

**Title:** Computational exploration of the global microbiome for antibiotic discovery

2 **Short title:** Discovery of antimicrobials in the global microbiome by machine learning

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## Summary

Novel antibiotics are urgently needed to combat the antibiotic-resistance crisis. We present a machine learning-based approach to predict prokaryotic antimicrobial peptides (AMPs) by leveraging a vast dataset of 63,410 metagenomes and 87,920 microbial genomes. This led to the creation of AMPSphere, a comprehensive catalog comprising 863,498 non-redundant peptides, the majority of which were previously unknown. We observed that AMP production varies by habitat, with animal-associated samples displaying the highest proportion of AMPs compared to other habitats. Furthermore, within different human-associated microbiota, strain-level differences were evident. To validate our predictions, we synthesized and experimentally tested 50 AMPs, demonstrating their efficacy against clinically relevant drug-resistant pathogens both in vitro and in vivo. These AMPs exhibited antibacterial activity by targeting the bacterial membrane. Additionally, AMPSphere provides valuable insights into the evolutionary origins of peptides. In conclusion, our approach identified AMP sequences within prokaryotic microbiomes, opening up new avenues for the discovery of antibiotics.

**Keywords:** metagenomics, antimicrobial peptides, antimicrobial activity, machine learning, global microbiome

## 44 Introduction

Antibiotic-resistant infections are becoming increasingly difficult to treat with conventional  
46 therapies. Indeed, such infections currently kill 1.27 million people per year<sup>1</sup>. Therefore, there is an  
urgent need to develop novel methods for antibiotic discovery. Computational approaches have been  
48 recently developed to accelerate our ability to identify novel antibiotics, including antimicrobial  
peptides (AMPs)<sup>2-5</sup>.

50 AMPs, found in all domains of life<sup>6-9</sup>, are short sequences (here operationally defined as 10-  
100 amino acid residues<sup>10</sup>) capable of disturbing microbial growth<sup>7,11</sup>. AMPs can target proteins, RNA,  
52 DNA, and other intracellular molecules; they most commonly interfere with cell wall integrity and cause  
cell lysis<sup>7</sup>. Natural AMPs can originate by proteolysis<sup>3,12</sup> or by non-ribosomal synthesis<sup>13</sup>, or they can  
54 be encoded within the genome<sup>14</sup>. Recently, proteome mining approaches have been developed to  
identify antimicrobials in extinct organisms<sup>15</sup>.

56 Bacteria live in an intricate balance of antagonism and mutualism in natural habitats. AMPs  
play an important role in modulating such microbial interactions and can displace competitor strains,  
58 facilitating cooperation<sup>16</sup>. For instance, pathogens such as *Shigella* spp.<sup>17</sup>, *Staphylococcus* spp.<sup>18</sup>,  
*Vibrio cholerae*<sup>19</sup>, and *Listeria* spp.<sup>20,21</sup> produce AMPs that eliminate competitors (sometimes from the  
60 same species) and occupy their niche.

AMPs hold promise as potential therapeutics and have already been used clinically as antiviral  
62 drugs (e.g., enfuvirtide and telaprevir<sup>22</sup>). AMPs that exhibit immunomodulatory properties are currently  
undergoing clinical trials<sup>23</sup>, as are AMPs that may be used to address yeast and bacterial infections<sup>24</sup>  
64 (e.g., pexiganan, LL-37, PAC-113). Although most AMPs display broad-spectrum activity, some can  
present narrow activity, having activity only against closely related members of the same species or  
66 genus<sup>25</sup>. Such AMPs are more targeted agents than conventional broad-spectrum antibiotics<sup>26,27</sup>. For  
example, the FDA-approved small peptide nisin has been shown to restore microbiome homeostasis  
68 in *Clostridioides difficile* infections in mice<sup>28</sup> and in *ex vivo* models of the human gut<sup>29</sup>. Furthermore,  
contrary to conventional antibiotics, the evolution of resistance to many AMPs occurs at low rates and  
70 is not related to cross-resistance to other classes of widely used antibiotics<sup>3,30,31</sup>.

The application of metagenomic analyses to the study of AMPs has been limited due to  
72 technical constraints, primarily stemming from the challenge of distinguishing genuine protein-coding

sequences from false positives<sup>32</sup>. As a consequence, the significance of small open reading frames (smORFs) has been historically overlooked in (meta)genomic analyses<sup>33–35</sup>. In recent years, significant progress has been made in metagenomic analyses of human-associated small open reading frames (smORFs)<sup>5,36</sup>. These advancements have incorporated machine learning (ML) techniques to identify smORFs encoding proteins belonging to specific functional categories<sup>37–40</sup>. Notably, a recent study uncovered approximately 2,000 AMPs from metagenomic samples of human gut microbiomes<sup>5</sup>. Nevertheless, it is important to note that the human gut represents only a fraction of the overall microbial diversity, suggesting that there remains an immense potential for the discovery of AMPs from prokaryotes in the diverse range of habitats across the globe.

In this study, we employed ML to predict and catalog the entire global microbiome. By computationally exploring 63,410 publicly available metagenomes and 87,920 high-quality microbial genomes<sup>41</sup>, we uncovered a vast array of AMP diversity. This resulted in the creation of AMPSphere, a collection of 863,498 non-redundant peptide sequences, encompassing candidate AMPs (c\_AMPs) derived from (meta)genomic data. Remarkably, the majority of these identified c\_AMP sequences had not been previously described. Our analysis revealed that these AMPs were specific to particular habitats and were predominantly not core genes in the pangenome.

Moreover, we synthesized 50 c\_AMPs from AMPSphere and found that 54% of them exhibited antimicrobial activity *in vitro* against clinically significant ESKAPEE pathogens, which are recognized as public health concerns<sup>42,43</sup>. These peptides demonstrated their ability to target bacterial membranes and were prone to adopting  $\alpha$ -helical and  $\beta$ -structures. Notably, the leading candidates displayed promising anti-infective activity in a preclinical animal model.

## Results

### ***AMPSphere comprises almost 1 million c\_AMPs from several habitats***

AMPSphere incorporates c\_AMPs predicted with ML using Macrel<sup>40</sup> from 63,410 worldwide distributed publicly available metagenomes (**Fig. 1A** and **Table SI1**) and 87,920 high-quality bacterial and archaeal genomes<sup>41</sup>. Sequences present in a single sample were removed<sup>40</sup>, except when they had a significant match (defined as amino acids identity  $\geq 75\%$  and E-value  $\leq 10^{-5}$ ) to a sequence in

DRAMP 3.0<sup>44</sup>. This resulted in 5,518,294 genes, 0.1% of the total predicted smORFs, coding for 863,498 non-redundant c\_AMPs (on average 37±8 residues long; **Fig. 1A** and **SI1**).

We subsequently estimated the quality of the smORF predictions and detected 20% (172,840) of the c\_AMP sequences in independent metaproteomes or metatranscriptomes (**Fig. SI2A** and **Methods - Quality control of c\_AMPs**). To further assess the gene predictions, we subjected the peptides to a bundle of *in silico* quality tests (see **Methods - Quality control of c\_AMPs**) and the subset of c\_AMPs that passed all of them (9.2%) is hereafter designated as high-quality.

Only 0.7% of the identified c\_AMPs (6,339 peptides) are homologous to experimentally validated AMP sequences in the DRAMP 3.0 database<sup>44</sup>. Moreover, most c\_AMPs were also absent from protein databases not specific to AMPs (**Fig. 1B**), such as SmProt 2<sup>45</sup> or GMGCv1<sup>46</sup>, suggesting that the c\_AMPs represent an entirely novel region of peptide sequence space. In total, we could find only 73,774 (8.5%) c\_AMPs with homologs in the tested databases. High-quality c\_AMPs were detected in public databases with higher frequency than general c\_AMPs (2.5-fold,  $P_{\text{Hypergeom.}} = 4.2 \cdot 10^{-250}$ , **Fig. 1B**).

To put c\_AMPs in an evolutionary context, we hierarchically clustered peptides using a reduced amino acid alphabet of 8 letters<sup>47</sup> and identity cutoffs of 100%, 85%, and 75% (**Fig. SI3**). At the 75% identity level, we obtained 521,760 protein clusters of which 405,547 were singletons, corresponding to 47% of all c\_AMPs from AMPSphere. A total of 78,481 (19.3%) of these singletons were detected in metatranscriptomes or metaproteomes from various sources, indicating that they are not artifacts. The large number of singletons suggests that most c\_AMPs originated from processes other than diversification within families, which is the opposite of the supposed origin of full-length proteins, in which singleton families are rare<sup>46</sup>. The 8,788 clusters with ≥8 peptides obtained at 75% of identity are hereafter named “families”, as in Sberro et al.<sup>36</sup>. Among them, we consider 6,499 as high-quality families because they contain evidence of translation or transcription, or ≥75% of their sequences pass all *in silico* quality tests (see Methods). These high-quality families span 15.4% of the AMPSphere (133,309 peptides).

All the c\_AMPs predicted here can be accessed at <https://ampsphere.big-data-biology.org/>. Users can retrieve the peptide sequences, ORFs, and predicted biochemical properties of each c\_AMP (*e.g.*, molecular weight, isoelectric point, and charge at pH 7.0). We also provide the distribution across geographical regions, habitats, and microbial species for each c\_AMP.

## 130 ***c\_AMPs are rare and habitat specific***

The AMPSphere spans 72 different habitats, which were classified into 8 high-level habitat groups, *e.g.*, soil/plant (36.6% of *c\_AMPs* in AMPSphere), aquatic (24.8%), human gut (13%) - (**Fig. 1A** and **Table S12**). Most of the habitats, except for the human gut, appear to be far from saturation in terms of newly discovered *c\_AMPs* (**Fig. 1C**). In fact, most AMPs are rare (median number of detections is 99, or 0.17% of the dataset), with 83.97% being observed in <1% of samples - see **Fig. S12**. Only 10.8% (93,280) *c\_AMPs* were detected in more than one high-level habitat (henceforth, “multi-habitat *c\_AMPs*”); this level is 7.25-fold less frequent than would be expected by a random assignment of habitats to samples ( $P_{\text{Permutation}} < 10^{-300}$ , see **Methods – Multi-habitat and rare *c\_AMPs***). Even within high-level habitat groups, *c\_AMP* contents overlap between habitats much less than expected by chance (2.4 to 192-fold less,  $P_{\text{Permutation}} \leq 5.4 \cdot 10^{-50}$ , see **Methods - Significance of the overlap of *c\_AMP* contents; Fig. 1D**), indicating the existence of cross-habitat boundaries.

## 142 ***Mutations in larger genes generate *c\_AMPs* as independent genomic entities***

Many AMPs are generated post-translationally by the fragmentation of larger proteins<sup>12</sup>. In parallel, encrypted peptides from protein sequences within the human proteome were also shown to be highly active. Those peptides have different physicochemical features compared to known AMPs<sup>3,31</sup>. However, AMPSphere only considered peptides encoded by dedicated genes. Nonetheless, we hypothesized that some of these have originated from larger proteins by fragmentation at the genomic level: about 7% (61,020) of AMPSphere *c\_AMPs* are homologous to full-length proteins in GMGCv1<sup>46</sup> (**Fig. 1B**), with 27% of hits sharing the start codon with the full-length protein, which suggests early termination of full-length proteins as one mechanism for generating novel *c\_AMPs* (**Fig. 2A** and **2B**).

To investigate the function of the full-length proteins homologous to AMPs, we mapped the matching proteins from GMGCv1<sup>46</sup> to their orthologous groups (OGs) from eggNOG 5.0<sup>48</sup>. We identified 3,792 (out of 43,789) OGs significantly enriched ( $P_{\text{Hypergeom.}} < 0.05$ , after multiple hypothesis corrections with the Holm-Sidak method) among the hits from AMPSphere. Although OGs of unknown function comprise 53.8% of all identified OGs, when considered individually, these OGs are, on average, smaller than OGs in other categories. Thus, despite each OG having a relatively small number of *c\_AMP* hits, when compared to the background distribution of the OGs in GMGCv1, OGs of

unknown function were the most enriched among the c\_AMP hits, with an average enrichment of  
160 10,857 fold ( $P_{\text{Mann}} \leq 3.9 \cdot 10^{-4}$ ; **Fig. 2C**; **Table SI3**).

### ***c\_AMP genes may arise after gene duplication events***

162 We next raised the question of whether c\_AMPs would be predominantly present in specific  
genomic contexts. To investigate the functions of the neighboring genes of the c\_AMPs, we mapped  
164 them against 169,484 high-quality genomes included in a study by del Río et al.<sup>49</sup>. 38.9% (21,465 out  
of 55,191) c\_AMPs with more than two homologs in different genomes in the database show  
166 phylogenetically conserved genomic context with genes of known function (see **Methods - Genomic  
context conservation analysis**). This proportion of c\_AMPs in conserved genomic context is slightly  
168 higher than for other gene family clusters calculated on the genomes' gene sets (1.1-fold,  $P_{\text{Permutation}} =$   
 $1.3 \cdot 10^{-111}$ ). This difference becomes more pronounced when comparing the genomic context of  
170 c\_AMPs and protein families composed of short (< 50 amino acids) peptides (11-fold increase,  
 $P_{\text{Permutation}} < 10^{-330}$ ).

172 Despite being involved in similar processes, AMPs were significantly depleted from conserved  
genomic contexts involving known systems of antibiotic synthesis and resistance, even when they  
174 were compared to small protein families (0.6-fold,  $P_{\text{Permutation}} = 1.7 \cdot 10^{-8}$ , **Fig. 3**). Instead, we found that  
c\_AMPs are encoded in conserved genomic contexts with ribosomal genes (24.1%) and ABC  
176 transporters (18%) at a higher frequency than other gene families (**Fig. 3A** and **Tables SI4** and **SI5**).

Most of the c\_AMPs (2,201 out of the 2,642) in conserved ribosomal genomic contexts are  
178 homologous to ribosomal proteins (**Fig. 3D**), congruent with the observation that, in some species,  
ribosomal proteins have antimicrobial properties<sup>50</sup>. Seventy-seven of the c\_AMPs that were  
180 homologous to ribosomal proteins were homologous to a gene in their immediate vicinity (up to 1 gene  
up/downstream). This phenomenon is not exclusive to ribosomal proteins: 2,309 c\_AMPs can be  
182 annotated to the same KEGG Orthologous Group (KO) as some of their conserved neighbors and  
may have originated from gene duplication events, the common annotation being interpreted, in this  
184 context, as evidence for a common evolutionary origin and not as a functional prediction for the  
c\_AMPs. Interestingly, 1,707 (73.9%) of these c\_AMPs are located downstream of the conserved  
186 neighbor with the same KO annotation. The livM family (branched-chain amino acid transport system  
permease protein, K01998), a transposase family (K07486), and a class of permeases (K03106) are

188 the most common KOs assigned to c\_AMPs and their neighbors (185, 128, and 67 c\_AMPs, respectively) - see **Table SI6**.

## 190 ***Most c\_AMPs are members of the accessory pangenome***

We observed that only a small portion ( $5.9\%$ ,  $P_{\text{Permutation}} = 4.8 \cdot 10^{-3}$ ,  $N_{\text{Species}} = 416$ ) of c\_AMP families present in ProGenomes2<sup>41</sup> show prevalence  $\geq 95\%$  in genomes from the same species (**Fig. 4**), here referred to as “core”<sup>51</sup>. This is consistent with previous work, in which AMP production was observed to be strain-specific<sup>52</sup>. In contrast, a high proportion (*circa* 68.8%) of full-length protein families are core in ProGenomes2<sup>41</sup> species. There is a 1.9-fold greater chance ( $P_{\text{Fisher}} = 2.2 \cdot 10^{-92}$ ) of finding a pair of genomes from the same species sharing at least one c\_AMP when they belong to the same strain ( $99.5\% \leq \text{ANI} < 99.99\%$ ).

198 One example of this strain-specific behavior is AMP10.018\_194, the only c\_AMP found in *Mycoplasma pneumoniae* genomes. *M. pneumoniae* strains are traditionally classified into two groups based on their P1 adhesin gene<sup>53</sup>. Of the 76 *M. pneumoniae* genomes present in our study, 29 were of type-1, 29 were of type-2, and the remaining 18 were of the undetermined type in this classification system<sup>54</sup> (**Methods - Determination of accessory AMPs**). Twenty-six of the 29 type-2 genomes contained AMP10.018\_194, as did 2 undetermined type genomes, but none of the type-1 genomes contained this AMP.

## 206 ***Bacterial strains from the human gut have more c\_AMP genes than conspecific strains from other human body sites***

We investigated the taxonomic composition of the AMPSphere by annotating contigs with the GTDB taxonomy<sup>55,56</sup> (see **Methods - Differences in the c\_AMP density in microbial species from different habitats**), which resulted in 570,187 c\_AMPs being annotated to a genus or species. The genera contributing the most c\_AMPs to AMPSphere were *Prevotella* (18,593 c\_AMPs), *Bradyrhizobium* (11,846 c\_AMPs), *Pelagibacter* (6,675 c\_AMPs), *Faecalibacterium* (5,917 c\_AMPs), and CAG-110 (5,254 c\_AMPs) (see **Fig. 4**). This distribution reflects the fact that these genera are among those that contribute the most assembled sequences in our dataset (all occupying percentiles above 99.75% among the assembled genera; see **Table SI7**). Therefore, we computed the c\_AMP density ( $\rho_{\text{AMP}}$ ) as the number of c\_AMP genes found per megabase pair of assembled sequence. The

216 densest genera were environmental microorganisms, such as *Algorimicrobium* ( $2.1 \frac{c_{AMP_{genes}}}{Mbp}$ ), as well  
as non-cultured taxa, *e.g.*, TMED78 (1.6), SFJ001 (1.5), STGJ01 (1.4), and CAG-462 (1.4). However,  
218 when we considered phylum-level annotations, we observed none of the above-mentioned genera  
belonging to the Methyloirabilota phylum, the group with the highest  $\rho_{AMP}$ , followed by  
220 Fusobacteriota. The absence of these genera is due to the presence of contigs assigned to these  
phyla, but not to a specific genus, likely because of a lack of representation in the database.

222 These environmental high c\_AMP density genera are, however, low abundance taxa (see  
**Methods - Differences in the c\_AMP density in microbial species from different habitats**) and,  
224 the average  $\rho_{AMP}$  of animal-host-associated samples is 1.6-fold higher than in samples from non-host-  
associated habitats ( $P_{Mann} < 10^{-330}$ ; **Fig. 5A** and **Fig. S14**). Differences between habitats reflect both  
226 changes in high-level taxonomic composition and subspecies variation. To disentangle these two  
effects, we analyzed the 3,930 microbial species that were present in at least 10 samples from two  
228 different habitats, comparing the AMP density in sequences from the same species in different  
habitats. This resulted in 1,531 species showing significant differences in at least one pair of habitats  
230 ( $FDR < 0.05$ , Holm-Sidak; see **Methods - Differences in the c\_AMP density in microbial species  
from different habitats**). For example, *Prevotella copri* has a higher density when found in the cat  
232 and human gut compared to other mammalian hosts or in wastewater ( $P_{Kruskal} = 4.9 \cdot 10^{-276}$ , **Fig. 5B** and  
**Table S18**). When comparing the human gut and oral cavity (the two body sites with the greatest  
234 overlap in species), in general, microbial strains have higher AMP density in the human gut ( $P_{Mann} =$   
 $1.2 \cdot 10^{-4}$ ,  $N_{Species} = 37$ , **Fig. 5C** and **Table S18**). We also checked non-animal hosts, such as plants, and  
236 observed that microbial species simultaneously observed in soil and plant-associated microbiome  
usually have higher AMP density in soil ( $P_{Mann} = 2.8 \cdot 10^{-4}$ ,  $N_{Species} = 130$ , **Fig. 5D** and **Table S18**).

