Determining the Relation of ASF Species Abundance to Sphingolipid Production
(Technical Topic)

Evaluating Social and Technological Influences on the Rise in Antibiotic Resistance
(STS Topic)

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Luke Malanga
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Technical Project Team Members
Luke Malanga
Nebiyu Solomon

On my honor as a student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Signature _____ Date _____
Luke Malanga

Signature _____ Date _____
Sean Travis Elliott, Associate Professor of STS, Department of Engineering and Society

Signature _____ Date _____
Sean R. Moore, Associate Professor of Pediatrics, School of Medicine

Technical Topic: Determining the Relation of ASF Species Abundance to Sphingolipid Production

Specific Aims

The altered Schaedler flora (ASF) are a gut microbiome model community of eight bacteria known to inhabit the GI tract of mice and used to explore microbial interactions in coculture. (Wymore Brand et al., 2015) ASF-519, one of the eight bacterial species of ASF, is commonly considered the most dominant microbe of the ASF consortia, but recent literature suggests sphingolipids, a class of lipids involved in tissue development and cell recognition, may play a key role in determining which microbe is dominant. (Geuking et al., 2011; Sarma-Rupavtarm et al., 2004) This discrepancy of knowledge stems from past microbiology's reliance on isolated bacteria and neglect of interspecies relationships. This is a problem because it limits therapeutic opportunities for microbiological diseases influenced by bacterial interspecies interactions. Therefore, a study is being carried out which aims to evaluate ASF pairwise interactions to rank the dominance of each strain, and to determine mutualistic, parasitic, commensalistic, or amensalistic relationships between ASF-519 and other ASF strains based off of computational growth curve measurements of the different strains. The impact of sphingolipids in manipulating both the dominance of ASF-519 and its interactions with other ASF strains will also be measured to determine the impact of external factors on ASF dominance and bacteria-bacteria interactions.

Aim 1: Evaluate ASF pairwise interactions to rank the dominance of each strain within a coculture by:

a) Measuring ASF optical density inside an anaerobic chamber using calibrated Cerillo plate readers

b) Using real time optical density measurements to compute relative abundance and to determine growth rate and carrying capacity from a growth model produced via Growthcurver in R

Aim 2: Determine relationships of interest between ASF-519 and other ASF strains and these relationships' dependence on chemical signaling by:

a) Determining dominance and relationships of interest by comparison and statistical analysis of abundance, and evaluating the dynamic aspects of these relationships through comparison and statistical analysis of growth rate and carrying capacity

b) Inhibiting sphingolipid production with myriocin to measure the impact of sphingolipid production in the interactions of interest between ASF-519 and other ASF strains

Growth curves will be produced for nine ASF samples per ASF-519 coculture with the other 7 ASF strains. The relatively small size of a Cerillo plate reader in comparison to a 96-well plate reader allows for the measurement of growth rate of ASF strains in a growth-promoting anaerobic environment. The ASF-519 growth rate and abundance will be compared when the other strains of ASF are present to test previous research that has determined that it is the most dominant ASF strain due to recent experiments that found this may not be the case always. Depending on the results of aim 1, cocultures of interest will be grown again, but some samples will be treated with myriocin, which inhibits sphingolipid production, indicating its role in the observed relationship. Cocultures of interest are those exhibiting growth which indicates high levels of interaction. In order to get a more comprehensive idea of the interaction of ASF-519 with other strains, the presence of sphingolipids and the abundance of ASF strains will be manipulated.

Overall, this project will help to fill an existing gap in traditional, modern microbiology by enhancing knowledge of bacteria to bacteria interactions, and moreover, having a more

developed idea of how these bacteria to bacteria interactions can be impacted by external signals. This could lead to positive implications in dealing with current microbiological diseases by generating more accurate models of ASF which account for the role of sphingolipids in determining each species growth and dominance. More accurate ASF models means more accurately interpreting results from relevant experiments and more accurately determining viable therapeutics for disorders of GI microflora.

