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Bacterial Wars—a tool for the prediction of bacterial predominance based on network analysis measures

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ABSTRACT

Bacterial Wars (BW) is a network-based tool that applies a two-step pipeline to display information on the competition of bacterial species found in the same microbiome. It utilizes antimicrobial peptide (AMP) sequence similarities to obtain a relationship between species. The working hypothesis (putative AMP defense) is that friendly species share sequence similarity among the putative AMPs of their proteomes and are therefore immune to their AMPs. This may not happen in competing bacterial species with dissimilar putative AMPs. Similarities in the putative AMPs of bacterial proteomes may be thus used to predict predominance. The tool provides insights as to which bacterial species are more likely to 'die' in a competing environmental niche.

INTRODUCTION

Interspecies competition between bacterial populations existing in microbiomes is complicated and multifactorial. Bacteria are known to employ AMPs in order to compete with neighbouring species in environments such as the human gut (1–3). AMPs may also provide a means for a 'natural' elimination of pathogenic microorganisms. Purified AMPs have been used in the treatment of infectious diseases (e.g. skin infections and wounds) (4) and against cancer (5). AMPs may be used as alternative therapeutics to antibiotics, with potential applications in drug resistant pathogens (6). In previous work (7), we proposed that sequence similarities between bacterial AMPs modulate interspecies competition. The assumption was that compatible bacteria would share high sequence similarities in the putative AMPs of their proteomes, while competing taxa would exhibit the opposite effect. We termed this hypothesis the 'putative AMP defense hypothesis'. We further implemented a computational methodology, termed Bacterial Wars (BW), to provide evidence to support the putative AMP defense hypothesis (7). The BW method allowed for (i) sequence similarity comparison of putative AMPs in the proteomes of bacterial taxa and (ii) prediction of interbacterial competition outcomes based on their AMPs. The BW methodology was assessed using 11 publicly available 16S rRNA datasets as well as a shotgun metagenomics dataset that ultimately provided evidence in favour of our putative AMP defense hypothesis (7). In the current work, we capitalize on our previously published BW methodology (7) and now provide a web application for this methodology to efficiently simulate bacterial predominance. The BW application tool allows researchers to assess the compatibility of bacterial species using a network-based methodology, with specific network measures, that highlight which species would be more likely to 'die' in a competitive microbiome niche. This may provide information to the experimental microbiologist for the selection and growth of unique mixtures of bacterial taxa and assess which species will prevail under specific wet lab conditions. The use of bacterial mixtures can help various fields of interest such as agriculture (8,9). The health industry may also potentially benefit from such bacterial mixtures as a replacement for antibiotics. Ultimately, it may be possible to regulate the population size of a specific bacterial species simply by manipulating its microenvironment and the composition of its participating taxa.

MATERIALS AND METHODS

The Bacterial Wars (BW) application

Querying the BW database. The BW application provides an interactive, user-friendly interface to allow for the prediction of which taxa are more likely to 'die' in an underlining microbiome. The BW database, detailed in our previous study (7), is now made accessible as a MySQL database that relates bacterial species with respect to their AMP sequence

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similarities. The database is available through the BW application and can be queried to generate networks of bacterial interactions based on the number of common AMPs.

A network measure to assess bacterial competition—the die *score.* The *Die* score, together with a mathematical explanation of its calculation for all nodes in a given network, is described in detail in our previous work (7). For completion the *Die* score formula is also provided below (see equation 1). It uses network topology measures, such as degree and strength of the edges, to calculate the relative likelihood for any given bacterial species (node) to 'die' in a specific microbiome. The higher (more positive) the *Die* score, the more likely a species is to 'die' in the underlining microbiome, whereas smaller (or negative) *Die* scores exhibit the opposite trend.

For a node i in a given BW network, the Die score (D) is calculated with equation (1):

$$D_i = \frac{N_i - S_i}{N_p} \quad (1)$$

where S_i represents the strength of the node (i.e. the weighted degree of the node i) and sums up the number of neighbouring bacteria that node i is linked to via an edge (e.g. shares common peptides), as well as the edge weight (e.g. number of common peptides). N_i represents the number of pairs with no edges (e.g. no common peptides). It is calculated using the expression $N_\mathrm{i} = N_\mathrm{p} - \deg_i$, where $\deg_i$ represents the degree of the node i. $N_p = (n^2 - n)/2$ represents the number of all possible pairs in the network.