### 238 **More transmissible species have lower AMP density**

To further establish the importance of AMP production in ecological processes, we investigated  
240 the role of AMPs in the mother-to-child transmissibility of bacterial species in a recently published  
dataset<sup>57</sup>, by correlating the  $\rho_{AMP}$  for each bacterial species to the published measures of microbial  
242 transmission. Human gut bacteria showed increased transmissibility at lower AMP densities ( $R_{Spearman} =$   
 $-0.44$ ,  $P_{Holm-Sidak} = 9.5 \cdot 10^{-3}$ ,  $N_{Species} = 45$ ). Similarly, in human oral microbiome bacterial species,

transmissibility from mother to offspring is consistently inversely correlated with their  $\rho_{AMP}$  for the first year ( $R_{\text{Spearman}} = -0.53$ ,  $P_{\text{Holm-Sidak}} = 1.2 \cdot 10^{-3}$ ,  $N_{\text{Species}} = 45$ ), 3 years ( $R_{\text{Spearman}} = -0.38$ ,  $P_{\text{Holm-Sidak}} = 3.9 \cdot 10^{-3}$ ,  $N_{\text{Species}} = 77$ ), and up to 18 years ( $R_{\text{Spearman}} = -0.35$ ,  $P_{\text{Holm-Sidak}} = 3.9 \cdot 10^{-3}$ ,  $N_{\text{Species}} = 91$ ). For the human oral datasets, we observed that lower  $\rho_{AMP}$  correlated to species transmission in the case of people cohabitating ( $R_{\text{Spearman}} = -0.33$ ,  $P_{\text{Holm-Sidak}} = 6.3 \cdot 10^{-3}$ ,  $N_{\text{Species}} = 91$ ). Thus, the AMP contents may influence microbial transmission success rates.

## Physicochemical features and secondary structure of AMPs

To investigate the properties and structure of the synthesized peptides, we first compared their amino acid composition to AMPs from available databases (DRAMP 3.0<sup>44</sup>, DBAASP<sup>58</sup>, and APD3<sup>59</sup>). Overall, the composition was similar, as was expected, given that Macrel's ML model was trained using known AMPs<sup>40</sup>. Notably, the AMPSphere sequences displayed a slightly higher abundance of aliphatic amino acid residues, specifically alanine and valine. However, these AMPSphere sequences consistently differed (**Fig. 6A**) from encrypted peptides (EPs), which are peptides previously identified within proteins, including those found in the human proteome<sup>3</sup>. The resemblances in amino acid composition between the identified c\_AMPs and known AMPs suggested similar physicochemical characteristics and secondary structures, both of which are recognized for their influence on antimicrobial activity<sup>60</sup>. The c\_AMPs exhibited comparable hydrophobicity, net charge, and amphiphilicity to AMPs sourced from databases (**Fig. S11**). Furthermore, they displayed a slight propensity for disordered conformations (**Fig. 6B**) and had a lower positive charge compared to EPs (**Fig. 6A**).

Subsequently, we conducted experimental assessments of the secondary structure of the active c\_AMPs using circular dichroism (**Fig. 6B** and **S16**). Similar to AMPs documented in databases, peptides derived from AMPSphere exhibited a pronounced propensity for adopting  $\alpha$ -helical structures. Notably, they also displayed an unusually high content of  $\beta$ -antiparallel structure in both water and methanol/water mixtures (**Fig. 6B**), despite their amino acid composition similarities to AMPs and EPs. We attribute these findings to the slightly elevated occurrence of alanine and valine residues, which are known to favor  $\beta$ -like structures with a preference for  $\beta$ -antiparallel conformation<sup>61</sup>.

### Validation of c\_AMPs as potent AMPs through in vitro assays

To evaluate the potential antimicrobial properties of c\_AMPs, we selected and chemically synthesized 50 peptide sequences based on their abundance, predicted solubility, and taxonomic diversity (**Methods - Selection of peptides to synthesis and activity testing**). Next, we subjected these peptides to testing against 11 clinically relevant pathogenic strains, encompassing *Acinetobacter baumannii*, *Escherichia coli* (including one colistin-resistant strain), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (including one methicillin-resistant strain), vancomycin-resistant *Enterococcus faecalis*, and vancomycin-resistant *Enterococcus faecium*. Our initial screening revealed that 27 AMPs (54% of the total synthesized) completely eradicated the growth of at least one of the pathogens tested (**Fig. 6C**). Remarkably, in some cases, the AMPs were active at concentrations as low as 1  $\mu\text{mol}\cdot\text{L}^{-1}$ . Several of the Gram-negative bacteria, *i.e.*, *A. baumannii*, *E. coli*, and *P. aeruginosa*, as well as the Gram-positive strain of vancomycin-resistant *E. faecium*, displayed higher susceptibility to the AMPs, with 22, 15, 4 and 15 peptide hits, respectively. However, none of the tested AMPs targeted methicillin-resistant *S. aureus* (MRSA) (**Fig. 6C**).

### The growth of human gut commensals is impaired by c\_AMPs

We screened the AMPs against eight of the most prevalent members of the human gut microbiota. We tested commensal bacteria belonging to four phyla (Verrucomicrobiota, Bacteroidota, Actinomycetota, and Bacillota), *i.e.*, *Akkermansia muciniphila*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Phocaeicola vulgatus* (formerly *Bacteroides vulgatus*), *Collinsella aerofaciens*, *Clostridium scindens*, and *Parabacteroides distasonis*.

While it is commonly observed that known natural AMPs do not target microbiome strains<sup>62</sup>, our study found that 30 of the synthesized AMPs (60%) demonstrated inhibitory effects on at least one commensal strain at low concentrations levels (8-16  $\mu\text{mol}\cdot\text{L}^{-1}$ ). Although this concentration range was higher than that required to inhibit pathogens (1-4  $\mu\text{mol}\cdot\text{L}^{-1}$ ), it still falls within the highly active range of AMPs based on previous studies<sup>63-65</sup> (**Fig. 6C**). Interestingly, all the analyzed gut microbiome strains were susceptible to at least two c\_AMPs, with strains of *A. muciniphila*, *B. uniformis*, *P. vulgatus*, *C. aerofaciens*, *C. scindens*, and *P. distasonis* exhibiting the highest susceptibility. In total, 36 AMPs (72% of the total synthesized peptides) demonstrated antimicrobial activity against pathogens and/or commensals.

## 300 **Depolarization and permeabilization of the bacterial membrane by AMPs from AMPSphere**

To gain insights into the mechanism of action responsible for the antimicrobial activity  
 302 observed in the peptides derived from AMPSphere (**Fig. 6C**), we conducted experiments to assess  
 their ability to permeabilize and depolarize the cytoplasmic and outer membranes of bacteria at their  
 304 Minimum Inhibitory Concentrations - MICs. Specifically, we investigated the effects of 22 peptides on  
*A. baumannii* (**Fig. 6D-E** and **Fig. S17A,D**) and 4 peptides on *P. aeruginosa* (**Fig. S17B-C,E**). For  
 306 comparison, we used polymyxin B, a peptide antibiotic known for its membrane permeabilization and  
 depolarization properties, as a control in these experiments<sup>3</sup>.

308 To investigate the potential permeabilization of the outer membranes of Gram-negative  
 bacteria by active AMPs derived from AMPSphere, we conducted 1-(N-phenylamino)naphthalene  
 310 (NPN) uptake assays (**Fig. S17A-B**). NPN is a lipophilic fluorophore that exhibits increased  
 fluorescence in the presence of lipids found in bacterial outer membranes. The uptake of NPN  
 312 indicates membrane permeabilization and damage. Among the 22 peptides evaluated for activity  
 against *A. baumannii*, 10 peptides caused significant permeabilization of the outer membrane,  
 314 resulting in fluorescence levels at least 50% higher than that of polymyxin B (**Fig. 6D**). Only three  
 peptides exhibited lower permeabilization than polymyxin B (**Fig. 6D**). In the case of *P. aeruginosa*  
 316 cells, two out of the four tested peptides showed higher permeabilization than polymyxin B (**Fig.**  
**S17C**).

318 To evaluate the potential membrane depolarization effect of the AMPs from AMPSphere, we  
 utilized the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide [DiSC<sub>3</sub>-(5)] (**Fig. S17D-E**). Among  
 320 the peptides tested against *A. baumannii* and *P. aeruginosa*, marinobacticin-1 (AMP10.321\_460) and  
 cagicin-2 (AMP10.014\_861) exhibited greater cytoplasmic membrane depolarization than polymyxin B  
 322 (**Fig. 6E; Fig. S17E-F**), respectively. Interestingly, all the tested AMPSphere peptides displayed a  
 characteristic crescent-shaped depolarization pattern compared to polymyxin B, with lower levels of  
 324 depolarization observed during the first 20 minutes of exposure, followed by an increase in  
 depolarization over time (**Fig. 6E** and **Fig. 7D-F**). Taken together, these results indicate that the  
 326 kinetics of cytoplasmic membrane depolarization are slower compared to the kinetics of outer  
 membrane permeabilization, which occurs rapidly upon interaction with the bacterial cells.

328 Our findings indicate that the AMPs from AMPSphere primarily exert their effects by  
 permeabilizing the outer membrane rather than depolarizing the cytoplasmic membrane, revealing a  
 330 similar mechanism of action to that observed for classical AMPs and EPs.

# **AMPs exhibit anti-infective efficacy in a mouse model**

Next, we tested the anti-infective efficacy of AMPSphere-derived peptides in a skin abscess murine infection model (**Fig. 7A**). Mice were subjected to infection with *A. baumannii*, a Gram-negative pathogen known for causing severe infections in various body sites including bloodstream, lungs, urinary tract, and wounds. This pathogen is recognized as one of the most dangerous<sup>66</sup>. Five lead AMPs from different sources displayed potent activity against *A. baumannii*: synechocin-1 (AMP10.000\_211, 8  $\mu\text{mol}\cdot\text{L}^{-1}$ ) from *Synechococcus* sp. (coral associated, marine microbiome), proteobactin-1 (AMP10.048\_551, 16  $\mu\text{mol}\cdot\text{L}^{-1}$ ) from Pseudomonadota (plant and soil microbiome), actinomycin-1 (AMP10.199\_072, 64  $\mu\text{mol}\cdot\text{L}^{-1}$ ) from *Actinomyces* (human mouth and saliva microbiome), lachnospirin-1 (AMP10.015\_742, 2  $\mu\text{mol}\cdot\text{L}^{-1}$ ) from *Lachnospira* sp. (human gut microbiome), and enterococcin-1 (AMP10.051\_911, 1  $\mu\text{mol}\cdot\text{L}^{-1}$ ) from *Enterococcus faecalis* (human gut microbiome).

The skin abscess infection was established with a bacterial load of 20  $\mu\text{L}$  of *A. baumannii* cells at  $1 \times 10^6$  CFU $\cdot\text{mL}^{-1}$  onto the wounded area of the dorsal epidermis (**Fig. 7A**). A single dose of each peptide, at their respective MIC (**Fig. 6A**), was administered to the infected area. Two days post-infection, synechocin-1 and actinomycin-1 peptides presented bacteriostatic activity, inhibiting the proliferation of *A. baumannii* cells, whereas lachnospirin-1 and enterococcin-1 presented bactericidal activity similar to that of the antibiotic polymyxin B (MIC = 0.25  $\mu\text{mol}\cdot\text{L}^{-1}$ ), reducing the colony-forming units (CFU) counts up to 3-4 orders of magnitude (**Fig. 7B**). Four days post-infection, none of the AMPs nor polymyxin B achieved a statistically significant reduction of *A. baumannii* growth at the infection site, although treatment with proteobactin-1, lachnospiracin-1, enterococcin-1, and polymyxin B reduced the CFU counts by 1-2 orders of magnitude compared to the untreated control. These results are promising since the AMPSphere peptides were administered only once immediately after the establishment of the abscess, highlighting their anti-infective potential.

Mouse weight was monitored as a proxy for toxicity and no significant changes were observed (**Fig. 7C**), suggesting that the peptides tested are not toxic.

# Discussion

Here, we use ML to identify thousands of novel candidate AMPs in the global microbiome. Building on previous studies that focused specifically on the human gut microbiome<sup>5,36,67</sup>, we cataloged AMPs from the global microbiome across 63,410 publicly available metagenomes, as well as 87,920 high-quality microbial genomes from the ProGenomes v2 database<sup>41</sup>, leading to the creation of AMPSphere (<https://ampsphere.big-data-biology.org/>), a publicly available resource encompassing 863,498 non-redundant peptides and 6,499 high-quality AMP families from 72 different habitats, including marine and soil environments and the human gut. We show that most of the c\_AMPs (91.5%) were previously unknown, lacking detectable homologs in other databases, and about one in five could be detected in independent sets of meta-transcriptomes or metaproteomes.

Two evolutionary mechanisms by which AMPs may be generated were explored. First, mutations in genes encoding longer proteins could generate gene fragments. Among the enriched ortholog groups of proteins from GMGCv1<sup>46</sup> homologous to c\_AMPs, we observed that a majority of groups had unknown function (53.8%), similar to what was reported by Sberro et al.<sup>36</sup> for small proteins from the human gut microbiome. The second mechanism is that a gene duplication could be followed by mutation, which we observed in the case of ribosomal proteins. Ribosomal proteins can harbor antimicrobial activity<sup>50</sup>, possibly due to their amyloidogenic properties<sup>68</sup>. Nonetheless, the majority of identified AMPs do not have detectable homology to other sequences, highlighting their novelty. The lack of observed homology, however, may be due to limitations in our ability to robustly detect these homology relationships in small sequences, but there is also the possibility that small proteins, such as AMPs, may be more likely to be generated *de novo* and may have repeatedly evolved in various taxa<sup>69</sup>.

Four out of the five genera with the most c\_AMPs present in AMPSphere share a host-associated lifestyle. Three of these (*Prevotella*, *Faecalibacterium*, and *CAG-110*) are common in animal hosts (**Fig. 4**). The greater density of c\_AMP genes in genera from the Bacillota and Bacillota A phyla is consistent with the well-known diversity of ABC transporters dedicated to the translocation of AMPs found in that group, resulting in improved resistance to compounds that bind extracellular targets; for a review, see Gebhard<sup>70</sup>.

We observed that c\_AMPs from AMPSphere are habitat-specific and mostly accessory  
 386 members of microbial pangenomes. Moreover, species-specific density ( $\rho_{AMP}$ ) shows that the habitat  
 plays an important role in shaping c\_AMP content. The  $\rho_{AMP}$  of strains from the same species can  
 388 differ even across body sites. In particular, we observed higher  $\rho_{AMP}$  in the human gut compared to the  
 human oral cavity, in agreement with a recent report of a strain-specific AMP (cutymicin), which is  
 390 present in only some of the hair follicles in the same human host<sup>71</sup>.

Valles-Colomer et al.<sup>57</sup>, who recently analyzed a large collection of human-associated  
 392 metagenomes, provide a species-specific index of transmissibility for the several transmission  
 scenarios they study (*e.g.*, mother to infant). Hypothesizing that AMP production may be related to  
 394 transmission, we correlated the species-specific  $\rho_{AMP}$  calculated in AMPSphere with transmission  
 scores. In both the human gut and oral microbiomes, species with higher  $\rho_{AMP}$  are less transmissible,  
 396 possibly because AMPs confer protection against strain replacement. Taken together, our results and  
 those of Valles-Colomer et al.<sup>57</sup> validate the AMP density concept and the applicability of AMPSphere  
 398 resources to study mechanisms of microbial establishment and competition.

Finally, we experimentally validated predictions made by our ML model and found that 72% (32  
 400 out of the 50) synthesized AMPs displayed antimicrobial activity against either pathogens or  
 commensals. Notably, four peptides (cagicin-1, cagicin-4, and enterococcin-1 against *A. baumannii*;  
 402 and cagicin-1 and lachnospirin-1 against vancomycin-resistant *E. faecium*) presented MIC values as  
 low as 1  $\mu\text{mol}\cdot\text{L}^{-1}$ , comparable to the MICs of some of the most potent peptides previously described in  
 404 the literature<sup>64,65</sup>.

We show that AMPs from the AMPSphere tended to target clinically relevant Gram-negative  
 406 pathogens and also showed activity against vancomycin-resistant *E. faecium*. Although conventional  
 AMPs do not target microbiome bacteria<sup>62</sup>, AMPs from AMPSphere showed efficacy against these  
 408 bacteria, suggesting potential ecological implications of peptides as protective agents for their  
 producing organisms. Notably, the amino acid composition and physicochemical characteristics of the  
 410 c\_AMPs in AMPSphere differed from those recently identified in EPs<sup>3</sup>.

Moreover, three peptides exhibited anti-infective efficacy in a murine infection model, with  
 412 lachnospirin-1 and enterococcin-1 being the most potent, resulting in a reduction of bacterial load by  
 up to four orders of magnitude. The active peptides included those derived from both human-  
 414 associated and environmental microbiota, validating our approach of investigating the global

microbiome. Overall, our findings unveil a wide array of novel AMP sequences, highlighting the  
416 potential of machine learning in the identification of much-needed antimicrobials.

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[*Escherichia coli* MG1655 phnE\_2::FRT (control strain for AIC 222)] and *Escherichia coli* AIC222  
426 [*Escherichia coli* MG1655 pmrA53 phnE\_2::FRT (polymyxin resistant)]. This work was funded by the  
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## **Declaration of interests**

438 Cesar de la Fuente-Nunez provides consulting services to Invaio Sciences and is a member of  
440 the Scientific Advisory Boards of Nowture S.L. and Phare Bio. The de la Fuente Lab has received  
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have any competing interests.

