FINE FOCUS

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An International Microbiology Journal for Undergraduate Research

Front cover and title page art is the original watercolor work of Sharon Cung, BSU Elementary Education major and artist. The image depicts the yeast *Saccharomyces cerevisiae*, after Sharon studied it for the first time microscopically (using the *Fine Focus* lens!). In addition to the use of this yeast in the beer and bread industry, this industrially important microorganism is also an ideal model organism for cloning & expression studies.

As a nod to undergraduate research, we featured this image on our cover design because the undergraduates working in the laboratory of a member of our faculty, Dr. Eric Rubenstein, study protein quality control mechanisms in *S. cerevisiae*. Their work has been featured in a prior issue of *Fine Focus* (Vol. 6:76-83 (2020)); for additional information on this important yeast species and details on Dr. Rubenstein's research, please visit vjrubenstein.wixsite.com/rubensteinlab

We are excited that Sharon has combined art and science and has transformed the look of our journal beginning with this issue.

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Objective Lens

John L. McKillip, Managing Editor, Fine Focus

Professor, Microbiology, Ball State University

Fine Focus was not only the first international journal for undergraduate microbiology research, but also the Ball State University Chapter of the American Society for Microbiology (BSUASM) – that is, a campus recognized student organization, and an honor's course at BSU. Students in both the club and the class manage the journal in all ways – from stewardship of our double-blind peer review system for manuscript management (via our OJS – Open Journal System) – to marketing, promotion, and design changes inside and out on the physical print copy of each issue. This new issue represents a completely new design for *Fine Focus*. For example, the artwork on the cover and on each title page is an original watercolor by one of our student Marketing Team conveners, Sharon Cung. This design represents the industrially important yeast *Saccharomyces cerevisiae*, as explained further inside the front cover. Since our inception in 2013, *Fine Focus* is managed as an "immersive learning" course at BSU – that is, a product-based course with the following unique attributes:

- A project offered to undergraduate students for academic credit
- Is conducted under the guidance of a faculty mentor
- Fills a need or solves a problem for a community partner
- Results in a specific outcome or deliverable
- Is conducted by a team or teams of undergraduate students (in classes of 10 or more) working collaboratively

These faculty-led, student-driven team model puts the students at the helm of day-to-day decision making (1). What better way to further introduce the effectiveness of this model than to introduce you to the team of students who put together the issue you are reading now. Their profiles are on the following pages.

Rylee Catey

Rylee is a pre-pharmacy student and has been part of the Review Team during spring 2022. Rylee was instrumental in reaching out to the Small World Initiative to establish a rapport with this dynamic organization and offer our journal as a source to prioritize and publish manuscript submissions dealing with crowdsourcing antibiotic discovery by undergraduates.

Harrison Clifton

Harrison, a Psychological Science major, was also a key member of our Review Team, serving as Team Convener and Lead Editor for one of our submissions over the spring semester.

Sharon Cung

An elementary education major, Sharon's legacy with *Fine Focus* is her cover art; this issue depicts the industrially important yeast Saccharomyces cerevisiae, also ideal as a host cell for heterologous protein expression. Sharon's artistic talents will continue in future issues as well.

Victoria Johnson

A journalism (magazine editing) major, Victoria's efforts led to an updated logo, on each page number of our new issue, and layout of multiple articles prior to going to press. Victoria proposed how each published article would appear in terms of style and font, and her design eye contributed greatly to our new look.

Laura Lemen

Laura is a biology (pre-vet) major and also is completing her honor's thesis research in microbiology – specifically investigating effects of lactoferrin on growth and survival of Listeria monocytogenes and *Bacillus* cereus in milk. Thus, Laura's insight and contributions on the Review Team were much appreciated.

Reece Malchow

An English major, Reece served as a Lead Editor on one of our manuscript submissions.

Alaina Marks

As a music education major, Alaina was courageous enough to serive as a Review Team Convener, and a Lead Editor on two manuscript submissions for this current issue. Alaina's meticulous nature and affable leadership set a positive tone for the entire class over the academic term.

Shannon McCloskey

Shannon brought her prior experience in print and digital media/journalism into *Fine Focus*, and developed much of our cover layout for this newly redesigned issue. Shannon's photography skills are on display, as she arranged for our head shots and photo shoot for this perspective feature.

Morgan Upton

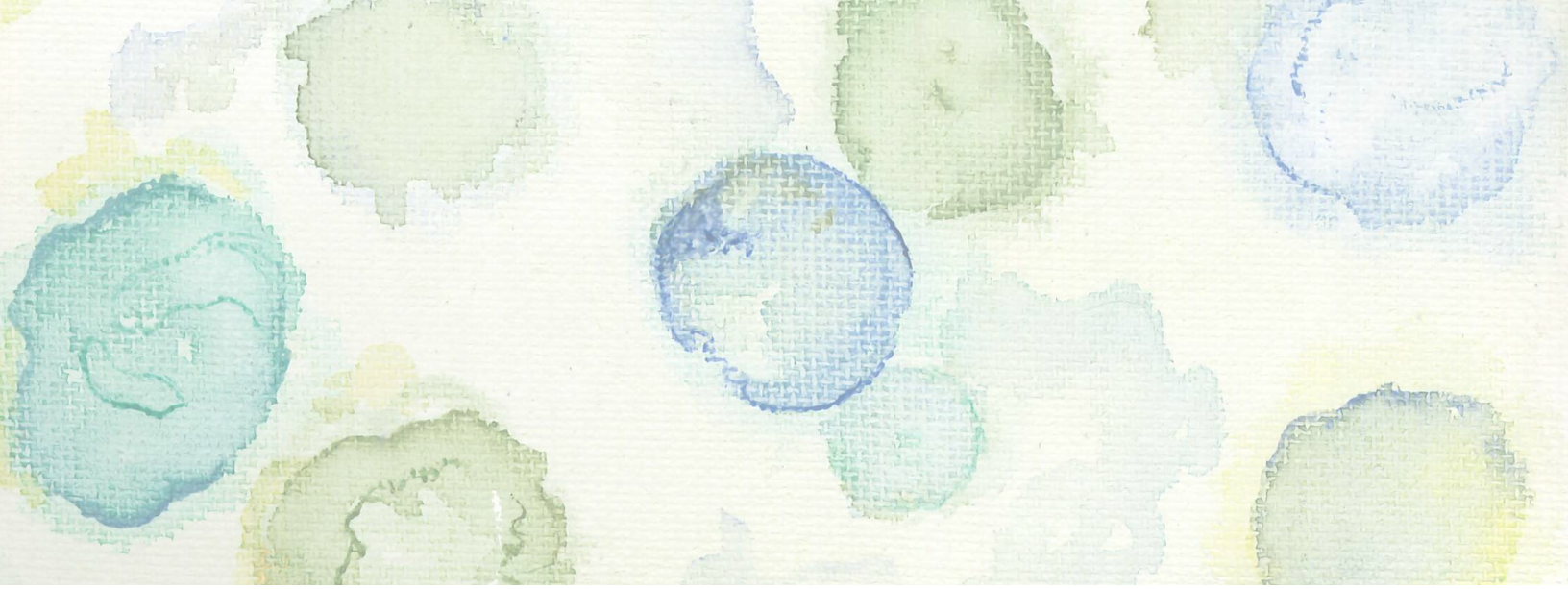
Morgan's biology major allowed her to serve as Review Team Convener, and contribute her scientific insight in our many manuscript review discussions for this issue.

Matt Welch

As an architecture major, Matt served as a liaison between our *Fine Focus* Honor's class, and the Student *Fine Focus* Club, particularly with regard to developing a fundraising idea to sell biology and chemistry themed stickers (available upon request, along with free print copies of this latest issue).

References

1. McKillip, J.L. 2009. Transformative Undergraduate Research: Students as the Authors of and Authorities on Their Own Education. Council on Undergraduate Research (CUR) Quarterly 30 (2):10-15.



Phage Therapy: Challenges and Opportunities

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Abstract

The development of antibiotic resistance in bacteria is a growing concern. This situation demands a search for antibiotic alternatives. Bacteriophages—natural viral predators of bacteria—are viewed as a possible alternative to treat bacterial infections. Many clinical trials today have not found phages effective as therapeutics. Some of the major challenges regarding usage of bacteriophage as a therapeutic have been: horizontal evolution of bacteria, limited host range of bacteriophage, removal of endotoxins in preparations, the technical feasibility of isolation, mode of administration, rapid clearance and immune rejection. These issues have been addressed in this review. Applications of genetic engineered phages and other remarkable non-human applications are also discussed.

Introduction

After the Golden Age of antibiotics (1950-60), use of antibiotics as the first line of defence has increased dramatically (1). Inappropriate prescription of antibiotics by clinicians is a major problem today (2). Seventy percent of antibiotic use in the USA is attributed to use on cattle (3). Indiscriminate usage and misuse of antibiotics have accelerated the emergence of antimicrobial resistance. A growing list of infections is becoming harder to treat, as antibiotics are becoming less effective (4). Sir Alexander Fleming expected the arrival of the antibiotic resistance era and was worried about the rise in antibiotic resistance by self-medication (5). It is estimated that by 2050, bacterial infections will cause 10 million deaths every year (6, 7). Alternatives are urgently needed to effectively treat these infections and prevent the return of pre-antibiotic era.

There is active research currently undertaken for the development of novel classes of antibiotics (8, 9). Bacteriophages (also known as phages) might provide us with a promising alternative for antibiotics. These are the viruses that infect bacteria and are the most abundant living entities in the world. It is predicted that every millimeter of a natural sample has 10^7 phage particles (10). The application of phage as therapeutics against bacteria is called phage therapy (Bacteriophage Therapy). Phage therapy is drawing global attention due to the rise in antimicrobial resistance. Early studies on phage therapy were conducted in Georgia (11). To date, only a few clinical trials have been conducted to modern standards (randomized, placebo-controlled, double-blinded) by the United States (US) Food and Drug Administration (FDA) as well as the European Medical Agency (EMA) jurisdictions.

It is necessary to re-evaluate the challenges involved in phage therapy. Here, a review of the challenges, possible solutions, safety and concerns for therapeutic phage applications is presented. Other potential applications of phages and their studies in humans are also discussed.

Why the Forgotten Magic?

Bacteriophages were first discovered independently by Frederick Twort in 1915 (12) and Félix d'Herelle in 1917 (13). d'Herelle realized the potential of these devourers of bacteria as a therapeutic and conducted further research on phages. One of the early investigations by d'Herelle was in India in 1927 (14). The mortality rates of cholera-infected study subjects decreased from 66.66% in control groups to 5.8% in phage treated groups. Phages offered a great scope of enquiry, but the simplicity in the production of antibiotics gave antibiotic therapy a lead over phage therapy.

The global spread of antibiotic-resistant bacteria and the comeback of the pre-antibiotic era alarmed the scientific community and warranted a search for antibiotic alternatives. Phage therapy is a superior alternative to antibiotics with many theoretical advantages. While antibiotics kill bacteria broadly, phages bind and infect the bacteria specifically. High specificity is an important advantage, as it might minimally impact beneficial microflora, making phages safer than antibiotics. The specificity also limits the number of bacterial types gaining specific phage-resistance mechanisms (15).

Bacteriophages follow lytic and lysogenic pathways of infection. Phages that follow the lysogenic pathway, integrate their genome into the bacterial genome, eventually lysing the cell. Phages following the lytic pathway enter bacteria, reproduce within and lyse the cell (Figure 1). These released phages infect other bacteria. In this process, the number of phages increases exponentially. This exponential growth of phage is advantageous, as theoretically, a smaller dose is needed. The exponential increase is seen specifically where hosts are present, making phages themselves contribute to the dosage at the required site (16).

Biofilm forming bacteria cause infections such as bacterial vaginosis, urinary tract infections, and middle-ear infections. Biofilms are polymeric matrices produced by bacteria as a defence mechanism that allows them to adhere to surfaces (17). Even when a bacterium is sensitive to an antibacterial agent, the antibiotic fails to penetrate through the biofilm matrix, increasing the resistance of the bacteria by 1000-fold (18). A phage has a distinctive capability of tackling biofilms efficiently (5) by encoding depolymerases that allow their direct penetration into the biofilm (19). Phages are also

known for stimulating an immune response. Receptor-binding proteins of a few phages display collagen motifs (20), which can co-stimulate the number and longevity of T cells (21). Furthermore, Van Belleghem *et al*. (22) demonstrated that phages can induce reproducible immune responses from monocytes. Recent studies have also shown that phages have antiviral properties and that phage therapy may also hold promise as a treatment for SARS-82 CoV-2 (23, 24).

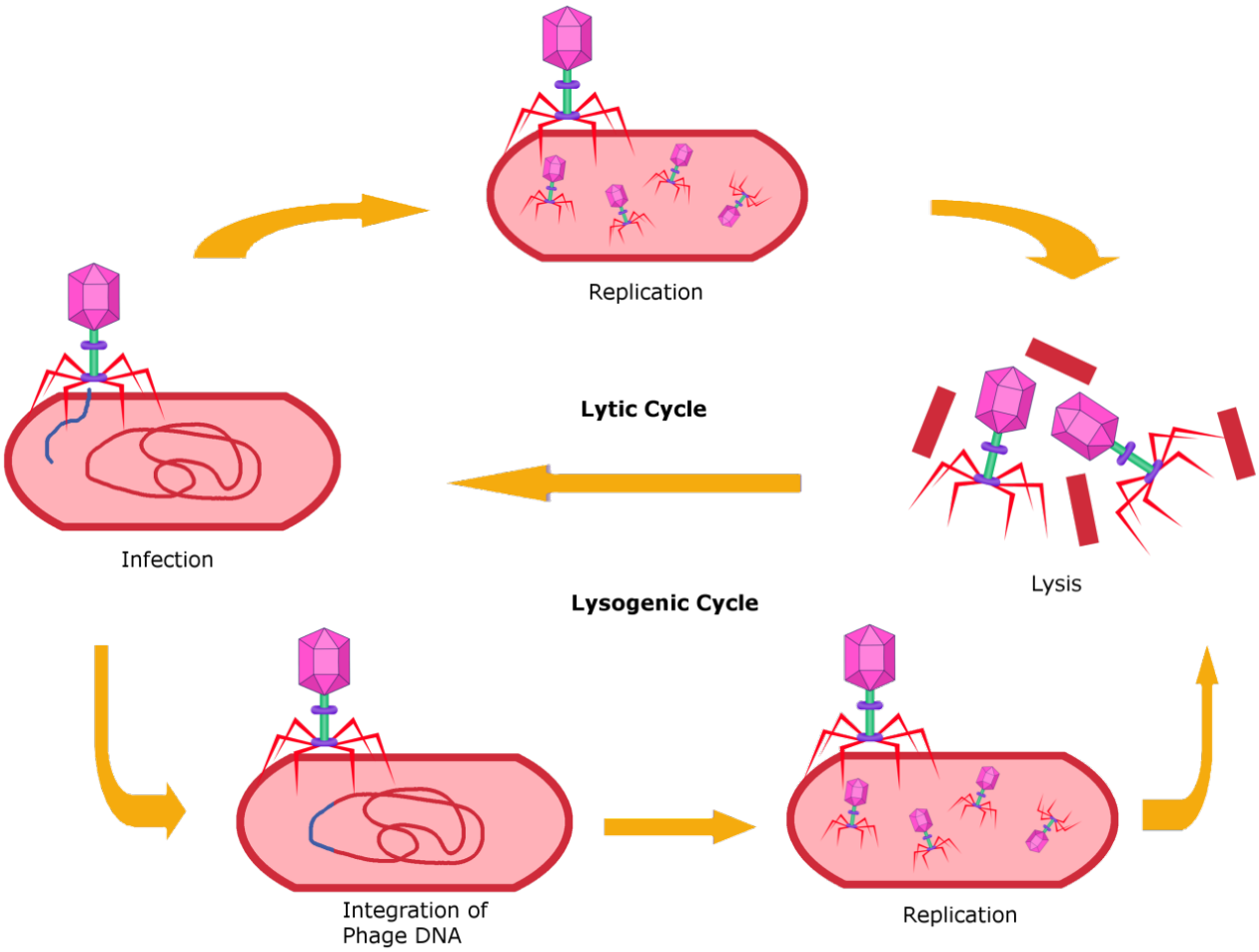

***Figure 1*: Phage attaches to the host cell and injects DNA to initiate the infection. In lytic cycle, phage DNA and proteins are synthesized and assembled into virions. These replicated phages lyse the cell wall and infect another host. Lysogenic cycle involves an additional step of integrating their genetic material to form an endogenous prophage, which compromises with the safety of the therapy.**

Challenges and Potential Solutions in Phage Therapy

1. Host Range

Challenge:

Bacteriophages selectively bind to specific receptors of bacteria, which confer a relatively narrow range of infectivity (25). This would narrow the infectivity range challenging the choice of phage for therapeutic use. As a minimum requirement, phages used in phage therapy should follow only the lytic pathway to ensure the safety of the patient (Figure 1). Bacterial infections, where the currently isolated phages lack a lytic cycle, are therefore not treatable with phages, and the bacteria include *Rickettsia, Coxiella africanum, Mycobacterium leprae, Proteuspenneri, Citrobacterkoseri, Salmonella arizonae, Porphyromonas* spp, and *Hafniaalvei* spp (26).

Potential Solutions:

Antibiotics: At present, phage therapy is generally considered as a last resort when a single bacterial strain dominates. In such situations, the synergistic use of antibiotics like ciprofloxacin in combination with phages can reduce the bacterial load by 10,000 times (27, 28). This combined therapy can boost bactericidal activity with their different mechanisms of attack. However, the choice of the combination is crucial. Antibiotics must not interfere with phage replication (29).

Phage Cocktails: Another possible solution is by employing a combination of phages (so-called phage cocktails), which cover a large spectrum of bacterial strains. Bacterial isolates of a patient are screened against a library of lytic phages for infection susceptibility. The infectious phages are administered together as multivalent phages (30). D'Herelle's pyophage and intestiphage (11) are a few well known accessible commercial phage cocktails.

Genetic Engineering: Genetic engineering can be used to improve the host range by modifying tail fibres (31). It can also improve the efficacy of phage therapy by converting a temperate phage to a lytic phage by removing its repressor genes (32).

2. Endotoxins

Challenge:

In the recent PhagoBurn clinical trial, a team of doctors had to reduce the dosage of phage administration from expected 10^6 PFU/ml to 10-100 PFU/ml due to high endotoxin concentrations in the phage preparations (33). Bacterial debris may remain in the phage preparations even after filtration. In the historic era (around 100 years ago) of phage therapy, not all the debris was removed, and the authors reported a few chemical contaminants which brought about death and illness (30, 34–36). The typical phage purification process (Ultracentrifugation in CsCl gradient) requires intensive labour, high expense and is time consuming (37).

Potential Solutions:

The bacterial debris, having pyrogens and toxins (38), can be cleared and high purity levels can be achieved with nanofibrillated filters (39). Endotoxin removal proteins are now available commercially (40). For a large scale production, usage of surrogate hosts could offer a superior solution(37).

Challenge:

In recent clinical trials, patients suffered many side effects due to increased concentrations of endotoxins among which abdominal pain, sudden fever and chills were common. Many biologists attribute endotoxins as the prime cause for these side effects (41). Expression of endotoxin genes in phages or rapid lysis of bacteria in patients can release toxins (38, 42).

Potential Solutions:

A therapeutic phage having a Lys^- gene (endolysin-deficient phage) cannot lyse the peptidoglycan layer of bacteria after infecting the bacteria. Phages attack the bacteria and do not lyse the host membrane, which does not lead to the release of endotoxins. The macrophages then eliminates these incapacitated bacteria (43).

3. Immune Rejection and Rapid Clearance

Challenge:

Large phage titers trigger the release of neutralizing antibodies in high amounts (44), which would hinder the action of phages. Being in continuous exposure with phages, 81% of healthy individuals show antibodies to T4 phage, prior to the treatment itself (45). Though the phage kinetics are much faster than the release of neutralizing antibodies (30, 46), the presence of such anti-phage antibodies before phage administration, and the release of anti-phage antibodies during the treatment brings concerns (47).

Potential Solutions:

Liposomal delivery of phages can decrease the clearance rate of phages by guarding the phages against anti-phage antibodies (48). Frequent administration of phages can reduce the rate of neutralizing antibodies (49). Cell-mediated immunity can be combated by making the phage protein coat express polyethylene glycols, which increase the phage circulation time in the blood (50).

Challenge:

Geier *et al.* (51) first observed rapid clearance of phages when Lambda phages were injected in high titers into transgenic mice lacking immune response. The administered phages are rapidly cleared by the reticuloendothelial system (RES) and are not available for therapeutic use in the body (52, 53).

Potential Solutions:

Longer circulating phages can be obtained by "serial passage" into the bloodstream of a mouse, and selecting phages with higher circulation time than the original wild-type phage (54).

According to Levin and Bull (55), phage treatment should only decrease the pathogen to an extent where the immune system can successfully clear the bacterial load. Phage engineering can help us generate phages that don't replicate or proliferate (56, 57). These can make an immune safe therapeutic phage.

4. Horizontal Gene Transfer

Once a phage infects a bacterium, the phage genome is replicated inside the host and eventually, phages assemble and lyse the bacterium (Figure 1). While the phage genome gets packed in the phage capsids, accidentally 1 in 10^7 phages receive the bacterial genome (58). This phage is now called a transducing particle. Infection of transducing particles in bacteria causes Horizontal Gene Transfer (HGT) or transduction (59). Transduction enhances HGT of virulent, resistant, metabolic and other fitness genes (60, 61), which enable the bacteria to rapidly adapt and evolve to changing environmental conditions.

