicted by our model, the refuge effect was eliminated even though there were cells growing next to the colicin producer (Fig. 4f). However, the BtuB mutation also makes the cells resistant to colicin (Supplementary Fig. 4b), such that the lack of protection could be linked in some way to the lack of cell death from E2 activity. To exclude this possibility, we engineered a strain that carries BtuB, but which is resistant to colicins via cytoplasmic expression of the E2 immunity protein (Table 1, Fig. 4b and Supplementary Fig. 4a). This strain was not affected by the colicin of the E2 producer (Supplementary Fig. 4b) but did provide protection (Fig. 4g). We conclude, therefore, that binding of difusing colicins by intoxicated cells is critical to the refuge effect. More generally, these experiments highlight the importance of processes that counteract the effects of diffusion at these scales, whether it be by binding, importing, or digesting solutes.

**Discussion**

The ecological interactions within microbial communities are central to their properties and impacts\(^57\). However, the fine spatial scales at which microbes position and interact are a challenge for experimental microbiology. Here we have presented a method for arranging microbial genotypes in defined 2D arrays with micron-scale resolution. This method allowed us to establish a causal link between the arrangement of bacterial strains at these fine scales and ecological outcomes. We find that spatial structure impacts the outcome of both interference and exploitative competition. Moreover, the fine-scale structure can shift the outcome of competition from one strain dominating to another, and it can be the difference between one strain thriving and mutual destruction. A key principle that emerges across our experiments is that spatial segregation is often protective and limits the effects of one strain on the other. We show that for colicin E2, this is explained by the ability of susceptible cells to bind the toxin rather than by diffusion limitation. The extent of such refuge effects—and whether coexistence is stable or transitory—is likely to depend on a number of factors that we have not explored here. These factors include the length of the time that strains grow together, the turnover in nutrients and the initial frequencies of strains, which are all interesting targets for future work.

We find that one cannot simply translate results on the effect of spatial structure from coarse to fine scales. The outcomes we see in our arrays can be very different to those seen at larger scales. In particular, the conditions under which we observe mutual destruction (Fig. 3c) correspond to conditions that allow coexistence at a larger scale\(^59\). Nevertheless, many of our other findings mirror the conclusions from experiments at larger spatial scales\(^23,60\) where spatial segregation is well known to limit competition and promote coexistence. Our work, therefore, demonstrates that space still matters when one considers patterning at the fine scales that appear more typical in nature. Our findings also fit well with a large body of work on bacterial biofilms, which emphasises how both the binding and breakdown of solutes can generate strong gradients over small spatial scales, even when diffusion is not itself limiting\(^12,3,33,61-66\). The commonness of these micron-scale solute gradients suggests that the effects we have documented here will be important across many species and ecological interactions. The manipulation of fine-scale spatial structure, therefore, has significant potential for both understanding and controlling microbial communities.

**Methods**

**Preparing aqueous phases.** Phosphate-buffered saline (PBS) (1×, pH 7.4, Gibco) was used as purchased. Lysogeny broth Miller (LB-Miller) medium was prepared by adding 10 g of tryptone (BD Difco\(^TM\)), 5 g of yeast extract (BD Difco\(^TM\)) and 10 g of sodium chloride (Fischer) to 1 L of Milli-Q\(^\circ\) water and autoclaving. 5× M9 medium (Sigma-Aldrich) without a carbon source (12.8 g L\(^-1\) Na\(_2\)HPO\(_4\), 3 g L\(^-1\) KH\(_2\)PO\(_4\), 0.5 g L\(^-1\) Na\(_2\)SO\(_4\), 1 g L\(^-1\) NH\(_4\)Cl, 2 mM MgSO\(_4\) and 0.1 mM CaCl\(_2\)) was dissolved in Milli-Q\(^\circ\) water, autoclaved and filtered through 0.22-μm poly-ethersulfone membrane (Millex-GP Syringe Filter Unit). LB-Miller agar solutions (molten) were prepared by combining LB-Miller medium with agar (final concentration of 1.5% w/v) (BD Difco\(^TM\)) and autoclaving. 2.0% w/v of ultra-low-gelling-temperature (ULGT) agarose (Sigma-Aldrich) was dissolved by autoclaving at 190 °C. LB-Miller medium (2 mL) and autoclaved. The broth was kept molten by keeping the solution in a static incubator at 37 °C. Molten solutions were only used for a week. Antibiotics were dissolved in Milli-Q\(^\circ\) water, filter-sterilised (0.22-μm polyethersulfone membrane) and frozen (−20 °C) as stock solutions at 100 mg mL\(^-1\) for ampicillin and 30 mg mL\(^-1\) for kanamycin (Sigma-Aldrich).