444 **Author contributions**

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- 450 6. **Methodology:** CDSJ YD JHC ARdR LPC MDTT CFN
7. **Project administration:** LPC XMZ PB CFN
- 452 8. **Resources:** LPC XMZ CFN
9. **Supervision:** LPC CFN
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## STAR Methods

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## 474    **KEY RESOURCES TABLE**

| <b>REAGENT or RESOURCE</b>         | <b>SOURCE</b>                                    | <b>IDENTIFIER</b> |
|------------------------------------|--------------------------------------------------|-------------------|
| <b>Bacterial and virus strains</b> |                                                  |                   |
| <i>Acinetobacter baumannii</i>     | American Type Culture Collection                 | ATCC 19606        |
| <i>Escherichia coli</i>            | American Type Culture Collection                 | ATCC 11775        |
| <i>Escherichia coli</i>            | <i>Escherichia coli</i><br>MG1655<br>phnE_2::FRT | AIC221            |
| <i>Escherichia coli</i>            | <i>Escherichia coli</i>                          | AIC222            |

|                                                    |                                                                                        |                                                     |
|----------------------------------------------------|----------------------------------------------------------------------------------------|-----------------------------------------------------|
|                                                    | MG1655 pmrA53<br>phnE_2::FRT<br>(polymyxin-resistant;<br>colistin-resistant<br>strain) |                                                     |
| <i>Klebsiella pneumoniae</i>                       | American Type<br>Culture Collection                                                    | ATCC 13883                                          |
| <i>Pseudomonas aeruginosa</i>                      |                                                                                        | PA01                                                |
| <i>Pseudomonas aeruginosa</i>                      |                                                                                        | PA14                                                |
| <i>Staphylococcus aureus</i>                       | American Type<br>Culture Collection                                                    | ATCC 12600                                          |
| <i>Staphylococcus aureus</i>                       | American Type<br>Culture Collection                                                    | ATCC BAA-1556<br>(methicillin-<br>resistant strain) |
| <i>Akkermansia muciniphila</i>                     | American Type<br>Culture Collection                                                    | ATCC BAA-635                                        |
| <i>Bacteriodes fragilis</i>                        | American Type<br>Culture Collection                                                    | ATCC 25285                                          |
| <i>Bacteroides thetaiotaomicron</i>                | American Type<br>Culture Collection                                                    | ATCC 29148                                          |
| <i>Bacteroides uniformis</i>                       | American Type<br>Culture Collection                                                    | ATCC 8492                                           |
| <i>Bacteroides vulgatus (Phocaeicola vulgatus)</i> | American Type<br>Culture Collection                                                    | ATCC 8482                                           |
| <i>Collinsella aerofaciens</i>                     | American Type<br>Culture Collection                                                    | ATCC 25986                                          |
| <i>Clostridium scindens</i>                        | American Type<br>Culture Collection                                                    | ATCC 35704                                          |
| <i>Parabacteroides distasonis</i>                  | American Type                                                                          | ATCC 8503                                           |

|                                        | Culture Collection          |                                                                                                                             |
|----------------------------------------|-----------------------------|-----------------------------------------------------------------------------------------------------------------------------|
| <b><i>Chemicals</i></b>                |                             |                                                                                                                             |
| Luria-Bertani broth                    | BD                          | 244620                                                                                                                      |
| Tryptic soy broth                      | Sigma                       | T8907-1KG                                                                                                                   |
| Agar                                   | Sigma                       | 05039                                                                                                                       |
| MacConkey agar                         | RPI                         | M42560-500.0                                                                                                                |
| Phosphate buffer saline                | Sigma                       | P3913-10PAK                                                                                                                 |
| Glucose                                | Sigma                       | G5767                                                                                                                       |
| 1-(N-phenylamino)naphthalene           | Sigma                       | 104043                                                                                                                      |
| 3,3'-dipropylthiadicarbocyanine iodide | Sigma                       | 43608                                                                                                                       |
| HEPES                                  | Fisher                      | BP310-100                                                                                                                   |
| Potassium chloride (KCl)               | Sigma                       | P3911                                                                                                                       |
| <b><i>Deposited data</i></b>           |                             |                                                                                                                             |
| Code for generation of AMPSphere       | This study                  | <a href="https://github.com/BigDataBiology/AMPSphere_manuscript">https://github.com/BigDataBiology/AMPSphere_manuscript</a> |
| AMPSphere database                     | This study                  | <a href="https://zenodo.org/record/4606582">https://zenodo.org/record/4606582</a>                                           |
| <b><i>Software and algorithms</i></b>  |                             |                                                                                                                             |
| NGLess 1.3.0                           | Coelho et al. <sup>72</sup> | <a href="https://github.com/ngless-">https://github.com/ngless-</a>                                                         |

|                   |                                     |                                                                                                 |
|-------------------|-------------------------------------|-------------------------------------------------------------------------------------------------|
|                   |                                     | toolkit/ngless                                                                                  |
| JUG 2.1.1         | Coelho <sup>73</sup>                | <a href="https://github.com/luispedro/jug">https://github.com/luispedro/jug</a>                 |
| Prodigal 2.6.3    | Hyatt et al. <sup>32</sup>          | <a href="https://github.com/hyattpd/Prodigal">https://github.com/hyattpd/Prodigal</a>           |
| Macrel v.1.0.0    | Santos-Júnior et al. <sup>40</sup>  | <a href="https://github.com/BigDataBiology/macrel">https://github.com/BigDataBiology/macrel</a> |
| CDHit 4.8.1       | Fu et al. <sup>74</sup>             | <a href="https://github.com/weizhongli/cdhit">https://github.com/weizhongli/cdhit</a>           |
| MMseqs2           | Steinegger and Söding <sup>75</sup> | <a href="https://github.com/soedinglab/MMseqs2">https://github.com/soedinglab/MMseqs2</a>       |
| python 3.8.2      | Van Rossum <sup>76</sup>            | <a href="https://www.python.org/">https://www.python.org/</a>                                   |
| matplotlib 3.4.3  | Hunter <sup>77</sup>                | <a href="https://matplotlib.org/">https://matplotlib.org/</a>                                   |
| numpy 1.21.2      | Harris et al. <sup>78</sup>         | <a href="https://numpy.org/">https://numpy.org/</a>                                             |
| pandas 1.3.2      | McKinney <sup>79</sup>              | <a href="https://pandas.pydata.org/">https://pandas.pydata.org/</a>                             |
| plotly 5.2.1      | Plotly Technologies Inc, 2015       | <a href="https://plot.ly">https://plot.ly</a>                                                   |
| scipy 1.7.1       | Virtanen et al. <sup>80</sup>       | <a href="https://www.scipy.org">https://www.scipy.org</a>                                       |
| scikit-learn 0.24 | Pedregosa et al. <sup>81</sup>      | <a href="https://scikit-">https://scikit-</a>                                                   |

|                                                       |                                                     |                                                                                                   |
|-------------------------------------------------------|-----------------------------------------------------|---------------------------------------------------------------------------------------------------|
|                                                       |                                                     | <a href="https://learn.org/">learn.org/</a>                                                       |
| scikit-bio 0.5.6                                      | The scikit-bio development team, 2020 <sup>82</sup> | <a href="http://scikit-bio.org/">http://scikit-bio.org/</a>                                       |
| BioPython 1.7.9                                       | Cock et al. <sup>83</sup>                           | <a href="https://biopython.org/">https://biopython.org/</a>                                       |
| eggnog-mapper v2                                      | Cantalapiedra et al. <sup>84</sup>                  | <a href="https://github.com/eggnogdb/eggnog-mapper">https://github.com/eggnogdb/eggnog-mapper</a> |
| HMMer 3.3+dfsg2-1                                     | Eddy <sup>85</sup>                                  | <a href="http://hmmer.org/">http://hmmer.org/</a>                                                 |
| modIAMP 4.2.2                                         | Müller et al. <sup>86</sup>                         | <a href="https://pypi.org/project/modlamp/">https://pypi.org/project/modlamp/</a>                 |
| FastTree 2.1                                          | Price et al. <sup>87</sup>                          | <a href="http://www.microbesonline.org/fasttree/">www.microbesonline.org/fasttree/</a>            |
| FastANI v.1.33                                        | Jain et al. <sup>88</sup>                           | <a href="https://github.com/ParBLiSS/FastANI">https://github.com/ParBLiSS/FastANI</a>             |
| Megahit 1.2.9                                         | Li et al. <sup>89</sup>                             | <a href="https://github.com/voutcn/megahit/">https://github.com/voutcn/megahit/</a>               |
| <b>Other</b>                                          |                                                     |                                                                                                   |
| ProGenomes2                                           | Mende et al. <sup>41</sup>                          | <a href="http://progenomes.embl.de/">http://progenomes.embl.de/</a>                               |
| DRAMP - Data repository of antimicrobial peptides 3.0 | Shi et al. <sup>44</sup>                            | <a href="http://dramp.cpu-bioinfor.org/">http://dramp.cpu-bioinfor.org/</a>                       |
| UniprotKB 2021_03                                     | The UniProt Consortium <sup>90</sup>                | <a href="https://www.uniprot.org/">https://www.uniprot.org/</a>                                   |

|                       |                                          |                                                                                                                                                                                               |
|-----------------------|------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Eggnog v.5.0          | Huerta-Cepas et al. <sup>48</sup>        | <a href="http://eggnog5.embl.de/">http://eggnog5.embl.de/</a>                                                                                                                                 |
| SmProt database v.2.0 | Hao et al. <sup>45</sup>                 | <a href="http://bigdata.ibp.ac.cn/SmProt/index.html">http://bigdata.ibp.ac.cn/SmProt/index.html</a>                                                                                           |
| StarPep45k            | Aguilera-Mendoza et al. <sup>91</sup>    | <a href="http://mobiosd-hub.com/starpep">http://mobiosd-hub.com/starpep</a>                                                                                                                   |
| PFAM 34.0             | Mistry et al. <sup>92</sup>              | <a href="http://pfam.xfam.org/">http://pfam.xfam.org/</a>                                                                                                                                     |
| AntiFAM v.6.0         | Eberhardt et al. <sup>93</sup>           | <a href="https://www.ebi.ac.uk/research/bateman/software/antifam-tool-identify-spurious-proteins">https://www.ebi.ac.uk/research/bateman/software/antifam-tool-identify-spurious-proteins</a> |
| GTDB 07-RS95          | Parks et al. <sup>55,56</sup>            | <a href="https://gtdb.ecogenomic.org/">https://gtdb.ecogenomic.org/</a>                                                                                                                       |
| NCBI release 207      | NCBI Resource Coordinators <sup>94</sup> | <a href="https://ftp.ncbi.nih.gov/refseq/release/">https://ftp.ncbi.nih.gov/refseq/release/</a>                                                                                               |
| DBAASP                | Pirtskhalava et al. <sup>58</sup>        | <a href="https://dbaasp.org/home">https://dbaasp.org/home</a>                                                                                                                                 |
| APD3                  | Wang and Wang <sup>59</sup>              | <a href="https://aps.unmc.edu/">https://aps.unmc.edu/</a>                                                                                                                                     |

# RESOURCE AVAILABILITY

## 476 **Lead contact**

Further information and requests for resources and reagents should be directed to and will be  
 478 fulfilled by the lead contacts: Cesar de la Fuente-Nunez (cfuente@upenn.edu) and Luis Pedro Coelho  
 (luispedro@big-data-biology.org).

## 480 **Materials availability**

Peptides were obtained from AAPPTec and synthesized using solid-phase peptide synthesis  
 482 and 9-fluorenylmethoxycarbonyl (Fmoc) strategy.

## **Data and code availability**

- 484 · Metagenomes and Genomes data are publicly available at the European Nucleotide Archives  
 (ENA) as of the date of publication. Accession numbers are listed in the supplementary tables.
- 486 · All original code has been deposited at GitHub in the link:  
[github.com/BigDataBiology/AMPSphere\\_manuscript](https://github.com/BigDataBiology/AMPSphere_manuscript)
- 488 · AMPSphere files have been deposited in Zenodo and are publicly available as of the date of  
 publication. DOIs are listed in the key resources table.
- 490 · AMPSphere is also available as a public online resource: <https://ampsphere.big-data-biology.org/>
- Any additional information required to reanalyze the data reported in this paper is available from  
 492 the lead contact upon request.

# EXPERIMENTAL MODEL AND SUBJECT DETAILS

## 494 **Bacterial strains and growth conditions**

The pathogenic strains *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 11775,  
 496 *Escherichia coli* AIC221 [*Escherichia coli* MG1655 phnE\_2::FRT (control strain for AIC 222)],  
*Escherichia coli* AIC222 [*Escherichia coli* MG1655 pmrA53 phnE\_2::FRT (polymyxin-resistant; colistin-  
 498 resistant strain)], *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* PA01,  
*Pseudomonas aeruginosa* PA14, *Staphylococcus aureus* ATCC 12600, *Staphylococcus aureus* ATCC  
 500 BAA-1556 (methicillin-resistant strain), *Enterococcus faecalis* ATCC 700802 (vancomycin-resistant

strain), and *Enterococcus faecium* ATCC 700221 (vancomycin-resistant strain) were grown and plated on Luria-Bertani (LB) agar plates and incubated overnight at 37°C from frozen stocks. After incubation, one isolated colony was transferred to 6 mL of medium (LB), and cultures were incubated overnight (16 h) at 37°C. The following day, inocula were prepared by diluting the overnight cultures 1:100 in 6 mL of the respective media and incubating them at 37°C until bacteria reached logarithmic phase (OD<sub>600</sub> = 0.3-0.5).

The gut commensal strains *Akkermansia muciniphila* ATCC BAA-635, *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29148, *Bacteroides uniformis* ATCC 8492, *Bacteroides vulgatus* ATCC 8482 (*Phocaeicola vulgatus*), *Collinsella aerofaciens* ATCC 25986, *Clostridium scindens* ATCC 35704, and *Parabacteroides distasonis* ATCC 8503 were grown in brain heart infusion (BHI) agar plates enriched with 0.1% (v:v) vitamin K3 (1 mg·mL<sup>-1</sup>), 1% (v:v) hemin (1 mg·mL<sup>-1</sup>, diluted with 10 mL of 1 N sodium hydroxide), and 10% (v:v) L-cysteine (0.05 mg·mL<sup>-1</sup>), from frozen stocks and incubated overnight at 37°C. Resazurin was used as oxygen indicator. After the incubation period, a single isolated colony was transferred to 3 mL of BHI broth and incubated overnight at 37°C. The next day, inocula were prepared by diluting the bacterial overnight cultures 1:100 in 3 mL of BHI broth and incubated at 37°C until cells reached the logarithmic phase (OD<sub>600</sub> = 0.3-0.5).

### **Skin abscess infection mouse model**

To assess the anti-infective efficacy of the peptides against *A. baumannii* ATCC 19606 in a skin abscess infection mouse model, the bacteria were cultured in tryptic soy broth (TSB) medium until an OD<sub>600</sub> of 0.5 was reached. Next, the cells were washed twice with sterile PBS (pH 7.4) and suspended to a final concentration of 5·10<sup>6</sup> colony-forming units (CFU) per mL<sup>-1</sup>. Six-week-old female CD-1 mice, after being anesthetized with isoflurane, were subjected to a superficial linear skin abrasion on their backs in an area that they could not touch with their mouth or limbs. An aliquot of 20 µL containing the bacterial load was then administered over the abraded area. A single dose of the peptides diluted in water at their MIC value was administered to the infected area two h after the infection. The animals were euthanized two- and four-days post-infection, and the infected area was extracted and homogenized for 20 min using a bead beater (25 Hz) and 10-fold serially diluted for CFU quantification on MacConkey agar plates for easy differentiation of *A. baumannii* colonies. The

experimental groups consisted of 3 mice per group ( $n = 3$ ), and each mouse was infected with an inoculum from a different colony to ensure variability.

## **METHOD DETAILS**

### ***Selection of metagenomes and high-quality microbial genomes***

Selection of metagenomes and genomes to compose the AMPSphere was similar to that adopted by Coelho et al.<sup>46</sup>. Only public metagenomes on 1 January 2020 produced with Illumina instruments (except for MiSeq), with at least 2 million reads and, on average, 75 bp long, were downloaded from the European Nucleotide Archive (ENA). These samples met two criteria: (1) tagged with taxonomy ID 408169 (for metagenome) or is a descendent of it in the taxonomic tree; and/or (2) experiments with the library source listed as “METAGENOMIC”. Samples were grouped by project and all projects with at least 20 samples were considered. Additionally, studies based on a whitelist, including metagenomes deposited by the Integrated Microbial Genomes System (IMG) missing from ENA, also were included. Metadata was manually curated from their describing literature and Biosamples database<sup>95</sup>. The habitat classification took into account the metadata, creating groups based on the similarity of habitat conditions, such as air, anthropogenic, aquatic, host-associated, ph:alkaline, sediment, terrestrial, and others. The sample origins and other relevant information related to host species were assessed using the NCBI taxonomic identification number. High-quality microbial genomes were selected from ProGenomes2 database<sup>41</sup>. The resulting 63,410 publicly available metagenomes and 87,920 high-quality microbial genomes are listed in **Table S11**.

### ***Reads trimming and assembly***

Reads were processed using NGLess<sup>72</sup>, trimming positions with quality lower than 25, and discarding reads shorter than 60 bp post-trimming. Metagenomes obtained from a host-associated microbiome passed through a filtering of reads mapping to the host genome when available. Reads were assembled with MEGAHIT 1.2.9<sup>89</sup> and the taxonomy of 16,969,685,977 contigs assembled from more than 14.7 trillion base pairs of sequenced DNA was inferred as previously described<sup>96</sup>, using MMSeqs2<sup>75</sup> to map the sequences against the GTDB release 95<sup>55,56</sup>, later manually curated to conform to the International Code of Nomenclature of Prokaryotes.

## 558 ***smORF and AMP prediction***

Analogously to Sberro et al.<sup>36</sup>, we used a modified version of Prodigal<sup>32</sup> to predict smORFs (33  
560 to 303 bp) from contigs. The 4,599,187,424 redundant smORFs, most of which (99.25%) originated in  
metagenomes were then de-duplicated, yielding 2,724,621,233 non-redundant smORFs. Macrel<sup>40</sup> was  
562 run on the de-duplicated smORFs to predict c\_AMPs. Singleton sequences (those appearing in a  
single sample or genome) were eliminated, except when they had a significant match (Amino acids  
564 identity  $\geq 75\%$  and E-value  $\leq 10^{-5}$ ) to a sequence from the Data Repository of Antimicrobial Peptides -  
DRAMP 3.0<sup>44</sup> using the 'easy-search' method from MMSeqs2<sup>75</sup>.

566 AMP genes originating from ProGenomes2<sup>41</sup> had the taxonomy of the original genome  
assigned to them, whereas the genes from metagenomes were assigned the taxonomy predicted for  
568 the contig where they were found. Insights about potential structural conformations were obtained by  
using the function secondary\_structure\_fraction from ProtParam module implemented in the SeqUtils  
570 in Biopython<sup>83</sup>. It calculates the fraction of amino acids which tend to assume conformations of helix  
[VIYFWL], turn [NPGS], and sheet [EMAL].

572 AMPSphere encompassed a total of 863,498 non-redundant predicted c\_AMPs encoded by  
5,518,294 redundant genes. AMP densities were estimated as the number of AMPs per assembled  
574 base pairs in a sample or a species.