4.1 Development of Phage Resistance

A complication of phage therapy is that the bacteria can gain resistance to the phage during the treatment (15, 62–64). Among 12 phage therapy clinical trials, seven studies reported phage resistance (65). Bacteria show phage resistance generally by modifying phage receptors (64). Often, such changes would affect bacterial fitness and could reduce its virulence (66, 67) as seen in Tom's case (see Human Trials- below).

The development of phage resistance is partially advantageous, but phages interfere with many cellular pathways, including translation, transcription, replication, and so, it is harder for bacteria to gain resistance against phages compared to antibiotics (68). While Khawaldeh *et al*. (69) did not find any development of phage resistance after administering a phage cocktail to a patient detected with *Pseudomonas aeruginosa* UTI (Urinary Tract Infection), Zhvania *et al*. (70) did report phage resistance in *Staphylococcus aureus* after phage administration to a patient with Netherton Syndrome.

5. Intracellular Treatment

Challenge:

Antibiotics can treat intracellular bacterial pathogens (like *M. tuberculosis*) as they have the capability of entering the

cell (71). A phage requires bacterial receptors to bind and kill a pathogen. The inability of phages to enter macrophages brings concern in tackling intracellular pathogens using phage therapy. Internalization of phages into the infected cells is a crucial step to treat intracellular pathogens.

Potential Solutions:

Targeting extracellular stage: Phage therapy was found to efficiently decrease pathology and prevent ulceration in *Mycobacterium ulcerans* infection, where phages targeted a temporary extracellular stage of the bacterium (72).

Using cell penetrating peptides: The model phage M13 decorated with cell penetrating peptides was localized in the ER, Endosomes, and Golgi within 6 hours of internalization in Hela cells (73). However, phages displaying such peptides might circulate for a lesser time (74).

Liposomal internalization: Liposomes with a positive charge fuses with the negatively charged cell membrane to deliver phages inside the cell membrane (75). Using non-pathogenic host: TM4 phage was utilized for intracellular drug delivery into infected macrophages. Non-pathogenic *M. smegmatis* was loaded with phages, and these were phagocytosed by macrophages. The phage lytic cycle then reduced intra-phagosomal bacterial counts (76).

6. Which Route Works Best?

6.1 Oral

Oral administration of a therapeutic is the most convenient and often desired mode of administration. Oral delivery of phage poses two major challenges regarding viability and gut transit.

The lower survival rates of phages (7%) meeting the hostile acidic environment of the stomach (77) presents a major challenge. Even, administering phages with alkali could increase the risk of opportunistic infections (78, 79). Administration of phages with yoghurt, or encapsulation of phages, are a possible solution to enhance the survival rate of phages (80, 81). Genetically modified phages could offer a simplified and cheaper methodology than encapsulation (82).

The gut transit of phages is a question without a clear understanding. There are examples of phages entering the bloodstream (83–85) and not entering the bloodstream (54, 86, 87) after oral administration. Furthermore, Majewska *et al*. (88) showed that phage-induced IgG and IgA hinder the gut transit of phages. Experiments by Międzybrodzki *et al*. (80) conclude that the phage entry into the bloodstream depends on the type of phage and the host. Therefore, more studies need to be conducted to find specific phage strains which can easily enter the human gut.

6.2 Other Routes

The most effective mode of phage administration is unclear yet. For pulmonary infections, phage delivery through inhalation seems a more convenient and effective administration (89, 90). Contradicting this view, few studies show greater effectiveness through intraperitoneal and intravenous routes of administration for pulmonary infections (91, 92).

Although intravenous administration of phages strongly elicits an immune response, Czaplewski *et al*. (93) believe that phage administration intravenously is also a promising alternative for antibiotics. The two famous success stories in phage therapy (see Human Trials) utilized systemic phage delivery. Considering simplicity and effectiveness, topical administration is highly advisable for eye, ear, nose and skin infections; oral administration for gut infections; intrarectal for prostate infections; and intravenous for systemic infections (94).

7. Side Effects in Phage Therapy

Until recently, phages were considered safe for human use as they selectively bind to only bacteria. Also, the abundance of phages in the human body indicates that phages are inherently safe since we are continuously exposed to them enterically and topically (34, 95). However, recent studies question this concept of phages "Generally Regarded as Safe" (96). A systematic review conducted by Steele *et al*. (97) concludes that there is limited evidence supporting the safety of phages. Tetz *et al*. (98) goes on to call phages "Potential Mammalian Pathogens."

The complex interactions of phages with the human body can cause serious side effects in phage therapy. Few such side effects could be chronic glomerulonephritis by the accumulation of antiphage-antibody complexes in the glomerular region (23, 99); increased concentration of endotoxins and inflammatory cytokines in the blood (84, 98); increased gut permeability, weight loss, messy hair (100); sudden fever and chills (101). Another potential side effect could be the unpredictable consequences of the human microbiome by the introduction of phages (102).

The most feared phenomenon by many phage biologists is the integration of virulence genes—like Cholera toxin, Staphylococcal enterotoxin and Shiga toxin—from the phage that can enhance the virulence armoury of bacteria. This could lead to the "evolution of new human pathogens" (103). It is therefore necessary that the therapeutic phages must be fully sequenced to confirm the absence of undesirable genes such as toxins.

8. Technical Feasibility

In a review by Czaplewski *et al*. (93) about alternatives for antibiotics, phage enzymes were thought to have the highest potential to replace antibacterials, while phages were scored relatively lower for their technical feasibility. Unlike antibiotics, phage (virus) preparation and storage is costlier. The shelf-life of phages must be long enough for laboratory study or commercial application (104). A therapeutic bacteriophage should not lose its activity before treating patients. Attainment of stability is a crucial part of development (105). The phage should grow well under industrial and laboratory cultures and must be easy to store and maintain (37). Since every phage strain has different optimal conditions (temperature, pH, buffer) for preservation, not all the diverse number of phages can presently be stored efficiently. Lyophilization and spray-drying (107) are current methods available to obtain phage powders, and the optimal conditions of storage for different therapeutic phages are yet to be explored. Zhang *et al*. (106) have successfully produced a freeze-dried phage powder of the model phage M13.

9. Other Challenges

To date, bioethical theories regarding phages have not been published; Intellectual Property protection is limited in the case of natural phages (108); Statistically evident double-blind clinical studies were not reported in adequate numbers. These factors create uncertainty in the development of a dedicated regulatory framework for phage therapy. The peculiar characteristics of phages have made their clinical assessment more complex, demanding further clinical research. Regardless of whether there is abundant research performed, commercial implementation of phage therapy would pose a significant challenge. Investment factors as well as profitability are exceedingly unknown (95), which hinders pharmaceutical investments (109).

Potential Solutions: In-depth research and large-scale phage production is possible through industrial investments. Phage producing centres and hospitals should have good collaboration (110) and be able to work together to provide positive evidence for the regulatory bodies which would grab the attention of pharmaceutical industries.

Human Trials

In vitro studies of phage therapy do not consider complex biological interactions, which influence the treatment. Our knowledge of phages *in vitro* is exceptional, but in vivo behaviour of phages is less well-known (111). This demands a need to study phages in a biological system (preferably in humans) to evaluate the efficacy and side effects of the treatment. Two major institutes conducting such investigations since the historical era (1917-1995) are Eliava Institute of Bacteriophages, Microbiology and Virology (IBMV, Georgia) and Hirfeld Institute of Immunology and Experimental Therapy (ITET, Poland). These institutes, along with Felix d'Herelle Reference Center for bacterial viruses (Canada), The Leibniz Institute DSMZ (Germany), and Queen Astrid Military Hospital (Belgium) are empowered with huge phage banks to store therapeutic phage cocktails (112–114). Eliava Institute has treated around a hundred foreign patients since 2012, and the numbers are expanding every year (115). The US Navy has also developed

a proprietary capacity to purify phage strains for specific infections (116). Phage therapy is now being studied globally due to the development of antibacterial resistance. One of the earliest and well-designed controlled trials in Georgia was during the 1960s (112). A total of 30,769 children less than 7 years were included. During the annual dysentery period, an anti-Shigella phage cocktail targeting Shigella boydi, S. newcastle, S. sonnei, S. flexenerei was administered to the children present on one side of a street. The children on the other side of the street received a placebo. A nurse reviewed the subjects once a week for 109 days. Dysentery was encountered by 1.76 in 1000 children receiving phage treatment, while 6.7 in 1000 from the controlled group were affected. But, many such studies conducted in the historic era (1917-1995) needed methodological evidence, and the results are not reliable and reproducible (30). Many phage biologists, therefore, believe these results to be spurious. A need for clinical trials set to modern standards—placebo-controlled, randomized, double-blind studies—are required to settle the debate on the efficiency of phage therapy. The first US-FDA approved phase I clinical trial was executed in 2009 (117), and numerous other studies followed. Most of the studies concluded phages as not a significant therapeutic (Table 1). Many of these studies failed to recruit statistically significant numbers, having small patient groups, which drastically limited the conclusions drawn (10). A recent Phase II clinical trial (Phagoburn) approved by France, Belgium, and Switzerland national health regulators was terminated

Table 1: **Data from clinical trials evaluating the efficacy of phages as therapeutic agents**

Year of Study	Problem and Etiologic agent	Route of Administration	Success Rate	Reference
1981 to 1986	Suppurative infections by *Staphylococcus, Pseudomonas, E. coli,, Klebsiella,* and *Salmonella*	Various	92% (n=550)	(41)
1987 to 1999	Suppurative infections by *Escherichia, Klebsiella, Proteus, Enterobacter, Pseudomonas* and *Staphylococcus*	Oral, Local, Intraperitoneal, Topical	86% (n=1307)	(84)
1987	Skin infections by *Pseudomonas, Staphylococcus, Klebsiella, Proteus* and *E. coli*	Oral and Local	74% (n=31)	(118)
1989	Post operative wound infections by *Pseudomonas* and *Staphylococcus*	Local	82% (n=65)	(119)

Year of Study	Problem and Etiologic agent	Route of Administration	Success Rate	Reference
1992	Skin and nasal mucosal infections by *K. ozaenae, K. rhinoscleromatis* and *K. pneumoniae*	Intraperitoneal	100% (n=109)	(120)
1995	Urinogenital inflamation by various agents	Oral and Local	92% (n=46) (8% more than antibiotic treated group)	(121)
2009 (Double Blind)	Otitis by *P. aeruginosa*	Local	76% decrease in bacterial count (n=12)	(122)
2009 (Double Blind)	Chronic venus leg ulcers by *P. aerginosa, S. aureus,* and *E. coli*	Local	No significant difference from control. (n=39)	(117)
2016 (Double Blind)	Diarrhea by *E. coli*	Oral	No significant decrease in diarrhea in different groups	(83)
2018 (Double Blind)	Urinary Tract Infection by *Staphylococcus aureus, E. coli, Streptococcus spp., Pseudomonas aaeruginosa, Enterococcus spp.*	Intravesical	Decrease of bacterial titers is 67% (n=9)	(101)
2019	Burn wounds by *P. aeruginosa*	Topical	69% Cured, 23%-adverse, 1 person died (n=13)	(33)
2020	Systemic infection with *Staphylococcus aureus*	Intravenous	61.5% cured within 7 days (n=13)	(123)

prematurely (several times before the trial had started), as the eligible patient recruitment was inadequate (33).

In light of the WMA-Declaration of Helsinki, which states "In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgment it offers hope of saving life, reestablishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published." (124), physicians offer phage therapy as the last resort. Often this includes combined therapy with antibiotics and in many cases, the patients recovered.One such case is that of Tom Patterson who is a 68-year-old professor in the Psychiatry Department, University of California Medical School. During his vacation to Egypt, he contracted a systemic infection (initially thought to be Food poisoning) by MDR (Multi Drug Resistant) A. baumannii. All standard antibiotic treatments failed. His wife Steffanie Strathdee—Associate Dean of Global Health Sciences, University of California—obtained an emergency authorization for treating her husband with phages. Tom was administered phage cocktails intravenously. There was a change in antibiotic resistance profiles. Bacteria had developed phage resistance. Tom finally got treated. The team then received a $1.2 million grant over three years and became the directors of IPATH (Innovative Phage Applications and Therapeutics) (79, 125, 126).

Another case study is that of Isabelle Holdaway, a 15-year-old girl who suffered from P. aeruginosa and *Mycobacterium* abscessus infection. Doctors performed a lung transplant, but the infection was still not cleared. One month post the transplant, *Mycobacterium* abscessus was isolated and the patient was diagnosed with a Mycobacterial infection. Though her survival chance was predicted to be less than 1%, doctors gave a try for phage therapy. Holdaway's Mycobacterial isolates were screened against more than 10,000 phages. Two of the 3 selected phages were following temperate life cycle. Bacteriophage Recombineering of Electroporated DNA (BRED) was used to prepare the lytic derivative of these phages. The cocktail of these phages was used for phage therapy. Holdaway received the intravenous phage treatment without any significant side effects. She was discharged after 9 days of her treatment. After 11 months of her treatment, virtually all her lesions disappeared (32, 127). Evaluation of clinical trials is necessary to evaluate the effectiveness of phage therapy (see Table-1). Phages are found to be safe in all the trials, but only a single double-blind clinical trial claims the efficacy of phage therapy. Thus, well-designed clinical trials are highly warranted to further evaluate the efficacy of phage therapy.

Other Applications

Bacteriophage has its application in varied arenas, from targeting MDR infections to targeting rot in harvested potatoes. Phages also may be used in decontaminating the hospital environment, which would decrease the incidence of nosocomial infections.

1. Agriculture and Food Safety

Bacteriophage application is becoming advanced in animal husbandry, food safety, and agriculture (128). It was in 2005 when for the first time, a bacteriophage product—Agriphage™—was formally approved by the regulatory agency of the US government to treat crop diseases (129). Since then, many phage products have hit the commercial markets (Table 2). The use of phages in agriculture was also exploited by OmniLytics Inc. When a customer sends in infected plant material, customized phage products are prepared and given to the customer (37).

Phages are also gaining popularity in the food industry to attack foodborne bacterial pathogens. In 2006, the FDA approved ListShield™, a phage cocktail targeting Listeria monocytogenes (which contaminates ready-made food products) as safe for consumption. Few other products include AgriPhage, BioTector, Ecoshield, Finalise, ListShield (130). Other than phages, phage-derived enzymes are also attractive investments. The usage of phage lytic enzymes in food conservation (as an antibacterial) was first reviewed in 2005 (131). Few commercially available enzymes are LISTEX™ & LMP-102 (from phages targeting Listeria) (132), ECP-100 (from *Escherichia coli* O157:H7 phage)

Table 2: **Examples of commercially available phage products**

Product Name	Company	Targeted Bacteria	Reference
LMP 102	Intralytix	*Listeria monocytogenes*	(135)
ListShield	Intralytix	*Listeria monocytogenes*	(136)
EcoShield	Intralytix	*E. coli O157:H7*	(137)
SalmoFresh	Intralytix	*Salmonella spp.*	(138)
Shiga Shield	Intralytix	*Shigella flexneri, S. sonnei, S. dysenteriae*	(139)
ListeX™ P100	Micreos Ltd	*Listeria monocytogenes*	(132)
SalmoPro	Phagelux Inc	*Salmonella enterica*	(140)
AgriPhage	Certis USA LLC	*Xanthomanas campestris*	(141)
PhageGuard	Micreos Food Safety	*L- Listeria monocytogene S- Salmonella E- E. coli O157*	(142)

(133), and SalmoFresh™ (from phage targeting Salmonella enterica) (134) are used in the food industry.

2. Promising Applications

Usage of phages is highly promising in eye drops and antiseptics, which follow topical administration. Such commercial phage products are highly effective and can attract high investments from pharmaceutical companies. Eye drops, for example, were found to be a statistically significant treatment for *P. aeruginosa* infection (143). Regarding antiseptics, Eliava Institute in Georgia has developed a commercial biopolymer bandage, with phage cocktails called "PhagoBioderm". For the development of other such commercial phage therapy products, projects like PhagoFlow (144) and PhagoMed (145) are implemented.

3. Genetic Engineered Phage

The lack of efficiency and other challenges met by phages in the therapeutic domain in the modern synthetic biology era can now be met by genetic engineering of phages (113, 146, 147). Genetically engineered phages have found their application in many other areas. Phage engineering is used for the targeted delivery of phages (148) and is also being exploited for protein and gene delivery. Tao P. *et al.* delivered proteins and genes in *vitro* and in vivo by using the T4 phage (149). Przystal *et al*. used phages as vectors to target orthotopic glioblastoma and suppressed the growth of glioblastoma by a systemic combination of temozolomide and suicide gene therapy (150). Folate-conjugated M13 coated by Poly(caprolactone-b-2-vinylpyridine) which encapsulated hydrophobic antitumor drug doxorubicin acted as a nanosized drug delivery vehicle (151). Phage coat protein can be modified by expressing immunogenic peptides that could deliver vaccines (152). Phages are also exploited to edit the microbiota by artificially synthesizing phages and modifying their tail fibers (153).

Summary

Though phage therapy is a promising and attractive source of treatment for emerging bacterial infections, further understanding of phage biology is essential before reimplementation of phage therapy. *In vivo* studies are required on liposomal phage delivery or delivery of genetically modified phages. There is no consensus view on the most effective route of phage administration, dosage and pharmacokinetics. Also, phage interaction with the immune system is not well known when compared to the knowledge we possess in regarding antibiotics.

The efficacy of genetically modified phages is to be evaluated *in vivo*. There is a need to be careful with genetically modified phages used in therapeutics. If phage resistance arises, switching to new phages with different technology, achieving the same efficacy would be difficult.

Programs like the "Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science" (SEA-PHAGES) trains students to isolate phages characterize genomes against a particular pathogen. Such programs need to be conducted with increased rigour across the globe. The establishment of more phage banks to store such newly found isolates can lessen the challenges faced by the host range.

Since phage therapy deals with viruses, the high cost involved in phage isolation is an obvious hurdle for commercial production. For industrial-scale production of phages, a search for surrogate hosts is necessary, which might marginally reduce the production cost. Despite these challenges, the author believes that bacteriophages can lead us into a progressive future with varied applications in agriculture, aquaculture, poultry, sewage treatment, and therapeutic use in humans aquaculture, poultry, sewage treatment, and therapeutic use in humans. aquaculture, poultry, sewage treatment, and therapeutic use in humans.

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References

1. Davies J. 2006. Where have all the antibiotics gone? Canadian Journal of Infectious Diseases and Medical Microbiology 17:287–290.

2. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. Molecules 23:795.

3. Landers TF, Cohen B, Wittum TE, Larson EL. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public health reports 127:4–22.

4. Shao Y, Wang Y, Yuan Y, Xie Y. 2021. A systematic review on antibiotics misuse in livestock and aquaculture and regulation implications in China. Science of The Total Environment 149205.

5. Fleming. 1945. PENICILLIN'S FINDER ASSAYS ITS FUTURE; Sir Alexander Fleming Says Improved Dosage Method Is Needed to Extend Use Other Scientists Praised Self-Medication Decried. The New York Times.

6. O'Neill J. 2014. AMR Review. https://amr-review.org/sites/default/files/One%20person%20every%203%20seconds%20white.jpg Accessed on 25/8/2021.

7. Drug-Resistant Infections: A Threat to Our Economic Future. World Bank. Text/HTML. https://www.worldbank.org/en/topic/health/publication/drug-resistant-infections-a-threat-to-our-economic-future. Accessed on 13/8/2021. World Bank. Text/HTML.

8. Chodisetti PK, Reddy M. 2019. Peptidoglycan hydrolase of an unusual cross-link cleavage specificity contributes to bacterial cell wall synthesis. PNAS 116:7825–7830.

9. Coates AR, Halls G, Hu Y. 2011. Novel classes of antibiotics or more of the same? British journal of pharmacology 163:184–194.

10. "Expert round table on acceptance and re-implementation of bacteriophage therapy," Sybesma W, Rohde C, Bardy P, Pirnay J-P, Cooper I, Caplin J, Chanishvili N, Coffey A, De Vos D, Scholz AH, McCallin S, Püschner HM, Pantucek R, Aminov R, Doškař J, Kurtboke Dİ. 2018. Silk Route to the Acceptance and Re-Implementation of Bacteriophage Therapy-Part II. Antibiotics (Basel) 7.

11. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon ST. 2010. Phage therapy in clinical practice: treatment of human infections. Current pharmaceutical biotechnology 11:69–86.

12. Twort FW. 1915. An investigation on the nature of ultra-microscopic viruses. The Lancet 186:1241–1243.

13. d'Hérelle F. 2007. On an invisible microbe antagonistic toward dysenteric bacilli: brief note by Mr. F. D'Herelle, presented by Mr. Roux. 1917. Research in microbiology 158:553–554.

14. d'Herelle F, Malone RH, Lahiri MN. 1930. Studies on Asiatic cholera. Studies on Asiatic Cholera, Memoir No 14.

15. Azam AH, Tanji Y. 2019. Bacteriophage-host arm race: an update on the mechanism of phage resistance in bacteria and revenge of the phage with the perspective for phage therapy. Appl Microbiol Biotechnol 103:2121–2131.

16. Bertani G. 1953. Lysogenic versus lytic cycle of phage multiplication, p. 65–70. In Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press.

17. López D, Vlamakis H, Kolter R. 2010. Biofilms. Cold Spring Harbor perspectives in biology 2:a000398.

18. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. Journal of clinical microbiology 37:1771–1776.

19. Cornelissen A, Ceyssens P-J, T'syen J, Van Praet H, Noben J-P, Shaburova OV, Krylov VN, Volckaert G, Lavigne R. 2011. The T7-related *Pseudomonas putida* phage 15 displays virion-associated biofilm degradation properties. PloS one 6:e18597.