**Preparing lipid/oil solutions.** Lipids were purchased from Avanti\(^\circ\) Polar Lipids in powder form and stored at −80 °C. Undecane and silicone oil AR20 (Sigma-Aldrich) were filtered before use through 0.22-μm filters (Corning\(^\circ\)) under vacuum. An optimised lipid/oil solution was prepared for printing\(^35\). Lipid films were prepared by bringing ampoules to room temperature and dissolving in anhydrous chloroform (Sigma-Aldrich) to make a chloroform/lipid solution at lipid concentrations of 25 mg mL\(^-1\). Chloroform/lipid stock solutions were used immediately. Using glass syringes (Hamilton\(^\circ\)), 45 μL and 20 μL of lipid stock solutions 1,2-dipytanylosn-glycerol-3-phosphocholine (DPhPC) and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), respectively, were transferred into a 7-mL, isopropanol-cleaned, Teflon-capped glass vial (Supelco\(^\circ\)). The lipid/chloroform solution was evaporated under a stream of nitrogen while rotating by hand to produce an even lipid film. The film was dried under vacuum for 24 h, then stored under house nitrogen at −80 °C until use. When required for printing, films were left at room temperature for 30 min, and 2 mL of a pre-mixed solution of undecane and silicone oil AR20 (35:65 by volume) was added to the film, followed by standing and controlling microbial communities.

**Construction of recombinant DNA.** Plasmids and primers used in this study are listed in Supplementary Tables 15 and 17, respectively. All PCRs were performed using KOD Hot Start DNA polymerase (Novagen\(^\circ\)), following standard protocols. Plasmids were assembled using one-step isothermal assembly (NEBuilder\(^\circ\) HiFi DNA Assembly Master Mix M3520A). All constructs were sequenced to verify whether sequences were correct (Source BioScience). The plasmids pGRG25-P\(_{\text{immE2}}\) and pGRG25-P\(_{\text{immE2}}\) were provided from references49,67. Chromosomally labelled (pC001-P\(_{\text{immE2}}\)) and mNeonGreen (pBC43-P\(_{\text{immE2}}\)) plasmids were provided from references66 by Nick Housden and Colin Kleanthous. The plasmids pGRG25-P\(_{\text{immE2}}\) were transferred before use through 0.22-μm filters (Corning\(^\circ\)) under vacuum. An optimised lipid/oil solution was prepared for printing\(^35\). Lipid films were prepared by bringing ampoules to room temperature and dissolving in anhydrous chloroform (Sigma-Aldrich) to make a chloroform/lipid solution at lipid concentrations of 25 mg mL\(^-1\). Chloroform/lipid stock solutions were used immediately. Using glass syringes (Hamilton\(^\circ\)), 45 μL and 20 μL of lipid stock solutions 1,2-dipytanylosn-glycerol-3-phosphocholine (DPhPC) and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), respectively, were transferred into a 7-mL, isopropanol-cleaned, Teflon-capped glass vial (Supelco\(^\circ\)). The lipid/chloroform solution was evaporated under a stream of nitrogen while rotating by hand to produce an even lipid film. The film was dried under vacuum for 24 h, then stored under house nitrogen at −80 °C until use. When required for printing, films were left at room temperature for 30 min, and 2 mL of a pre-mixed solution of undecane and silicone oil AR20 (35:65 by volume) was added to the film, followed by standing and controlling microbial communities.