## ***AMP families***

576 Protein families have a relationship between the structure, function, and origin of proteins<sup>97</sup>.  
We used a reduced amino acids alphabet of 8 letters<sup>47</sup> - [LVIMC], [AG], [ST], [FYW], [EDNQ], [KR] - to  
578 cluster together sequences which are likely to have similar functions<sup>47,98</sup>. These c\_AMPs were  
hierarchically clustered in this reduced alphabet using three sequential identity cutoffs (100%, 85%,  
580 and 75%) with CD-Hit<sup>74</sup> to improve the profiling and pattern searching at the protein space<sup>99</sup>. A cluster  
was considered an AMP family when it consisted of at least 8 sequences<sup>36</sup>. Representative sequences  
582 of peptide clusters were selected according to their length (taking the longest) with ties being broken  
by considering alphabetical order.

584 To validate the clustering procedure, we used a sample of 3,000 thousand sequences  
randomly drawn from AMPSphere, excluding the representatives. These sequences were aligned  
586 against the representative sequence of their cluster using the Smith-Waterman algorithm<sup>100</sup> with the  
blocks substitution matrix 62 (BLOSUM 62), and a gap open and extension penalties of -10 and -0.5,

588 respectively. The alignment score was then converted to an E-value, according to the model by Karlin  
and Altschul<sup>101</sup>, using the values of  $\kappa$  (0.132539) and  $\lambda$  (0.313667) constants adjusted to search for a  
590 short input sequence as implemented in the BLAST algorithm<sup>102,103</sup>. Alignments were considered  
significant when their E-value was less than  $10^{-5}$ . We found that more than 95.3% of alignments  
592 produced in the first two levels (100% and 100-85% of identity) were significant along with 77.1% of  
those from the third level (100-85-75% of identity) – see **Fig. S13**.

#### 594 **Quality control of c\_AMPs**

The genes of c\_AMPs were subjected to 5 different quality tests to reduce the likelihood that  
596 the observed peptides were artifacts or fragments of larger proteins. Initially, the peptides were  
searched against AntiFam v.7.0<sup>93</sup> using HMMSearch<sup>85</sup> with the option “--cut\_ga”, and significant hits  
598 were classified as spurious. We observed that 99.9% of the c\_AMPs in AMPSphere do not belong to  
AntiFAMs.

600 A test for the terminal positioning in contigs checked if there were in-frame stop codons  
upstream to the smORF coding for a given c\_AMP. When no stop codon is found, we cannot rule out  
602 the possibility that the smORF is part of a larger gene due to a fragmentary assembly. Most (68.4%) of  
the c\_AMPs are encoded by at least a gene that is not terminally placed.

604 The RNAcode program<sup>104</sup> predicts protein-coding regions based on evolutionary signatures  
typical for protein genes. This analysis depends on a set of homologous and non-identical genes.  
606 Therefore, AMP clusters containing at least 3 gene variants were aligned. Given that an extensive  
portion of the AMPSphere candidates - 53% (459,910 out of 863,498) is not part of such a cluster,  
608 they could not be tested. Of the tested c\_AMPs, 53% (215,421 out of 403,588) were considered genes  
with evolutionary traits of protein-coding sequences.

610 To further verify the experimental evidence of c\_AMPs, we checked for evidence of  
transcription and/or translation using a set of 221 publicly available metatranscriptome sets,  
612 comprising human gut (142), peat (48), plant (13), and symbionts (17); and 109 publicly available  
metaproteomes from 37 habitats - **Table S19**. Using bwa v.0.7.17<sup>105</sup>, AMP genes were mapped against  
614 the reads from the metatranscriptomes, and with NGLess<sup>72</sup>, we selected those genes with at least 1  
read mapped across a minimum of two samples. Using Regex methods implemented in Python 3.8<sup>76</sup>,  
616 k-mers of all AMPSphere peptides (with length equal to at least half the length of the sequence) were  
checked for peptide sequences in metaproteomics data. In the case of perfect matches between a k-

mer and a metaproteomic peptide for more than half the length of the sequence, it was considered that there is additional evidence that this c\_AMP is likely to be expressed, as described by Ma et al.<sup>5</sup>. Briefly, the number of mapped peptides against the set of samples was counted and those peptides with at least 1 match covering more than 50% of the peptide were marked as detected. c\_AMPs with experimental evidence in metatranscriptomes and/or metaproteomes accounted for 1/5 of the AMPSphere.

We separated AMPs passing all quality-control tests into those with experimental evidence of translation/transcription (17,115 c\_AMPs, ~2% of AMPSphere) and those without it (63,098 c\_AMPs, ~7%). Quality filters for families consisted of keeping only those with  $\geq 75\%$  of its c\_AMPs passing all quality control tests or having at least 1 c\_AMP with experimental evidence of translation/transcription.

### 628 ***Sample-based c\_AMPs accumulation curves***

For each habitat and group of habitats, we computed the sample-based accumulation curves by randomly sampling metagenomes 32 times in steps of 10 metagenomes. At each step, the number of unique c\_AMPs found was computed, and the curves were drawn with the average obtained across the permutations.

### ***Multi-habitat and rare c\_AMPs***

The c\_AMPs present simultaneously in  $\geq 2$  habitat groups were computed. To test the significance of this number, we opted for a similar approach to that described in Coelho et al.<sup>46</sup>. The number of c\_AMPs present in more than 1 habitat (high-level or general), here considered “multi-habitat”, was determined by counting the hits for each c\_AMP in each sample. After that, the habitat labels for each sample were shuffled 100 times and the number of randomly obtained multi-habitat c\_AMPs was counted. Shapiro-Wilks test was used to check the data distribution as normal (for general habitats -  $p = 0.49$ ; and high-level habitats -  $p = 0.1$ ) and this resulted in  $676,489.7 \pm 4281.8$  multi-habitat c\_AMPs by chance for high-level habitat groups, and in  $685,477.17 \pm 4,369.6$  multi-habitat c\_AMPs by chance for general habitats. High-level habitat groups presented 93,280 multi-habitat c\_AMPs, while general habitats presented 173,955 multi-habitat c\_AMPs. Both cases were 136.21 and 117.1 standard deviations below the value expected by chance, respectively. This was significant for the two cases with low estimated p-values ( $p < 10^{-300}$ ).

646 To determine the rarity of c\_AMPs, the non-redundant genes in AMPSphere were mapped  
against the reads of metagenome samples using NGLess<sup>72</sup>. We considered only uniquely mapped  
648 reads. From the mapping, we computed the c\_AMPs detected per sample and the number of  
detections per c\_AMP, considering “rare”, the c\_AMPs detected less than the average of the entire  
650 AMPSphere (682 detections or 1% of all samples). This approach was adopted to overcome the high  
computational costs involving a competitive mapping procedure. We expect our approach  
652 overestimates how prevalent the c\_AMPs are, and because of that, it is a robust way to estimate the  
rarity observed in c\_AMPs.

#### 654 ***Significance of the overlap of c\_AMP contents***

Similar to the significance testing of multi-habitat c\_AMPs, the number of overlapping c\_AMPs  
656 was computed for each pair of habitats (general and high-level). We shuffled the sample labels 1,000  
times, counting the number of randomly overlapping c\_AMPs for each pair of habitats, and used the  
658 Shapiro-Wilk test to verify normal distributions. Then, we estimated the probability of observing the  
overlap by Chebyshev's inequality, which does not rely on normal distributions:  $p \leq \frac{1}{Z^2}$ , where Z stands  
660 for the Z-score computed from the average and standard deviations estimated by the shuffling  
procedure. The probabilities were adjusted using Holm-Sidak implemented in multiplanetests from the  
662 statsmodels package<sup>106</sup>, and those below 5% were considered significant.

#### ***Differences in the c\_AMP density in microbial species from different habitats***

664 The c\_AMP density was defined as  $\rho_{AMP} = n_{c\_AMPs} / L$ , where  $n_{c\_AMPs}$  is the number of c\_AMP  
redundant genes and L is the assembled base pairs. It was computed per sample to verify the  
666 differences between animal host- and non-host-associated samples, including only habitats with  $\geq 100$   
samples. The calculated densities were filtered by using Tukey's fences calculated with  $k=1.5$  to  
668 eliminate outliers.

This density was also calculated at phylum, species, and genus levels, summing all assembled  
670 base pairs for contigs assigned to each one of those taxonomy levels in the samples used in  
AMPSphere. In that case, we assume, as an approximation, that in a large assembled segment, the  
672 start positions of AMP genes are independent and uniformly random. Thus, we calculated the

standard sample proportion error, with the formula:  $STDerr = \sqrt{\frac{\rho^*(1-\rho)}{L}}$ . The error was used to

calculate the margin of error at a 95% confidence interval ( $Z=1.96$ ). Genera, phyla and species  $\rho_{AMP}$  within a margin of error superior to 10% of the calculated value were eliminated along with outliers according to Tukey's fences ( $k=1.5$ ). To verify the effects of different habitats in the  $\rho_{AMP}$  of species, we took the density calculated per species per sample from species present in  $\geq 2$  habitats in  $\geq 10$  samples per habitat and tested their medians using the Mann-Whitney U test implemented in the scipy package<sup>80</sup>.

We verified the species abundances in each sample using mOTUs 2<sup>107</sup>. None of the genera found as those with the highest  $\rho_{AMP}$  (*Algorimicrobium*, TMED78, SFJ001, STGJ01, and CAG-462) were also verified throughout the mOTUs, which show them as not abundant microbes.

To evaluate the effects of potential fragments in our density analysis, all tests and comparisons were also performed restricting the set of c\_AMPs used to only those with at least one stop codon upstream the gene. The results showed that results related to the c\_AMP density observed for all AMPSphere did not modify when controlling for quality (**Fig. S15**).

Differences between habitats for each species, genus, or sample groups were tested using Mann-Whitney U and Kruskal-Wallis tests implemented in the scipy package<sup>80</sup>. P-values were adjusted using the Holm-Sidak method implemented in multipletests from the statsmodels package<sup>106</sup>.

## Determination of accessory AMPs

Core, shell, and accessory c\_\_AMP clusters were determined using the subset of c\_AMPs obtained from ProGenomes v2<sup>41</sup> because of their high-confidence assigned taxonomies and the genomically-defined species (specl). To increase confidence in our measures, only species containing  $\geq 10$  genomes were considered. AMPs and families ( 8 c\_AMPs) present in fewer than 50% of the genomes from a microbial species were classified as accessory. Those c\_AMPs and families present in 50% - 95% of the genomes in the cluster were classified as shell<sup>108</sup>, and those present in  $>95\%$  of the genomes were classified as core genes<sup>51</sup>.

We used FastANI v.1.33<sup>88</sup> to cluster genomes in species in the ProGenomes v2<sup>41</sup>, keeping one randomly selected representative for each clonal complex ( $ANI \geq 99.99\%$ ) and inferring strains ( $99.5\% \leq ANI < 99.99\%$ ) as in Rodriguez et al.<sup>109</sup>. Only species with  $\geq 10$  genomes after elimination of clonal

redundancy were kept. To verify the propensity of AMPs being shared between genomes belonging to the same strains, we computed all possible pairs of genomes per species and computed the pairs sharing AMPs, testing the results with the Fisher's Exact test implemented in the scipy package<sup>80</sup>. We also extracted the predicted full-length proteins from the ENA database for each genome and hierarchically clustered them after alphabet reduction in a similar fashion to that described in the topic "**AMP families**", keeping those clusters with  $\geq 8$  sequences for each species. The prevalence of full-length protein families within a species was computed as above mentioned and the number of core families was compared to the number of c\_AMP core families using the probability calculated as number of species with proportion of core full-length protein families less or equal to that observed for c\_AMPs divided by the total of assessed species.

To determine the genotype of *Mycoplasma pneumoniae* samples in ProGenomes2<sup>41</sup>, the gene coding for P1 adhesin<sup>53</sup> was mapped against the genomes using the reference gene NZ\_LR214945.1:c568695-567307 with bwa<sup>105</sup>, and later extracted with SAMtools<sup>110</sup> and BEDtools<sup>111</sup>. The extracted genes were aligned using Clustal Omega<sup>112</sup>, and a phylogenetic tree was built using nucleotide sequences and FastTree 2<sup>87</sup> with the restricted time-reversible substitution model and a bootstrapping procedure with 1,000 pseudo-replicates to determine node support. The tree was used to segregate and classify genomes taking the strain type of reference genomes from Diaz et al.<sup>54</sup> and was consistent with the previously established groups.

### **Annotation of AMPs using different datasets**

Databases used in the annotation were the small protein sets in SmProt 2<sup>45</sup>, the bioactive peptides database starPepDB 45k<sup>91</sup>, the small proteins from the global data-driven census of *Salmonella*<sup>113</sup>, the global microbial gene catalog GMGCv1<sup>46</sup>, and a specific AMP database - DRAMP 3.0<sup>44</sup>. To only have sequences that were unlikely to be artifacts of assembly for the analysis, only c\_AMPs passing the terminal placement test were searched against the GMGCv1<sup>46</sup>. The AMPs were annotated using MMseqs2<sup>75</sup> with the 'easy-search' method, retaining hits with an E-value maximum of  $10^{-5}$ . To normalize coordinates of hits to the full-length protein, we corrected for the elimination of the initial methionine performed by Macrel<sup>40</sup>, so that hits starting at the second amino acid were considered as if they matched the first one (as the peptide has had its initial methionine removed).

We used the hypergeometric test implemented in the scipy package<sup>80</sup> to model the association between c\_AMPs and the background distribution of ortholog groups from GMGCv1<sup>46</sup>. To that, the

number of genes in the redundant GMGCv1<sup>46</sup> for each ortholog group was computed along with the  
 732 counts for ortholog groups in the top hits to AMPSphere. The enrichment was given as the proportion  
 of hits presenting a given ortholog group divided by the proportion of that ortholog group among the  
 734 redundant sequences in GMGCv1<sup>46</sup> and was considered significant when  $p < 0.05$  after a correction  
 with Holm-Sidak method implemented in multiplanetests from the statsmodels package<sup>106</sup>. With a robust  
 736 approach, filtering the OGs by the number of c\_AMP hits and GMGCv1<sup>46</sup> hits associated with them,  
 using a minimum of 10, 20, or even 100 proteins, the results were kept similar to those obtained with  
 738 all data.

To check for genomic entities generated after gene truncation, we screened for c\_AMP  
 740 homologs using the default settings for Blastn<sup>103</sup> against the NCBI database<sup>94</sup>, keeping only significant  
 hits with a maximum E-value of  $10^{-5}$ . We selected the AMP10.271\_016, predicted to be produced by  
 742 *Prevotella jejuni*, which shares the start codon with the gene coding for a NAD(P)-dependent  
 dehydrogenase (WP\_089365220.1). Using Biopython<sup>83</sup>, we codon-aligned the fragments from  
 744 metagenomic contigs assembled from samples SAMN09837386, SAMN09837387, and  
 SAMN09837388, and genomic fragments of different strains of *Prevotella jejuni* CD3:33  
 746 (CP023864.1:504836-504949), F0106 (CP072366.1:781389-781502), F0697 (CP072364.1:1466323-  
 1466436), and from *Prevotella melaninogenica* strains FDAARGOS\_760 (CP054010.1:157726-  
 748 157839), FDAARGOS\_306 (CP022041.2:943522-943635), FDAARGOS\_1566  
 (CP085943.1:1102942-1103055), and ATCC 25845 (CP002123.1:409656-409769).

## 750 **Positive selection tests**

The genes of c\_AMPs belonging to 100 high-quality families randomly sampled were codon-  
 752 aligned, excluding identical sequences and the stop codons. Selection tests were run using HyPhy  
 version 2.5.1 ([www.hyphy.org](http://www.hyphy.org))<sup>114</sup> on the codon alignments and trees obtained as previously  
 754 mentioned. Alignment-wide episodic diversification tests were computed on the gene family clusters  
 with the BUSTED method<sup>115</sup>. Genes with Holm-Bonferroni multiple-test corrected p-values lower than  
 756 0.05 were considered as positively selected. Only a small fraction (~15%) of the tested families  
 presented evidence of episodic diversification.

## 758 **Genomic context conservation analysis**

We mapped the 863,498 AMP sequences against a collection of 169,632 reference genomes, MAGs and SAGs curated elsewhere<sup>49</sup> with DIAMOND<sup>116</sup> in blastp mode. Hits with identity > 50% (amino acid) and query and target coverage > 90% were considered significant. A total of 107,308 AMPs have homologs in at least one genome. We built gene families from the hits of each AMP detected in the prokaryotic genomes, and calculated a conservation score of the functional annotation of the neighboring genes in a window of 3 genes up and downstream. The vertical conservation score of each OG, KEGG pathway, KEGG orthology, KEGG module – from the Kyoto Encyclopedia of Genes and Genomes (KEGG)<sup>117</sup>, PFAM 33.1<sup>92,97</sup>, and CARD<sup>118</sup> at each position was calculated as the number of genes with a given functional annotation divided by the number of genes in the family. AMPs with more than 2 hits and a vertical conservation score > 0.9 with any functional term were considered to have conserved genomic contexts.

For testing whether the number of AMPs with conserved genomic neighbors is higher than in other gene families within the 169,632 genomes curated by del Río et al.<sup>49</sup>, we used MMSeqs2<sup>75</sup> for building *de novo* gene families (establishing the minimum amino acid identity of 30%, coverage of the shorter sequence of at least 50%, and maximum E-value of  $10^{-3}$ ). We then took 10,000 random sets of 55,191 gene families with more than 2 members (number of AMPs with more than two homologs in the genomes) composed of: (i) small (< 50 residues) proteins, and (ii) no length-restricted proteins. Then, we computed the number of gene families showing conserved genomic contexts with known functions within each set and confirmed their normal distribution using the Shapiro-Wilks test<sup>80</sup>. Later, the conservation values obtained for AMPSphere were compared to those sets using P-values computed from their respective Z-scores.

To verify the ortholog groups from c\_AMPs and the gene neighborhood, the peptides were also annotated using EggNOG-mapper v2<sup>84</sup> using default settings and selecting the best hit for each c\_AMP, by filtering the lowest E-value and highest best score. Their KEGG ortholog groups (KOs) were used as functional labels to cluster and verify the gene neighborhood in terms of functional similarity. It was possible to annotate 56.1% (60,173 out of 107,308) of c\_AMPs with hits to the genome set tested using the EggNOG5 database<sup>48</sup>, with 9.1% of them missing COG categories, and about 18.1% of them belonging to translation-related functions (J), 14.4% belonging to unknown function proteins (S), and 9% of them belonging to replication, recombination, and repair (L).

## 788 ***c\_AMPs and bacterial species transmissibility***

We used the species taxonomy and transmissibility indexes calculated by Valles-Colomer et al.<sup>57</sup> to demonstrate the effect of AMPs on the transmission of bacterial species from mother to children. Only those species overlapping AMPSphere and the datasets from Valles-Colomer et al.<sup>57</sup> were kept for this analysis, and their AMP densities were calculated separately for samples from the human gut and human oral cavity as mentioned in the section ***Differences in the c\_AMP density in microbial species from different habitats***. The AMP density and the coefficient of transmissibility were correlated using Spearman's method implemented in the scipy package<sup>80</sup> to keep robust comparisons and were tested for different situations, e.g. following children's microbiome after 1, 3, and up to 18 years, as well as, cohabitation and intra-datasets. Missing data was completely omitted for calculus purposes.