Bacterial compatibility using the BW application. The BW application is able to extract and analyze relationships among different species and/or genera. The specific analysis process involves two steps: (i) a list of user-defined bacterial species/genera is used as input to query the BW database. If the user supplies information at the genus level in the input list, BW will retrieve all the species under this genus and include them in the downstream analysis. BW will then extract information on the number of shared common peptides between all pairs of bacterial taxa in the list. A network of bacteria species, termed the BW network, is constructed using the information available from the BW database. Species are denoted as network nodes and the number of common AMPs define their edge weights. (ii) *Die* scores are calculated for all the nodes in the BW network.

As an alternative function in Step 1, the BW application accepts a list of user-defined bacterial UniProt IDs. The tool then extracts the species associated with the UniProt IDs and continues to assess for similarities in the putative AMPs found in these species. This allows for users to obtain networks of bacterial interactions and their corresponding *Die* scores by initiating the search using proteins that are of interest to their research.

RESULTS

Case studies to highlight the functionality of BW

A simple case-study with a user-defined list of 3 example species/genera: *Alcaligenes, Corynebacterium* and *Escherichia coli* is presented an indicative example via the BW web application (see Figure 1). The list generates 14 different species belonging to the *Alcaligenes, Corynebacterium* genera as well as *Escherichia coli*. The number of common protein IDs is displayed and the AMPs which reside in these proteins can be viewed (down to the actual amino acid sequence) by clicking on an individual row. A network depicting the connections based on the pairwise relationships (edgelist) can be generated (see Figure 1, Step 1) and the *Die* score calculations obtained directly form the network are visualized using a bar chart (see Figure 1, Step 2). The species which is least likely to 'die' in this microbiome environment is *Corynebacterium glutamicum* (*Die* score: –1.46), as shown by the labels in the chart (see Figure 1, Step 2). While the species with the highest *Die* scores and hence most likely to 'die' are: *Alcaligenes* sp., *Corynebacterium crenatum* and *Alcaligenes xylosoxydans* (*Die* score: 1.22). To further showcase the potential utility of the BW application we examined two enclosed microbiomes (gut and lung) as case studies (see below). These simulated experiments should not be confused with a validation of the BW methodology. This has been addressed in detail in our previous publication (7). These simulations simply provide an assessment of the actual practical application and functionality of the BW web tool.

Gut microbiome simulation. To showcase the functionality of BW, we considered a specific example using taxa from the gut microflora. We initially added a list of genera that are commonly found in the human gut microbiome as input to BW. These included *Faecalibacterium, Clostridium, Roseburia, Lactobacillus, Staphylococcus, Bacteroides, Prevotella, Enterococcus.* The *Escherichia coli* (*E. coli*) species is also commonly found in the human gut microbiome and was also included in the list (10). The species obtained from the BW database generated a network (Supplementary Information Figure S1A) that outlines the bacterial interactions in the natural gut microflora (based on the putative AMP defense hypothesis). The respective *Die* scores for each species indicate which taxa are more likely to 'die' in the underlining microbiome (Supplementary Information Figure S1B and Table S1).

We next sought to simulate the infection of a pathogenic species, namely *Salmonella enteritidis* (*S. enteritidis*), which is the main cause of food-borne salmonellosis in humans. The natural gut microflora is disadvantageous for *S. enteritidis* as shown by the network generated by BW (Figure 2A) and the corresponding *Die* score value of 1.16 (see Figure 2B). *S. enteritidis* does not appear to have multiple connections (common AMPs) with other species in the natural gut microbiome (with the exception of *E. coli*), thereby rendering it incompatible and highly likely to 'die' within the bounds of this specific, enclosed bacterial niche (in accordance to the putative AMP hypothesis—Oulas *et al.*, 2021).

It is interesting to observe how an imbalance of the natural gut microbiome with the addition of another pathogenic genus such as *Shigella*, resulted in an increase in number of connections for *S. enteritidis* (degree) in the network containing both pathogens of the gut (Supplementary Information Figure S2A). This indicates that there are multiple common/similar AMPs between these two pathogens and is also reflected in the *Die* scores observed for the