Significance

Sphingolipids and the Gut Microbiome: Dysbiosis of the GI microbiome is linked to a number of diseases, including inflammatory bowel diseases (IBD), such as crohn's disease (CD) and ulcerative colitis (UC). An abundance of host produced sphingolipids and deficiency of microbe produced sphingolipids has also been associated with CD and UC (Brown et. al, 2019). Recent literature suggests sphingolipids play a role in determining microbe dominance (Lee et. al, 2021). Elucidating what factors determine microbe dominance is a current need in research, since coculture interactions cannot be straightforwardly determined from growth in monoculture. ASF-519 is of particular interest to this experiment since it has been measured to be dominant in vivo in the GI tract of 6-week-old germfree C.B-17 SCID mice inoculated with ASF (Sarma-Rupavtarm et. al) and in stool samples of germfree mice inoculated with ASF(Geuking et. al, 2011), and so we suspect its growth will exhibit a dependence on sphingolipid levels in coculture.

Applications: Therefore the study aims (1) to evaluate ASF pairwise interactions to rank the dominance of each strain within a coculture and (2) to evaluate these microbe-microbe relationships' dependence on chemical signaling of sphingolipids. Determining microbe-microbe relationships of ASF bacteria in coculture will test the findings of previous studies and add to our understanding of the ASF community as a model, which may help to design and interpret experiments which use ASF. Evaluating these relationships' dependence on sphingolipid production may provide insight to the

growth mechanisms of the ASF community. To be able to modulate the growth pattern of a community via chemical signaling could be a powerful research tool and therapeutic. Additionally, if the pathogenesis of CD and UC is due in part to an imbalance of microbe produced sphingolipids, then characterizing their effect on growth rate may lead to new treatments. Finally, the technical innovations of this project aim to streamline the determination of abundance, growth rate, and carrying capacity for anaerobic bacteria in coculture using commercially available devices, which may benefit future research which relies on the accessibility and repeatability of the methods implemented here.

Innovation

Determining Dominance, Prior Art: The dominance of bacteria in coculture can be determined from their relative abundance (cells/mL) which in turn can be estimated from optical density (OD) measurements (AU/cm) for a dilute solutions bacterium and spectrophotometer for which a best fit conversion equation is known (Mira et. al, 2022). Alternatively, bacteria of similar shapes and sizes may be assumed to have similar absorbances at the equal concentrations, although this can introduce error (Young et. al, 2006). If our concern is relative biomass, and not cell count per se, then OD suffices for dilute solutions, since a linear relationship has been demonstrated for OD and biomass at low concentrations (below 0.25 gDW/1 for the UV-VIS and below 0.5 gDW/1 for the fiber optics spectrophotometer) (Willaert et. al). It is also possible to apply machine learning to automatically segment bacteria and produce a cell count, but this is usually done by diluting and performing a pour so that images are clear and all cells lie near the same plane, again restricting the bacterial concentration over which cell counts are accurate.

Determining Dominance, Innovation: Many past co culture experiments have used CFU and grid counts of dilutions to measure abundance (Acai et. al, 2019), but we aim to utilize recent advancements in coculture duets and spectrophotometry devices to provide real time

growth data. Neither the use of OD to measure abundance and model growth nor the equations to be used are novel, and the use of these methods has even been carried out on ASF by miniature plate readers in anaerobic chambers (Jensen et, al, 2014), but the use of the Cerillo stratus and Cerillo coculture duets for real time optical density measurements of ASF bacteria in coculture has not been published to our knowledge. We hope a streamlined process using commercially available tools will provide a more easily repeatable protocol.

Approaches in Running ASF experiments: One previous approach that was used to run Altered Schaedler Flora experiments was through the process of systems-level metabolism (Biggs et. al, 2016). Where Altered Schaedler Flora were grown in different in vivo conditions and dominance was identified. It was found that cross-feeding interactions were relatively rare but critically relevant in determining dominance (Morgan et. al, 2020), whilst non-growth associated and emergent metabolism was very common. Another approach that was performed was using sphingolipids as a tool to advance microbiological knowledge and explore potential therapeutic opportunities due to their functionality in cell signaling, membrane organization, and host-microbe interactions. Relevant to this experiment as well, it was found that sphingolipids play a role in influencing the composition of the gut microbiota, which can have wide ranging effects on host health. (Brown et. al, 2019)

Speciality of Our Approach: Our approach is special as unlike previous experiments as optical density will be used as a tool to measure ASF dominance which minimizes error that may have occurred when attempting to design in vivo conditions using metabolite production and consumption measurements. As also mentioned before, this project utilizes coculture duets which allow for the growth of both the ASF and presence of sphingolipids to be measured in a

collaborative manner which minimizes the possible errors that may occur when measuring them separately.