### ***AMPSphere web resource***

800 AMPSphere is found at the address <https://ampsphere.big-data-biology.org/>. The implementation is based on Python<sup>76</sup> and Vue Javascript. The database was built with sqlite, and 802 SQLAlchemy was used to map the database to Python objects. Internal and external APIs were built using FastAPI and Gunicorn to serve them. In the front end, Vue 3 was used as the backbone and 804 Quasar built the layout. Plotly was used to generate interactive visualization plots, and Axios to render content seamlessly. LogoJS (<https://logojs.wenglab.org/app/>) is used to generate sequence logos for 806 AMP families; while the helical wheel app (<https://github.com/clemlab/helicalwheel>) generates AMP helical wheels.

## 808 ***Selection of peptides to synthesis and activity testing***

Only high-quality (see the topic “***Quality control of c\_AMPs***”) c\_AMPs were considered for 810 synthesis. They were filtered according to 6 criteria for solubility and 3 criteria for synthesis, as used before in PepFun<sup>119</sup>. A peptide approved for at least 6 of these criteria was then filtered by predicting 812 AMP activity with 6 methods in addition to Macrel<sup>40</sup>: AMPScanner v2<sup>120</sup>, ampir<sup>38</sup>, amPEPpy<sup>121</sup>, APIN<sup>122</sup>, and AMPLify<sup>123</sup>. Peptides predicted to be AMPs by all methods were filtered by length, discarding 814 sequences longer than 40 amino acid residues, for which conventional solid-phase peptide synthesis using Fmoc strategy has lower yields and many recoupling reactions<sup>124–127</sup>. AMPs were sorted by their 816 abundance (the number of redundant genes), keeping the most abundant peptide per AMP family.

After this process, we obtained 364 candidate AMPs, belonging to 166 families and 198 clusters with  
818 <8 c\_AMPs. Of those, 30 candidates were homologous to sequences from the databases used in  
annotation (*e.g.*, SmProt 2<sup>45</sup>). To compose the list of 50 peptides proceeding to synthesis and testing:  
820 (i) we selected 34 of the most abundant peptides, eliminating peptides that overlap with other  
candidates and replacing them with the next most abundant ones; (ii) randomly selecting c\_AMPs with  
822 homologs to a final proportion of 30% of our set, more specifically 14 peptides that matched  
GMGCv1<sup>46</sup>, and 1 that matched SmProt 2<sup>45</sup>; and (iii) 1 peptide also found in the MAGs binned from  
824 stool samples used to investigate fecal transplantations<sup>128</sup>.

### ***Minimal inhibitory concentration determination***

826 The 50 AMPs were tested for antimicrobial activity using the broth microdilution method<sup>129</sup>. MIC  
values were considered as the concentration of the peptides that killed 100% of cells after 24 h of  
828 incubation at 37°C. First, peptides diluted in water were added to untreated flat-bottom polystyrene  
microtiter 96-well plates in two-fold dilutions ranging from 64 to 1  $\mu\text{mol}\cdot\text{L}^{-1}$ , and then peptides were  
830 exposed to an inoculum of  $2\cdot 10^6$  cells in LB or BHI broth, for pathogens and gut commensals,  
respectively. After the incubation time, the absorbance of each well representing each of the  
832 conditions was analyzed using a spectrophotometer at 600 nm. The assays were conducted in three  
biological replicates to ensure statistical reliability.

### ***Circular dichroism assays***

834 Circular dichroism experiments were conducted using a J1500 circular dichroism  
spectropolarimeter (Jasco) at the Biological Chemistry Resource Center (BCRC) of the University of  
Pennsylvania. The experiments were carried out at a temperature of 25°C. Circular dichroism spectra  
838 were obtained by averaging three accumulations using a quartz cuvette with an optical path length of  
1.0 mm. The spectra were recorded in the wavelength range from 260 to 190 nm at a scanning rate of  
840  $50\text{ nm}\cdot\text{min}^{-1}$  with a bandwidth of 0.5 nm. The peptides were tested at a concentration of  $50\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ .  
Measurements were performed in water, a mixture of water and trifluoroethanol (TFE) in a ratio of 3:2,  
842 and a mixture of water and methanol in a ratio of 1:1. Baseline measurements were recorded prior to  
each measurement. To minimize background effects, a Fourier transform filter was applied. The  
844 helical fraction values were calculated using the single spectra analysis tool available on the BeStSel  
server<sup>130</sup>.

846

# **Outer membrane permeabilization assays**

848 Membrane permeability was analyzed using the 1-(N-phenylamino)naphthalene (NPN) uptake  
 assay. NPN demonstrates weak fluorescence in an extracellular environment but displays strong  
 850 fluorescence when in contact with lipids from the bacterial outer membrane. Thus, NPN will show  
 increased fluorescence when the integrity of the outer membrane is compromised. *A. baumannii*  
 852 ATCC 19606 and *P. aeruginosa* PA01 were cultured until cell numbers reached an OD<sub>600</sub> of 0.4,  
 followed by centrifugation (10,000 rpm at 4°C for 3 min), washing, and resuspension in buffer (5  
 854 mmol·L<sup>-1</sup> HEPES, 5 mmol·L<sup>-1</sup> glucose, pH 7.4). Subsequently, 4 μL of NPN solution (working  
 concentration of 0.5 mmol·L<sup>-1</sup>) was added to 100 μL of bacterial solution in a white flat bottom 96-well  
 856 plate. The fluorescence was monitored at λ<sub>ex</sub> = 350 nm and λ<sub>em</sub> = 420 nm. The peptide solutions in  
 water (100 μL solution at their MIC values) were introduced into each well, and fluorescence was  
 858 monitored as a function of time until no further increase in fluorescence was observed (30 min). The  
 relative fluorescence was calculated using a non-linear fit. The positive control (antibiotic polymyxin B)  
 860 was used as baseline. The following equation was applied to reflect % of difference between the  
 baseline (polymyxin B) and the sample:

$$Relative fluorescence = \frac{100 \times (fluorescence_{sample} - fluorescence_{polymyxinB})}{fluorescence_{polymyxinB}}$$

# **862 Cytoplasmic membrane depolarization assays**

The ability of the peptides to depolarize the cytoplasmic membrane was assessed by  
 864 measuring the fluorescence of the membrane potential-sensitive dye 3,3'-dipropylthiadicarbocyanine  
 iodide [DiSC<sub>3</sub>-(5)]. This potentiometric fluorophore fluoresces upon release from the interior of the  
 866 cytoplasmic membrane in response to an imbalance of its transmembrane potential. *A. baumannii*  
 ATCC 19606 and *P. aeruginosa* PA01 cells were grown with agitation at 37°C until they reached mid-  
 868 log phase (OD<sub>600</sub> = 0.5). The cells were then centrifuged and washed twice with washing buffer (20  
 mmol·L<sup>-1</sup> glucose, 5 mmol·L<sup>-1</sup> HEPES, pH 7.2) and re-suspended to an OD<sub>600</sub> of 0.05 in 20 mmol·L<sup>-1</sup>  
 870 glucose, 5 mmol·L<sup>-1</sup> HEPES, 0.1 mol·L<sup>-1</sup> KCl, pH 7.2. An aliquot of 100 μL of bacterial cells was added  
 to a black flat bottom 96-well plate and incubated with 20 nmol·L<sup>-1</sup> of DiSC<sub>3</sub>-(5) for 15 min until the  
 872 fluorescence stabilized, indicating the incorporation of the dye into the cytoplasmic membrane. The

membrane depolarization was monitored by observing the change in the fluorescence emission  
874 intensity of the dye ( $\lambda_{\text{ex}} = 622 \text{ nm}$ ,  $\lambda_{\text{em}} = 670 \text{ nm}$ ), after the addition of the peptides (100  $\mu\text{L}$  solution at  
their MIC values). The relative fluorescence was calculated using a non-linear fit. The positive control  
876 (antibiotic polymyxin B) was used as baseline. We estimated the % of difference between the baseline  
(polymyxin B) and the sample using the same mathematical approach as in the “***Outer membrane***  
878 ***permeabilization assays***”.

## **QUANTIFICATION AND STATISTICAL ANALYSIS**

880 Graphs for the experimental results were created and statistical tests conducted in GraphPad  
Prism v.9.5.1 (GraphPad Software, San Diego, California USA).

## 882 **ADDITIONAL RESOURCES**

AMPSphere is freely available for download in Zenodo<sup>131</sup> and as a web server  
884 (<https://ampsphere.big-data-biology.org/>). The code related to the AMPSphere generation and  
analysis is available in: <https://github.com/BigDataBiology/AMPSphere>.

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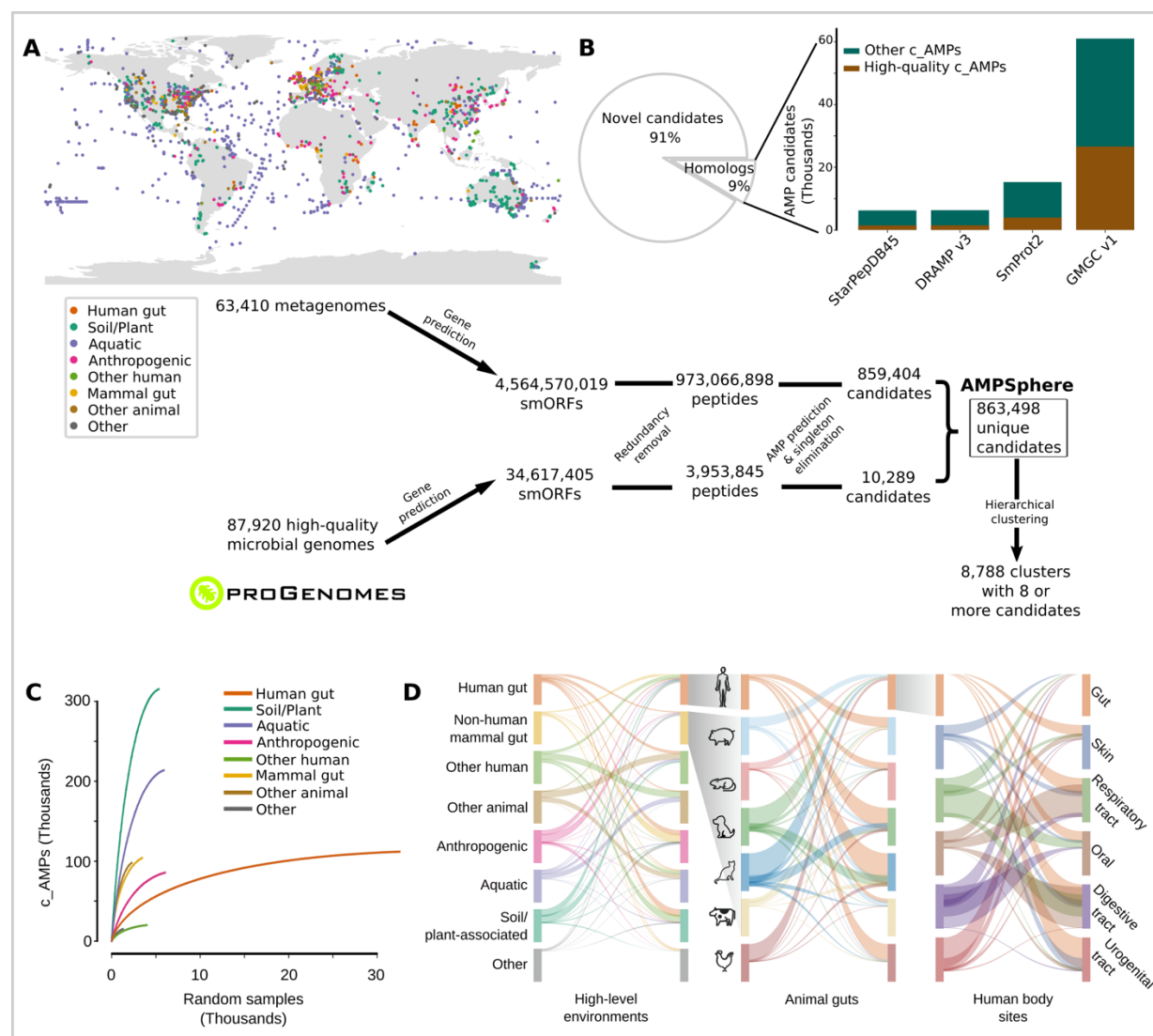
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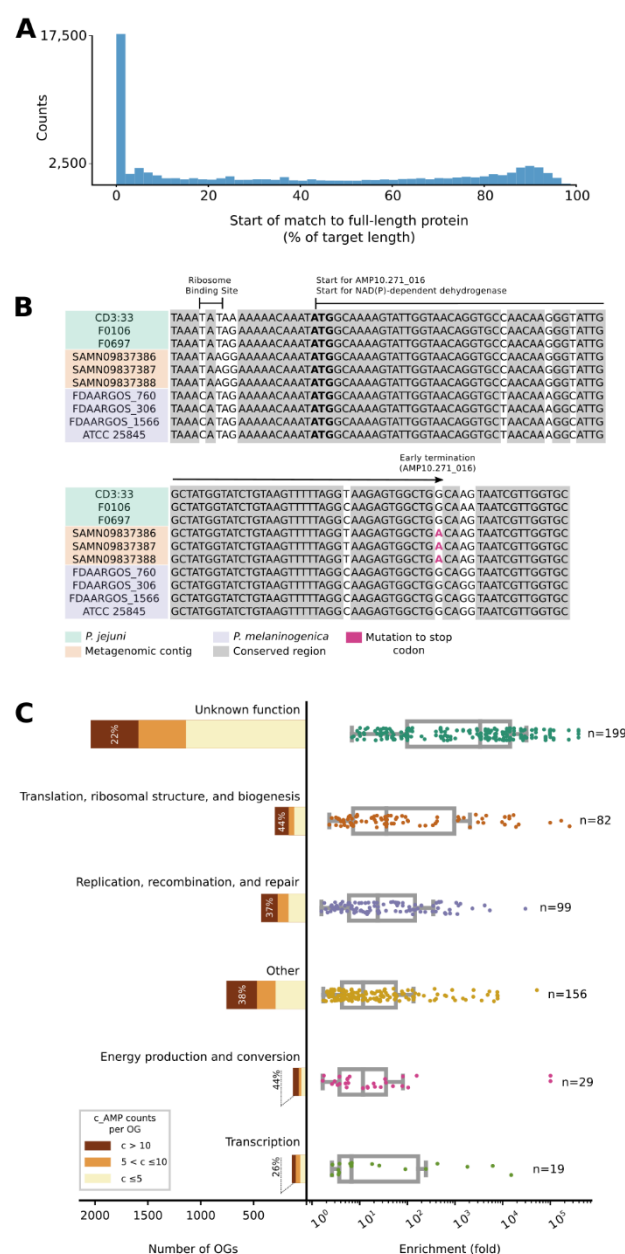
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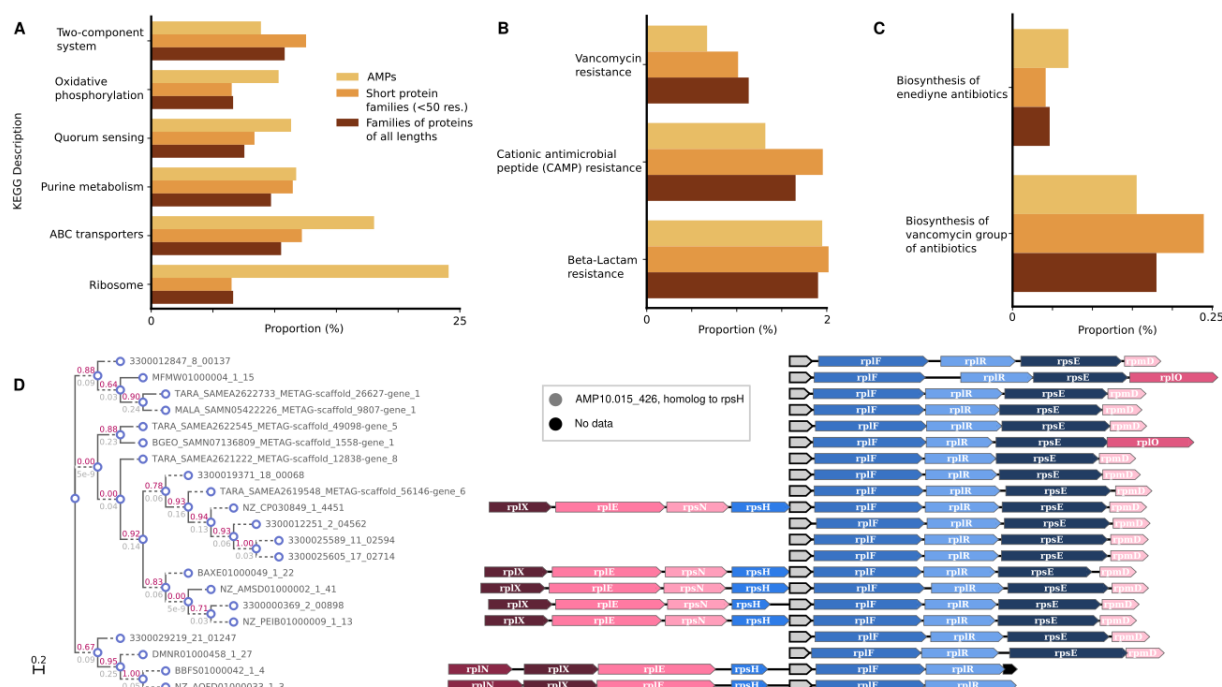
# 1268 **Figures**