**Construction of bacterial strains.** Bacterial strains used in this study are outlined in Table 1 and Supplementary Table 16. E. coli BZB1011 and BZB1011 pCol genotypes were provided from reference49,67. Chromosomally labelled (Tr7 insertion) sGFP and mRFP1 BZB1011 strains and BZB1011 pCol genotypes were

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**Fig. 4 Susceptible cells protect clonemates by binding incoming toxin.** a Schematic of the printing map used for testing clonemate absorption. The producer strip (E2) consists of $7 \times 4 \times 1$ droplets (black), the changeable strip consists of $7 \times 2 \times 1$ droplet (grey) and the sensitive strip consists of $7 \times 2 \times 1$ droplets (orange) in the $x$, $y$ and $z$ dimensions, respectively. b A schematic of the two resistance strategies used for creating $R^1$ and $R^2$. $R^1$ is resistant to colicins because of deletion of the BtuB receptor (which normally binds colicins). $R^2$ is resistant to colicins through constitutive expression of the cognate immunity protein, which binds and inactivates the toxin when it enters the cytosol. c–g Printing maps, competition schematics, composite bright-field and fluorescence microscopy images and bar charts of printed communities comprising three bioinks. c The control printed arrays containing three S strains ($n = 7$). A Wilcoxon test showed that the median productivities of S-skyblue (S rfp) and S-orange (S gfp) were non-significantly different ($P > 0.9999$, see Supplementary Table 5 for the statistical test). d Printed arrays containing E2 cells (black) and two S strains ($n = 13$). A Wilcoxon test showed that the median productivities of S-skyblue and S-orange were significantly different ($P = 0.0002$, see Supplementary Table 6 for the statistical test). e Printed arrays containing E2 cells, a strip of agarose not containing bacteria and S cells ($n = 4$). Mann–Whitney tests showed the median productivities of S-orange in d and e were significantly different ($P = 0.0029$) and non-significantly different ($P = 0.1143$) different, respectively (see Supplementary Tables 7 for the statistical test). f Printed arrays containing E2 cells, $R^1$ cells (reddish-purple) and S cells ($n = 4$). A Mann–Whitney test showed that the median productivities of S-orange in d and f were significantly different ($P = 0.008$, see Supplementary Table 8). g Printed arrays containing E2 cells, $R^2$ cells (red) and S cells ($n = 12$). A Mann–Whitney test showed that the median productivities of S-orange in d and g were non-significantly different ($P = 0.604$, see Supplementary Table 9). In d–g, arrows on schematics represent the predicted colicin reach during the competition experiments (not to scale). In the bar charts, each data point is a biologically independent replicate and the height of the bars are mean productivity values. In c–g, the term ‘productivity’ ($p$) of a genotype $a$ in a printed array after 18 h of competition is defined as $p = A_a/A_S$, where $A_a$ is the total cross-sectional area that a susceptible genotype occupies after 18 h of competition (at the same starting density and spatial pattern as genotype $a$), but without interference competition.
provided from Mavridou et al.49. The BZB1011 Δbtb::yfp:N7 or mrfp1::N7 strains were created by using the method described by Cianfanelli et al.46. Briefly, the regions (700 base pairs) upstream and downstream of the btb gene were amplified by PCR and joined together in the pFOK plasmid using the NEB® HiFi DNA Assembly Master Mix. In all, 5 µL of the reaction was then transformed into chemically competent E. coli JDS201 (recovery in 1 mL of LB medium + 100 µM dexamethasone (DAP) and 50 µg mL⁻¹ kanamycin). Transformants were screened by colony PCR (GoTag G2 polymerase) and sequencing (Source Bioscience) using the primers oOPC-613 and oOPC-615. Three clones carrying the correct plasmid (pTMB) were then conjugated with BZB1101 yfp::N7 and BZB1011 mrfp1::N7 plasmid-conjugants for which a second recombination event had occurred were then counter-selected. Clones were finally verified by colony PCR and sequencing (by using the primers TML-P and TML-P10) for deletion of the btb gene. The BZB1101 imm36::N7 pCoE2 pcB11-Pho3yphat or pB3pa-Pho3yphat-NF F3 YST2 strains were generated by transforming competent BZB1101 imm36::N7 pCoE2 cells (from Mavridou et al.49) with the respective plasmids, and by selecting for transformants on LB-Miller agar plates (1.5% w/v) supplemented with 30 µg mL⁻¹ of kanamycin. The BZB1101 mrfp1::N7 pCoE2::N7 strains were created by the method described by Cianfanelli et al.68. Briefly, 100 µL of BZB1101 mrfp1::N7 cells (feeding in 1 mL of LB-Miller medium overnight) were inoculated in LB-Miller medium and incubated overnight at 37 °C (shaking at 225 rpm). The following day, the overnight cultures were diluted in LB-Miller medium to an OD₆₀₀ of 0.05 and incubated at 37 °C (shaking at 225 rpm). After this, cultures were again centrifuged at 4 °C for 10 min at 1734 × g. After the addition of these solutions, cultures were left on ice for 45 min. Following this, the cultures were centrifuged at 4 °C for 10 min at 1734 × g and centrifuged at 4 °C for 10 min at 1734 × g. The remaining 900 µL of fresh LB-Miller medium and cultured for 3 h (shaking at 250 rpm). Cultures were then normalised to 10⁶ cells mL⁻¹ by centrifugation at 8000 × g for 5 min and resuspended in 100 µL of fresh LB-Miller medium. Centrifugation, removal of the supernatant and addition of fresh LB medium (100 µL) were repeated twice to remove any colicin in the supernatant. If 1:1 competitions were investigated at an SI of 0.50, the two normalised bacteria cultures were combined at a 1:1 ratio (50 + 50 µL). At any other SI (that required two or three nozzles for printing), a bioink was created per nozzle. To create the bioinks, 150 µL of molten 20% w/v ULGT agarose in LB-Miller medium (37 °C), 30 µL of LB-Miller medium and 20 µL of normalised culture were combined in PCR tubes and mixed by gently pipetting back and forward using a 200-µL pipette to homogenise the solution for printing. Pipette tips (Corning® 1–200 µL Filtered Isotip®) and PCR tubes were maintained at 37 °C before homogenising the bioink.