Approach

Liquid robot protocol: To advance the aims of this study, we will write python scripts compatible with the Opentrons software and OT-2 liquid pipetting robot. The protocol requires serial dilutions, pipetting of media into plates, and plate cleaning. Each of these can be achieved via scripts produced with the online Opentrons protocol designer. We will write in each experiment's protocols and available command library to make manual adjustments to the script as needed. Use of the OT-2 liquid robot aims to save time and decrease user error.

Measuring abundance: Dominance is determined by measuring relative abundance. The experiment aims to maintain dilute cell concentrations at which OD can be used to estimate relative biomass (Willaert et. al). Abundance can be inferred from relative biomass, and so dominance will be measured from the OD of each coculture pair. OD will be measured using the Cerillo coculture duet in the Cerillo stratus, which is a portable OD600 spectrophotometer small enough to be used in an anaerobic chamber. Spectrophotometers of the same optical configuration should produce consistent measurements, but a quality control experiment will be carried out with serial dilutions of Avicell mixed in brain heart infusion to confirm this of the devices to be used. If the devices cannot be recalibrated to measure the same OD from equal Avicell dilutions with sufficient accuracy, then we propose to calibrate each stratus in order to convert its OD measurements into cell density, as proposed by Mira et al.

Modeling growth rate: However, to rank the abundance of each strain without characterizing growth kinetics would be to exclude important aspects of the microbe-microbe interaction. So, in order to determine coculture relationships of interest and these relationships' dependence on chemical signaling, the experiment aims to evaluate key growth characteristics of

ASF bacteria in coculture, including but not limited to the carrying capacity and growth rate, and compare these characteristics for each bacteria in coculture to those of its coculture mate and to previous literature on such characteristics in monoculture. There are a number of mathematical primary growth curve models that can be made from optical density data and which have fits and accuracy that vary with bacteria. Some have been reparameterized to measure biological parameters, including but not limited to the growth rate and carrying capacity. This project will only consider primary models, which predict $Log(N)$ over time in constant environmental conditions (Longhi et. al, 2017). We aim to implement a program which will model growth from OD and produce metrics, such as the growth characteristics listed above, for comparing bacterial growth of each bacterium in coculture. To begin, we propose to use the Growthcurver package in R to model growth using the following basic form of the logistic equation: (Sprouffske et. al, 2016)

$$N(t) = \frac{K}{1+(\frac{K-N_0}{N_0})e^{-rt}}$$

Where $N(t)$ is population size as a function of time, t; K is the carrying capacity; N_0 is the initial population size; and r is the growth rate . r is related to the specific growth rate (which is the fractional increase in biomass over time), μ, by the equation $\mu = e^r - 1$ (Kosseva, 2013). Given a set of constant environmental conditions, which we assume with a primary model, r and μ are constant. If time allows, the project will implement other primary models such as the Huang, Giménez, and Dalgaard coculture model (Acai et. al, 2021) or a reparameterized Gompertz and Richards (Zwietering et. al) and evaluate the models' bias and uncertainty to suggest a model best fit for the coculture pair and experimental conditions. This would be a new innovation, since to our knowledge there is no program made to simulate a number of models for each experiment and to

measure their accuracy and bias, from which one model can be determined better fit. At the very least, a pipeline will be established to use Growthcurver to determine the growth rate and carrying capacity for use in this study.

Having measured the abundance over time, growth rate, and carrying capacity of each bacteria in coculture, microbe-microbe relationships and dominance can be determined. With mutualistic, parasitic, commensalistic, or amensalistic relationships between ASF-519 and the other microbes characterized, pairs which exhibit greatly increased or stunted growth for one or both bacteria will be chosen for second round of experiments where some samples will be treated with myriocin, an inhibitor of sphingolipid production. The same pipeline will be used to determine abundance, growth rate, and carrying capacity so that the study can evaluate the effect of sphingolipid production on growth and dominance.

Alternative Approaches: In the case that the Stratus readers cannot be calibrated to give equal readings, then an experiment can be run for each device to determine an equation to convert the measurement from OD to cell density (Mira et al., 2022). If it is not feasible to accurately measure OD in real time, then one of the other above growth assays, such as a dilution and CFU count, could be performed manually. For modeling, if the logistic model does not provide reliable estimates of growth rate and carrying capacity, one of the other listed models will be tried.