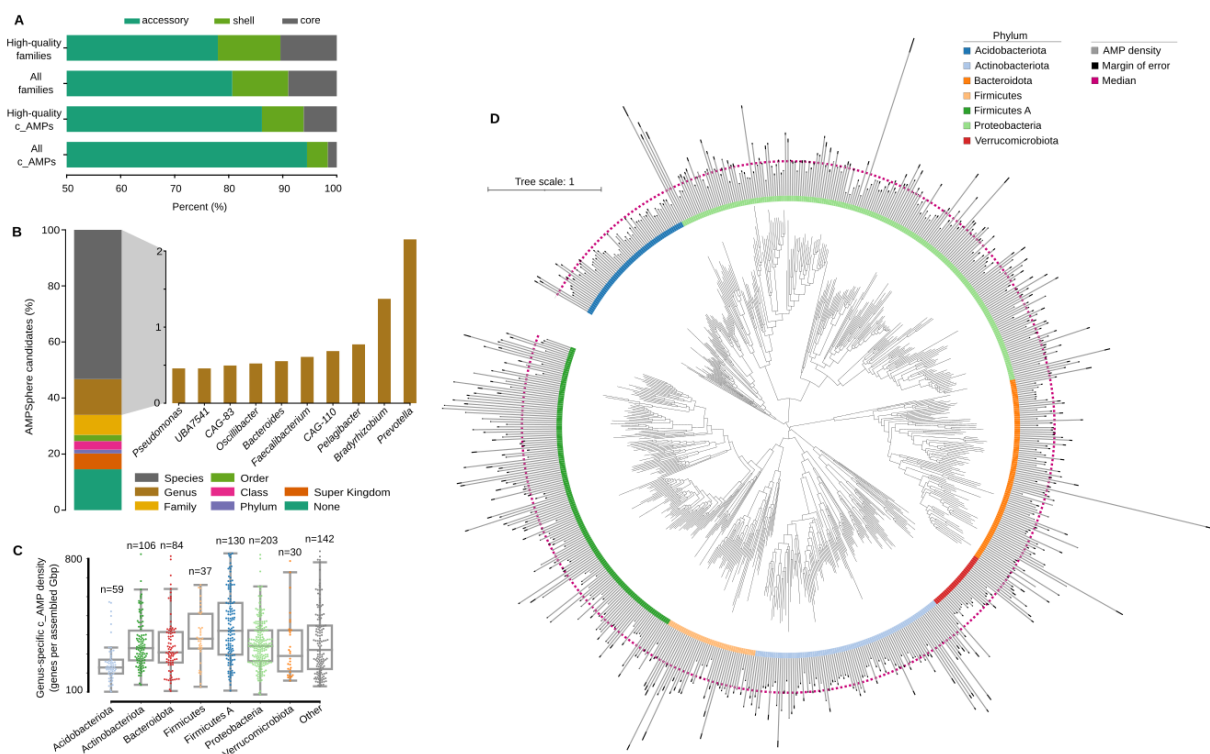
**Figure 1. AMPSphere comprises 836,498 non-redundant c\_AMPs from thousands of metagenomes and high-quality microbial genomes. (A)** To build AMPSphere, we first assembled 63,410 publicly available metagenomes from diverse habitats. A modified version of Prodigal<sup>32</sup>, which can also predict smORFs (30-300 bp), was used to predict genes on the resulting metagenomic contigs as well as on 87,920 microbial genomes from ProGenomes2<sup>41</sup>. Macrel<sup>40</sup> was applied to the 4,599,187,424 predicted smORFs to obtain 863,498 non-redundant c\_AMPs (see also **Fig. S11**). c\_AMPs were then hierarchically clustered in a reduced amino acids alphabet using 100%, 85%, and 75% identity cutoffs. We observed at 75% of identity 118,051 non-singleton clusters, and 8,788 of them were considered families ( $\geq 8$  c\_AMPs). **(B)** Only 9% of c\_AMPs have detectable homologs in other peptides (SmProt 2<sup>45</sup>, DRAMP 3.0<sup>44</sup>, starPepDB 45k<sup>91</sup>, STsORFs<sup>113</sup>) and general protein datasets (GMGCv1<sup>46</sup>) - see also **Fig. S12B**. **(C)** AMP discovery is impacted by the sampling, with most of the habitats presenting steep sampling curves, e.g., soil. **(D)** Overall, c\_AMPs are habitat-specific - see also **Fig. S12C-D** and **Tables S11 and S12**.



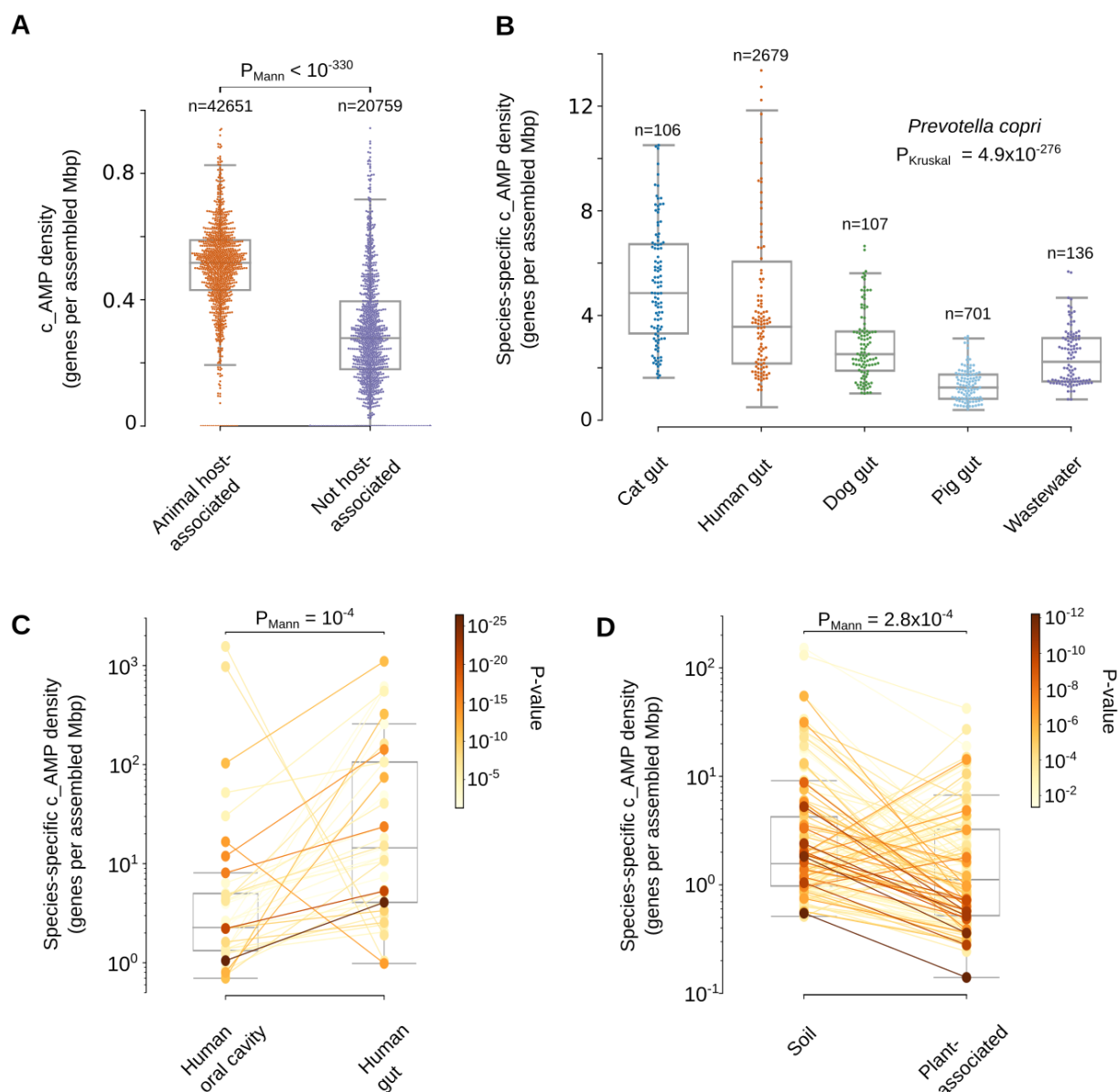
**Figure 2. Mutations in genes encoding large proteins generate c\_AMPs as independent genomic entities.** (A) About 7% of c\_AMPs are homologous to proteins from GMGCv1<sup>46</sup>, with almost one-fourth of the hits sharing start positions with the larger protein. (B) As an illustrative example, AMP10.271\_016 was recovered in three samples of human saliva from the same donor<sup>132</sup>. AMP10.271\_016 is predicted to be produced by *Prevotella jejuni*, sharing the start codon (bolded) of an NAD(P)-dependent dehydrogenase gene (WP\_089365220.1), the transcription of which was stopped by a mutation (in red; TGG > TGA). (C) The OGs of unknown function represent the largest (2,041 out of 3,792 OGs) and most enriched ( $P_{\text{Kruskal}} = 2.66 \cdot 10^{-39}$ ) class with homologs to c\_AMPs in GMGCv1<sup>46</sup>. Interestingly, when considered individually, the number of c\_AMP hits to unknown OGs was the lowest ( $P_{\text{Kruskal}} = 6 \cdot 10^{-3}$ ). These results do not change when underrepresented OGs are excluded by using different thresholds (e.g., at least 10, 20, or 100 homologs per OG) - see also **Table S13**.



**Figure 3. The genome context of c\_AMPs shows a preference for neighborhoods containing ABC transporters and ribosome assembly proteins** - see *Tables S14* and *S15*. **(A)** Compared to other proteins, c\_AMPs tend to be closer to ABC transporters and ribosomal machinery-related genes than families of proteins with different sizes ( $\leq 50$  amino acids and all lengths). **(B)** The proportion of c\_AMPs in a genome context involving antibiotic resistance genes is lower than families of proteins shorter than 50 amino acids and, in the case of CAMP and vancomycin resistance, than all proteins. **(C)** The proportion of c\_AMPs in neighborhoods with antibiotic synthesis-related genes is very small ( $<0.25\%$ ). **(D)** AMP10.015\_426 is an example of a c\_AMP homologous to the ribosomal protein rpsH, found in the context of other ribosomal protein genes.



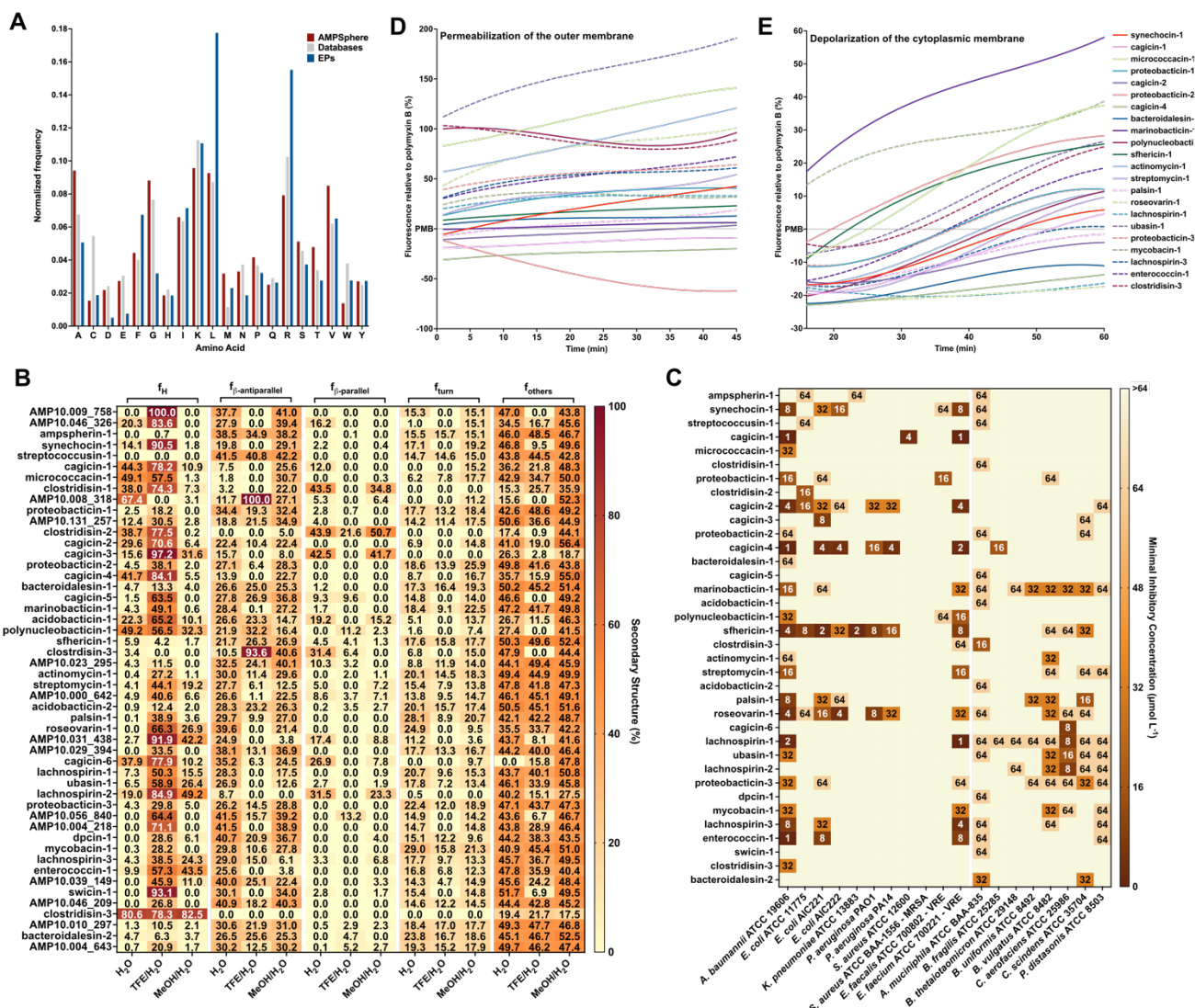
**Figure 4. AMP variation is taxonomy-dependent.** (A) Most of c\_AMPs and families from ProGenomes2<sup>41</sup> are classified as core genes (defined as  $\geq 95\%$  genomes within a species). (B) The majority of the c\_AMPs were classified down to the level of genus and species. Animal-associated genera (e.g. *Prevotella*, *Faecalibacterium*, *CAG-110*) contribute the most c\_AMPs, possibly reflecting data sampling. (C) Using the  $\rho_{AMP}$  per genus, we observed the distribution of c\_AMPs per phyla, with Bacillota A as the densest. (D) The  $\rho_{AMP}$  distribution (gray bars, confidence interval of 95% shown as black bars) with respect to taxonomy shows Bacillota A, Actinomycetota, and Pseudomonadota as the densest phyla in c\_AMPs. As a reference, the median of  $\rho_{AMP}$  for the presented genera is indicated by a magenta dashed line (see **Tables S17** and **S18**).



**Figure 5. Habitats differ in their  $c_{\text{AMP}}$  densities, with differences being observed between conspecific strains.** (A) Host-associated samples presented a higher  $\rho_{\text{AMP}}$  (calculated at a genus and samples levels) than samples from environmental samples (a random sample of 1 thousand dots for each group was drawn excluding outliers – see **Differences in the  $c_{\text{AMP}}$  density in microbial species from different habitats** in **Methods**). (B) *Prevotella copri* has a higher  $\rho_{\text{AMP}}$  in cat and human guts compared to the same species in the guts of pigs and dogs. 106 randomly selected points are shown for each host. (C) Investigating the species-specific  $\rho_{\text{AMP}}$  of microbes found in samples from the human gut and human oral cavity, we observed 34 out of the 37 tested species presenting a higher  $c_{\text{AMP}}$  density in the gut. (D) The effect of a host when it is not an animal was investigated by verifying the species-specific  $\rho_{\text{AMP}}$  of microbes happening in samples from soil and plants. We observed 85 out of the 130 tested species presenting a higher density in soils. For panels C and D the

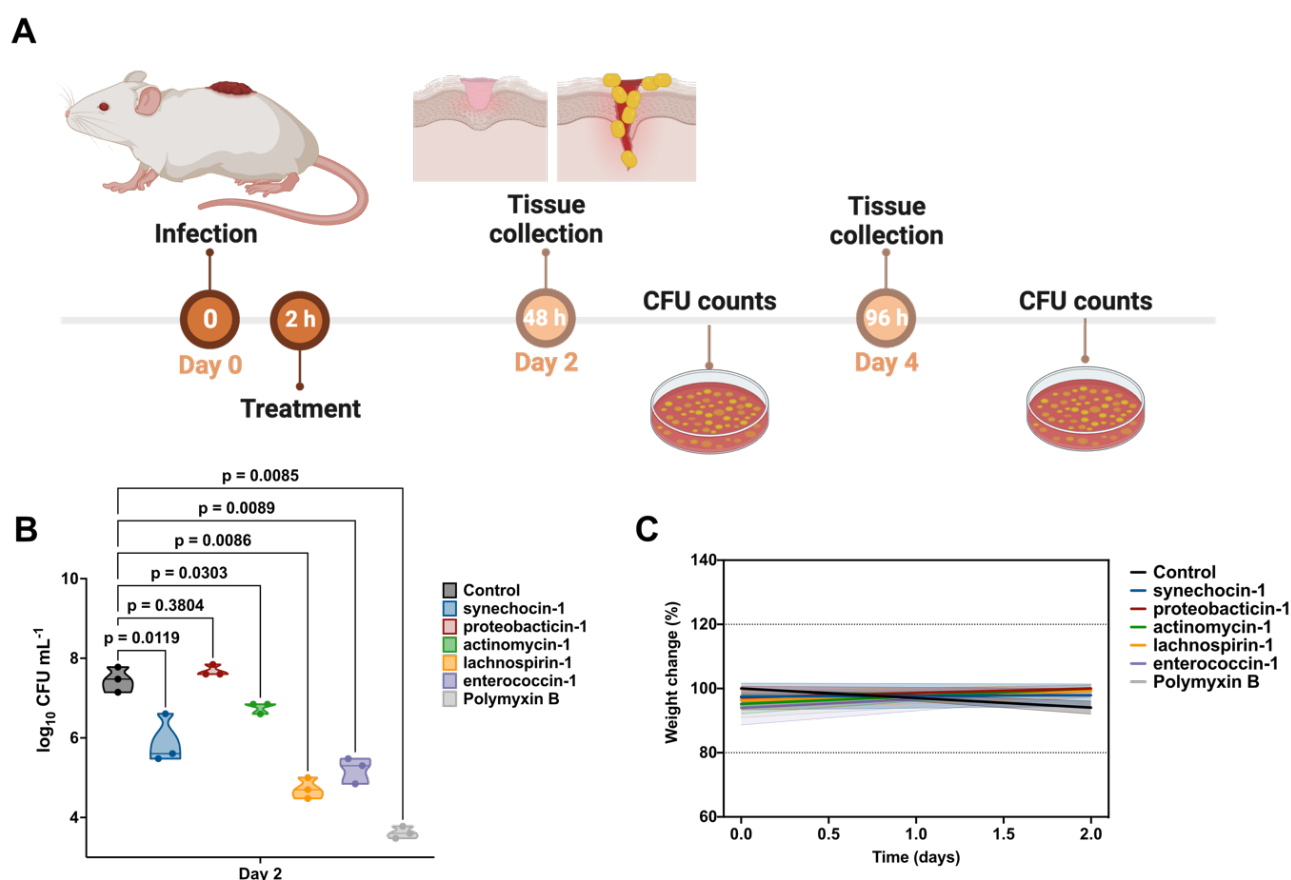
significance was color-encoded using a  $\text{Log}_{10}(P_{\text{Mann}})$  scale. See also **Fig. S14**, **Fig. S15**, and **Table S18**.

1324 Our results also showed that differences in  $\rho_{\text{AMP}}$  observed also were kept even when restricting the c\_AMP genes by controlling their quality (**Fig. S15**).