**Preparation of bioinks.** Genotypes were cultured directly from glycerol stocks (50% v/v glycerol) in 4 mL of LB-Miller medium for 15 h (shaking at 250 rpm). From the overnight cultures, 20 µL of each was added to 2 mL of fresh LB-Miller medium and cultured for 3 h (shaking at 250 rpm). Cultures were then normalised to 10⁶ cells mL⁻¹ by centrifugation at 8000 × g for 5 min and resuspended in 100 µL of fresh LB-Miller medium. After this, both pellets were recombined pairwise by resuspending one of the pellets in 5 mL of water), bringing the initial OD₇₀₀ to 0.01. The layout of the plate was randomised and two last columns, which were deleted with Milli-Q® water. The chamber is connected to a glass nozzle (with a tip diameter of 150 µm) from which the aqueous printing solution is ejected (Supplementary Fig. 8). To prevent mixing of the printing solution with the water inside the chamber and nozzle, an undecane oil plug of 5.5 µL was used to separate the two solutions. A quartz cuvette (Starna Scientific Ltd) with dimensions of 20 × 10 × 10 mm (length, width and height) was filled with 1 mL of lipid/oil solution and mounted on the micromanipulator. An IR heater (Beurer Ltd., 150 W) was used to equilibrate the temperature of the nozzle and the quartz cuvette (from a fixed distance) to approximately 37 °C over 30 min. After equilibration, the bioink was loaded into the nozzle, and the tip was immersed into the lipid/oil solution contained in the quartz cuvette, which was positioned on a digitally controlled micromanipulator (PatchStar micromanipulator, Scientifica, 20-nm resolution). The micromanipulator movements and the piezo actuation were synchronised using a custom-made software developed in LabVIEW (National Instruments®). Printing was monitored using a side-on stereomicroscope (Nikon® SMZ40T) and videos and pictures were acquired using a digital camera (Thorlabs DCC1645C) mounted on the microscope. For creating patterns at SI = 0.75 and 0.94, a second nozzle was used to print the second pattern. The second nozzle was normalised to the offset of the first nozzle so that the pattern could be printed line-by-line. For three bioinks, the two smaller strips were printed first using two nozzles followed by replacing the first nozzle with a new nozzle containing the third bioink for printing the larger strip. The third nozzle was aligned with a control droplet to complete the pattern.