20. Lévesque C, Duplessis M, Labonté J, Labrie S, Fremaux C, Tremblay D, Moineau S. 2005. Genomic Organization and Molecular Analysis of Virulent Bacteriophage 2972 Infecting an Exopolysaccharide-Producing Streptococcus thermophilus Strain. Appl Environ Microbiol 71:4057–4068.

21. Sturm A, Krivacic KA, Fiocchi C, Levine AD. 2004. Dual function of the extracellular matrix: stimulatory for cell cycle progression of naive T cells and antiapoptotic for tissue-derived memory T cells. Journal of Immunology 173:3889–3900.

22. Van Belleghem JD, Clement F, Merabishvili M, Lavigne R, Vaneechoutte M. 2017. Pro-and anti-inflammatory responses of peripheral blood mononuclear cells induced by Staphylococcus aureus and Pseudomonas aeruginosa phages. Scientific reports 7:1–13.

23. Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Gorski A. 2005. Bacterial viruses against viruses pathogenic for man? Virus research 110:1–8.

24. Mishra VN, Kumari N, Pathak A, Chaturvedi RK, Gupta AK, Chaurasia RN. 2020. Possible Role for Bacteriophages in the Treatment of SARS-CoV-2 Infection. International journal of microbiology 2020.

25. Hyman P, Abedon ST. 2010. Bacteriophage host range and bacterial resistance. Advances in applied microbiology 70:217–248.

26. Oliveira H, Sillankorva S, Merabishvili M, Kluskens LD, Azeredo J. 2015. Unexploited opportunities for phage therapy. Frontiers in pharmacology 6:180.

27. Oechslin F, Piccardi P, Mancini S, Gabard J, Moreillon P, Entenza JM, Resch G, Que Y-A. 2017. Synergistic interaction between phage therapy and antibiotics clears Pseudomonas aeruginosa infection in endocarditis and reduces virulence. The Journal of infectious diseases 215:703–712.

28. Bedi MS, Verma V, Chhibber S. 2009. Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of Klebsiella pneumoniae B5055. World Journal of Microbiology and Biotechnology 25:1145–1151.

29. Knezevic P, Curcin S, Aleksic V, Petrusic M, Vlaski L. 2013. Phage-antibiotic synergism: a possible approach to combatting Pseudomonas aeruginosa. Research in microbiology 164:55–60.

30. Carlton RM. 1999. Phage therapy: past history and future prospects. ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS-ENGLISH EDITION- 47:267–274.

31. Le S, He X, Tan Y, Huang G, Zhang L, Lux R, Shi W, Hu F. 2013. Mapping the tail fiber as the receptor binding protein responsible for differential host specificity of Pseudomonas aeruginosa bacteriophages PaP1 and JG004. PloS one 8:e68562.

32. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, Gilmour KC, Soothill J, Jacobs-Sera D, Schooley RT. 2019. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus.* Nature medicine 25:730.

33. Jault P, Leclerc T, Jennes S, Pirnay JP, Que Y-A, Resch G, Rousseau AF, Ravat F, Carsin H, Le Floch R, Schaal JV, Soler C, Fevre C, Arnaud I, Bretaudeau L, Gabard J. 2019. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by Pseudomonas aeruginosa (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. The Lancet Infectious Diseases 19:35–45.

34. Kutter E, Sulakvelidze A, Sulakvelidze A. 2004. Bacteriophages : Biology and Applications. CRC Press.

35. Merril CR, Scholl D, Adhya SL. 2003. The prospect for bacteriophage therapy in Western medicine. Nature Reviews Drug Discovery 2:489.

36. Pirisi A. 2000. Phage therapy--advantages over antibiotics? Lancet 356:1418.

37. Gill JJ, Hyman P. 2010. Phage choice, isolation, and preparation for phage therapy. Current Pharmaceutical Biotechnology 11:2–14.

38. Brüssow H, Canchaya C, Hardt W-D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiology and Molecular Biology Reviews 68:560–602.

39. Szekeres GP, Németh Z, Schrantz K, Németh K, Schabikowski M, Traber J, Pronk W, Hernádi K, Graule T. 2018. Copper-coated cellulose-based water filters for virus retention. ACS omega 3:446–454.

40. Petsch D, Anspach FB. 2000. Endotoxin removal from protein solutions. Journal of biotechnology 76:97–119.

41. Slopek S, Weber-Dabrowska B, Dabrowski M, Kucharewicz-Krukowska A. 1987. Results of bacteriophage treatment of suppurative bacterial infections in the years 1981-1986. Archivum Immunologiae et Therapie Experimentalis (Warsz) 35:569–583.

42. McGrath S, Fitzgerald GF, van Sinderen D. 2004. The impact of bacteriophage genomics. Current opinion in biotechnology 15:94–99.

43. Ramachandran J, Padmanabhan S, Sriram B. August 2006. Lysin-deficient bacteriophages having reduced immunogenicity. US7087226B2.

44. Kucharewicz-Krukowska A, Slopek S. 1987. Immunogenic effect of bacteriophage in patients subjected to phage therapy. Archivum Immunologiae et Therapie Experimentalis (Warsz) 35:553–561.

45. Dąbrowska K, Miernikiewicz P, Piotrowicz A, Hodyra K, Owczarek B, Lecion D, Kaźmierczak Z, Letarov A, Górski A. 2014. Immunogenicity studies of proteins forming the T4 phage head surface. Journal of Virology 88:12551–12557.

46. Duckworth DH, Gulig PA. 2002. Bacteriophages: potential treatment for bacterial infections. BioDrugs 16:57–62.

47. Saha D, Mukherjee R. 2019. Ameliorating the antimicrobial resistance crisis: phage therapy. IUBMB life 71:781–790.

48. Chhibber S, Kaur J, Kaur S. 2018. Liposome Entrapment of Bacteriophages Improves Wound Healing in a Diabetic Mouse MRSA Infection. Frontiers of Microbiology 9:561.

49. Sulakvelidze A, Alavidze Z, Morris JG. 2001. Bacteriophage Therapy. Antimicrobial Agents and Chemotherapy 45:649–659.

50. Kim K-P, Cha J-D, Jang E-H, Klumpp J, Hagens S, Hardt W-D, Lee K-Y, Loessner MJ. 2008. PEGylation of bacteriophages increases blood circulation time and reduces T-helper type 1 immune response. Microbial Biotechnology 1:247–257.

51. Geier MR, Trigg ME, Merril CR. 1973. Fate of bacteriophage lambda in non-immune germ-free mice. Nature 246:221–223.

52. Łusiak-Szelachowska M, Żaczek M, Weber-Dąbrowska B, Międzybrodzki R, Kłak M, Fortuna W, Letkiewicz S, Rogóż P, Szufnarowski K, Jończyk-Matysiak E, Owczarek B, Górski A. 2014. Phage Neutralization by Sera of Patients Receiving Phage Therapy. Viral Immunology 27:295–304.

53. Westwater C, Kasman LM, Schofield DA, Werner PA, Dolan JW, Schmidt MG, Norris JS. 2003. Use of Genetically Engineered Phage To Deliver Antimicrobial Agents to Bacteria: an Alternative Therapy for Treatment of Bacterial Infections. Antimicrobial Agents and Chemotherapy 47:1301–1307.

54. Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, Adhya S. 1996. Long-circulating bacteriophage as antibacterial agents. PNAS USA 93:3188–3192.

55. Levin BR, Bull JJ. 2004. Population and evolutionary dynamics of phage therapy. Nature Reviews Microbiology 2:166–173.

56. Hagens S, Habel A, von Ahsen U, von Gabain A, Bläsi U. 2004. Therapy of experimental pseudomonas infections with a nonreplicating genetically modified phage. Antimicrobial Agents and Chemotherapy 48:3817–3822.

57. Matsuda T, Freeman TA, Hilbert DW, Duff M, Fuortes M, Stapleton PP, Daly JM. 2005. Lysis-deficient bacteriophage therapy decreases endotoxin and inflammatory mediator release and improves survival in a murine peritonitis model. Surgery 137:639–646.

58. Bushman F. 2001. Lateral DNA Transfer: Mechanisms and Consequences1 edition. Cold Spring Harbor Laboratory Pr, Cold Spring Harbor, NY.

59. Zinder ND. 1955. Bacterial transduction. Journal of Cellular and Comparative Physiology 45:23–49.

60. Hatfull GF. 2008. Bacteriophage genomics. Current Opinion in Microbiology 11:447–453.

61. Waddell TE, Franklin K, Mazzocco A, Kropinski AM, Johnson RP. 2009. Generalized transduction by lytic bacteriophages. Methods in Molecular Biology 501:293–303.

62. Alves DR, Gaudion A, Bean JE, Esteban PP, Arnot TC, Harper DR, Kot W, Hansen LH, Enright MC, Jenkins ATA. 2014. Combined Use of Bacteriophage K and a Novel Bacteriophage To Reduce *Staphylococcus aureus* Biofilm Formation. Applied and Environmental Microbiology 80:6694–6703.

63. Castillo DE, Nanda S, Keri JE. 2019. Propionibacterium (Cutibacterium) acnes Bacteriophage Therapy in Acne: Current Evidence and Future Perspectives. Dermatology Therapy (Heidelb) 9:19–31.

64. Labrie SJ, Samson JE, Moineau S. 2010. Bacteriophage resistance mechanisms. Nature Reviews Microbiology 8:317–327.

65. El Haddad L, Harb CP, Gebara MA, Stibich MA, Chemaly RF. 2019. A systematic and critical review of bacteriophage therapy against multidrug-resistant ESKAPE organisms in humans. Clinical Infectious Diseases 69:167–178.

66. Capparelli R, Nocerino N, Lanzetta R, Silipo A, Amoresano A, Giangrande C, Becker K, Blaiotta G, Evidente A, Cimmino A, Iannaccone M, Parlato M, Medaglia C, Roperto S, Roperto F, Ramunno L, Iannelli D. 2010. Bacteriophage-Resistant *Staphylococcus aureus* Mutant Confers Broad Immunity against Staphylococcal Infection in Mice. PLOS ONE 5:e11720.

67. Denes T, Bakker HC den, Tokman JI, Guldimann C, Wiedmann M. 2015. Selection and Characterization of Phage-Resistant Mutant Strains of *Listeria monocytogenes* Reveal Host Genes Linked to Phage Adsorption. Applied and Environment Microbiology 81:4295–4305.

68. Loc-Carrillo C, Abedon ST. 2011. Pros and cons of phage therapy. Bacteriophage 1:111–114.

69. Khawaldeh A, Morales S, Dillon B, Alavidze Z, Ginn AN, Thomas L, Chapman SJ, Dublanchet A, Smithyman A, Iredell JR. 2011. Bacteriophage therapy for refractory Pseudomonas aeruginosa urinary tract infection. Journal of Medical Microbiology 60:1697–1700.

70. Zhvania P, Hoyle NS, Nadareishvili L, Nizharadze D, Kutateladze M. 2017. Phage Therapy in a 16-Year-Old Boy with Netherton Syndrome. Frontiers in Medicine (Lausanne) 4:94.

71. Lutwyche P, Cordeiro C, Wiseman DJ, St-Louis M, Uh M, Hope MJ, Webb MS, Finlay BB. 1998. Intracellular delivery and antibacterial activity of gentamicin encapsulated in pH-sensitive liposomes. Antimicrobial agents and chemotherapy 42:2511–2520.

72. Trigo G, Martins TG, Fraga AG, Longatto-Filho A, Castro AG, Azeredo J, Pedrosa J. 2013. Phage therapy is effective against infection by *Mycobacterium* ulcerans in a murine footpad model. PLoS neglected tropical diseases 7:e2183.

73. Kim A, Shin T-H, Shin S-M, Pham CD, Choi D-K, Kwon M-H, Kim Y-S. 2012. Cellular internalization mechanism and intracellular trafficking of filamentous M13 phages displaying a cell-penetrating transbody and TAT peptide. PloS one 7:e51813.

74. Hodyra-Stefaniak K, Lahutta K, Majewska J, Kaźmierczak Z, Lecion D, Harhala M, Kęska W, Owczarek B, Jończyk-Matysiak E, Kłopot A, Miernikiewicz P, Kula D, Górski A, Dąbrowska K. 2019. Bacteriophages engineered to display foreign peptides may become short-circulating phages. Microbial Biotechnology

75. Singla S, Harjai K, Katare OP, Chhibber S. 2016. Encapsulation of Bacteriophage in Liposome Accentuates Its Entry in to Macrophage and Shields It from Neutralizing Antibodies. PLoS ONE 11:e0153777.

76. Broxmeyer L, Sosnowska D, Miltner E, Chacón O, Wagner D, McGarvey J, Barletta RG, Bermudez LE. 2002. Killing of *Mycobacterium* avium and *Mycobacterium* tuberculosis by a mycobacteriophage delivered by a nonvirulent *mycobacterium*: a model for phage therapy of intracellular bacterial pathogens. Journal of Infectious Diseases 186:1155–1160.

77. Ramirez K, Cazarez-Montoya C, López-Moreno H, Campo N. 2018. Bacteriophage cocktail for biocontrol of *Escherichia coli* O157:H7: Stability and potential allergenicity study. PLOS ONE 13:e0195023.

78. Belitsos PC, Greenson JK, Yardley JH, Sisler JR, Bartlett JG. 1992. Association of gastric hypoacidity with opportunistic enteric infections in patients with AIDS. Journal of Infectious Diseases 166:277–284.

79. Patey O, McCallin S, Mazure H, Liddle M, Smithyman A, Dublanchet A. 2019. Clinical Indications and Compassionate Use of Phage Therapy: Personal Experience and Literature Review with a Focus on Osteoarticular Infections. Viruses 11:18.

80. Międzybrodzki R, Kłak M, Jończyk-Matysiak E, Bubak B, Wójcik A, Kaszowska M, Weber-Dąbrowska B, Łobocka M, Górski A. 2017. Means to facilitate the overcoming of gastric juice barrier by a therapeutic staphylococcal bacteriophage A5/80. Frontiers in microbiology 8:467.

81. Vinner GK, Vladisavljević GT, Clokie MR, Malik DJ. 2017. Microencapsulation of Clostridium difficile specific bacteriophages using microfluidic glass capillary devices for colon delivery using pH triggered release. PLoS One 12:e0186239.

82. Nobrega FL, Costa AR, Santos JF, Siliakus MF, van Lent JWM, Kengen SWM, Azeredo J, Kluskens LD. 2016. Genetically manipulated phages with improved pH resistance for oral administration in veterinary medicine. Scientific Reports 6:39235.

83. Sarker SA, Sultana S, Reuteler G, Moine D, Descombes P, Charton F, Bourdin G, McCallin S, Ngom-Bru C, Neville T, Akter M, Huq S, Qadri F, Talukdar K, Kassam M, Delley M, Loiseau C, Deng Y, El Aidy S, Berger B, Brüssow H. 2016. Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh. EBioMedicine 4:124–137.

84. Weber-Dabrowska B, Dabrowski M, Slopek S. 1987. Studies on bacteriophage penetration in patients subjected to phage therapy. Archivum Immunologiae et Therapiae Experimentalis (Warsz) 35:563–568.

85. Keller R, Engley FB. 1958. Fate of Bacteriophage Particles Introduced into Mice by Various Routes. Proceedings of the Society for Experimental Biology and Medicine 98:577–580.

86. Bruttin A, Brüssow H. 2005. Human Volunteers Receiving *Escherichia coli* Phage T4 Orally: a Safety Test of Phage Therapy. Antimicrobial Agents and Chemotherapy 49:2874–2878.

87. Weiss M, Denou E, Bruttin A, Serra-Moreno R, Dillmann M-L, Brüssow H. 2009. In vivo replication of T4 and T7 bacteriophages in germ-free mice colonized with *Escherichia coli*. Virology 393:16–23.

88. Majewska J, Beta W, Lecion D, Hodyra-Stefaniak K, Kłopot A, Kaźmierczak Z, Miernikiewicz P, Piotrowicz A, Ciekot J, Owczarek B. 2015. Oral application of T4 phage induces weak antibody production in the gut and in the blood. Viruses 7:4783–4799.

89. Huff WE, Huff GR, Rath NC, Balog JM, Xie H, Moore PA, Donoghue AM. 2002. Prevention of *Escherichia coli* respiratory infection in broiler chickens with bacteriophage (SPR02). Poultry Science 81:437–441.

90. Semler DD, Lynch KH, Dennis JJ. 2012. The promise of bacteriophage therapy for Burkholderia cepacia complex respiratory infections. Frontiers in cellular and infection microbiology 1:27.

91. Carmody LA, Gill JJ, Summer EJ, Sajjan US, Gonzalez CF, Young RF, LiPuma JJ. 2010. Efficacy of Bacteriophage Therapy in a Model of Burkholderia cenocepacia Pulmonary Infection. Journal of Infectious Diseases 201:264–271.

92. Malik DJ, Sokolov IJ, Vinner GK, Mancuso F, Cinquerrui S, Vladisavljevic GT, Clokie MRJ, Garton NJ, Stapley AGF, Kirpichnikova A. 2017. Formulation, stabilisation and encapsulation of bacteriophage for phage therapy. Advances in Colloid and Interface Science 249:100–133.

93. Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, Foster S, Gilmore BF, Hancock RE, Harper D. 2016. Alternatives to antibiotics—a pipeline portfolio review. The Lancet infectious diseases 16:239–251.

94. Luong T, Salabarria A-C, Roach DR. 2020. Phage therapy in the resistance era: where do we stand and where are we going? Clinical Therapeutics.

95. Henein A. 2013. What are the limitations on the wider therapeutic use of phage? Bacteriophage 3:e24872.

96. US FDA. 2006. Food Additives Permitted for Direct Addition to Food for Human Consumption; Bacteriophage Preparation. Federal Register.

97. Steele A, Stacey HJ, de Soir S, Jones JD. 2020. The safety and efficacy of phage therapy for superficial bacterial infections: a systematic review. Antibiotics 9:754.

98. Tetz G. V., Ruggles, K.V., Zhou H., Heguy A., Tsirigos A. 2017. Bacteriophages as potential new mammalian pathogens. Scientific Reports, 7(1).

99. Plaa GL, Hook JB. 1976. Target organ toxicity: liver and kidney. Environmental Health Perspectives 15:1.

100. Tetz G, Tetz V. 2016. Bacteriophage infections of microbiota can lead to leaky gut in an experimental rodent model. Gut Pathogens 8:33.

101. Ujmajuridze A, Chanishvili N, Goderdzishvili M, Leitner L, Mehnert U, Chkhotua A, Kessler TM, Sybesma W. 2018. Adapted Bacteriophages for Treating Urinary Tract Infections. Frontiers in Microbiology 9:1832.

102. 2016. Microbiome: Phage community in the gut. Nature Reviews Microbiology 14:605.

103. Madhusudana Rao B, Lalitha KV. 2015. Bacteriophages for aquaculture: Are they beneficial or inimical. Aquaculture 437:146–154.

104. Withington R. 2001. Regulatory issues for phage-based clinical products. Journal of Chemical Technology & Biotechnology 76:673–676.

105. Ackermann H-W, Tremblay D, Moineau S. 2004. Long-term bacteriophage preservation. WFCC Newsletter 38:35–40.

106. Zhang Y, Peng X, Zhang H, Watts AB, Ghosh D. 2018. Manufacturing and ambient stability of shelf freeze dried bacteriophage powder formulations. International Journal of Pharmaceutics 542:1–7.

107. Matinkhoo S, Lynch KH, Dennis JJ, Finlay WH, Vehring R. 2011. Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. Journal of Pharmaceutical Sciences 100:5197–5205.

108. Pirnay J-P, Verbeken G, Rose T, Jennes S, Zizi M, Huys I, Lavigne R, Merabishvili M, Vaneechoutte M, Buckling A. 2012. Introducing yesterday's phage therapy in today's medicine. Future Virology 7:379–390.

109. Oliveira H, Sillankorva S, Merabishvili M, Kluskens LD, Azeredo J. 2015. Unexploited opportunities for phage therapy. Frontiers in Pharmacology 6.

110. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon ST. 2010. Phage therapy in clinical practice: treatment of human infections. Current pharmaceutical biotechnology 11:69–86.

111. Parracho HM, Burrowes BH, Enright MC, McConville ML, Harper DR. 2012. The role of regulated clinical trials in the development of bacteriophage therapeutics. Journal of Molecular and Genetic Medicine 6:279–286.

112. Llanos-Chea, A., Citorik, R.J., Nickerson, K.P., Ingano, L., Serena, G., Senger, S., Lu, T.K., Fasano, A. and Faherty, C.S. 2019. Bacteriophage therapy testing against Shigella flexneri in a novel human intestinal organoid-derived infection model. Journal of pediatric gastroenterology and nutrition 68(4):509.

113. "Expert round table on acceptance and re-implementation of bacteriophage therapy." 2016. Silk route to the acceptance and re-implementation of bacteriophage therapy. Biotechnol J 11:595–600.

114. Djebara S, Maussen C, De Vos D, Merabishvili M, Damanet B, Pang KW, De Leenheer P, Strachinaru I, Soentjens P, Pirnay J-P. 2019. Processing Phage Therapy Requests in a Brussels Military Hospital: Lessons Identified. 3. Viruses 11:265.

115. Kropinski AM. 2018. Bacteriophage research–what we have learnt and what still needs to be addressed. Research in microbiology 169:481–487.

116. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, Barr JJ, Reed SL, Rohwer F, Benler S, Segall AM, Taplitz R, Smith DM, Kerr K, Kumaraswamy M, Nizet V, Lin L, McCauley MD, Strathdee SA, Benson CA, Pope RK, Leroux BM, Picel AC, Mateczun AJ, Cilwa KE, Regeimbal JM, Estrella LA, Wolfe DM, Henry MS, Quinones J, Salka S, Bishop-Lilly KA, Young R, Hamilton T. 2017. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection. Antimicrobial Agents and Chemotherapy 61:e00954-17.

117. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. 2009. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. Journal of Wound Care 18:237–238, 240–243.

118. Cisło M, Dabrowski M, Weber-Dabrowska B, Woytoń A. 1987. Bacteriophage treatment of suppurative skin infections. Archivum Immunologiae Therapiae Experimentalis (Warsz) 35:175–183.

119. Kochetkova VA, Mamontov AS, Moskovtseva RL, Erastova EI, Trofimov EI, Popov MI, Dzhubalieva SK. 1989. Phagotherapy of postoperative suppurative-inflammatory complications in patients with neoplasms. Sovetskaia meditsina 23–26.

120. Bogovazova GG, Voroshilova NN, Bondarenko VM, Gorbatkova GA, Afanas'eva EV, Kazakova TB, Smirnov VD, Mamleeva AG, Glukharev IA, Erastova EI. 1992. [Immunobiological properties and therapeutic effectiveness of preparations from Klebsiella bacteriophages]. Zh Mikrobiol Epidemiol Immunobiol 30–33.

121. Perepanova TS, Darbeeva OS, Kotliarova GA, Kondrat'eva EM, Maĭskaia LM, Malysheva VF, Baĭguzina FA, Grishkova NV. 1995. [The efficacy of bacteriophage preparations in treating inflammatory urologic diseases]. Urol Nefrol (Mosk) 14–17.

122. Wright A., Hawkins C.H, Anggard E.E, Harper D.R. 2009. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clinical Otolaryngology 34(4) 39–357.

123. Fabijan AP, Lin RC, Ho J, Maddocks S, Zakour NLB, Iredell JR. 2020. Safety of bacteriophage therapy in severe *Staphylococcus* aureus infection. Nature microbiology 5:465–472.

124. World Medical Association. (n.d.). Declaration of Helsinki 2008. Wma.Net. Retrieved August 12, 2021, from https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/doh-oct2008/.

125. Dublanchet, A.; Patey, O.; Mazure, H.; Liddle, M.; Smithyman, A.M. Indications and Limitations of Phage Therapy in Human Medicine: Personal Experience and Literature Review. Preprints 2018, 2018070091.

126. Strathdee S, Patterson T. 2019. The Perfect Predator: A Scientist's Race to Save Her Husband from a Deadly Superbug: a Memoir. Hachette Books.

127. Charlotte J. 2019. Genetically engineered phage therapy has rescued a teenager on the brink of death. MIT Technology Review.

128. Monk AB, Rees CD, Barrow P, Hagens S, Harper DR. 2010. Bacteriophage applications: where are we now? Lett Appl Microbiol 51:363–369.

129. US EPA. 2005. Xanthomonas Campestris pv. Vesicatoria and Pseudomonas Syringae pv. Tomato Specific Bacteriophages; Exemption from the Requirement of a Tolerance. Federal Register.

130. Ryan EM, Gorman SP, Donnelly RF, Gilmore BF. 2011. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. J Pharm Pharmacol 63:1253–1264.

131. Loessner MJ. 2005. Bacteriophage endolysins—current state of research and applications. Current opinion in microbiology 8:480–487.

132. Chibeu A, Agius L, Gao A, Sabour PM, Kropinski AM, Balamurugan S. 2013. Efficacy of bacteriophage LISTEXTMP100 combined with chemical antimicrobials in reducing *Listeria monocytogenes* in cooked turkey and roast beef. International Journal of Food Microbiology 167:208–214.

133. Abuladze T, Li M, Menetrez MY, Dean T, Senecal A, Sulakvelidze A. 2008. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. Applied and Environmental Microbiology 74:6230–6238.

134. Soffer N, Abuladze T, Woolston J, Li M, Hanna LF, Heyse S, Charbonneau D, Sulakvelidze A. 2016. Bacteriophages safely reduce Salmonella contamination in pet food and raw pet food ingredients. Bacteriophage 6:e1220347.

135. Lang LH. 2006. FDA Approves Use of Bacteriophages to be Added to Meat and Poultry Products. Gastroenterology 131:1370.

136. Soni KA, Nannapaneni R, Hagens S. 2010. Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. Foodborne Pathogens and Disease 7:427–434.

137. Boyacioglu O, Sharma M, Sulakvelidze A, Goktepe I. 2013. Biocontrol of *Escherichia coli* O157: H7 on fresh-cut leafy greens. Bacteriophage 3:e24620.

138. Sharma CS, Dhakal J, Nannapaneni R. 2015. Efficacy of lytic bacteriophage preparation in reducing Salmonella *in vitro*, on turkey breast cutlets, and on ground turkey. Journal of food protection 78:1357–1362.

139. Soffer N, Woolston J, Li M, Das C, Sulakvelidze A. 2017. Bacteriophage preparation lytic for Shigella significantly reduces Shigella sonnei contamination in various foods. PLoS One 12:e0175256.

140. Phagelux Inc. SalmoPro®. Available online: https://www.fda.gov/media/95017/download. Accessed on 12/8/2020.

141. USA C. AgriPhageTM - Bactericide - Certis Biologicals. https://www.omnilytics.com/agriculture/ Accessed on 3/10/2019.

142. PhageGuard – The natural solution for food safety.

143. Fukuda K, Ishida W, Uchiyama J, Morita T, Harada Y, Sumi T, Matsuzaki S, Daibata M, Fukushima A. 2012. Therapeutic Effects of Topical Bacteriophage KPP12 Administration on Pseudomonas aeruginosa Keratitis in Mice. Investive Ophthalmology and Visual Science 53:6201–6201.

144. PhagoFlow. 2017. Liste-gefoerderter-Projekte-VSF-FBK_20-10-2017.pdf. https://innovationsfonds.g-ba.de/downloads/media/112/Liste-gefoerderter-Projekte-VSF-FBK_20-10-2017.pdf Accessed on 1/5/2019.

145. PhagoMed. 2018. €4 million funding for PhagoMed – PhagoMed | Accelerating phage therapy.

146. Abdelkader K, Gerstmans H, Saafan A, Dishisha T, Briers Y. 2019. The Preclinical and Clinical Progress of Bacteriophages and Their Lytic Enzymes: The Parts are Easier than the Whole. Viruses 11:96.

147. Citorik RJ. 2018. Development of natural and engineered bacteriophages as antimicrobials. Thesis, Massachusetts Institute of Technology.

148. Li J, Feng L, Fan L, Zha Y, Guo L, Zhang Q, Chen J, Pang Z, Wang Y, Jiang X, Yang VC, Wen L. 2011. Targeting the brain with PEG–PLGA nanoparticles modified with phage-displayed peptides. Biomaterials 32:4943–4950.

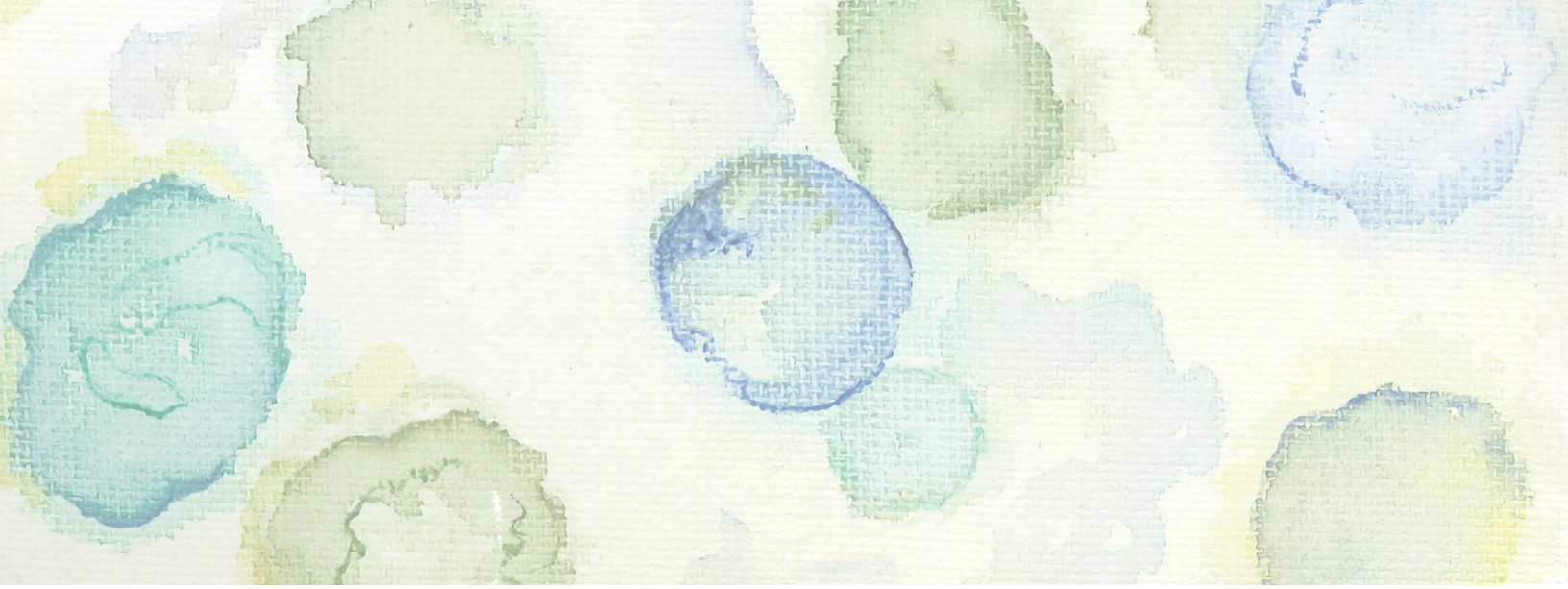
149. Tao P., Mahalingam M., Marasa B.S., Zhang Z., Chopra A.K, Rao V.B. 2013. *In vitro* and in vivo delivery of genes and proteins using the bacteriophage T4 DNA packaging machine. Proceedings of the National Academy of Sciences 110(15):5846–5851.

150. Przystal JM, Waramit S, Pranjol MZI, Yan W, Chu G, Chongchai A, Samarth G, Olaciregui NG, Tabatabai G, Carcaboso AM, Aboagye EO, Suwan K, Hajitou A. 2019. Efficacy of systemic temozolomide- activated phage-targeted gene therapy in human glioblastoma. EMBO Molecular Medicine e8492.

151. Suthiwangcharoen N, Li T, Li K, Thompson P, You S, Wang Q. 2011. M13 bacteriophage-polymer nanoassemblies as drug delivery vehicles. Nano Research 4:483–493.

152. Aghebati-Maleki L, Bakhshinejad B, Baradaran B, Motallebnezhad M, Aghebati-Maleki A, Nickho H, Yousefi M, Majidi J. 2016. Phage display as a promising approach for vaccine development. J Biomedical Science 23.

153. Ando H, Lemire S, Pires DP, Lu TK. 2015. Engineering Modular Viral Scaffolds for Targeted Bacterial Population Editing. Cell Systems 1:187–196.



Oxidative Stress Response in Bacteria: A Review

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Abstract

Oxidative Stress Response (OSR) is a defense mechanism used to maintain redox homeostasis after an increase in levels of Reactive Oxygen Species (ROS). Due to ROS, cell components are vulnerable to damage including the membrane and DNA - which can impact essential functions and lead to cellular death. Without repair, damages caused by ROS have the potential to disrupt cell function in an irreparable manner. Bacterial cells respond to ROS using both endogenous and exogenous pathways depending on their method of metabolism and evolutionary ability. Bacteria have developed regulatory mechanisms to contain damage and are also known to use antioxidants as defense. In this review we will cover the damage induced by ROS to different cellular structures, and mechanisms of OSR used by bacterial cells to promote survival.

Introduction

The term Oxidative Stress (OS) was coined by Helmut Sies *et al*, in 1985 [42], and is used to describe the stress an organism can experience when there is a disruption in its redox homeostasis due to reactive oxygen species (ROS). ROS are oxygen-containing chemicals that are highly reactive, such as hydrogen peroxide (H2O2), ozone (O3) and their radicals. When they interact with cellular components, those components can become oxidized. Interactions with ROS can result in damaged DNA, RNA, lipid membranes, and proteins which may result in cellular death. In contrast, though counter-intuitive, this stress can also act as a cellular signal to encourage proliferation or survival [35]. OS affects all types of organisms, including bacteria. The presence of ROS and oxidative stress in bacteria is connected to the evolution of microbes in an aerobic environment millions of years ago [25]. Defenses against ROS and OS are complex, including genome regulation to reduce levels of ROS, and cellular damage repair. The mechanisms by which bacteria experience, defend against, and repair oxidative damage provides crucial conceptual insight into an organism and its metabolism. The purpose of this review article is to outline both endogenous and exogenous oxidative stress causes and responses to that stress in bacteria.

Causes of Oxidative Stress

The way different bacteria respond to oxygen is mostly defined by their method of metabolism and reflects how that microbe evolved to respond to oxygen and its potentially harmful byproducts. Oxidative stress is created by the production and accumulation of reactive oxygen species and the production of ROS and can be driven by numerous endogenous and exogenous factors [49]. Bacteria may be exposed to many environmental stressors, which may lead to the production of ROS and subsequent oxidative stress.

The production of endogenous ROS can result from a cell's own metabolic processes. ROS can be produced through the oxidation of respiratory enzymes during cellular respiration [40]. In the bacterium model *Escherichia coli*, ROS were confirmed to be produced as a byproduct of both aerobic and anaerobic cellular respiration with the simultaneous generation of both superoxide and hydrogen peroxide. This occurs when molecular oxygen collides with redox enzymes and flavoenzymes. NADH dehydrogenase II, involved in bacterial respiratory metabolism, lipoamide dehydrogenase, involved in glycine catabolism, and fumarate reductase, involved in anaerobic bacterial respiration, are each known for the production of ROS due to their particular ability to transfer their electrons to molecular oxygen [29, 30]. While healthy aerobic cells have evolved to efficiently scavenge the produced ROS, obligate anaerobes may experience increased damage from ROS produced if exposed to oxygen.

Exogenous factors also have the potential to produce ROS within the cell. If bacteria are in an environment with a high concentration of oxygen, they will experience OS (discussed in depth in a review by Haugaard [14]). Other environmental factors can include toxic chemicals and substances introduced into the environment inhabited by the bacteria, such as herbicides, industrial additives, and medications. Paraquat, a lethal herbicide, as well as menadione and phenazines (industrial additives), are redox cycling compounds, molecules that can accept electrons as well as donate electrons to oxygen, producing ROS. They are exogenous causes of bacterial oxidative stress due to this ability [28]. Noticing how ROS are generated exogenously is important to understand why prokaryotes face OS. One of the first observations made by scientists when beginning to study the causes of ROS was realizing that radiation usually preceded the formation of ROS. Radiation has been observed to break water down to hydroxyl radicals, which may then externally damage the cell [45, 55].

Membrane damage

Reactive oxygen species can interact with structures found in the bacterial cell membrane. Cellular viability, membrane selective permeability, and proton motive force, rely on an intact membrane structure [26]. ROS-mediated stress can disrupt these by causing intracellular damage such as lipid peroxidation, which can be detrimental to the cell membrane structure [15]. Lipid peroxidation occurs when ROS takes away electrons from the lipids, disrupting associated functions of the membrane.

Lipid peroxidation starts a cascade down the rest of the membrane until the entire membrane is affected. The created lipid radicals react with oxygen to form lipid peroxyl radicals. They can react with other lipids to form hydroperoxide which can be broken down into lipid peroxide. These reactions can affect the structure of the lipids by shortening their tails. These chemical mechanisms are covered in detail in the review by Girotti [12]. It is thought that as the number of double bonds in the fatty acid increases, then the sensitivity to oxidation also increases [53].

The alterations of lipids in bacterial membranes cause morphological changes to bacteria. When *Campylobacter jejuni* interacts with ROS, the spiral-shaped bacteria change to a coccoid form. ROS increases the cell membrane permeability, which results in the structural change seen in *C. jejuni* [57]. *E. coli* also experiences morphological changes from the *bacillus* form to a coccoid form [18]. These morphological changes occur when the bacterium tries to heal itself from the damage caused by ROS by removing pieces of the membrane [58]. If this damage to the membrane is not repaired, it can lead to damage of the DNA and eventually the death of the cell [18].

Another damaging effect of oxidative stress is a decrease in proton motive force (PMF) from inhibition of transport across the cell membrane. The reduction of PMF due to oxidative damage of the membrane structure interferes with the ability of obligate aerobes to create ATP [11]. As a result, when their membranes are damaged this can lead to an inhibition of cell growth and eventually cell death [8]. Without a fully functional membrane, obligate aerobes cannot produce enough ATP to carry out necessary cellular functions.

Protein damage

Proteins are also affected by ROS due to their structure. Amino acids can be modified by ROS which can damage proteins. Proteins containing cysteine and methionine are particularly susceptible to being oxidized. Both can become oxidized to form sulfenic acids, while further reduction can become an irreversible modification [17, 56]. These modifications can affect the functionality of the associated protein. For example, when methionine is oxidized, proteins become denatured and the hydrophilic properties of methionine are lost, which results in structural alterations [2]. It is important to note that although methionine and cysteine are more readily oxidized, other amino acids can also become damaged by ROS. Amino acids such as arginine, lysine, proline, histidine, and threonine can be carbonylated [39, 50]. When amino acids are carbonylated, this is an irreversible sign of aging and oxidative damage as shown in *E. coli* [8]. These damages, unless repaired, ultimately lead to loss of function of proteins and cause protein misfolding.

The degradation of proteins can be repaired by enzymes and chaperone proteins. Heat shock proteins (Hsp), a group of chaperone proteins, can aid in the refolding, repair, and recycling of damaged proteins. A redox regulated chaperone protein, Hsp33, is specific to prokaryotes and is inactive under reduced conditions. This chaperone protein is activated when the environment becomes oxidized. Upon exposure to protein oxidation, Hsp33 also unfolds but instead of losing activity and aggregating, it uses the structural rearrangements to activate its chaperone function [16]. Additional heat shock chaperone proteins that are not redox regulated can also refold proteins that have been affected by oxidative stress, thus helping restore protein function.

DNA damage

Overproduction of ROS can lead to modification of nucleotides or the sugar phosphate backbone of the DNA helix. Damage to the DNA can potentially result in mutations, or changes in the genetic sequence. When ROS interacts with DNA, it will oxidize the structure, generating damage to the DNA in the form of strand breaks and base modifications [10]. Similar to other endogenously caused OS, oxidative DNA damage is an inevitable consequence of aerobic cellular metabolism (discussed in reviews by Storz & Imlay and Sigler *et al*. [43, 46]), and threatens the survival and growth of bacterial cells.

ROS can interact with both purine and pyrimidine bases, and the deoxyribose sugar backbone of the DNA molecule. The likelihood of the oxidation of DNA bases depends on the redox potential of the individual nucleotide. It is shown that purines are more likely to oxidize because of their low redox potential. When there is a single oxygen from ROS reacting with a purine, it results in the addition of a carbonyl

group - this is the most common damage to purines. For example, guanine has a low oxidation potential and that makes it a prime target for ROS, resulting in the formation of 8-oxo-7, 8-dihydroguanine (8-oxoG) [22]. After hydroxyl radicals react with DNA, the lesions are going to result in a damaged site that, when replicated, causes mutations. The oxidation product of guanine, 8-oxo-G, can base pair with either adenine or cytosine. The base pairing of 8-oxo-G with adenine in bacteria is seen to cause G:C to T:A base transversion mutations [31, 32]. If these transversion mutations happen in sites responsible for protein function, the mutations may decrease the bacterial cell's survival.

Base excision repair (BER) is the most common DNA repair mechanism to address oxidative damage. Glycosylases are a ubiquitous family of enzymes that catalyze the removal of damaged bases from the DNA strand in the BER pathway. DNA repair mechanisms however, often overlap. Oxidized bases can be excised through both the BER pathway, which removes a single lesion using glycosylase, and the nucleotide excision repair (NER) pathway, which removes a lesion-containing segment of the DNA strand [7]. The state of the bacteria, however, may influence the use of these repair pathways. For example, in starving *P. putida*, 8-oxo-G repair is limited, increasing mutations for the purpose of adaptation [37].

Regulatory modifications

Oxidative stress influences how much mRNA from genes not required for stress response is transcribed. A study by Muthukrishnan *et al*. performed in *E. coli* cells noted a decrease in the transcription rates specifically for genes not related to stress when the cells were experiencing OS. The researchers noted that under oxidative stress, there was a 76% decrease in the cells ability to transcribe compared to cells under normal conditions. This is likely caused by a decrease in the number of available inducers, regulators, and transcriptional components, extending the time frame in which it takes transcription to occur and decreasing growth rate [33, 60].

Translational control under oxidative stress conditions is focused on producing proteins necessary to negate OS [3, 51]. Oxidative stress negatively impacts the ability of *E. coli* cells to translate proteins in a timely manner.

As concentrations of H2O2 increase, the rate of ribosome translational elongation decreases and the time it takes for the translation elongation rate to recover increases. The cause of this reduced translational elongation rate is the downregulation of tRNAs by degradation within *E. coli* under oxidative stress [60, 61]. Demonstrated by Zhong *et al*., translation elongation rates under continual experimental OS conditions recovered by an increase in tRNA species 75 minutes after the initial drop [60].