**Figure 6. Amino acid composition, structure, antimicrobial activity, and mechanism of action of c\_AMPs.** (A) Amino acid frequency in c\_AMPs from AMPSphere, AMPs from databases (DRAMP v3, APD3, and DBAASP), and encrypted peptides (EPs) from the human proteome. (B) Heat map with the percentage of secondary structure found for each peptide in three different solvents: water, 60% trifluoroethanol in water, and 50% methanol in water. Secondary structure was calculated using BeStSel server<sup>130</sup>. (AC) Activity of c\_AMPs assessed against ESKAPEE pathogens and human gut commensal strains. Briefly,  $10^6$  CFU·mL<sup>-1</sup> was exposed to c\_AMPs two-fold serially diluted ranging from 64 to 1  $\mu\text{mol} \cdot \text{L}^{-1}$  in 96-wells plates and incubated at 37 °C for one day. After the exposure period, the absorbance of each well was measured at 600 nm. Untreated solutions were used as controls and minimal concentration values for complete inhibition were presented as a heat map of antimicrobial activities ( $\mu\text{mol} \cdot \text{L}^{-1}$ ) against 11 pathogenic and eight human gut commensal bacterial strains. All the assays were performed in three independent replicates and the heatmap shows the mode obtained within the two-fold dilutions concentration range studied. (D) Fluorescence values relative to polymyxin B (PMB, positive control) of the fluorescent probe 1-(N-phenylamino)naphthalene (NPN) that indicate

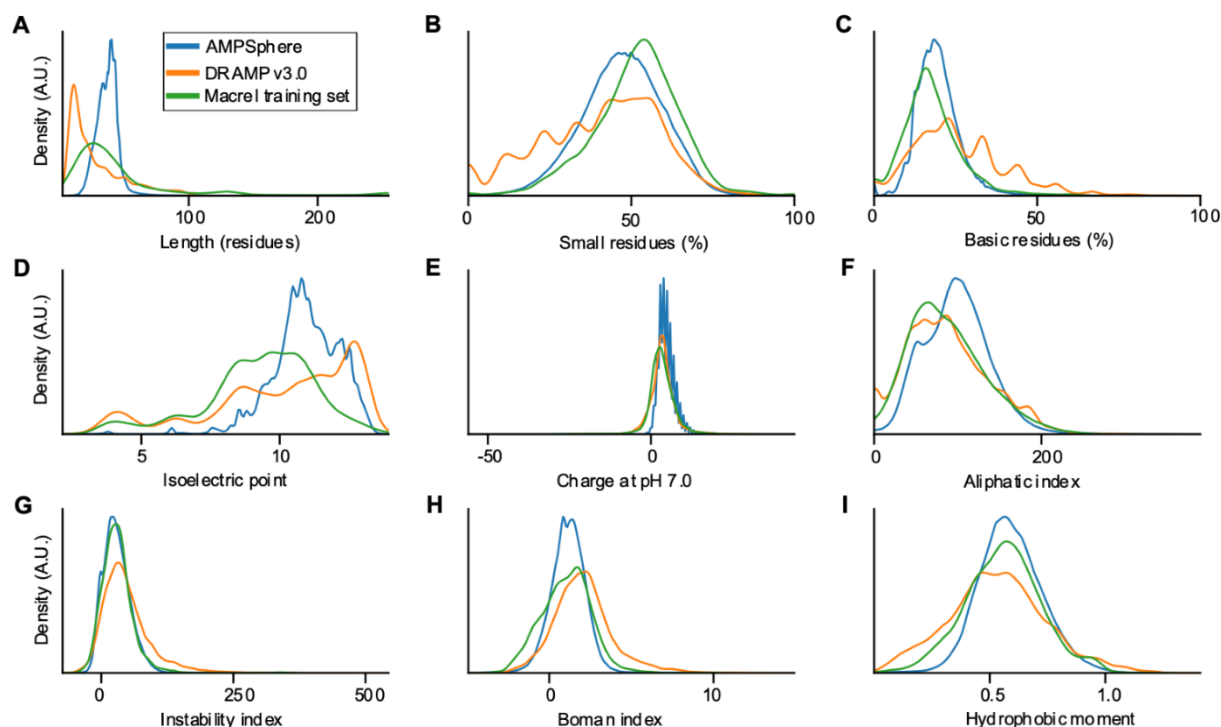
1340 outer membrane permeabilization of *A. baumannii* ATCC 19606 cells. (**E**) Fluorescence values relative  
 1342 to PMB (positive control) of 3,3'-dipropylthiadicarbocyanine iodide [DiSC3-(5)], a hydrophobic  
 1344 fluorescent probe, used to indicate cytoplasmic membrane depolarization of *A. baumannii* ATCC  
 19606 cells. Depolarization of the cytoplasmic membrane occurred with a slow kinetics compared to  
 the permeabilization of the outer membrane and took approximately 20 min to stabilize.



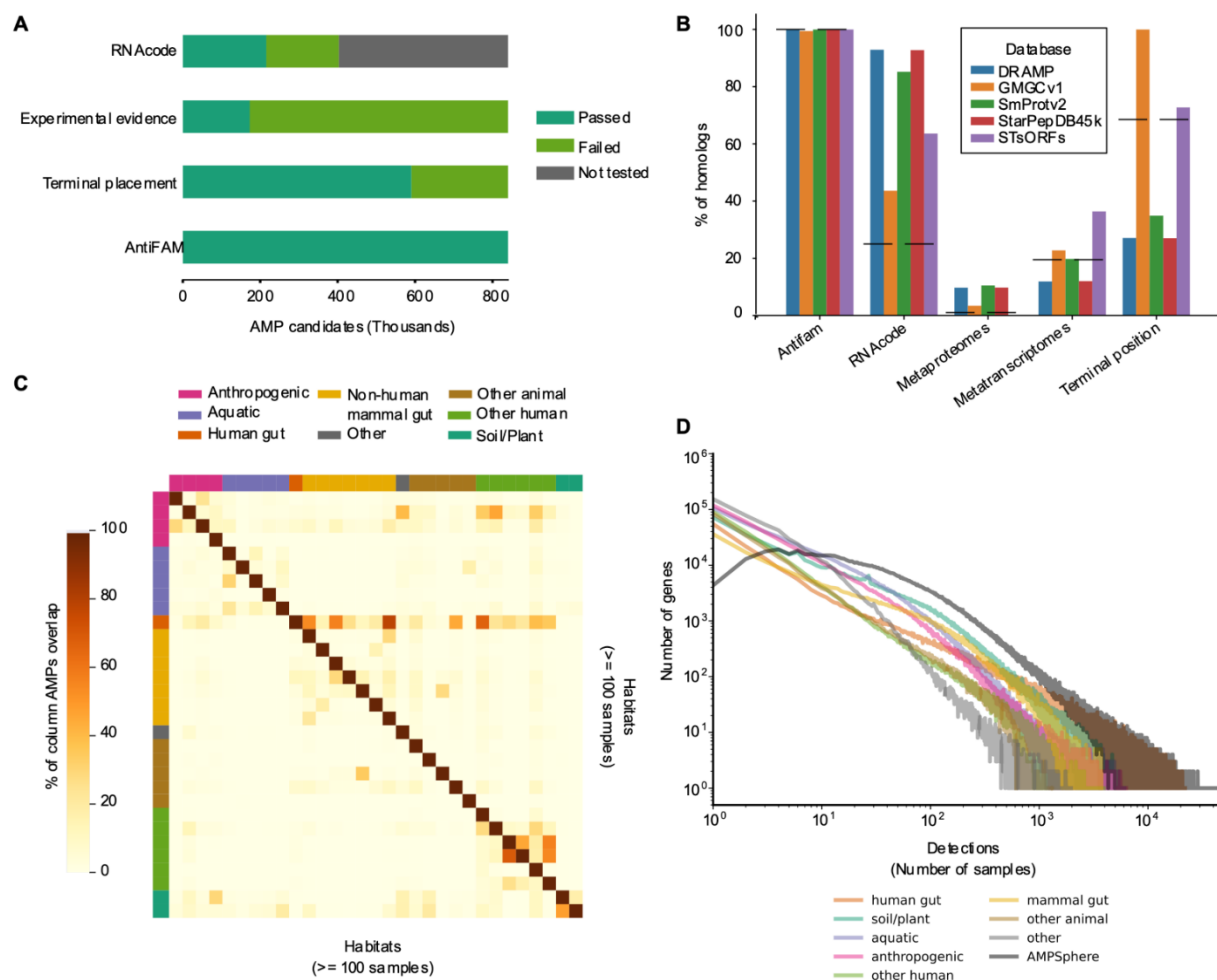
**Figure 7. Anti-infective activity of AMPs in pre-clinical animal model.** (A) Schematic of the skin abscess mouse model used to assess the anti-infective activity of the peptides against *A. baumannii* cells. (B) Peptides were tested at their MIC in a single dose one hour after the establishment of the infection. Each group consisted of three mice (n=3) and the bacterial loads used to infect each mouse derived from a different inoculum. (C) To rule out toxic effects of the peptides, mouse weight was monitored throughout the experiment. Statistical significance in (B) was determined using one-way ANOVA where all groups were compared to the untreated control group; P-values are shown for each of the groups. Features on the violin plots represent median and upper and lower quartiles. Data in (C) are the mean  $\pm$  the standard deviation. Figure created in BioRender.com.

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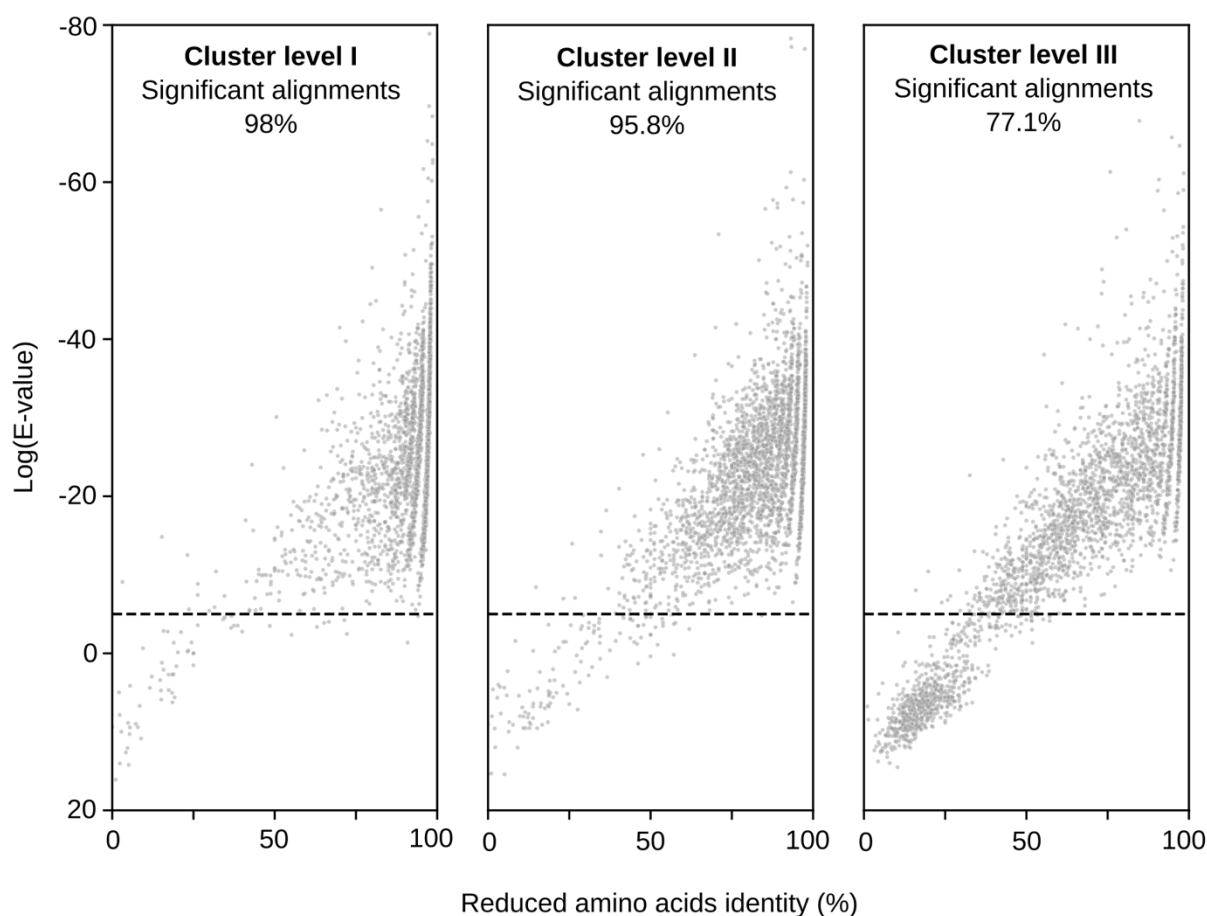
# Supplemental Information



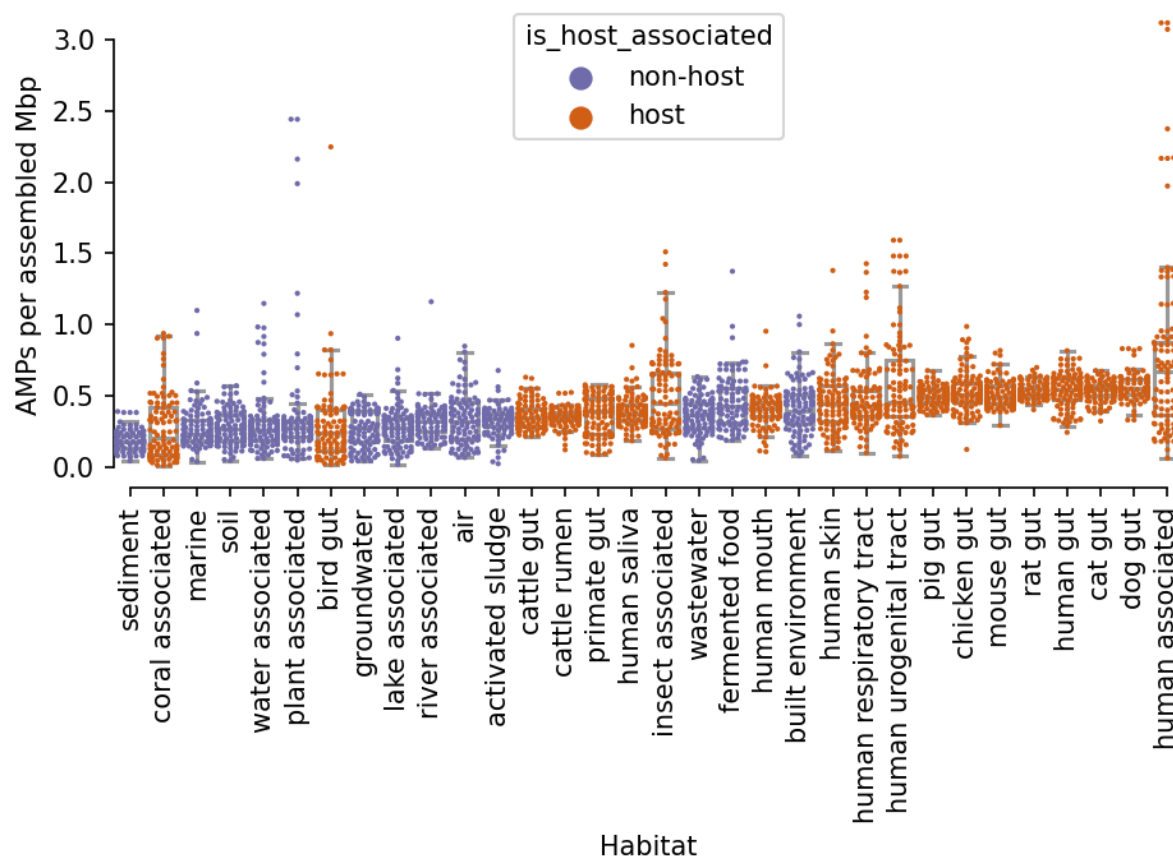
1356 **Figure S1. General physical-chemical features of c\_AMPs in AMPSphere and validated**  
1358 **databases of antimicrobial peptides.** All graphs are drawn using the continuous probability density  
1360 curve and given as functions of the arbitrary density units. The c\_AMPs from AMPSphere retain the  
1362 same predicted biochemical properties of those from validated AMPs, although with an average length  
1364 longer than the peptides in the training set of Macrel [S1] and DRAMP 3.0 [S2]. **(A)** The distribution of  
1366 peptide length in residues across the AMPSphere. **(B)** Distribution of proportions of residues with  
small side chains [A, B, C, D, G, N, P, S, T, V] per AMP. **(C)** Distribution of proportions of basic  
residues [H, R, K] per AMP. **(D)** Distribution of isoelectric points in AMPSphere peptides. **(E)**  
Distribution of peptide charges at pH 7.0. **(F)** Distribution of aliphatic index in peptides from  
AMPSphere. **(G)** Distribution of instability index in AMPSphere. **(H)** Distribution of Boman index in  
AMPSphere. **(I)** Distribution of hydrophobic moments in AMPSphere.



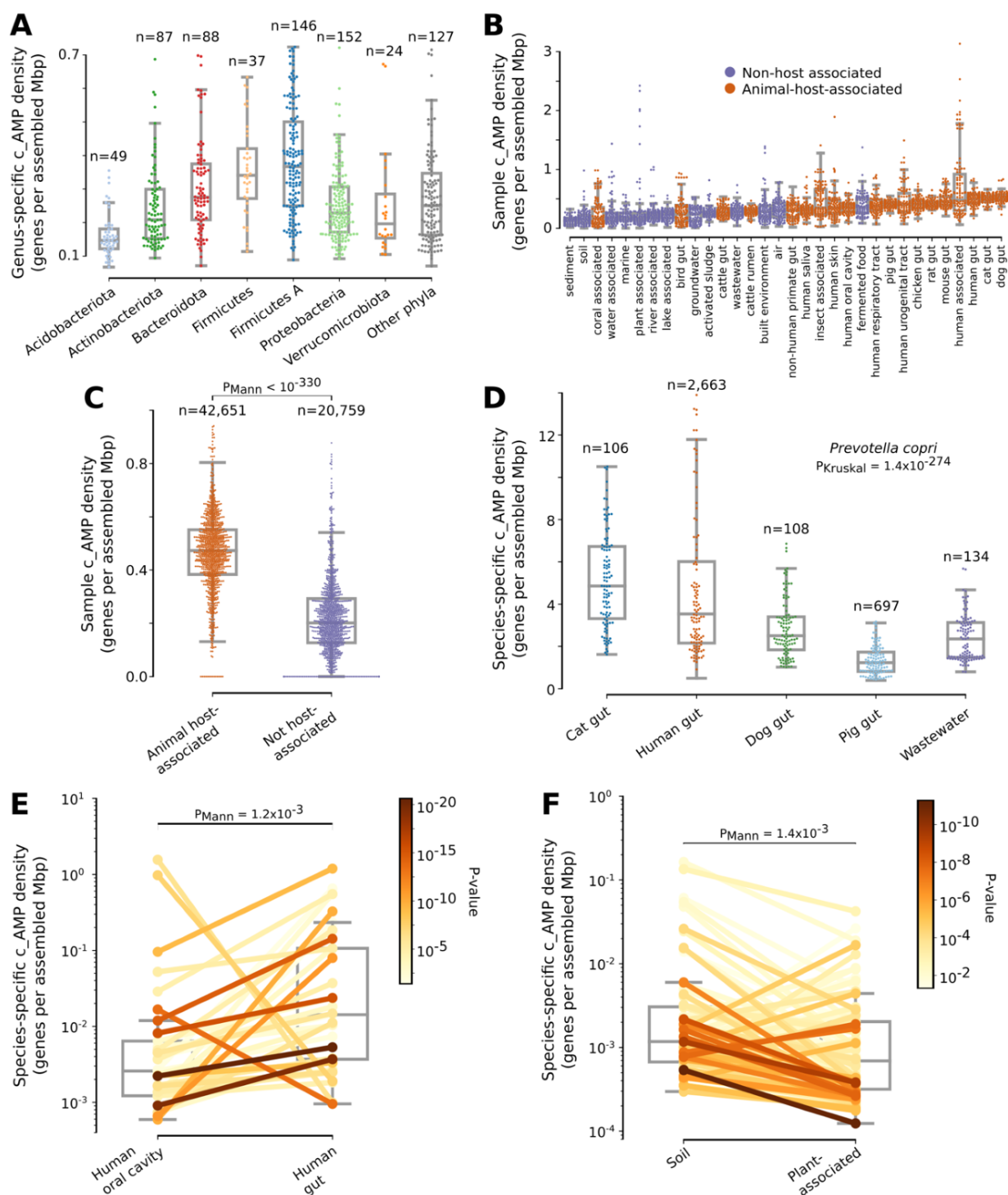
**Figure S12. c\_AMPs quality and habitat distribution.** (A) Quality assessment of AMPSphere reveals most of the peptides passing at least 1 of the tests. The RNACode test depends on gene diversity, which is very low for AMPSphere, and therefore, determines a low rate of positives among our candidates. (B) c\_AMPs homologous to databases of validated bioactive peptides also showed a higher average quality of these datasets. (C) The limited overlap of c\_AMPs among habitats argues in favor of using habitat groups to gain resolution. Note that the group of habitats with the highest paired overlaps belong to human body sites and samples from human guts and non-human mammalian guts. Only habitats with at least 100 samples were shown. (D) It is also possible to observe the great proportion of rare genes in AMPSphere from different habitat groups, in which few genes are largely detected.