**Resulting printing and imaging**

Creating printed arrays containing bacteria. The functioning of the 3D droplet printer is outlined in our previous work41. In brief, a custom-built piezoelectric transducer (+130 V) transmits controlled pressure impulses to a chamber filled with Milli-Q® water. The chamber is connected to a glass nozzle (with a tip diameter of 150 µm) from which the aqueous printing solution is ejected (Supplementary Fig. 8). To prevent mixing of the printing solution with the water inside the chamber and nozzle, an undecane oil plug of 5.5 µL was used to separate the two solutions. A quartz cuvette (Starna Scientific Ltd) with dimensions of 20 × 10 × 10 mm (length, width and height) was filled with 1 mL of lipid/oil solution and mounted on the micromanipulator. An IR heater (Beurer Ltd., 150 W) was used to equilibrate the temperature of the nozzle and the quartz cuvette (from a fixed distance) to approximately 37 °C over 30 min. After equilibration, the bioink was loaded into the nozzle, and the tip was immersed into the lipid/oil solution contained in the quartz cuvette, which was positioned on a digitally controlled micromanipulator (PatchStar micromanipulator, Scientifica, 20-nm resolution). The micromanipulator movements and the piezo actuation were synchronised using a custom-made software developed in LabVIEW (National Instruments®). Printing was monitored using a side-on stereomicroscope (Nikon® SMZ40T) and videos and pictures were acquired using a digital camera (Thorlabs DCC1645C) mounted on the microscope. For creating patterns at SI = 0.75 and 0.94, a second nozzle was used to print the second pattern. The second nozzle was normalised to the offset of the first nozzle so that the pattern could be printed line-by-line. For three bioinks, the two smaller strips were printed first using two nozzles followed by replacing the first nozzle with a new nozzle containing the third bioink for printing the larger strip. The third nozzle was aligned with a control droplet to complete the pattern.
incubated at 37 °C for 18 h. After 18 h, printed arrays were washed with M9 medium to remove excess bacteria that grew out into solution from the printed arrays during the competition experiment. Within each compartment containing a printed array, 400 μL of LB-Miller medium were removed and 200 μL of M9 medium were added. Following this, 200 μL of the medium were removed and another 200 μL of M9 medium were added to further wash and remove the bacteria. Finally, printed arrays were picked up using a cut pipette tip (20 μL) and transferred into individual containers containing 400 μL of M9 medium (Nunc™ Lab-Tek™ II Chambered Coverglass (eight wells))). Printed arrays were left in M9 media for 10 h to allow the exchange of LB medium with M9 medium, required for imaging. Printed arrays were imaged using a Leica® SPS confocal microscope using a HCX Plano-Fluar x10/0.30 objective (0.3 numerical aperture), at excitation wavelengths of 405 nm and 546 nm and emission cut-offs at 420–520 nm and 625 nm, respectively (exciting superfolder GFP, mRFP1, Ypet and mNeonGreen), at a z-step of 2.73 μm and scanning speed of 400 Hz.

Colony-forming units of printed arrays. Printed arrays were washed and transferred to 400 μL of M9 medium after 8 h of competition. This time point was chosen because space in the printed arrays was saturated with E. coli within 8 h. Next, printed arrays were moved to individual 1-mL centrifuge tubes containing 200 μL of M9 medium and sealed at 55 °C for 1 min in a thermostainer (Eppendorf ThermoMixer™ C) shaking at 1000 rpm. Following this melting, the E. coli-containing solutions were serially diluted by 10,000-fold (100 μL of 900 μL of M9 medium). Following this, 10,000× diluted solutions were plated by beads on 1.5% w/v LB-Miller agar plates and then left to grow for 24 h at 37 °C in a static incubator. After 24 h, colonies were counted using a Zeiss PlanApo Z ×0.5 objective on an AxioZoom.V16 Zeiss microscope. Genotypes were recognised by fluorescence.