In *E. coli*, small RNAs (sRNAs) are shown to help cells adjust to environmental changes by controlling expression of key proteins, causing a competition for binding which can result in repressed translation. Genome regulation under oxidative stress is also affected by the number of functional and available small RNAs (as discussed in a review by Van Assche *et al*. [51]). For example, in *Salmonella enterica*, small RNAs RyhB-1 and RyhB-2 participate in the oxidative stress response and deletion of these sRNA lead to an increase of ROS in the cell. These sRNAs are upregulated by OxyR, an important regulon later discussed, a result of OxyR interacting with the sRNA promoters [3]. Small RNAs help the cell adjust in response to oxidative stress.

Another example of sRNA involved in managing oxidative stress is found in the bacterium *Deinococcus radiodurans* R1, where a specific sRNA, OsiA, is required for helping the bacterium handle varying levels of oxidative stress. When the sRNA is knocked out, the mutant bacteria are more sensitive to H2O2 and produce less catalase, an enzyme that breaks down H2O2. Chen *et al*. show that in the OsiA mutant, the mRNA of a catalase gene, katA, has a decreased half-life. The regulation of katA by OsiA helps D. *radiodurans* R1 to cope with oxidative stress, induced by H2O2 [4].

Removal of ROS

In order to neutralize ROS, bacteria are equipped with antioxidant molecules (AOX) [1]. AOX are responsible for protecting bacteria from and fighting against ROS and include enzymatic and non-enzymatic antioxidant defenses. Enzymatic defenses directly target ROS molecules, inactivating them and converting them into molecules that are significantly less reactive [44]. Non-enzymatic defenses such as vitamins C and E and thiols are molecules

that naturally behave as reducing agents. Non-enzymatic antioxidants become oxidized in place of sensitive cellular components [38]. (Discussed in reviews by J.G. Scandalios and Staerck *et al*. [38, 44].) Bacteria use both enzymatic and non-enzymatic methods jointly to address ROS and OS [1].

Enzymatic inactivation of ROS includes enzymes such as dismutases, catalases, peroxidases, and reductases, commonly found in aerotolerant bacteria [44]. These enzymes, such as superoxide dismutase (SOD), an enzymatic inactivator of superoxide, help to prevent varying types of damage from OS. Loss of these enzymes have been shown to coincide with increased oxidative damage [19, 47]. One example function of SOD is in the periplasm of *E. coli*. SodC is thought to be a dismutase that detoxifies the superoxide anions that are released from the oxidative phosphorylation process [21]. This process allows for ROS damage to be prevented in the membrane. Another enzyme involved in ROS inactivation is catalase which deactivates hydrogen peroxide into oxygen and water. Catalase has been shown to increase survivability of bacteria in the presence of hydrogen peroxide [36]. These enzymatic defenses continue to evolve to help bacteria survive in the presence of ROS.

Enzymatic antioxidants are often regulated by systems called regulons. Regulons are groups of bacterial genes that are regulated together to control specific responses. A prominently known regulon, seen in many Gram-negative and some Gram-positive bacteria is the OxyR regulon. An influx of hydrogen peroxide (H2O2) in the cell will initiate a response from the OxyR transcription factor, causing it to convert to its oxidized form. Once oxidized, the transcription factor will positively regulate the genes associated with the OxyR regulon [54]. The RpoS and PerR regulons also respond to H2O2. RpoS behaves as a sigma factor, recruiting RNA polymerase, while PerR is similar to OxyR and is more often found in Gram-positive cells [52, 9]. While OxyR, RpoS, and PerR regulons tend to respond to H2O2, the SoxRS regulon functions to counteract an increase in superoxide radical anions. The OhrR regulon is another antioxidant regulon which functions to identify and destroy organic peroxides during oxidative stress [9]. Although every antioxidant regulon is not present in all types of bacteria, more than one regulon can contribute to bacterial stress responses [9, 52].

Non-enzymatic antioxidants can be acquired from the environment, synthesized biologically, or both. Vitamin C, or ascorbic acid, is a water soluble, non-enzymatic scavenger of free radicals. Ascorbic acid is taken from the environment, though some bacterium, such as *Streptomyces antibioticus* and *Acetobacter suboxydans* are able to produce it [59, 48]. Additionally, vitamin C is capable of assisting in the regeneration of oxidized vitamin E, another important antioxidant [34]. Vitamin E or alpha tocopherol is lipid-soluble and resides in the hydrophobic region of the cell membrane; it works to defend the membrane from oxidative stress injuries. Vitamin E acts to reduce lipid peroxyl radicals and will eliminate the chances of lipid peroxidation of the cell [13, 20]. Non-enzymatic defenses can also work jointly with enzymes. Glutathione (GSH) is a thiol that can detoxify hydrogen peroxide and lipid peroxides in conjunction with glutathione peroxidase (GSH-Px) and will reduce hydrogen peroxide into water and oxygen by donating an electron to hydrogen peroxide. This antioxidant will also protect the cell from lipid peroxidation and convert vitamins C and E back into their active forms [1, 6, 27].

Thiols, such as glutathione, thioredoxin, and glutaredoxin, become oxidized to decrease OS within the cell. Gram-negative bacteria both synthesize and import active thiols like glutathione from the environment using dedicated transporter systems. In contrast, most Gram-positive bacteria do not synthesize glutathione, but can import it [5]. Once cellular thiols are inactivated through oxidation, they must be returned to their active, reduced form. This maintenance is performed by enzymes such as glutathione reductase, thioredoxin reductase, and glutaredoxin [5]. Glutathione reductase helps to keep glutathione in its reduced form to respond to an increase of ROS when the cell is under oxidative stress [5]. Thioredoxin is reduced by thioredoxin reductase (discussed in this review by Lu & Holmgren [23]). The importance of thioredoxin reductase can be noted in a study by Serrano *et al*. of *Lactobacillus plantarum* where they found that overexpression of trxB1, a thioredoxin reductase, results in a high resistance to ROS [41]. The thioredoxin system also plays a role in DNA repair and is highly conserved among various bacteria including, *E. coli, Helicobacter pylori, Bacillus subtilis,* and *Mycobacterium tuberculosis* [23].

Glutaredoxin is important in that it will deactivate OxyR when H2O2 levels are reduced to a normal range [54]. Thiol maintenance is vital for the survival of bacteria in oxidative stress environments.

Conclusion

While oxidative stress can be an important signaling strategy for bacteria, it can also be a dangerous source of cellular damage [35]. ROS of both endogenous and exogenous creation threaten to oxidize key components of the cell [49]. This review has discussed the range of damage that can be caused. ROS can oxidize proteins resulting in both reversible and irreversible damage, lipid peroxidation can threaten the integrity of the cell membrane, and DNA damage can induce mutations within the genome [17, 56, 15, 10]. Fortunately, microbial evolution has provided bacteria with mechanisms to counteract OS and its damage.

Although more abundant in aerobic bacteria, all bacteria have some mechanisms for addressing ROS. Regulatory modifications can be made to both transcription and translation to modulate the use of resources and the production of antioxidants [3, 33, 51, 60]. ROS can then be neutralized using enzymatic and non-enzymatic mechanisms and the damage repaired [38, 44]. Bacterial genomes have entire regulons dedicated to these responses. Antioxidants and their maintenance enzymes are closely regulated to both evaluate the oxidative state of the cell and ensure bacterial survival. While bacteria have the capability to respond to oxidative damage, the process is not infallible if the stress outweighs the capacity of the cell to respond.

References

1. Birben E, Sahiner UM, Sackesen CS, Erzurum S, Kalayci O (2012) Oxidative stress and antioxidant defense.
2. Brot N, Weissbach H (1983) Biochemistry and physiological role of methionine sulfoxide residues in proteins. Arch Biochem Biophys 223:271–281
3. Calderón IL, Morales EH, Collao B, Calderón PF, Chahuán CA, Acuña LG, Gil F, Saavedra CP (2014) Role of Salmonella Typhimurium small RNAs RyhB-1 and RyhB-2 in the oxidative stress response. Res Microbiol 165:30–40
4. Chen Y, Xue D, Sun W, Han J, Li J, Gao R, Zhou Z, Zhang W, Chen M, Lin M, Wang J, Zuo K (2019) sRNA OsiA Stabilizes Catalase mRNA during Oxidative Stress Response of Deincoccus radiodurans R1. Microorganisms. doi: 10.3390/microorganisms7100422
5. Couto N, Wood J, Barber J (2016) The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. Free Radic Biol Med 95:27–42
6. Curello S, Ceconi C, Bigoli C, Ferrari R, Albertini A, Guarnieri C (1985) Changes in the cardiac glutathione status after ischemia and reperfusion. Experientia 41:42–43
7. Demple B, Halbrook J (1983) Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature 304:466–468
8. Desnues B, Cuny C, Grégori G, Dukan S, Aguilaniu H, Nyström T (2003) Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable *Escherichia coli* cells. EMBO Rep 4:400–404
9. Dubbs, J. M., & Mongkolsuk, S. (2012). Peroxide-sensing transcriptional regulators in bacteria. Journal of Bacteriology, 194(20), 5495-5503.
10. Eisen JA, Hanawalt PC (1999) A Phylogenomic Study of DNA Repair Genes, Proteins, and Processes. Mutat Res 435:171–213
11. Farr SB, Touati D, Kogoma T (1988) Effects of oxygen stress on membrane functions in *Escherichia coli*: role of HPI catalase. J Bacteriol 170:1837–1842
12. Girotti AW (1985) Mechanisms of lipid peroxidation. J Free Radic Biol Med 1:87–95
13. Han R-M, Zhang J-P, Skibsted LH (2012) Reaction Dynamics of Flavonoids and Carotenoids as Antioxidants. Molecules 17:2140–2160
14. Haugaard N (1968) Cellular mechanisms of oxygen toxicity. Physiol Rev 48:311–373
15. Hong Y, Zeng J, Wang X, Drlica K, Zhao X (2019) Post-stress bacterial cell death mediated by reactive oxygen species. Proc Natl Acad Sci 116:10064–10071
16. Jakob U, Muse W, Eser M, Bardwell JCA (1999) Chaperone Activity with a Redox Switch. Cell 96:341–352
17. Jocelyn PC (1967) The Standard Redox Potential of Cysteine-Cystine from the Thiol-Disulphide Exchange Reaction with Glutathione and Lipoic Acid. Eur J Biochem 2:327–331

18. Joshi SG, Cooper M, Yost A, Paff M, Ercan UK, Fridman G, Friedman G, Fridman A, Brooks AD (2011) Nonthermal Dielectric-Barrier Discharge Plasma-Induced Inactivation Involves Oxidative DNA Damage and Membrane Lipid Peroxidation in *Escherichia coli*. Antimicrob Agents Chemother 55:1053–1062

19. Keyer K, Gort AS, Imlay JA (1995) Superoxide and the production of oxidative DNA damage. J Bacteriol 177:6782–6790

20. Kojo S (2004) Vitamin C: Basic Metabolism and Its Function as an Index of Oxidative Stress. Curr Med Chem 11:1041–1064

21. Korshunov S, Imlay JA (2006) Detection and Quantification of Superoxide Formed within the Periplasm of *Escherichia coli*. J Bacteriol 188:6326–6334

22. Liu M, Gong X, Alluri RK, Wu J, Sablo T, Li Z (2012) Characterization of RNA damage under oxidative stress in *Escherichia coli*. Biol Chem 393:123–132

23. Lu J, Holmgren A (2014) The thioredoxin antioxidant system. Free Radic Biol Med 66:75–87

24. Lugtenberg EJJ, Peters R (1976) Distribution of lipids in cytoplasmic and outer membranes of *Escherichia coli* K12. Biochim Biophys Acta BBA - Lipids Lipid Metab 441:38–47

25. Lyons TW, Reinhard CT, Planavsky NJ (2014) The rise of oxygen in Earth's early ocean and atmosphere. Nature 506:307–315

26. Maness P-C, Smolinski S, Blake DM, Huang Z, Wolfrum EJ, Jacoby WA (1999) Bactericidal Activity of Photocatalytic TiO2 Reaction: toward an Understanding of Its Killing Mechanism. Appl Environ Microbiol 65:4094–4098

27. Masella R, Di Benedetto R, Varì R, Filesi C, Giovannini C (2005) Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. J Nutr Biochem 16:577–586

28. Massey V, Singer TP (1957) STUDIES ON SUCCINIC DEHYDROGENASE. J Biol Chem 228:263–274

29. Messner KR, Imlay JA (1999) The Identification of Primary Sites of Superoxide and Hydrogen Peroxide Formation in the Aerobic Respiratory Chain and Sulfite Reductase Complex of *Escherichia coli*. J Biol Chem 274:10119–10128

30. Messner KR, Imlay JA (2002) Mechanism of Superoxide and Hydrogen Peroxide Formation by Fumarate Reductase, Succinate Dehydrogenase, and Aspartate Oxidase. J Biol Chem 277:42563–42571

31. Moriya M (1993) Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-Oxoguanine in DNA induces targeted GC TEA transversions in simian kidney cells. Proc Natl Acad Sci USA 5

32. Moriya M, Ou C, Bodepudi V, Johnson F, Takeshita M, Grollman AP (1991) Site-specific mutagenesis using a gapped duplex vector: A study of translesion synthesis past 8-oxodeoxyguanosine in *E. coli*. Mutat Res Repair 254:281–288

33. Muthukrishnan A-B, Martikainen A, Neeli-Venkata R, Ribeiro AS (2014) In Vivo Transcription Kinetics of a Synthetic Gene Uninvolved in Stress-Response Pathways in Stressed *Escherichia coli* Cells. PLoS ONE. doi: 10.1371/journal.pone.0109005

34. Packer, J.E., Slater, T. F., and Willson, R. L. (1979) Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 278:737-738

35. Ray PD, Huang B-W, Tsuji Y (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal 24:981–990

36. Rocha ER, Selby T, Coleman JP, Smith CJ (1996) Oxidative stress response in an anaerobe, Bacteroides fragilis: a role for catalase in protection against hydrogen peroxide. J Bacteriol 178:6895–6903

37. Saumaa S, Tover A, Tark M, Tegova R, Kivisaar M (2007) Oxidative DNA Damage Defense Systems in Avoidance of Stationary-Phase Mutagenesis in *Pseudomonas putida*. J Bacteriol 189:5504–5514

38. Scandalios JG (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res 38:995–1014

39. Schonberg A, Moubasher R, Mostafa A (1948) Degradation of alpha-amino-acids to aldehydes and ketones by interaction with carbonyl compounds. J Chem Soc 2:176–182

40. Seaver LC, Imlay JA (2001) Alkyl Hydroperoxide Reductase Is the Primary Scavenger of Endogenous Hydrogen Peroxide in *Escherichia coli*. J Bacteriol 183:7173–7181

41. Serrano M, Molenaar D, Wels M, Teusink B, Bron P, De Vos W, Smid E (2007) Thioredoxin reductase is a key factor in the oxidative stress response of *Lactobacillus plantarum* WCFS1. Microb. Cell Factories

42. Sies H, Cadenas E, Symons MCR, Scott G (1985) Oxidative stress: damage to intact cells and organs. Philos Trans R Soc Lond B Biol Sci 311:617–631

43. Sigler K, Chaloupka J, Brozmanová J, Stadler N, Höfer M (1999) Oxidative stress in microorganisms—I: Microbialvs. higher cells—Damage and defenses in relation to cell aging and death. Folia Microbiol (Praha) 44:587–624

44. Staerck C, Gastebois A, Vandeputte P, Calenda A, Larcher G, Gillman L, Papon N, Bouchara J-P, Fleury M (2017) Microbial antioxidant defense enzymes. Microb Pathog 110:56–65

45. Stein G, Weiss J (1948) Chemical Effects of Ionizing Radiations. Nature 161:650–650

46. Storz G, Imlay J (1999) Oxidative Stress. Curr Opin Microbiol 2:188–194

47. Tappel AL (1962) Vitamin E as the Biological Lipid Antioxidant. In: Harris RS, Wool IG, Marrian GF, Thimann KV (eds) Vitam. Horm. Academic Press, pp 493–510

48. Teruhide Sugisawa, Setsuko Ojima, P. K. Matzinger & Tatsuo Hoshino (1995) Isolation and Characterization of a New Vitamin C Producing Enzyme (L-Gulono-γ-lactone Dehydrogenase) of Bacterial Origin, Bioscience, Biotechnology, and Biochemistry, 59:2, 190-196, DOI: 10.1271/bbb.59.190

49. Toyokuni S (1999) Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 49:91–102

50. Traoré DAK, Ghazouani AE, Jacquamet L, Borel F, Ferrer J-L, Lascoux D, Ravanat J-L, Jaquinod M, Blondin G, Caux-Thang C, Duarte V, Latour J-M (2009) Structural and functional characterization of 2-oxo-histidine in oxidized PerR protein. Nat Chem Biol 5:53–59

51. Van Assche E, Van Puyvelde S, Vanderleyden J, Steenackers HP (2015) RNA-binding proteins involved in post-transcriptional regulation in bacteria. Front Microbiol. doi: 10.3389/fmicb.2015.00141

52. Vijayakumar, S. R. V., Kirchhof, M. G., Patten, C. L., & Schellhorn, H. E. (2004). RpoS-regulated genes of *Escherichia coli* identified by random lacZ fusion mutagenesis. Journal of Bacteriology, 186(24), 8499–8507.

53. Wagner BA, Buettner GR, Burns CP (2002) Free Radical-Mediated Lipid Peroxidation in Cells: Oxidizability Is a Function of Cell Lipid bis-Allylic Hydrogen Content. doi: 10.1021/bi00181a003

54. Wang X, Mukhopadhyay P, Wood MJ, Outten FW, Opdyke JA, Storz G (2006) Mutational Analysis To Define an Activating Region on the Redox-Sensitive Transcriptional Regulator OxyR. J Bacteriol 188:8335–8342

55. Weiss J (1944) Radiochemistry of Aqueous Solutions. Nature 153:748–750

56. Wood PM (1981) The redox potential for dimethyl sulphoxide reduction to dimethyl sulphide. FEBS Lett 124:11–14

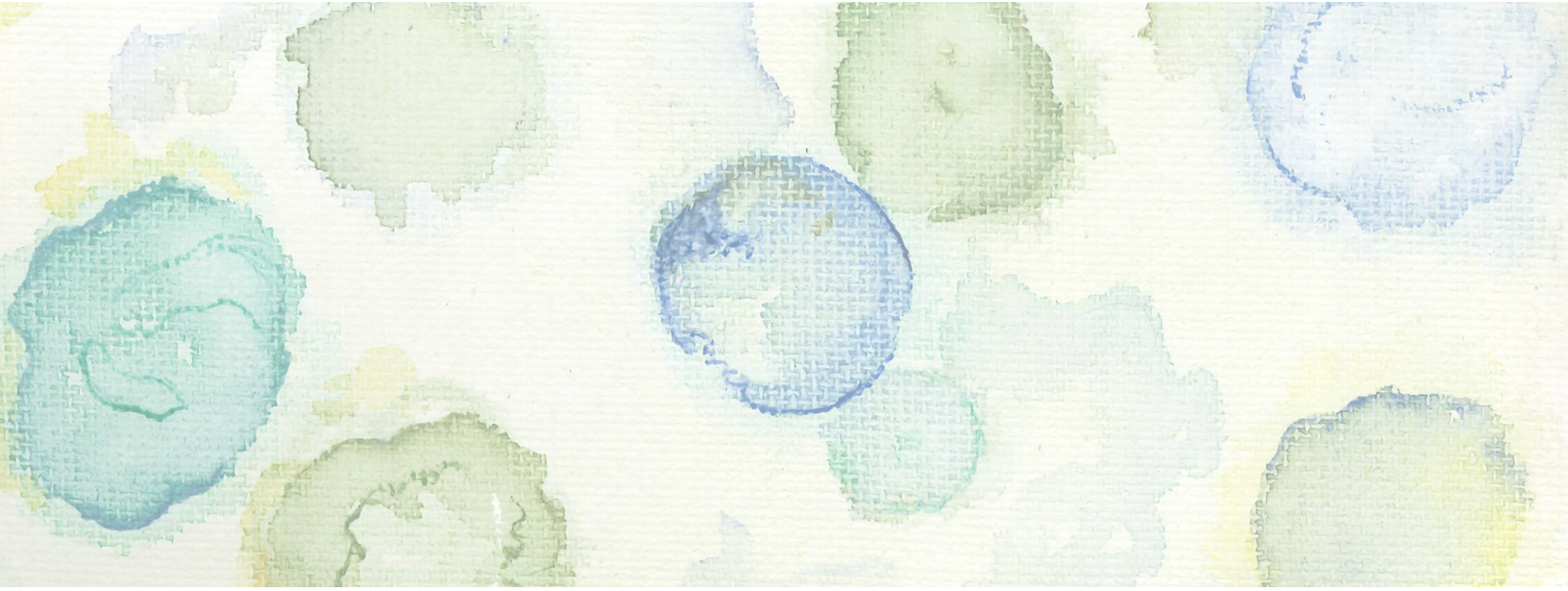
57. Xie Y, He Y, Irwin PL, Jin T, Shi X (2011) Antibacterial Activity and Mechanism of Action of Zinc Oxide Nanoparticles against Campylobacter jejuni. Appl Environ Microbiol 77:2325–2331

58. Zeng H, Guo G, Mao XH, De Tong W, Zou QM (2008) Proteomic Insights into Helicobacter pylori Coccoid Forms Under Oxidative Stress. Curr Microbiol 57:281

59. Zhang, Z., Aboulwafa, M., Smith, M. H., & Saier, M. H., Jr (2003). The ascorbate transporter of *Escherichia coli*. Journal of bacteriology, 185(7), 2243–2250. https://doi.org/10.1128/JB.185.7.2243-2250.2003

60. Zhong J, Xiao C, Gu W, Du G, Sun X, He Q-Y, Zhang G (2015) Transfer RNAs Mediate the Rapid Adaptation of *Escherichia coli* to Oxidative Stress. PLoS Genet. doi: 10.1371/journal.pgen.1005302

61. Zhu M, Dai X (2019) Maintenance of translational elongation rate underlies the survival of *Escherichia coli* during oxidative stress. Nucleic Acids Res 47:7592–7604



The Influence of Infant Formulae on the Growth of Commensal and Pathogenic Streptococcus Species in the Infant Oral Cavity

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Abstract

The oral microbiome is a complex community of microorganisms that influences the health of the human host. A number of diseases are associated with dysbiotic oral microflora in infants and children, including dental and gastrointestinal diseases. A variety of factors can influence the composition of the oral microbial community in infants, including mode of delivery, feeding method, and diet. This study focuses on the effect of nutritional differences in infant formulae on the growth of a commensal species (*Streptococcus mitis*) and a pathogenic species (*Streptococcus mutans*) that are commonly found in the infant oral cavity. A culture-dependent model was utilized to test the effects of one infant formula (Nutramigen Enflora) supplemented with a probiotic (*Lactobacillus rhamnosus*) and a similar infant formula without probiotic supplementation (Enfamil NeuroPro) on the growth of each species. A Snyder's media test was used to assess acidogenic potential of each species. Bacterial growth in each formula was assessed by measuring colony forming units (CFUs) and by measuring the pH of the culture media over an 8 hour incubation. Results indicate that the probiotic formula may selectively inhibit the growth of the pathogen and aid in producing more favorable conditions for the commensal. These findings may make Nutramigen Enflora the preferred infant formula for overall health. The results of this study may assist parents in selecting alternatives to breastmilk that will support the proper development of the infant oral microbiome by favoring the growth of commensal bacteria.