**Figure SI3. Clustering validation of families.** To validate the clustering procedure using a reduced amino acid alphabet, samples of 1,000 peptides were randomly drawn from AMPSphere (excluding representative sequences) and aligned against their cluster representatives. Three different levels (I, II, and III) of clustering were tested. The E-values were computed per alignment and plotted against the corresponding alignment identity. The averaged proportion of significant alignments is shown above each graph.

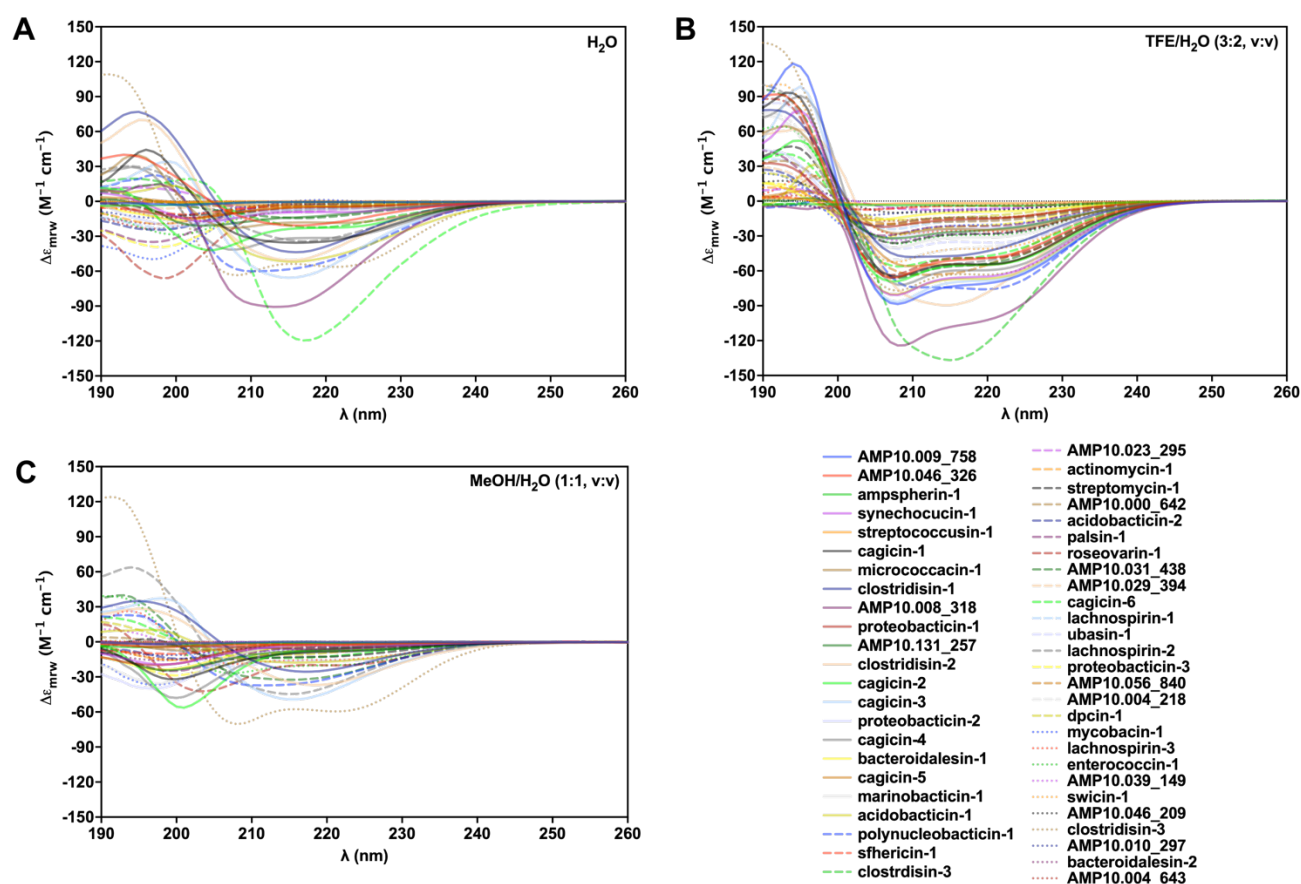


**Figure SI4. Host-associated habitats are denser in c\_AMPs.** The c\_AMP density (given as c\_AMP genes per assembled Mbp) was computed per sample and plotted per habitat. Only habitats with at least 100 samples were used. To favor a good visualization, we sampled 2,000 dots to plot their distribution trends. habitats were colored by their ontology, orange for the animal host-associated samples and purple for the non-animal-host-associated samples. Host-associated habitats cluster at the right portion of the graph with some anthropogenic habitats, which are closer to animal host-associated samples than habitat samples. Few exceptions, such as coral-associated and bird gut habitats cluster with non-animal-host associated habitats.



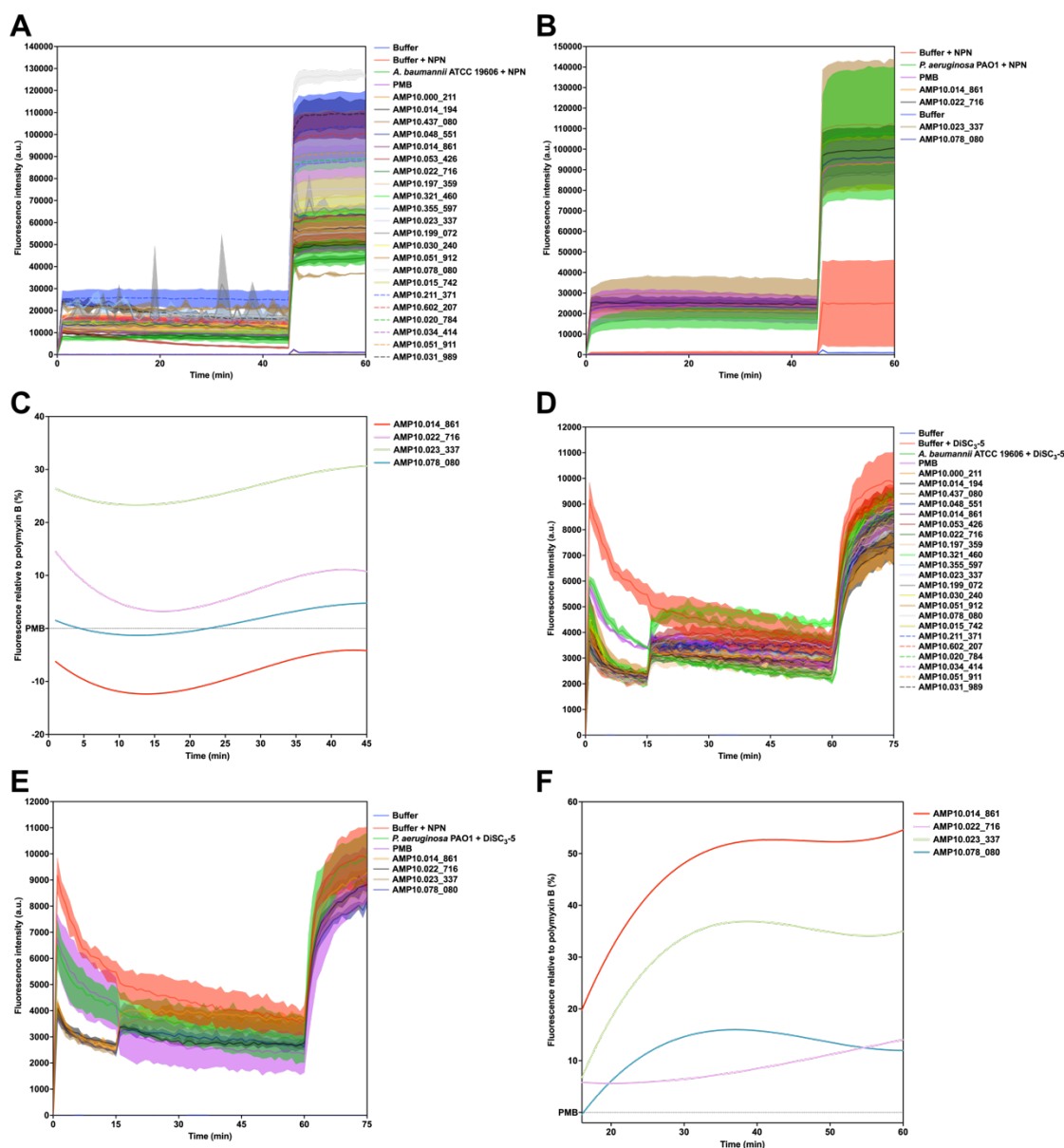
**Figure SI5. Quality-controlled c\_AMPs do not change the differences observed for c\_AMP density across several bases of comparison.** To evaluate the effects of possible gene fragments, we filtered off genes not having a stop codon upstream of them and recalculated the c\_AMP density (given as c\_AMP genes per assembled Mbp). The same results could be observed in the correspondent analysis using all c\_AMPs. **(A)** The main phyla contributing with more c\_AMPs in AMPSphere and the distribution of these densities in each of those groups by genera - correspondent

to **Fig. 4C**. **(B)** Animal host-associated habitats are denser in c\_AMPs - correspondent to **Fig. S14**. **(C)** Animal host-associated habitats have a higher sample c\_AMP density when compared to those non-host-associated - correspondent to **Fig. 5A**. **(D)** Hosts are a factor for variation of c\_AMP density in *Prevotella copri*, presenting a higher pAMP in cat and human guts compared to the same species in guts of pigs and dogs - correspondent to **Fig. 5B**. 106 randomly selected points are shown for each host. **(E)** Species-specific  $\rho_{AMP}$  of microbes from the human gut are higher, when compared against the same species found in the human oral cavity - correspondent to **Fig. 5C**. **(F)** For non-animal hosts, the species-specific  $\rho_{AMP}$  of microbes from the soil is higher when compared against the same species found in plant-associated samples - correspondent to **Fig. 5D**. For panels **E** and **F** the significance was color-encoded using a  $\text{Log}_{10}(P_{\text{Mann}})$  scale.



**Figure S16. Circular dichroism spectra of the c\_AMPs.** c\_AMPs secondary structural tendency was analyzed using three different solvents: **(A)** water, **(B)** trifluoroethanol (TFE) and water mixture (3:2, V:V), and **(C)** methanol (MeOH) and water mixture (1:1, V:V). The experiments were carried out at 25

1410 °C, and the circular dichroism spectra shown are an average of three accumulations obtained using a  
 1412 quartz cuvette with an optical path length of 1.0 mm, ranging from 260 to 190 nm at a rate of 50  
 1414 nm·min<sup>-1</sup> and a bandwidth of 0.5 nm. All peptides were tested at a concentration of 50 μmol·L<sup>-1</sup>, with  
 respective baselines recorded prior to measurement. A Fourier transform filter was applied to minimize  
 background effects.



1416 **Figure S17. Mechanism of action of AMPSphere peptides.** Permeabilization assays with the  
 1418 fluorescent probe 1-(N-phenylamino)naphthalene (NPN) showing the effect of AMPSphere peptides  
 1420 on (A) *A. baumannii* ATCC 19606 and (B) *P. aeruginosa* PA01 cells, and (C) Fluorescence values  
 relative to polymyxin B (PMB, positive control) of the fluorescent probe 1-(N-phenylamino)naphthalene  
 (NPN) that indicate outer membrane permeabilization of *P. aeruginosa* PA01 cells. Depolarization  
 assays with the hydrophobic probe 3,3'-dipropylthiadicarbocyanine iodide [DiSC<sub>3</sub>-5], effect of  
 AMPSphere peptides in (D) *A. baumannii* ATCC 19606 and (E) *P. aeruginosa* PA01 cells. (F)

1422 Fluorescence values relative to PMB (positive control) of 3,3'-dipropylthiadicarbocyanine iodide  
[DiSC3-(5)], a hydrophobic fluorescent probe, used to indicate cytoplasmic membrane depolarization  
1424 of *P. aeruginosa* PA01 cells. Data in (A), (B), (D), and (E) are the mean plus and minus the standard  
deviation.

## 1426 **Excel Tables**

**Table SI1. Metadata and description of (meta)genomes used in AMPSphere.** The sample is  
1428 identified by its access code in ENA, the habitat shows the type of habitat this sample was retrieved  
from. Other data about the sequencing, such as the number of raw inserts and the number of  
1430 assembled base pairs (bp) are also available along with the information on N50. The number of  
predicted complete large ORFs (>100 amino acids) and smORFs (10-100 amino acids) is shown  
1432 (ORFs+smORFs) along with the number of smORFs alone and the predicted non-redundant c\_AMPs.

**Table SI2. c\_AMP distribution in the habitat groups.** The habitats grouped under each class are  
1434 shown along with the number of genes encoding the non-redundant c\_AMPs, the number of c\_AMP  
clusters in total, and the number of clusters containing  $\geq 8$  c\_AMPs (c\_AMP families).

**Table SI3. Ortholog groups (OGs) enrichment in the hits to the GMGCv1** [S3]. Top hits were  
1436 assessed and the proportion of OGs from eggNOG 5 [S4] was compared using the number of  
c\_AMPs affiliating to homologs of a given OG and the total number of OGs found in the homologs of  
1438 c\_AMPs (156,711) in the comparison to the GMGCv1 [S3]. As a background measure, we used the  
counts of a given OG in the redundant set of genes belonging to GMGCv1 [S3] and the total number  
1440 of OGs found in the redundant GMGC catalog [S3] (9,180,087,363). Enrichment in the c\_AMPs set  
was given as the fold-change calculated for each given OG in relation to that expected in the GMGCv1  
1442 [S3]. P-values were adjusted using Holm-Sidak and only significant hits ( $P < 0.05$ ) were shown.

**Table SI4. c\_AMP genome context in comparison to families with proteins of different sizes.**  
1444 The proportion of families of proteins of different sizes (all lengths and only  $\leq 50$  amino acids) is  
presented in comparison to the proportion of mapped AMPs (55,191) with genome contexts involving  
1446 a given Kyoto Encyclopedia of Genes and Genomes – KEGG ortholog pathway [S5] shown with their  
accession code and description.  
1448

**Table SI5. Permutations with random families of different sizes show that the genome context  
1450 of c\_AMPs is different from other protein families.** We performed 10,000 random samplings of  
55,191 protein families of all lengths and only smaller than 50 amino acids. These families were  
1452 assessed regarding their genome neighborhood conservation. It was found that the values found in  
AMPSphere are mostly different from those observed for any other protein families across genome  
1454 contexts involving known functions, antibiotics synthesis/resistance, and antibiotic resistance genes  
from CARD [S6].

1456 **Table SI6. AMPs with conserved genome contexts sharing KEGG ortholog groups (KO) with**  
**gene neighbors.** AMPs were annotated with eggNOG mapper [S7] and those harboring KOs were  
1458 included in this analysis. It is shown for each KO a brief description of its activity, the total number of  
AMPs annotated, the number of those with conserved neighbors (conservation score > 0.9), and the  
1460 number of AMPs assigned to a given KO inserted in a conserved neighborhood containing other  
genes annotated to that KO (the intersection before the 2 previous columns).

1462 **Table SI7. c\_AMP density across different genera.** The number of redundant c\_AMP genes as well  
as their respective assembled base pairs is presented with the calculated AMP density. The error on  
1464 the AMP density measure is also shown evidence that most of the genera present an error above 10%  
(our cutoff).

1466 **Table SI8. Differential species-specific c\_AMP density across habitats.** The tested species had  
their densities (as c\_AMP genes per assembled gigabase pairs) compared across samples from  
1468 different habitats (Habitats A and B), and the number of samples for each habitat was also registered  
after eliminating the outliers using Tukey's fences with  $k = 1.5$  (# Samples A and B). The average  
1470 c\_AMP density for each species in each habitat was registered along with its standard deviation (Avg.  
c\_AMP density and Std. c\_AMP density). The Mann-Whitney U test was applied to each pair of  
1472 habitats and later corrected using the Holm-Sidak method, shown as the 'Adjusted P-value'.  
Comparisons considered species present in at least 10 samples for each habitat with  $\geq 100$  samples  
1474 presenting c\_AMPs. Only significant comparisons were shown.

**Table SI9. Metatranscriptomes and metaproteomes used in the verification for experimental**  
1476 **signals of transcription and/or translation of c\_AMP genes from AMPSphere.**  
Metatranscriptomes from EMBL-ENA were used in the comparisons with genes in AMPSphere to  
1478 verify signals of transcription in datasets *ad hoc*. The datasets from the Proteomics Identification  
Database (PRIDE) - EMBL-EBI were also used in the comparison to c\_AMPs and verified the same  
1480 peptides in datasets *ad hoc*.

### Supplemental references

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- 1494 S6. Alcock BP, Raphenya AR, Lau TTY, *et al.* CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res* 2020; **48**:D517–D525.
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