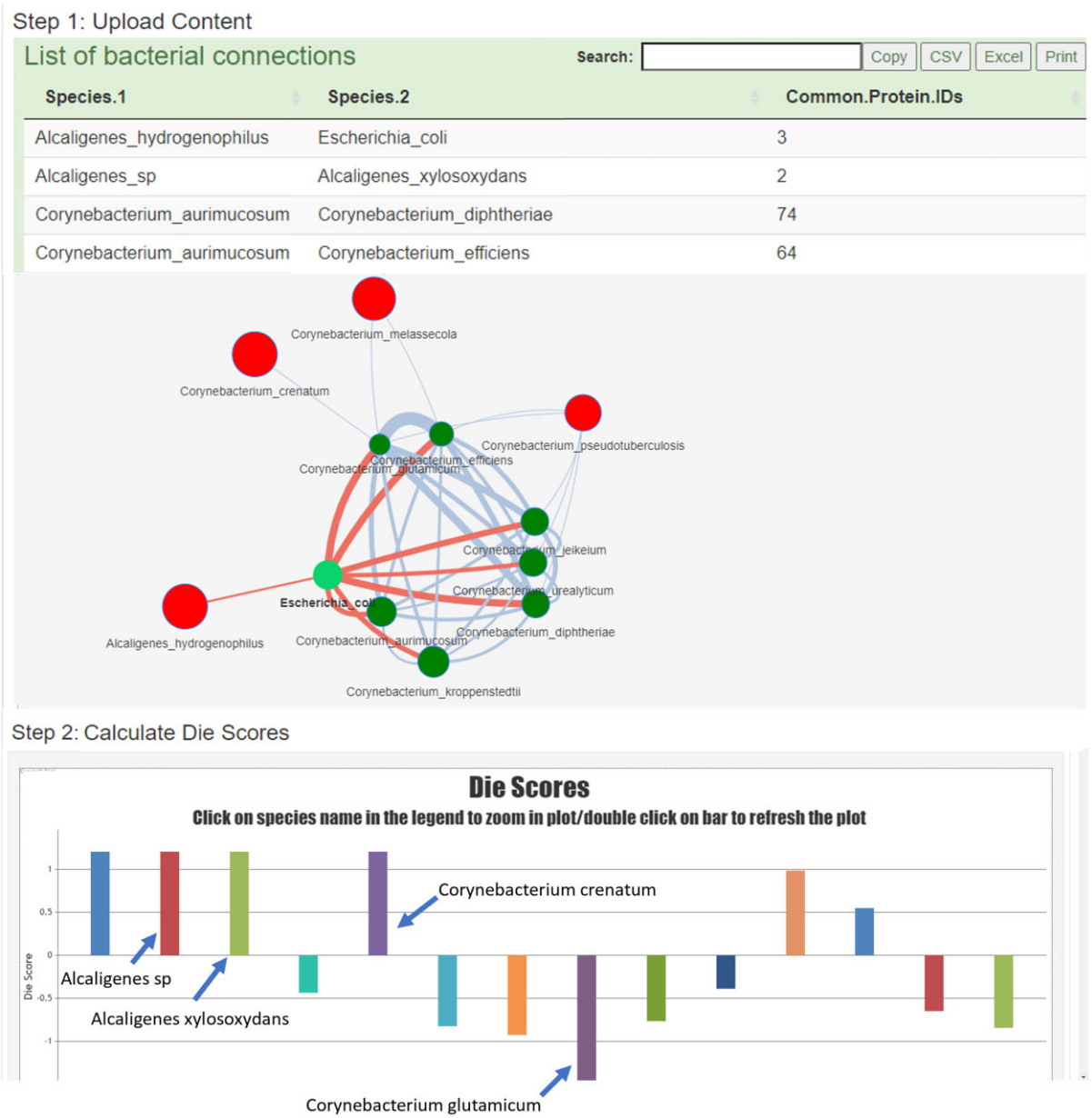

Figure 1. BW web interface—taxa upload example. Step 1 shows the loaded list of taxa as a table of pairwise species relationships with respect to common AMPs (from BW database). The BW network generated from the table is also shown. Clicking on a node (species) highlights all neighboring species edges (shown as red edges). The node size and color reflect the *Die* scores (larger nodes - most likely to 'die', small nodes—less likely to 'die', green color—negative *Die* score, red color - positive *Die* score). Step 2 shows the bar chart output of the predicted *Die* scores. Species with the highest and lowest *Die* scores are labelled and shown with arrows in the bar chart.

double-infection simulation. The *Die* score of *S. enteritidis* was dramatically reduced from 1.16 to 0.32 (Supplementary Information Figure S2B) thereby, favoring its overall endurance in the niche. The BW results appear to be in line with recent evidence supporting the notion that bacterial co-infection in the intestine appears to affect host immunity (11).