Introduction

The human microbiome is defined as the totality of microorganisms which inhabit the human body (25). Estimates indicate that there are about as many bacterial cells as there are human cells in the body (46). The gut microbiome alone harbors about 1,000 species of bacteria with a combined total of 2 million genes, which is 100× the number of genes in the human genome (55). Our microbial symbionts are intimately intertwined with both systemic and specific bodily function (22), and each niche in the human body nurtures a unique community of microbes.

The microbiome is inherently linked to the health of the human host. The effects that the microbiome exerts on the human body are directly linked to the state of the microbiome itself: changes in species composition and relative abundance can be characterized on a spectrum from health to dysbiosis. Many endogenous and exogenous factors (e.g. immune function, body site, diet, antibiotic use, lifestyle) influence the environmental conditions of the niches within the human body (Figure 1), which in turn influence microbial community composition and thus overall host health (22).

Dysbiosis, a state in which the microbial community composition becomes unbalanced, is triggered by alterations in factors such as diet, immune function, hygiene, and hormone levels (27). It is well known that a dysbiotic microbiome is associated with a multitude of disease states of the body and the mind, such as cancers, obesity, cardiovascular disease, psoriasis, and major depressive disorder (57, 20, 22). The impacts of the microbiome on health are plentiful and significant, and thus the ways in which we humans care for our microbiomes is of high importance.

The oral cavity is a distinct niche within the overall human microbiome that is of interest due to its association with diseases in adults and children such as dental caries, periodontal disease, various cancers (i.e. oral, esophogeal, pancreatic, and colorectal) and gastrointestinal diseases (56).

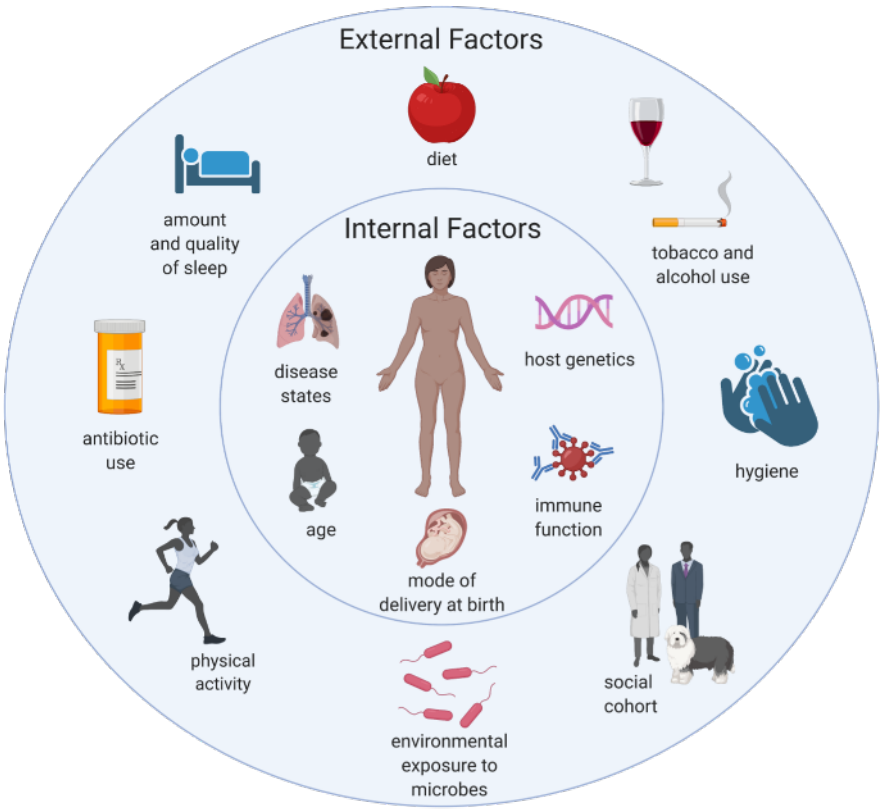

Figure 1: **Internal and external factors that influence the human microbiome. Factors that shape the human microbiome can be internal (a condition of the body that is not easily changed, such as age, genetics, and mode of delivery at birth) or external (including lifestyle choices such as diet, exercise, and drug use).**

The process of acquiring the oral microbiome begins during gestation, continues through parturition and infancy, and is highly influenced by factors such as genetics, gestation length, delivery mode, and diet (59). Following birth, the infant is exposed to the multitude of microbes present within their environment (27). The oral cavity is colonized in a sequential manner, first with pioneer colonizers attaching and enabling subsequent colonizers to adhere and form a biofilm (52). The most common pioneer colonizers of the oral cavity are Gram-positive bacteria including Streptococci species such as *S. mitis* and *S. salivarius*, because of their ability to adhere to previously uncolonized epithelial tissue and their presence in breastmilk. Pathogenic species like *S. mutans* are less abundant than commensal species, but their overgrowth is linked with a dysbiotic oral microbiome and diseases of the oral cavity like dental caries (57). Typically, pathogenic species such as *S. mutans* disrupt the normal balance of the oral microbiome by producing acids that lower the pH within the environment to a level that favors aciduric, pathogenic species. This triggers the overgrowth of the pathogenic species and the simultaneous inhibition of commensal species like *Streptococcus sanguinis* that are less tolerant to low pH conditions (57, 59). The composition of the oral microbiome fluctuates preceding the first tooth eruption, after which it begins to stabilize. Because of this, more research is needed to determine if the composition of the predentate oral microbiome influences the more permanent oral microbiome later in life (59). The acquisition and development of a healthy oral microbiome is important for a child's overall health as several childhood diseases, as mentioned above, are associated with a dysbiotic oral microbiome (59).

Nutrition is an important factor that can influence the trajectory of the development of the early oral microbiome (59) and thus the health of the individual as a whole. During infancy, the primary source of nutrition and early microbial exposure is either breastmilk or infant formula. Breastfeeding is widely considered to be the optimal nutrition source for infants (3; 54). However, there are many reasons why breastfeeding may not be a suitable dietary choice. For instance, preterm or low birth weight infants may need a more nutritionally dense food source such as infant formula to achieve a healthy weight (40). Similarly, infant formulae are desirable for infants suffering from malnutrition due to famine (40). HIV-positive mothers also rely on infant formulae to avoid transmission of the virus to the infant (40), and the advent of soy-based formulae in the 1920's provided a solution for lactose-intolerant infants (40). Infant formula utilizes either cow's milk, soy, amino acids, or goat's milk as a replacement for breastmilk. Some infant formulae containing probiotics tout claims of boosting the microbiome, including Lactobacilli and Bifidobacteria in the formulations (18). However, these claims have not been clinically supported, and the effects of these formulations on the composition of the oral mıcrobiome have not yet been thoroughly investigated

This study aims to elucidate the relationship between the nutritional profile of infant formulae with the growth of one commensal and one pathogenic constituent of the infant oral microbiome.. A commensal species, *S. mitis*, and a pathogenic species, *S. mutans*, were used to represent beneficial and pathogenic *Streptococcus* species commonly found in the infant oral cavity.

S. mitis is a Gram-positive commensal species of the oral cavity and is understood to be one of the primary colonizers of the oral cavity (19). Like *S. mutans*, *S. mitis* metabolizes carbohydrates such as lactose and sucrose into lactic acid (30, 38). *S. mitis* produces an enzyme known as neuraminidase in addition to a number of adhesins which may aid in the adherence and subsequent colonization of surfaces within the oral cavity (31). The results from some studies suggest that *S. mitis* may supplement host immunity through modulating the expression of various immune markers (61). Additionally, one study has shown that *S. mitis* induced the expression of an antimicrobial peptide that not only aids in deterring pathogenic microbes, but that *S. mitis* itself is tolerant to (17, 42). The production of antimicrobials that target pathogenic microbes in turn may boost host health by limiting the growth of pathogens.

S. mutans is a Gram-positive facultative anaerobe that is known for its role in the development of dental caries, especially in infants and children (24). Two characteristics of *S. mutans* that contributes to its pathogenicity are its acidogenity and acidurity: the ability to metabolize sugars

(glucose, fructose, lactose, sucrose) to lactic acid and thrive in low pH conditions, respectively (36). This increase in acidity resulting from *S. mutans*' metabolism erodes the dental enamel and leads to dental caries. Therefore, a decrease in pH is indicative of the growth of *S. mutans* and the dysbiotic state that can be associated with its growth (36).

These species were grown on media containing one of two infant formulae that were chosen for their similarity in nutritional composition to elucidate the effect of probiotic supplementation on the growth of the commensal and pathogenic species: a cow's milk-based formula without probiotic supplementation (Enfamil NeuroPro) and a cow's milk based formula with probiotics (Enfamil Nutramigen with Enflora LGG). The overall growth of the two species was assessed by counting CFUs to determine the cell density of the culture media every hour throughout the duration of the experiment. The metabolic activity of acidogenic bacteria was measured by pH level of the culture media to determine the rate of converting sugars into lactic acid. Metabolic responses indicative of commensals thriving suggest that infant formula is beneficial for the establishment of a microbiome on a healthy trajectory, while the opposite is true with the opportunistic pathogens.

Methods

Species Descriptions

Two species of *Streptococcus*-group bacteria were chosen for this study: one commensal (*S. mitis*) and one opportunistic pathogen (*S. mutans*) that are commonly found within the infant oral cavity (59). Both cultures were sourced from the American Type Culture Collection (atcc.org, Manassas, VA). *S. mitis* type strain ATCC 49456 was the sole strain available from ATCC. *S. mutans* type strain ATCC 25175 was selected for its origin from dental caries.

Snyder's Media Test

A Snyder's media test was used to determine the cariogenic potential of both species in the study. Snyder's media (Thomas Scientific, Swedesboro, NJ) contains a pH indicator that is used to qualitatively assess a decline in pH over time, which is indicative of cariogenic potential as acid erodes dental enamel and causes dental caries. The faster the inoculated media changes color, the greater the cariogenic potential of a species (48). A change in media color from blue-green to yellow within 24 hours indicates high cariogenic potential, while a change in color to orange indicates moderate cariogenic potential and no color change indicates low cariogenic potential. In this study, the color of the media for each species was compared after 24 hours of incubation. Both species were incubated at 37°C in Snyder's media in triplicate for 24 hours followed by comparisons of the media color to the uninoculated control.

Growth Curves under Baseline Conditions

Growth curves were created for each species under baseline conditions to describe the general growth kinetics of both species. The approximate time of the mid-log phase (i.e. the midpoint of the logarithmic phase) was needed for this study. The experimental cultures were inoculated with mid-log starting cultures because the mid-log phase is indicative of the fastest growth rate (i.e. maximum slope). The amount of time needed to reach the plateau phase for each species was used to determine the length of time for incubation during the experiment.

Growth curves were produced by measuring the optical density of the culture grown in the control media (Tryptic Soy Broth, Thermo Fisher Scientific, Waltham, MA) in triplicate until the plateau phase of growth was reached (i.e. until growth became steady). For each species, three fresh cultures were inoculated in 10 mL TSB and allowed to reach turbidity (24 hours). Uninoculated media was placed in a cuvette to be used as a blank for the spectrophotometer (Vernier SpectroVis Plus, Vernier, Beaverton, OR). The batch culture was inoculated with a 1:20 dilution (Ashley Hawkins, personal communication) of turbid culture and media. The optical density of each batch culture was measured at 600 nm (51) every 30 minutes for the first 4 hours, then every hour until a plateau in the growth curve was reached. These measurements were plotted against time to create growth curves (Figures 2 and 3) from which the mid-log and plateau phase could be determined.

Preparation of Culture Media

Once baseline growth curves were established, both species were maintained in tryptic soy broth (TSB) at 37°C. TSB was chosen as a control media because it was an ATCC recommended growth media for each species. Two types of infant formula (Table 1) were compared in this study: a cow's milk-based formula (Enfamil NeuroPro, Mead Johnson, Chicago, IL) and a cow's milk-based formula supplemented with probiotics (Nutramigen Enflora, Mead Johnson, Chicago, IL). The Nutramigen Enflora formula is supplemented with *Lactobacillus rhamnosus*, one of the most widely used probiotic strains utilized as a dietary supplement (26). A 1:5 (v/v) dilution of infant formula to TSB without dextrose was prepared for each formula following Hinds *et al*. (2016). Infant formulae were diluted with TSB without dextrose (Thermo Fisher Scientific, Waltham, MA) to ensure bacterial growth without adding another carbohydrate source.

Assessment of Cell Density under Experimental Conditions

For both species, cultures in TSB at the mid-log phase were prepared for the experiment in triplicate. Each of the infant formula media and the control media was inoculated with 100 μL of starting culture and vortexed to ensure homogeneity in replicates of three, for a total of 18 culture tubes including the control. Plate counts were performed by diluting the starting culture with fresh TSB to dilutions of 10^{-4}, 10^{-5}, and 10^{-6}. 100 μL of each of these dilutions were plated onto tryptic soy agar (TSA) plates in replicates of three. The TSA plates were incubated at 37°C for 24 hours, after which colony forming units were counted in plates that had between 30-300 colony forming units (CFUs) (33). The following formula was used to calculate the original cell density of the starting culture:

Original cell density=(colony forming units)/(original sample volume)

Assessment of Metabolic Activity under Experimental Conditions

Every hour over the course of each incubation, the pH of the culture media was measured to monitor the change in acid production using a YSI Ecosense pH 100A meter (YSI Incorporated, Yellow Springs, OH). The meter was calibrated according to manufacturer's instructions before use. A decrease in pH indicates acid production, which is related to the cariogenic potential of a culture.

Statistical Analyses

Statistical analyses were conducted using JASP (Version 0.11.1, JASP Team 2019). Shapiro-Wilks tests were used to confirm a normal distribution of data. Normally distributed data were analyzed using independent t-tests; when assumptions for parametric statistics were violated, data were analyzed using the non-parametric Mann-Whitney test. Independent t-tests and Mann-Whitney tests were performed on pH data to compare the effect of each species on the pH of each media at a given time point. Analysis of Variance (ANOVA) was used to assess the change in pH over time within the same media and species and to assess the change in pH between media types for a given species at a given point in time. Tukey's post-hoc tests were performed for all ANOVAs with significant p-values ($\alpha = 0.05$).

The same tests were utilized on data reporting CFUs, except for time points where one of the three replicate data points were missing due to inadequate growth during plate counts. In these cases, mean values will be used for data reporting, as there were not enough replicates at each time point to conduct statistical analyses. CFU and pH data were compared over time for observable trends between the two measurements for each species in each media type. Data from hours 0 and 8 were used as time points in the analysis because they represented the start-point and plateau phase of each incubation, respectively.

Results

Growth Curves under Baseline Conditions

The growth curves produced for both *S. mitis* (Figure 2) and *S. mutans* (Figure 3) showed a logarithmic curve with the lag phase from hours ca. 0-3 hours, a log phase from ca. 3-6 hours, a plateau phase from ca.6-8 hours, and the mid-log point ca. hour 4.5.

Snyder's Media Test

The degree of color change in the inoculated Snyder's Media was compared relative to the uninoculated test media (Figure 4a). After 24 hours, a color change in the media inoculated with *S. mutans* (Figure 4b) indicated a positive result and a lack of color change in the media inoculated with *S. mitis* (Figure 4c) indicated a negative result.

Colony Forming Units

The mean CFUs of *S. mutans* increased over time in TSB, with a ca. 10X increase between hour 0 and 8 (Table 2; Figure 5a), whereas the mean CFUs of *S. mitis* decreased over time in TSB, with a ca. 16X decrease between hour 0 and 8 (Table 2, Figure 5a). The mean CFUs of *S. mutans* decreased over time in NeuroPro and Enflora, by ca. 0.18X and 6.8X,

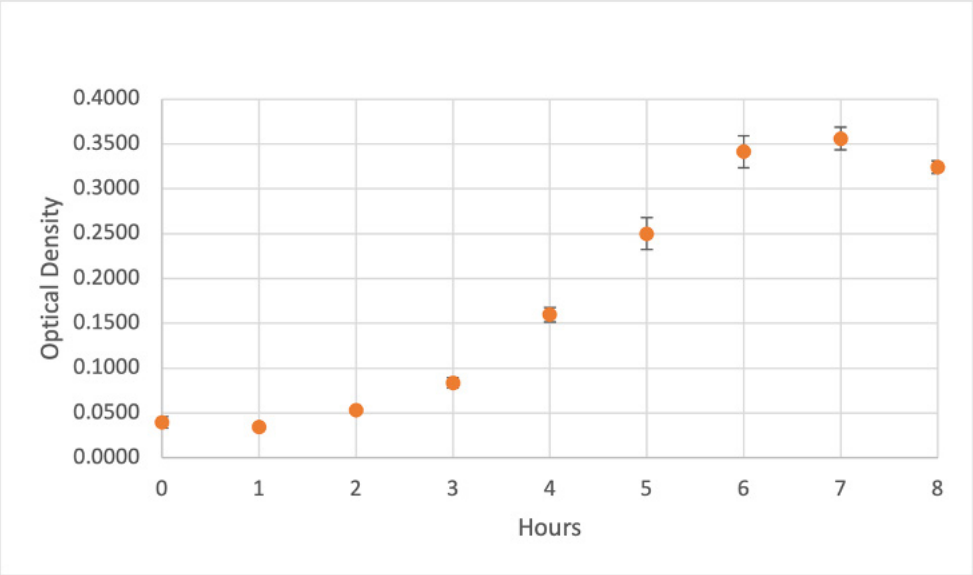

Figure 2: **The growth curve of *S. mitis* under baseline conditions. Growth in TSB was quantified by optical density, measured by spectrophotometery, over 8 hours. Error bars represent standard deviation of the mean (n= 3).**

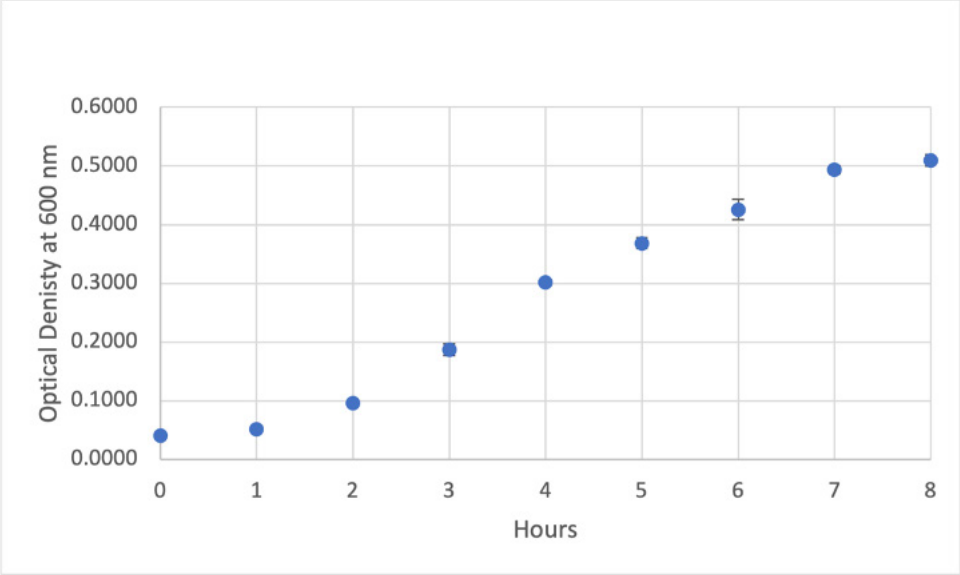

Figure 3: **The growth curve of *S. mutans* under baseline conditions. Growth in TSB was quantified by optical density, measured by spectrophotometery, over 8 hours. Error bars represent standard deviation of the mean (n= 3).**

respectively. The mean CFUs of *S. mitis* increased over time in NeuroPro and Enflora, with an increase of ca. 3X and 2.1X respectively between hour 0 and 8 (Table 2, Figures 5b and 5c). At all time points in each media, *S. mitis* had greater CFUs than *S. mutans*, except at hour 8 in TSB (Table 2, Figure 5).