STS Research Topic: Evaluating Social and Technological Influences on the Rise in Antibiotic Resistance

Such research on bacterial growth normally translates to antibacterial therapeutics. Antibiotics stand as an exemplar of human innovation reapplying biological tools to revolutionize our way of life. However, data shows that bacteria are reclaiming these tools— and

fast. The following examines the influence of social and technological factors on the usage of antibiotics and the resulting rise in resistance, employing the social construction of technology and technological determinism as frameworks.

Progress in Antibiotics

Antibiotics are substances used to treat bacterial infection by interfering with cellular mechanisms and integrity, but many bacteria have defenses which guard against specific types of natural antibiotic, either because they themselves produce the antibiotic or because they've evolved to survive its usage. The resulting ability to survive in the presence of antibiotic is called antibiotic resistance.

At the turn of the twentieth century, one third of all deaths were due to pneumonia, tuberculosis, diarrhea/enteritis (which would have normally been caused by a bacterial infection), and diphtheria. (Dodds, 2017) By contrast, in 2014, pneumonia accounted for less than 4.5% of deaths, and enteritis wasn't even in the top ten. (Dodds, 2017) Deaths due to enteritis and diphtheria, the former of which now seem to be increasing, amounted to less than a percent (CDC Online Newsroom - Deaths from Gastroenteritis Double, 2012; Clarke et al., 2019). While better hygiene and healthcare certainly play a role, few think the role of antibiotics is overemphasized. These developments are inestimably valuable for society. So, is the world cured of bacteriogenic diseases forever? Speaking broadly, no, or at least not without much continued effort.

The Rise of antimicrobial resistance

This is because microbes evolve in response to antibiotics. "The end of the 20th century and beginning of the 21st saw the beginning and rapid rise of advanced microbial resistance to antibiotics." (Dodds, 2017) Projecting future morbidity is a subject of some controversy, but one

model predicts that by 2050 antimicrobial resistance (AMR) will raise the annual death toll from bacteriogenic illnesses to 10 million. (Sutherland & Barber, 2017) For reference, cancer was responsible for 9.56 million deaths in 2017 and, by the same model as above, is projected to be 8.2 million in 2050. While some argue that this model is sensationalized and carries a lot of uncertainty (Kraker et al., 2016) none deny that AMR is a serious threat to public health.

The rise in AMR is due in considerable part— if not entirely— to the medicinal use of antibiotics, including the misuse of antibiotics by users. Alexander Fleming, who discovered penicillin, said at his Nobel prize address: "I would like to sound one note of warning… The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant." (The Nobel Prize in Physiology or Medicine 1945, n.d.) Others will argue that resistance increases in proportion to the evolutionary pressure resulting simply from so many available antibiotics being in use, which few deny, but most do not consider this a comprehensive account of the rise in AMR. (Abushaheen et al., 2020) Generally physicians agree that the rise in AMR is due in large part to the "misuse of antimicrobials, inappropriate prescribing patterns, [and the] lack of new novel antibiotics." (Abushaheen et al., 2020)

While these factors which promote the rise of AMR involve a number of actors and relationships, this paper will consider patients and their families primarily and the others only secondarily. For this purpose, "patients" does not include animals, although much of what's said can be applied to farmers and their livestock. Patients are only involved in one of the above three practices, the "misuse of antimicrobials," and that only in part. In general, over the counter oral antibiotics are not approved in the U.S. (Can You Buy Antibiotics over the Counter?, 2023)

However, doctors do prescribe antibiotics to be self-administered, and "if the treatment regime is not completed, or the dose is too low, then the opportunity for developing antibiotic resistance will occur." (Dodds, 2017) In this way, the misuse of antibiotics which leads to AMR is partially a social problem, so an account of the social dynamic will be given first using the social construction of technology (SCOT) and then using technological determinism.