Lung microbiome simulation. The lung microbiome is inevitable different from the gut. Notably, the most prominent bacterial phyla are Bacteroides, Firmicutes, Proteobacteria, and Actinobacteria. At the genus level, *Pseudomonas, Streptococcus, Prevotella, Fusobacteria* and *Veillonella* predominate, with lesser contributions from potential pathogens including *Haemophilus* and *Neisseria* (12).

Species such as, *Pseudomonas aeruginosa, Streptococcus pneumoniae* and *Streptococcus pyogenes* are known to cause common respiratory infections. Although these are feared hospital pathogens and some of the main causes of respiratory infections, they are actually considered to be part of the normal lung microbiota in healthy adults (13–16). These species are often resistant to many classes of antibiotics and

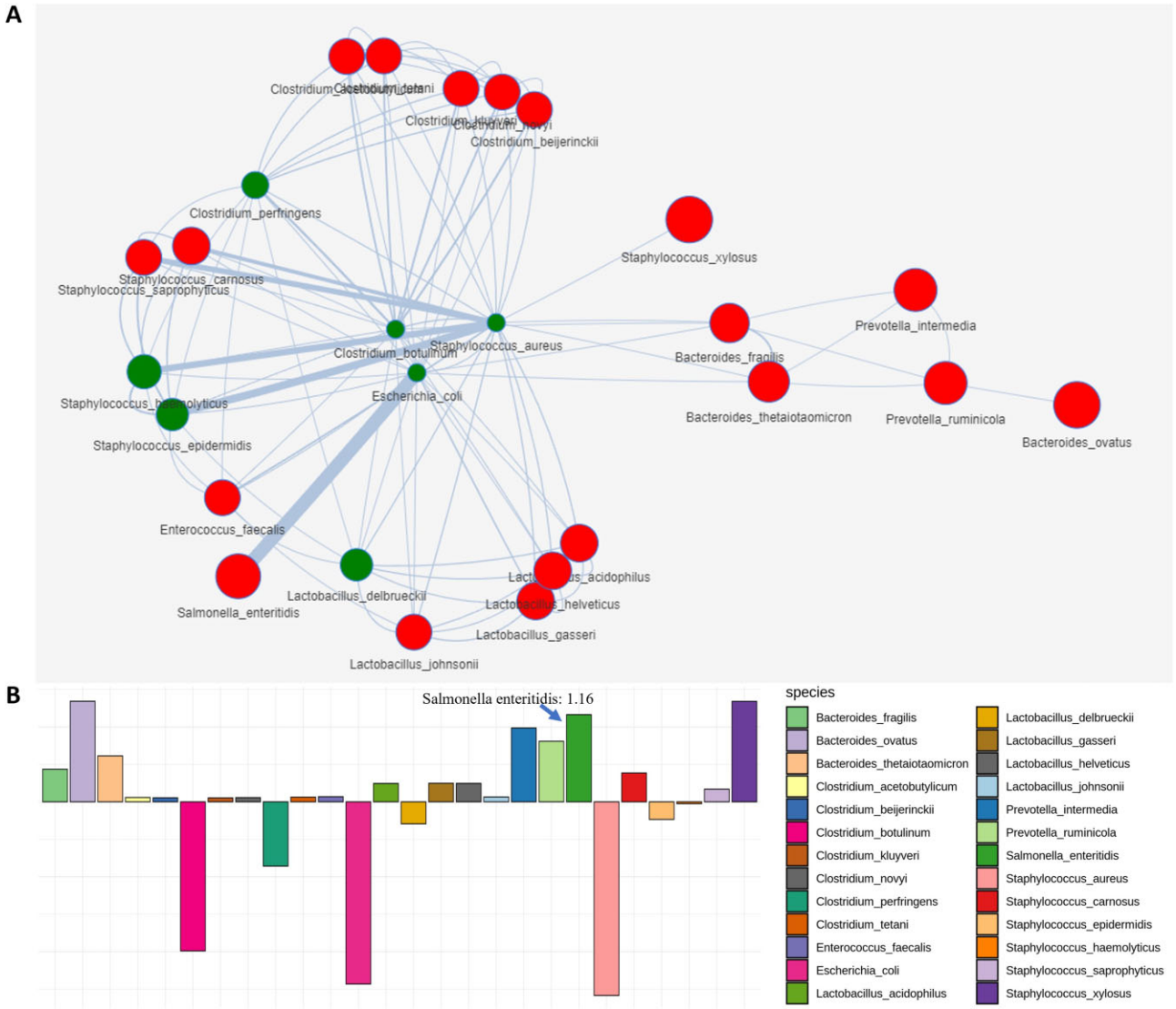

Figure 2. BW output for taxa in the gut microflora simulating infection by *S. enteritidis*. (**A**) Network of species with *S. enteriditis* showing large node with a negative *Die* score. (**B**) BW *Die* score bar chart for taxa in the gut microflora simulating infection by *S. enteritidis*.

therapeutic agents, thus rendering them problematic during infection as they can be difficult to treat. They are often termed 'opportunistic' pathogens because they rarely infect healthy individuals (17). They constitute a primary risk for patients with compromised immune systems including those with cancer, acquired immune deficiency syndrome (AIDS) and cystic fibrosis (CF). (17).