Flow cytometry of printed arrays. Printed arrays were washed and transferred to 400 μL of M9 medium after 18 h of competition. Next, printed arrays were moved to individual 1-mL centrifuge tubes containing 200 μL of M9 medium and sealed. The cell suspensions were diluted a further tenfold in M9 medium. Diluted cell suspensions were analysed on a BD Accuri C6 Flow Cytometer (Version 1.0.264.21) using 10,000 and 8000 as thresholds for the forward and side scatter parameters, respectively. For each experimental condition, 20,000 events were quantified per sample. A custom-written script was written on Rstudio (Version 1.3.959) to analyse data. The population of bacteria was gated by considering only the events within the core of the cell population in order to minimise count biases (Supplementary Fig. 7c). This was calculated from the forward and side scatter dimensions. Cells that had a fluorescence intensity above 29.5 were assigned as microcolonies76,77. However, as we were not concerned with the specific type of feature separating microcolonies, we generated a binary mask to extract each microcolony. Local microcolony data were performed using a generalisation of the algorithm used during 2D segmentation. Specifically, a rough segmentation of the initial array of voxel intensities K(x, y, z) was first generated by applying a simple intensity threshold, allowing the bright microcolony-containing regions to be separated from the dark background. However, variations in background intensity and intermediate intensities of voxels between closely packed microcolonies prevented this approach from reliably isolating individual microcolonies within these regions.

To separate these closely packed microcolonies, we located 3D ridge-like features (e.g., blobs, sheets or tubes) that were dark relative to nearby microcolonies76,77. Initially, H(x, y, z) was convolved with a Gaussian kernel G(x, y, z, σ) of spatial scale σ to isolate features at that scale:

\[ H(x, y, z, σ) = G(x, y, z, σ) \]

The second-order derivatives of \( H \) at each voxel were used to construct the Hessian \( H \) of the image at each voxel:

\[ \nabla^2 H(x, y, z, σ) = \begin{bmatrix} I_{xx} & I_{xy} & I_{xz} \\ I_{yx} & I_{yy} & I_{yz} \\ I_{zx} & I_{zy} & I_{zz} \end{bmatrix} \]

\( H \) contains information on the 3D features of scale \( σ \) present within the image around position \( (x, y, z) \). These features can be classified by finding the eigenvalues \( λ_x, λ_y, \) and \( λ_z \) of \( H \), where \( |λ_x| ≥ |λ_y| ≥ |λ_z| \). However, as we were not concerned with the specific type of feature separating microcolonies, we generated a binary segmentation of the image simply by finding all voxels for which \( λ_z \) was above a threshold value. This fine-grained mask was then combined with the initial rough segmentation using a voxelwise AND filter, generating the final segmentation.

Calculating segregation indices. We used a SI to measure genetic mixing within printed arrays, and to quantify printing fidelity. SI was computed for printed-colony micrographs as follows:

1. Segmentation: Each micrograph pixel was assigned a genetic identity (‘A’, ‘B’, or ‘None’) by comparing its fluorescence channel intensities, thresholded separately using Otsu’s method78. Pixels in which both channels exceeded the intensity threshold were classified as containing neither cell type (‘None’).
2. Local SI: We computed a local segregation index \( s_i \) for each A- or B-type pixel \( i \) by comparing its genetic identity with those of neighbouring pixels. For a square neighbourhood of size \( h \), \( s_i(h) \) is defined as

\[ s_i(h) = \frac{1}{N(h)} \sum_{j=1}^{N(h)} g(j) \cdot g(i) \]

where \( N(h) \) is the number of \( A- \) or \( B- \)type pixels within \( h \) rows or columns of focal pixel \( i \). Thus, \( s_i(h) \) is the fraction of \( i \)‘s neighbours that share its genotype. Note: for ease of image processing, here we are considering square neighbourhoods instead of the circular ones used previously76,77.
3. Global SI: We defined the global segregation index \( S(h) \) for a community micrograph as the arithmetic mean of local segregation indices for each of \( M \) focal pixels:

\[ S(h) = \frac{1}{M} \sum_{i=1}^{M} s_i(h) \]

\( S(h) \) varies in value from 0.5 (high genetic mixing on spatial scale \( h \)) to 1.0 (no genetic mixing).

To assess printing fidelity, we generated reference images representing the idealised community structure (Supplementary Fig. 2e) and compared the resulting series of values, resulting in a function \( d(φ) \) that was positive at values of \( φ \) where the outer boundary of the print was further-than-average away from the centre and negative at values of \( φ \) where it was closer than average. \( d(φ) \) has peaks at values of \( φ \) corresponding to each of the four corners of the print interleaved with troughs at values of \( φ \) corresponding to the sides.