An Account Using the Social Construction of Technology

"The social constructionist argument… proposes that social factors and actors lead to technological innovation." (Humphreys, 2005) Acknowledging that the word used here is "innovation" and not "usage," I will extend these principles to argue that social factors influence the usage of antibiotics. SCOT analyzes conflicts, such as the misuse of antibiotics that leads to AMR, in the context of interpretive flexibility and closure. Interpretive flexibility emphasizes the capacity for technology to be understood differently by different social groups. (Pinch & Bijker, 1984) Closure refers to diminished need for an improved design resulting from a closure mechanism such as "rhetorical closure," which is when "relevant social groups see the problem as being solved," although it may only be a *perceived* resolution. (Pinch & Bijker, 1984)

The social construction of technology can account for this conflict, namely the misuse of antibiotics which leads to AMR, by identifying patients as a relevant social group that might reach rhetorical closure without actually addressing the rise of AMR. A patient may want to complete their treatment if they're aware of the rise of AMR and feel an obligation towards society to protect this shared, nonrenewable, and precious resource. (Langford & Morris, 2017) However, this aligns with the responsible use of antibiotics, so it does not contribute to the conflict we are considering. On the other hand, a patient may value the remission of symptoms regardless of whether they have completed their regimen. This would be one perspective

resulting from the interpretive flexibility of antibiotics, but there are more social reasons for misuse which result from aspects of the technology's design that will be explained below and which still apply to the SCOT framework. A patient who does not consider, or does not value, the impact of their actions have on AMR, might perceive the conflict as resolved, either for lack of consideration or because they view it as a purely technical problem to be solved by scientists, resulting in a "rhetorical closure" of the conflict for many patients. Patients who believe the conflict is resolved may not follow their regimen which may lead to the development of AMR as described above.

An Account Using Technological Determinism

Proponents of technological determinism (TD), by contrast, might account for this conflict by identifying the effects of antibiotic design aspects, effects such as the ability of antibiotics to be shared or the inconveniences of the regimen, which influence a patient's usage. TD can refer to the "subtle but profound social and psychological influences at the microsocial level of the regular use of particular kinds of tools." (Technological Determinism, n.d.) This expression is a form of soft TD, as opposed to "hard TD," which "argues that technology is the main or the only significant driver" and "that social influences have little effect on the nature of technology." (Adler, 2006) Identifying aspects of design which enable or exclude certain usage, such as the ability of antibiotics to be saved and shared, aligns with hard TD, where identifying other aspects of design which influence but do not determine usage, such as the inconveniences of an antibacterial regimen, aligns with soft TD, although the two theories are not entirely exclusive.

The way an antibiotic is distributed and administered can allow patients to save an incomplete regimen and share it with others, "determining" the technology's use in accordance

with hard TD. Physicians are normally apprehensive when prescribing antibiotics, (1) because it can have negative side effects (Cunha, 2001) and (2) because they know the threat AMR poses, which may in turn make some patients want to save antibiotics because they can be difficult to obtain by prescription. When the patient's symptoms begin improving a week into treatment, they may think less about their own infection and begin thinking more about what they'll do when their children get strep. Are they supposed to let a doctor decide whether to withhold an antibiotic which would certainly hasten their kids' recovery? They may choose to save the remainder of the drug instead of using it to complete their treatment. This point is not entirely technical, as it represents a social problem, viz. a parent's desire to prioritize their children's health over public health, but the conflict is enabled by an effect of the technology's design, viz. the therapeutic's ability to be saved and shared.

The inconveniences of an antibiotic's use can encourage patients to end an incomplete regimen, subtly influencing the technology's usage in accordance with soft TD. A patient may decide not to finish the prescribed regimen if they experience any of the possible negative, albeit manageable, side effects. (Cunha, 2001) They may also wish to end treatment so that they can enjoy the milk, citrus, and alcohol patients are sometimes told to avoid mixing with antibiotics. (The Do's and Don'ts of Taking Antibiotics | St. Luke's Health, n.d.) Poor antibiotic usage, therefore, is a technically driven problem insofar as a patient may decide against completing a prescribed regimen both because of negative side effects and because of the antibiotic's ability to be saved and shared.

Conclusion

The preceding analysis provides many ways in which social and technological factors, individually and in conjunction, influence the patients' usage of antibiotics and the rise of AMR.

These factors include the value a patient attributes to personal, family, or public health, as well as the deficiency of antibiotic based therapeutics regarding side effects and the ability to be saved and shared. SCOT can be used to frame the problem as a conflict interpreted by patients who may perceive a resolution that hasn't taken place, and both soft and hard TD respectively can be used to frame the problem as arising directly from design aspects and as resulting from the design's influence on the user. As the problem clarifies and the solution is just out of sight, one thing remains clear: the way forward will require more social and technological innovation—and a lot more appreciation for the little guy.

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