To show which media yielded the highest cell density for each species at a given time, the mean values for CFUs in different media types were compared within a species (Table 2, Figure 6b). NeuroPro had the highest CFU count for *S. mitis* of ca. 2.59 x 107 compared to ca. 5.07 x 106 in TSB and ca. 2.40 x 107 in Enflora at hour 0 (Table 2, Figure 6a). At the end of the incubation, CFUs of *S. mitis* in NeuroPro was 347X and 2X higher than TSB and Enflora respectively. For *S. mutans* at hour 0, TSB yielded the highest CFU count of ca. 4.31 x 106 compared to ca. 2.40 x 105 in NeuroPro and ca. 3.90 x 105 in Enflora (Table 2, Figure 6b). At the end of the incubation, CFUs of *S. mutans* in TSB were 208X and 841X higher than NeuroPro and Enflora respectively. CFUs of *S. mutans* decreased ca. 15% in NeuroPro and ca. 87% in Enflora over the course of the incubation.

pH

The mean pH of *S. mitis* significantly decreased over time in all three media types (Table 4, Figure 7b). Between hour 0 and hour 8, the pH decreased by ca. 2.6% in TSB, ca. 1.9% in NeuroPro, and ca. 1.2% in Enflora. The mean pH of *S. mutans* significantly decreased over time in TSB but increased slightly over time in NeuroPro and Enflora (Table 3, Figure 7a). Between hour 0 and hour 8, the pH decreased by ca. 13.2% in TSB and increased by ca. 0.3% and ca. 0.4% in NeuroPro and Enflora respectively.

Results of the independent t-tests comparing pH levels of *S. mutans* to *S. mitis* showed a significant difference in each of the media types at two time points (Table 4, Figure 7). None of the Mann-Whitney tests were significant (Table S1). At hour 0 and 6, there was no significant difference in pH between the two species when cultured in all three of the media types (Table 4, Table S1). At hour 8, there was a significant difference in pH between the two species when cultured in all three of the media types. In NeuroPro and Enflora, *S. mutans* had a higher pH than *S. mitis*. In TSB, *S. mutans* had a lower pH than *S. mitis*.

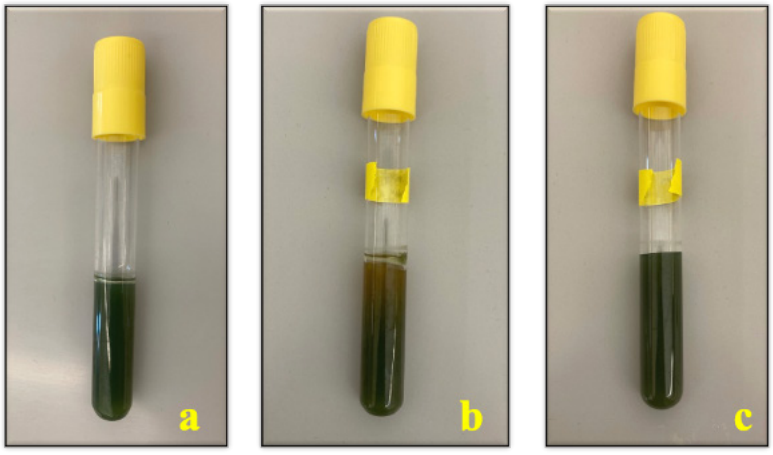

Figure 4: **Results of Snyder's Media Test. A change in the color of the media from dark green to orange within 24 hours indicates a positive result. Cultures pictured are representative of all three replicates for each species: a) uninoculated media, b) media inoculated with *S. mutans*, c) media inoculated with *S. mitis*.**

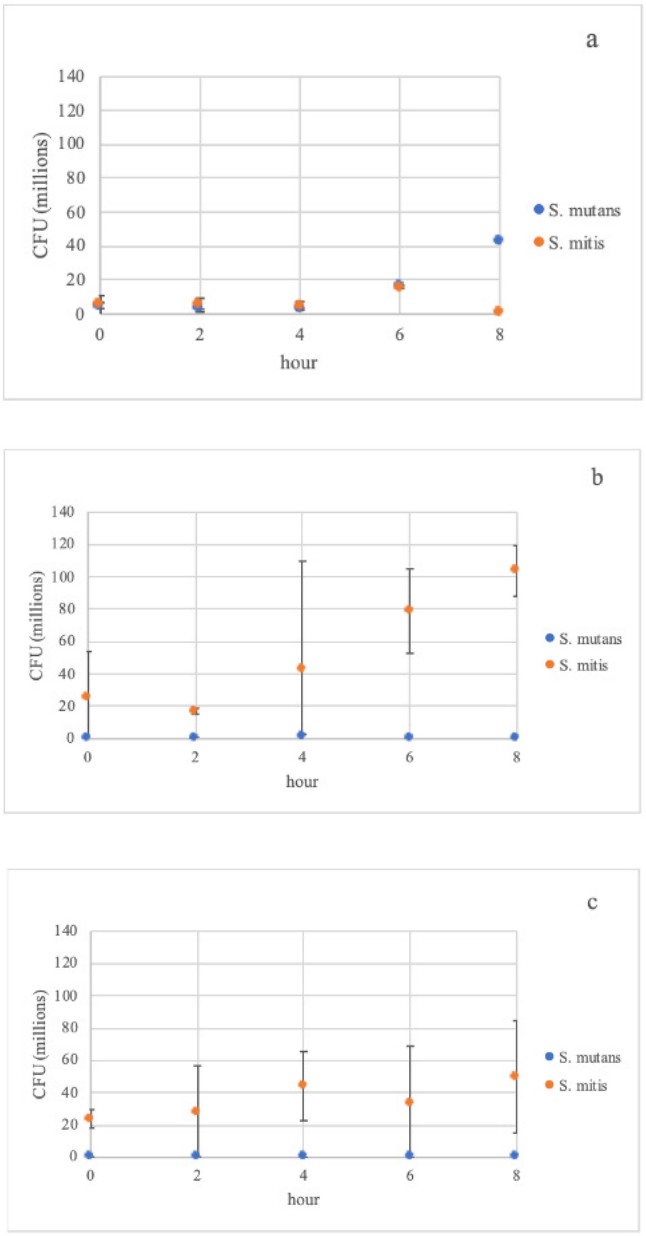

Figure 5: **Mean Colony Forming Units (CFU) of *S. mutans* and *S. mitis* over time. a) in TSB, b) in NeuroPro, c) in Enflora. Error bars representing standard deviation of the mean are included when n = 3.**

The results of the ANOVAs comparing the pH levels over the course of the incubation were significant ($p < 0.001$) for both species in all three media (Figure 8, Tables S2, S3, and S4), except for *S. mutans* in NeuroPro ($p = 0.753$). For *S. mutans* and *S. mitis*, there was a significant decline in the pH levels at 0, 4, and 8 hours when cultured in TSB. In NeuroPro, the pH levels at hours 0 and 8 significantly declined for *S. mitis*. In Enflora, the pH levels at hours 0 and 8 were significantly different in *S. mutans* and *S. mitis*, with an increase in pH for *S. mutans* and a decrease in pH for *S. mitis*. For *S. mutans*, the pH increased by ca. 0.3% and 0.4% in NeuroPro and Enflora, respectively, and dropped by ca. 15% in TSB. For *S. mitis*, the pH decreased by ca. 2.7%, 2%, and 1% in TSB, NeuroPro, and Enflora, respectively.

The Relationship between CFUs and pH

For the species and media that resulted in increased bacterial growth over time, there were notable trends between CFUs and pH over time. For *S. mutans* grown in TSB (Figure 9), *S. mitis* grown in NeuroPro (Figure 10), and *S. mitis* grown in

Enflora (Figure 11), the general trend is as the CFUs increase, the pH also decreases. In optimal growth conditions (Figure 6 & 7), there appears to be a steeper slope of the trendline relating CFUs to pH as compared to less optimal growth conditions (Figure 11). For the two conditions with the highest growth, *S. mutans* in TSB (Figure 9) and *S. mitis* in NeuroPro (Figure 10), the inflection point where CFUs increase sharply and pH decreases sharply was around the 4.5 hour mark (ca. mid-log phase). Figure 11 is representative of the remaining conditions: no inflection point in the slopes of the CFU and pH graphs was observed. The trends outlined above indicate that the high-growth conditions (*S. mutans* in TSB, *S. mitis* in NeuroPro and Enflora) had a stronger relationship between an increase in CFUs and decrease in pH than the low-growth conditions (*S. mutans* in NeuroPro and Enflora, *S. mitis* in TSB).

Discussion

Snyder's Media Test Confirms the Cariogenic Potential of S. mutans

The results of the Snyder's media test showed that *S. mutans* has a greater cariogenic potential than *S. mitis*. This reaffirms that *S. mutans* is an acidogenic and aciduric species. *S. mutans* produces lactic acid (36) which lowers the pH of the surrounding microenvironment, limits the growth of non-aciduric species (which are commonly commensal species) and produces conditions that favor the growth of other aciduric pathogens (32). In addition, the results of the Snyder's test showed that *S. mitis* does not have a high cariogenic potential. Although *S. mitis* is known to metabolize carbohydrates to lactic acid like the pathogenic *S. mutans* (30), *S. mitis* is generally considered to act as a commensal species when its growth is kept in check

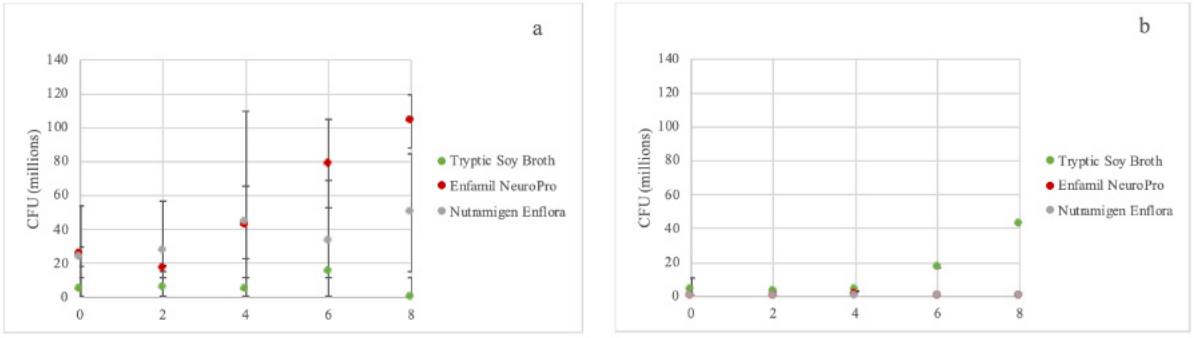

Figure 6: **Mean Colony Forming Units (CFU) of (a) *S. mitis* and (b) *S. mutans* in TSB, NeuroPro, and Enflora over time. Error bars representing standard deviation of the mean are included when n = 3.**

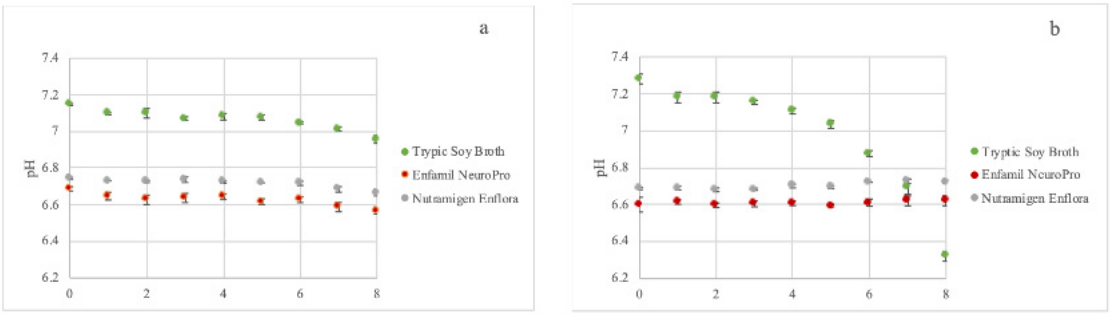

Figure 7: **Mean pH values in each media type over time: a) *S. mitis*, b) *S. mutans*. Error bars represent standard deviation of the mean.**

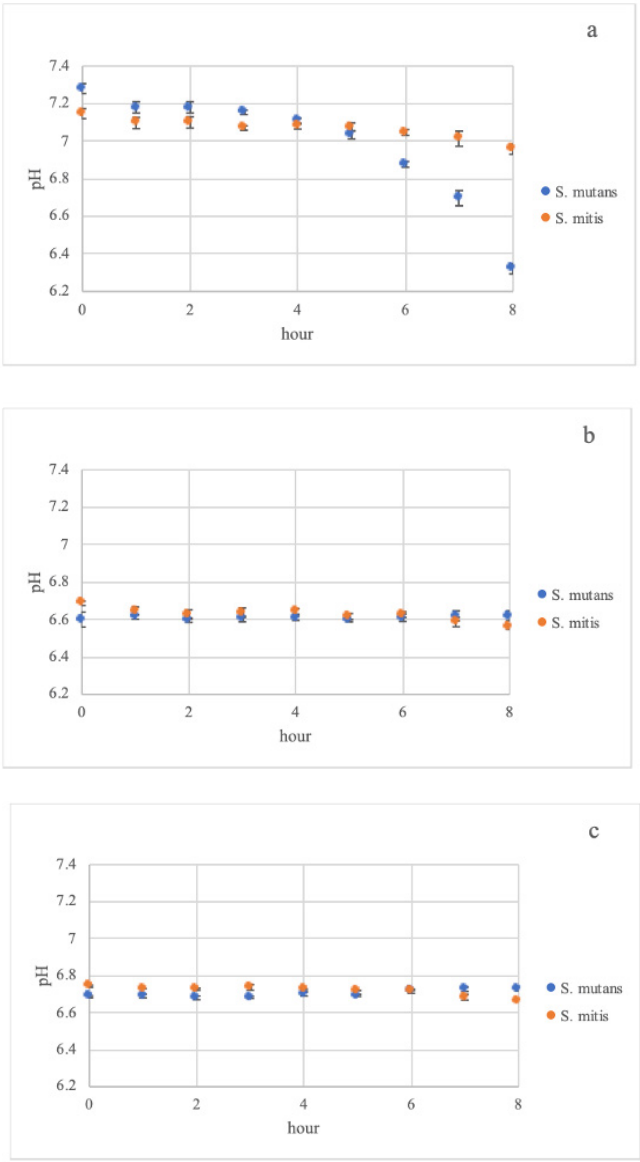

Figure 8: **Mean pH values of *S. mutans* and *S. mitis* over time in a) TSB, b) NeuroPro, and c) Enflora. Error bars represent standard deviation of the mean (n = 3).**

by competing commensals. To ensure the establishment of a beneficial microbial community early in life, it is important for infant formulae to have a nutritional composition that both inhibits the growth of pathogens like the cariogenic *S. mutans* and enhances the growth of commensals such as *S. mitis*.

Cell Density Reveals Lactose-based Formula Favors the Growth of S. mitis *over* S. mutans

S. mutans had ca. 141X more CFUs in TSB than *S. mitis* at the end of the 8 hour incubation, therefore TSB favored the growth of the pathogenic *S. mutans* over *S. mitis*.

When cultured in infant formula, there was a decrease in CFU counts of *S. mutans* over time, suggesting that the infant formulae did not provide optimal growing conditions for *S. mutans* compared to *S. mitis* and that the infant formulae may have an inhibitory effect on the growth of this opportunistic pathogen. This may be in part due to the different types and concentrations of carbohydrates found in the infant formulae and TSB. TSB contains 0.025 g/ml of dextrose while NeuroPro contains 0.015 g/ml of lactose and Enflora contains 0.014 g/ml of dextrose. Previous research examined the effects of infant formula composition on the growth of the pathogen *S. mutans* and found that

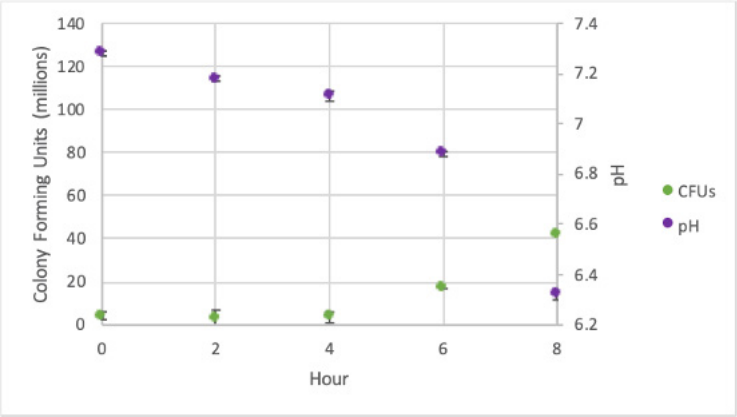

Figure 9: **The relationship between CFUs and pH for *S. mutans* grown in Tryptic Soy Broth. Error bars representing standard deviation of the mean when n = 3.**

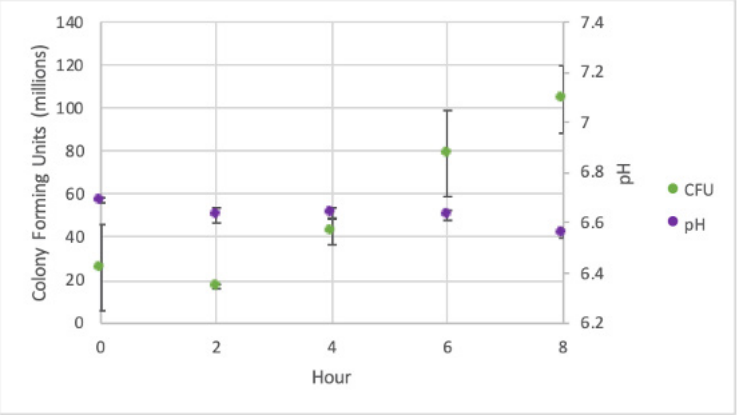

Figure 10: **The relationship between CFUs and pH of *S. mitis* grown in NeuroPro. Error bars representing standard deviation of the mean are included when n = 3.**

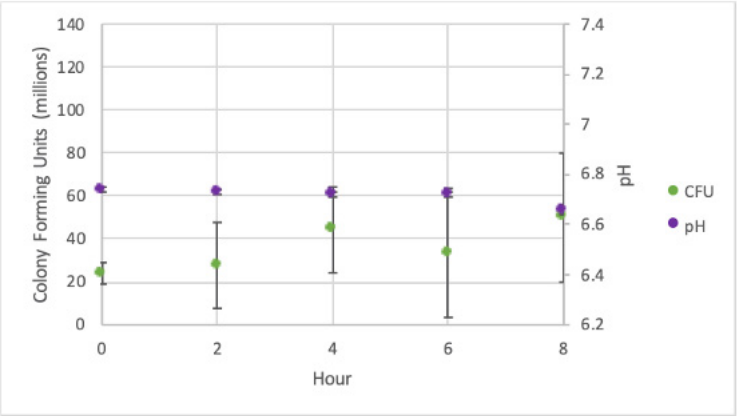

Figure 11: **The relationship between CFUs and pH of *S. mitis* grown in Enflora. Error bars representing standard deviation of the mean are included when n = 3.**

sucrose-based formulae yielded more growth of *S. mutans* when compared to formulae containing lactose (24). This is possibly due to sucrose being more readily fermentable, leading to a lowering of pH and sucrose being a substrate for the production of polysaccharides that allow bacterial adhesion and biofilm formation (45). Because *S. mutans* is known for its rapid consumption of carbohydrates (24, 34), the higher concentration of dextrose in TSB may have allowed for greater proliferation of *S. mutans* compared to the infant formulae due to sucrose being more readily fermentable than lactose and rapidly lowering the pH of the formula, creating a low pH environment in which *S. mutans* thrives.

The small difference between the CFU count of *S. mutans* in NeuroPro versus Enflora at the end of the incubation (CFUs for NeuroPro was 4X that of Enflora compared to CFUs for TSB being 208X and 841X higher than NeuroPro and Enflora, respectively) is likely not due to the different carbohydrates found in each formula, since the dextrose in Enflora is preferred to the lactose in Neuropro (40). The magnitude of decrease in CFUs of *S. mutans* was greater in Enflora (ca. 6.8X decrease) than in NeuroPro (ca. 0.18X decrease) over the course of the incubation; therefore, Enflora had a greater inhibitory effect on the growth of *S. mutans* than NeuroPro. Since the concentrations of carbohydrates are similar between NeuroPro and Enflora and previous research shows that *S. mutans* does not prefer lactose over dextrose, it may be the presence of the probiotic *L. rhamnosus* that caused this differential magnitude of decrease in CFUs between the two infant formulae.

One hypothesis for why the probiotic formula controlled the growth of both species is that the probiotic *L. rhamnosus* prevented uncontrolled growth by competing with the other species for resources. Previous research has shown that supplementation with *L. rhamnosus* resulted in a decrease in dental caries and amount of *S. mutans* in children (ages 1-6 years), indicating that the probiotic may have the ability to outcompete the pathogen and significantly inhibit its growth and acid production (41). This same degree of interspecies competition has not been shown between *L. rhamnosus* and *S. mitis*, perhaps because commensal species both prefer similar environmental conditions (i.e. neutral pH as opposed to the low pH that *S. mutans* produces and thrives in) and do not threaten each other by altering the environment to a state that would inhibit the other species' growth. This is good news in terms of how the infant formula impacts the community structure of the oral microbiome, because it indicates that the probiotic infant formula can potentially control both the overall growth of microbes (i.e. it controlled the growth of both the commensal and pathogen) and differentially (selectively) control the growth of the commensal and pathogen, with the commensal being favored and the pathogen being more strongly inhibited.