Furthermore, \( d(φ) \) is circular, with \( d(φ) = d(φ+π) \). Thus, it could therefore be found from the phase of the Discrete Fourier Transform (DFT) on \( d(φ) \) with wavenumber \( k = 4 \). Explicitly,

\[ \theta = \frac{4}{π} \arctan \left( \sum_{n=-N/2}^{N/2} \frac{d(\phi_n) - c_n}{\phi_n} \right) \]

The position of each microcolony was then rotated about \( (\bar{x}, \bar{y}) \) by \( \theta \) and the size of the print in each direction estimated as the difference between the minimum and maximum \( x \) and \( y \) coordinates of the registered microcolonies.

Microcolony neighbour–neighbour distance estimation: Delaunay triangulation75 was applied to the positions of all microcolonies in the print. Microcolonies directly connected in the resulting triangulation were assigned as neighbours. Microcolonies forming the outer boundary of the print were therefore excluded when calculating the radial neighbour–neighbour distance profile.
SI(h) values with those measured for printed communities (Fig. 1h). This comparison revealed a good fit between measured and reference SI(h) values for a range of spatial scales (Fig. 1h; h = 5, 10, 20, 50, 100 px, equivalent to 7.6, 15.2, 30.3, 75.8, 151.5 μm), confirming that community printing is capable of modulating genetic mixing. Image processing and SI calculations were carried out using MATLAB® (MathWork®) (R2017a) and code is available at https://zenodo.org/record/4354822#.X9ArJPT7rPU.

Definitions and calculations

The term ‘frequency’ (f) of a genotype, a, in a printed array containing genotype a and b after 18 h of competition is defined as

\[ f = \frac{A_a}{A_a + A_b} \] (7)

is the total cross-sectional area that a susceptible genotype occupies after 18 h of competition at the same starting density and spatial pattern as genotype a, but without interference competition. For competition between two genotypes (Fig. 3), this would be the total cross-sectional area occupied by a susceptible strain at the given SI, competing against another susceptible strain. For competition between three genotypes (Fig. 4), this would be the total cross-sectional area occupied by a susceptible genotype in the strip (Fig. 4c), competing against the other two susceptible genotypes in the other strips.

Data availability

Raw datasets, including image-processing data and micrographs, generated and/or used during this study, are available in the gshare repository https://doi.org/10.6084/m9.fghshare.13375957. Source data are provided with this paper.

Code availability

Code used for 3D segmentation and subsequent analysis of z-projected and z-stacked confocal microscopies of the printed array, f is used in Figs. 1, 2, and 3. In Supplementary Fig. 2a, A = total volume occupied by a strain, number of cells and number of CFUs for 3D segmentation, flow cytometry and CFU, respectively. The term ‘productivity’ (p) of a genotype, a, in a printed array after 18 h of competition is defined as

\[ p = \frac{A_a}{A_a} \] (8)

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Raw datasets, including image-processing data and micrographs, generated and/or used during this study, are available in the gshare repository https://doi.org/10.6084/m9.fghshare.13375957. Source data are provided with this paper.

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\[ p = \frac{A_a}{A_a} \] (8)

A is the total cross-sectional area that a susceptible genotype occupies after 18 h of competition at the same starting density and spatial pattern as genotype a, but without interference competition. For competition between two genotypes (Fig. 3), this would be the total cross-sectional area occupied by a susceptible strain at the given SI, competing against another susceptible strain. For competition between three genotypes (Fig. 4), this would be the total cross-sectional area occupied by a susceptible genotype in the strip (Fig. 4c), competing against the other two susceptible genotypes in the other strips.
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Author contributions

R.K.K., D.G., W.K., G.S.P., H.B. and K.R.F. conceived and designed experiments. R.K.K. and K.R.F. wrote the paper, with comments from other authors. R.K.K., T.A.M.-L. and A.A. performed experiments. O.J.M. and W.P.I.S. wrote custom software for image analysis of printed arrays and calculation of segregation indices, respectively. D.A.M. and D.G. designed and generated the colicin toolbox. I.Z. designed and built the high-power piezo driver.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to H.B. or K.R.F.

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