We first selected a list of bacterial genera that are commonly found in lung microbiomes under healthy (control) circumstances (12,18–20). These included *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacteria* and *Veillonella*. The search in the BW database retrieved a set of species that generated a network simulation of the natural lung microbiota (Supplementary Information Figure S3A). The *Die* scores reveal which species are most likely to prevail in this microbiome (Supplementary Information Figure S3B and Table S2). All potential pathogens (*Streptococcus pneumonia*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*) attained low *Die* score as they are naturally co-existing with other species in the underlining lung microbiota.

Next, we sought to simulate a microbiome that is immunocompromised or representing patients that often develop respiratory infections, such as patient with Cystic Fibrosis (CF). The microbiomes of these patients are often characterized by the overabundance of certain additional genera. These include *Rothia*, *Porphyromonas*, *Bergeyella*, *Butyrivibrio*, *Lachnospira* and *Terrimonas* (21). When these taxa were included in the list of species/genera for BW, the network showed an increase in the number of 'friendly' connections with the opportunistic pathogen (see Figure 3A) thus strengthening their presence in the underlining microbiome. It appears that inclusion of the additional CF taxa further caused a decrease in the *Die* scores for all three of the opportunistic pathogens residing in the natural lung microbiota (NLM). *Pseudomas aeruginosaDie* scores: NLM: –0.8, CF: –0.88; *Streptococcus pneumoniaeDie* scores: NLM:

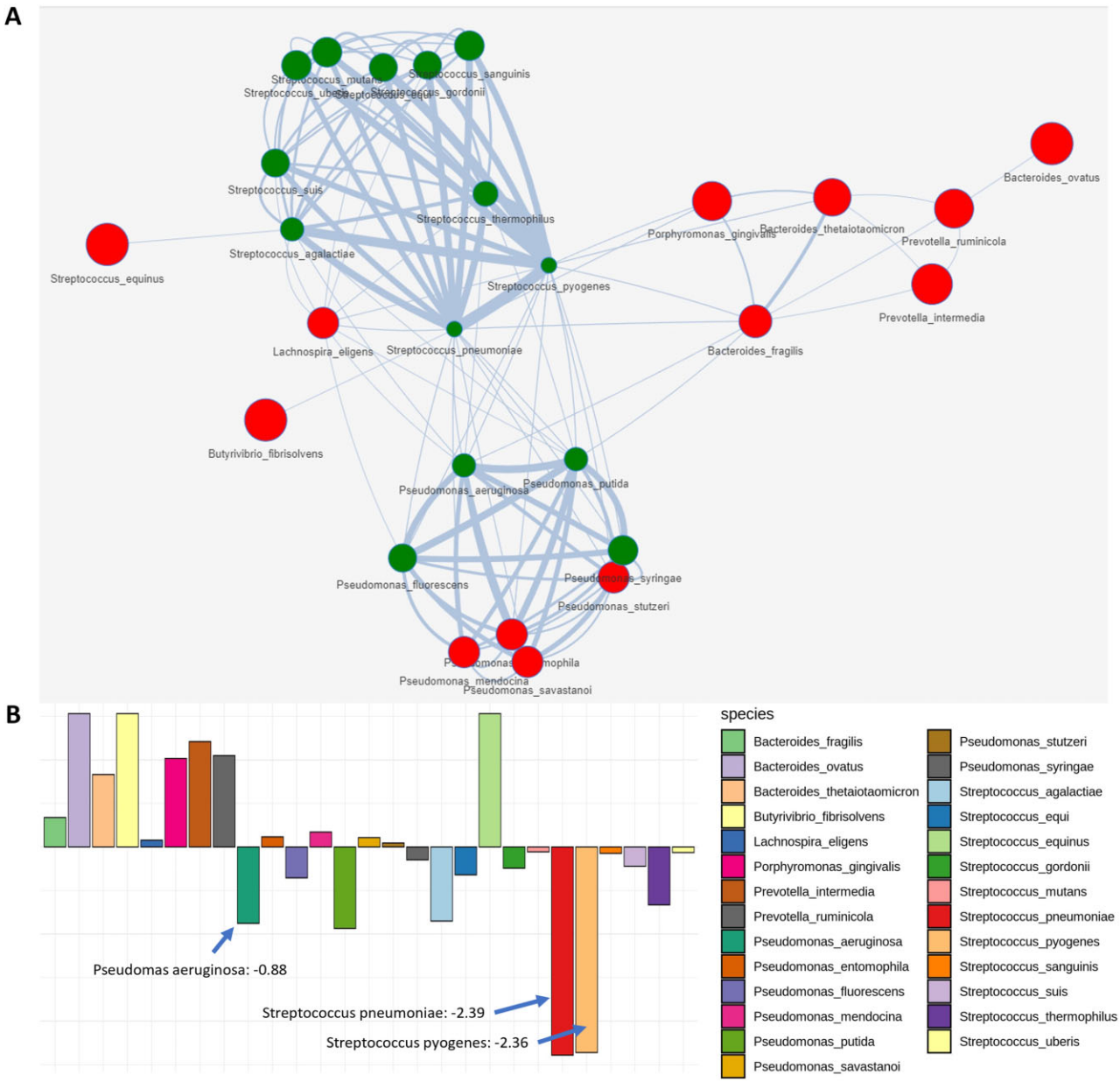

Figure 3. BW output for taxa simulating the lung microbiota of CF patients. (**A**) Network of species. (**B**) BW *Die* score bar chart for taxa simulating the lung microbiota of CF patients.

–1.80, CF: 2.39; *Streptococcus pyogenesDie* scores: NLM: –2.09, CF: –2.36 (see Figure 3B, see also Supplementary Information Tables S3 and S4).

DISCUSSION

The natural balance of enclosed microbiomes is a delicate homeostatic process that is dynamic and subject to change by internal as well as external factors. Naturally occurring bacteria are often shifted from their 'normal' abundance by the introduction of additional species that upset the overall equilibrium of the niche. One way that bacteria achieve this is by direct combat with their neighbors and AMPs are a main form of bacterial warfare. Based on our hypothesis, increased similarities among putative AMPs between bacterial taxa establish more friendly connections in a 'social' bacterial network thus increasing their chances of survival. The opposite case, where AMPs differ, results in the overall alienation of taxa and hence decreasing their chances of survival in a competitive environment. The case studies shown herein are examples of how enclosed bacterial niches, such as the gut and lung, can be simulated using the BW tool. Results can provide insights as to which bacterial taxa will thrive when co-existing with others and which will perish. The tool can simulate infections such as the *S. enteritidis* in the gut, and also disease-control microbiomes such as the CF versus normal lung microbiota. The tool is intended to aid in designing wet lab conditions to verify bacterial

interactions and overall bacterial predominance. As a limitation it should be mentioned that the BW portal works on a limited set of microbes, namely those that are present in UniProt *release 2022_02* and for which AMP information has been successfully extracted and stored in the BW online database.

Undoubtedly, the use of AMPs for bacterial competition is but one of many mechanisms that bacteria have in their armory. Additional tools may include: (a) Type 6 secretion system (T6SS) (22)—this is a sort of piston-like structure that is used for punching holes in neighboring cells to inject killing compounds into those cells. (b) Biofilms (23)—these are communities of bacteria that adhere to both manmade and natural surfaces. These biofilms act like bunkers, shielding bacteria from threats, e.g. immune system, antibiotics, threats from the environment, such as lack of water, UV radiation, or limitations in nutrients. Biofilms also protect bacteria from other bacterial competitors and may enable bacteria in close proximity to share nutrients with each other, but also to share traits that give these bacteria a competitive advantage over their neighbors. These traits can include antibiotic resistance and metabolic traits. In order to fully simulate bacterial competition and warfare all of these mechanisms (and more) should be taken under consideration. BW currently provides a simplified approach of simulating bacterial prevalence and we are currently investigating modes of action by which to include additional bacterial competition mechanisms in our application.

DATA AVAILABILITY

BW is available as a web interface at the following url: https://bioinformatics.cing.ac.cy/bacterialwars (source code available in the help page).

SUPPLEMENTARY DATA

Supplementary Data are available at NARGAB Online.

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Conflict of interest statement. None declared.

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