The decreased amount of *Streptococcus* growth may also have been influenced by the antimicrobial substances produced by *L. rhamnosus*. Existing literature shows that *L. rhamnosus* has multiple avenues of controlling microbial growth in co-culture, including the production of various antimicrobial substances. *L. rhamnosus* produces microcine, a small antimicrobial peptide along with another 7 different antibacterial peptides (35). *L. rhamnosus* also produces two kinds of lectin proteins (antimicrobial molecules that target pathogenic microbes) which have been shown to successfully inhibit the growth and biofilm formation of Salmonella species and *E. coli* (44). These lectin proteins have strong carbohydrate-recognition capabilities, which allows them to distinguish pathogenic microorganisms from nonpathogens based on the types and configurations of polysaccharides on the cell surfaces (44). The specificity of these probiotic lectin proteins for pathogenic microbes may explain why *L. rhamnosus* differentially affected the growth of *S. mutans* and *S. mitis*. These pathogen specific antimicrobial proteins may have targeted the pathogenic *S. mutans*, thus exerting a greater inhibitory effect on the pathogen compared to the commensal *S. mitis*. Competitive exclusion and the production of antimicrobial compounds present potential explanations for how the probiotic suppressed growth of the two *Streptococcus* species in this study.

In addition, *L. rhamnosus*' competition with *S. mitis* may also enhance host health by preventing the overgrowth of *S. mitis*. While *S. mitis* is generally considered a commensal in the oral microbiome, some studies have shown that an overabundance may cause the species to act as an

opportunistic pathogen (39). As previously mentioned, *S. mitis* can produce lactic acid like *S. mutans*, which in excess may lead to the formation of dental caries, giving it the potential to act as an opportunistic pathogen (1). In a study comparing the species composition of oral microbiomes of individuals with and without dental fluorosis, a dental disease associated with dental caries, individuals with dental fluorosis had a higher abundance of *S. mitis* than those without dental fluorosis (58). Therefore, it is understood that while *S. mitis* typically acts as a commensal when kept in check by the cohabitants of the oral microbial community, the overgrowth of *S. mitis* may lead to decreased health of the oral microbiome. By competing with *S. mitis* and preventing the domination of the oral microbiome, *L. rhamnosus* may bolster host health by maintaining balance within the oral microbiome. In fact, studies on the effects of probiotic consumption on the composition of the oral microbiome show promise for boosting host health. In a study comparing the oral microbiome composition of adults administered dietary *Lactobacillus* and *Streptococcus* probiotics and a control group, it was found that there was a short-term increase of overall diversity of the oral microbiome (13). However, the species diversity reverted to baseline (pre-treatment) levels after discontinuing probiotic intake.

S. mutans *Thrives in Sucrose-dense TSB,* S. mitis *Thrives in Lactose-based Formulae*

The significant decline in pH of *S. mutans* over time in TSB compared to in the infant formula media suggests that TSB provided better growing conditions for *S. mutans*, allowing the organism to metabolize sugars into lactic acid at a higher rate.

When *S. mutans* was cultured in both infant formula media, the pH actually increased slightly over time, which indicates *S. mutans*' lack of growth in the media. This lack of growth observed in the infant formulae suggests that these two infant formulae limit the growth of *S. mutans*, perhaps in part due to lower carbohydrate concentration in the infant formulae compared to TSB.

One explanation for the increasing pH of the infant formulae is *S. mutans*' ability to produce alkali under stress (47). *S. mutans* has been shown to produce alkali by converting arginine into ammonia, CO2, putrescine, and ATP (34). The production of ATP through this agmatine deiminase system is a protective response against starvation, among other stressors (7, 34). This stress-induced ATP production provides energy for the starving cell. It is plausible that starvation caused by low carbohydrate levels in the infant formulae induced alkali production as a protective measure in *S. mutans*. This alkali production may have in turn caused the small increase in pH over the course of the incubation. When comparing between the two infant formula media, *S. mutans* had a lower pH in the non-probiotic formula, indicating that *S. mutans* fared better in the nonprobiotic formula than in the probiotic formula. The lower pH of the non-probiotic formula suggests that *S. mutans*' growth was not as strictly inhibited as in the probiotic formula, indicating that the non-probiotic formula is less effective at preventing pathogenic growth.

S. mitis' relatively high pH in comparison to *S. mutans* when grown in TSB is another indication of the commensal's low cariogenic potential, despite its shared ability to produce lactic acid as a metabolite like *S. mutans*. The small magnitude of the drop in pH over the course of the incubation in TSB (ca. 2.7%) compared to the ca. 15% pH drop of *S. mutans* shows that TSB is better suited for the growth of *S. mutans* than *S. mitis*. The fact that *S. mitis* had a greater pH drop over the course of the incubation when grown in nonprobiotic formula compared to the probiotic formula may indicate that *S. mitis* also competed with *L. rhamnosus* for resources. The presence of the probiotic *L. rhamnosus* controlled the growth of both the pathogen *S. mutans* and the commensal *S. mitis*, although to differing degrees. The probiotic inhibited the growth of the pathogen more strictly and allowed for more growth of the commensal. The manner in which the probiotic differentially affected the growth of the pathogen and commensal demonstrates that *L. rhamnosus* may support the establishment of a healthy oral microbial community by controlling the overall level of microbial growth while simultaneously favoring the growth of commensals over pathogens.

Relationship between CFUs and pH

Over the course of the incubation, an increase in CFUs is generally accompanied by a decrease in pH. This simultaneous increase in cell density and acid production relates to the overall growth of each species. For instance, when *S. mutans* experienced an increase in CFUs between 4-6 hr and 6-8 hr over the course of the incubation in TSB, this was accompanied by a sharp decline in pH at both intervals. This shows the relationship between the increase in cell number and the increase in acid production, a byproduct of the organism's metabolism. Therefore, an increase in CFUs and decrease in pH can be interpreted as an overall increase in growth rate and cellular metabolism. This pattern that connects CFUs to pH is mirrored in the growth of *S. mitis* in both NeuroPro and Enflora.

Similarly, the decrease in CFUs of *S. mutans* grown in both of the infant formula media was accompanied by a slight increase in pH. The slow increase in pH shows that *S. mutans* was struggling to survive in these media, as there was no lactic acid production occurring to decrease the pH, and it is possible that the aforementioned agmatine deiminase system induced the production of alkali and ATP to cope with stress. This indicates that these media did not provide optimal growing conditions for this species to enable it to be a cariogenic threat. The suboptimal conditions of the infant formulae did not allow *S. mutans* to reach its full cariogenic potential, indicating that the infant formulae may help reduce the risk of developing dental caries over time. This pattern was more prominent in the probiotic formula, which provided the least optimal conditions for *S. mutans* out of all three media types, indicating that the probiotic formula had the best potential for both inhibiting the growth of the pathogen and therefore preventing it from producing enough lactic acid to induce tooth decay (enamel demineralization begins around a pH of 5.5) (37).

Conclusions and Directions for Future Research

The community structure of the oral microbiome is constructed over time, beginning during gestation and continuing into adulthood (1, 59). The colonization of the oral cavity occurs sequentially: newly introduced species are dependent on the species already present in the oral cavity (52). Since the established microbial community determines the species that are subsequently acquired, the state of the oral microbiome earlier in life shapes the trajectory of the microbiome later in life (59), especially since commensals and pathogens tend to exclude each other via competition (32).

This study showed that a probiotic-supplemented infant formula (Nutramigen Enflora) was more successful at inhibiting the growth of the pathogen (*S. mutans*) than the non-probiotic formula (Enfamil NeuroPro). The growth of *S. mitis* was greater in the non-probiotic formula, this along with the additional health benefits to the gut (i.e. preventing infection and diseases and aiding in maintaining a healthy weight) conferred by the probiotic may make Nutramigen Enflora the preferred infant formula for overall health. This information may aid parents in choosing an infant formula for their child if breastfeeding is not a viable option or choice. Since the nutrition during infancy is a key factor in determining the structure of the oral microbiome for the rest of the child's life (59), it is of parents' great concern that their child is consuming the best possible nutrition source for supporting the healthy development of the oral microbiome.

Although this study provides preliminary data to help parents in choosing an infant formula for their child if breastfeeding is not a viable option, it is limited in its ability to predict the behavior of oral microbiota *in vivo*. The health of the oral microbiome is affected by a large variety of influences, including internal factors such as host genetics (23) and external factors such as diet (29). The confluence of these factors works together to mold each individual's unique oral microbiome community (27). Between 700-1,200 different species of bacteria cohabitate within the oral cavity of humans, alongside numerous fungi, archaea, and viruses that all interact with one another to shape the oral microbial community (15, 57). The commensal and pathogenic species selected for this study are merely representative of the hundreds of different species that colonize the oral cavity. Future research on other prominent species, such as the commensals *Lactobacillus plantarum* and *Streptococcus sanguinis* and other pathogens including *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (27) is needed to expand the understanding of the full range of interspecific interactions that shape the oral microbiome.

In addition, there are many different kinds of infant formulae on the market that may differentially affect the growth of oral microorganisms. While this study sought to understand if and how one probiotic infant formula would differentially affect the growth of *Streptococcus* species compared to one non-probiotic infant formula of similar nutritional composition, there are many more brands and formulations of infant formula that could be studied. The two infant formulae used in this study were cow's milk based, but many other infant formulae use alternatives such as soy or goat milk and have varying nutritional composition that may affect the growth of oral microorganisms. A variety of carbohydrates (i.e. dextrose, sucrose, lactose), lipids (i.e. milk fats, palm, coconut, & sunflower oils), and proteins (i.e. casein, whey, soy protein isolate) are present in different infant formulae (18, 43, 49). Additionally, comparing how breastmilk affects the growth of oral microorganisms versus infant formulae is of interest, as breastmilk is recognized as being superior in supporting a healthy microbiome over infant formulae (40). Therefore, comparing how different kinds of infant formulae compare to breastmilk in terms of supporting microbiome health may help parents choose an infant formula that is most comparable to breastmilk in that aspect.

Finally, while this study examined the growth of oral microorganisms over a period of 8 hours, the oral microbiome is acquired sequentially over the years of childhood (52). Therefore, additional longitudinal studies are needed to capture how the community changes over time. One example of an important time point in the establishment of the oral microbiome is the eruption of the first tooth at ca. 6 months of age (59). The dental surface provides a new substrate for the colonization of the oral microflora and also happens to be *S. mutans*' preferred colonization niche within the mouth. Therefore, the time of primary dental eruption may be a critical inflection point in the trajectory of the development of the oral microbiome (12). Therefore, a deeper understanding of factors that influence the oral microflora during the predentate and primary dental eruption stages of the infant oral microbiome are of great importance.

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References

1. Abranches, J., Zeng, L., Kajfasz, J.K., Palmer, S.R., Chakroborty, B., Wen, Z.T., Richards, V.P., Brady, L.J. & Lemos, J.A. 2019. Biology of oral streptococci. Microbiol Spectr. 6:426-434. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6287261/

2. Al-Shehri, S., Knox, C., Liley, H., Cowley, D., Wright, J., Henman, M., Hewavitharana, A., Charles, B., Shaw, P., Sweeney, E. & Duley, J. 2015. Breastmilk-saliva interactions boost innate immunity by regulating the oral microbiome in early infancy. PLoS ONE 10:e0135047. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0135047

3. Al-Shehri, S., Sweeney, E., Cowley, D., Liley, H., Ranasinghe, P., Charles, B., Shaw, P., Vagenas, D., Duley, J. & Knox, C. 2016. Deep sequencing of the 16S ribosomal RNA of the neonatal oral microbiome: a comparison of breast-fed and formula-fed infants. Sci Rep. 6:38309. https://www.nature.com/articles/srep38309

4. Benedict, C., Vogel, H., Jonas, W., Woting, A., Blaut, M., Schurmann, A. & Cedernaes, J. 2016. Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. Mol Metab. 5:11751186. https://pubmed.ncbi.nlm.nih.gov/27900260/

5. Bowen, W.H., Burne, R.A., Wu, H., & Koo, H. 2018. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. Trends in Microbiol. 26:229-242. https://pubmed.ncbi.nlm.nih.gov/29097091/

6. Bravo J., Forsythe, P., Chew, M., Escaravage, E., Savignac, H., Dinan, T., Bienenstock, J. & Cryan, J. 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci. 108:16050-16055. https://www.pnas.org/content/108/38/16050

7. Burne, R. & Marquis, R. 2000. Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol let. 193:1-6. https://pubmed.ncbi.nlm.nih.gov/11094270/

8. Buyukeren, M., Yigit, S., Buyukcam, A., Kara, A., Tolga, H. & Murat Yurdakok M. 2020. A new use of *Lactobacillus rhamnosus* GG administration in the NICU: colonized vancomycin-resistant enterococcus eradication in the gastrointestinal system. J Mat-Fetal & Neonat Med. https://www.tandfonline.com/doi/abs/10.1080/14767058.2020.1743671

9. Cho, I. & Blaser, M.J. 2012. The human microbiome: at the interface of health and disease. Nature, 13:260-270. https://www.nature.com/articles/nrg3182

10. Cook, M., Allen, J., Pence, B., Wallig, M., Gaskins, H., White, B. & Woods, J. 2016. Exercise and gut immune function: evidence of alterations in colon immune cell homeostasis and microbiome characteristics with exercise training. Immunol Cell Biol. 94:158-163. https://onlinelibrary.wiley.com/doi/10.1038/icb.2015.108

11. Craig, S., Blankenberg, D., Parodi, A., Paul, I., Birch, L., Savage, J., Marini, M., Stokes, J., Nekrutenko, A., Riemherr, M., Chiaromonte, F. & Makova, K. 2018. Child weight gain trajectories linked to oral microbiota composition. Sci Rep. 8:14030. https://www.nature.com/articles/s41598-018-31866-9

12. Crielaard, W. *et al*. 2011. Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. BMC Med Genomics 4:22. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3058002/

13. Dassi, E., Ferretti, P., Covello, G., Bertorelli, R., Denti, M. A., De Sanctis, V., *et al*. 2018. The short-term impact of probiotic consumption on the oral cavity microbiome. Sci Rep. 8:1-8. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6041349/

14. Deo, P. N., & Deshmukh, R. 2019. Oral microbiome: Unveiling the fundamentals. JOMFP. 23:122-128. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6503789/

15. Dewhirst, F.E., Chen, T., Izard, J., Paster, B.J., Tanner, A.C.R., Yu, W.H., *et al*. 2010. The human oral microbiome. J Bacteriol. 192:2002-5017. https://pubmed.ncbi.nlm.nih.gov/20656903/

16. Dominguez-Bello, M.G., De Jesus-Laboy, K.M., Shen, N., Cox, L.M., Amir, A., Gonzalez, A., Bokulich, N., Song, S., Hoashi, M., Rivera-Vinas, J., Mendez, K., Knight, R. & Clemente, J. 2016. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. Nat med. 22:250. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5062956/

17. Eberhard, J., Pietschmann, R., Falk, W., Jepsen, S. & Dommisch, H. 2009. The immune response of oral epithelial cells induced by single-species and complex naturally formed biofilms. Oral Microbiol Immunol. 24:325-330. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1399-302X.2009.00518.x

18. Enfamil NeuroPro infant formula. 2020. Retrieved April 30, 2021, from https://www.enfamil.com/products/enfamil-neuropro-infant-formula/

19. Engen, S., Rørvik, G., Schreurs, O. *et al*. (2017). The oral commensal *Streptococcus mitis* activates the aryl hydrocarbon receptor in human oral epithelial cells. Int J Oral Sci. 9:145-150. https://www.nature.com/articles/ijos201717

20. Farrell, J.J., Zhang, L., Zhou, H., Chia, D., Elashoff, D., & Akin, D. 2012. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. Gut. 61:582-588. https://pubmed.ncbi.nlm.nih.gov/21994333/

21. Ferrer M, Méndezgarcía C, Rojo D, Barbas C, & Moya A. 2017. Antibiotic use and microbiome function. Biochem Pharmacol. 134:114-126. https://pubmed.ncbi.nlm.nih.gov/27641814/

22. Gilbert, J., Blaser, M.J. Caporaso, J.G. Jansson, J. Lynch, & S.V. Knight, R. 2018. Current understanding of the human microbiome. Nat Med. 24:392-400. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7043356/

23. Goodrich, J., Waters, J., Poole, A., Sutter, J., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J., Spector, T., Clark, A. & Ley, Ruth. 2014. Human genetics shape the gut microbiome. Cell. 159:789-799. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4255478/

24. Hinds, L.M., Moser, E.A.S., Eckert, G., & Gregory, R.L. 2016. Effect of Infant Formula on *Streptococcus mutans* Biofilm Formation. J Clin Pediatr Dent. 40:401-408. https://pubmed.ncbi.nlm.nih.gov/27472563/

25. Huttenhower, C. & Gevers, D. 2012. Structure, function and diversity of the healthy human microbiome. Nature. 486:207-214. https://www.nature.com/articles/nature11234

26. Isolauri E., Kirjavainen P.V., & Salminen S. 2002. Probiotics: a role in the treatment of intestinal infection and inflammation? Gut. 50:iii54-iii59. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1867676/

27. Jia, G., Zhi, A., Lai, P.F.H., Wang, G., Xia, Y., Xiong, Z., Zhang, H., Che, N., & Ai, L. 2017. The oral microbiota—a mechanistic role for systemic diseases. Brit Dent J. 224:447-455. https://pubmed.ncbi.nlm.nih.gov/29569607/

28. Karczewski, J., Poniedziałek, B., Adamski, Z. & Rzymski, P. 2014. The effects of the microbiota on the host immune system. Autoimmun. 47:494:504. https://pubmed.ncbi.nlm.nih.gov/25019177/

29. Kato, I., Vasquez, A., Moyerbrailean, G., Land, S., Djuric, Z., Sun, J., Lin, H., & Ram, J. 2016. Nutritional Correlates of Human Oral Microbiome. J Am Coll Nutr. 36:88-98. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5477991/

30. Kilian, M., Mikkelsen, L., & Henrichsen, J. 1989. Taxonomic study of viridans streptococci: description of *Streptococcus* gordonii sp. nov. and emended descriptions of *Streptococcus* sanguis (White and Niven 1946), *Streptococcus* oralis (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int J Syst and Evolut Microbiol. 39:471-484. https://www.researchgate.net/publication/242219755_Taxonomic_Study_of_Viridans_Streptococci_Description_of_*Streptococcus*_gordonii_sp_nov_and_Emended_Descriptions_of_*Streptococcus*_sanguis_White_and_Niven_1946_*Streptococcus*_oralis_Bridge_and_Sneath_1982

31. Kirchherr, J.L., Bowden, G.H., Richmond, D.A., Sheridan,M.J., Wirth, K.A., & Cole, M.F. 2005. Clonal diversity and turnover of *Streptococcus mitis* bv shedding and nonshedding oral surfaces of human infants during the first year of life. Clin Diagn Lab Immunol. 10:1184-1190. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1247832/

32. Lamont, R.J., Koo, H., & Hajishengallis, G. 2018. The oral microbiota: dynamic communities and host interactions. Nat Rev: Microbiol. 16:745-759. https://pubmed.ncbi.nlm.nih.gov/30301974/

33. Leboffe, M. & Pierce, B. 2016. Microbiology: Laboratory Theory and Application, Brief. 3rd ed. Morton Publishing. Englewood, CO. pg. 412.

34. Lemos, J. A., Palmer, S. R., Zeng, L., Wen, Z. T., Kajfasz, J. K., Freires, I. A., Abranches, J., & Brady, L. J. 2019. The Biology of *Streptococcus mutans*. Microbiol Spectr. 7: 10.1128. https://pubmed.ncbi.nlm.nih.gov/30657107/

35. Lu R., Fasano S., Madayiputhiya N., *et al*. 2009. Isolation, identification, and characterization of small bioactive peptides from *Lactobacillus* GG conditional media that exert both anti- Gram-negative and Gram-positive bactericidal activity. J Pediatr Gastroenterol Nutr. 49:23-30. https://pubmed.ncbi.nlm.nih.gov/19465870/

36. Madigan M., Martinko J., eds. 2005. Brock Biology of Microorganisms (11th ed.). Prentice Hall. ISBN 978-0-13-144329-7

37. Marsh P.D. 2009. Dental plaque as a biofilm: the significance of pH in health and caries. Compend Contin Educ Dent. 30:76-78. https://pubmed.ncbi.nlm.nih.gov/19301526/

38. Mashimo P., Yamamoto Y., Nakamura M., Reynolds H., & Genco R. 1985. Lactic acid production by oral *Streptococcus mitis* inhibits the growth of oral Capnocytophaga. J Periodontol. 56:548-52.

39. Mitchell, J. 2011. *Streptococcus mitis*: walking the line between commensalism and pathogenesis. Mol. Oral Microbiol. 26:89-98. https://pubmed.ncbi.nlm.nih.gov/21375700/

40. More, S., Sankeshwari, R., Patil, P.A., Jalihal, S.S., & Ankola, A.V. 2018. Infant formula and early childhood caries. J Dent Res Rev. 5:7-11. https://www.jdrr.org/article.asp?issn=2348-2915;year=2018;volume=5;issue=1;spage=7;epage=11;aulast=More

41. Naase L., Hatakka K., Savilahti E., *et al*. 2001. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res. 35:412–20. https://pubmed.ncbi.nlm.nih.gov/11799281/

42. Nishimura, E., Eto, A. & Kato, M. 2004. Oral streptococci exhibit diverse susceptibility to human beta-defensin-2: antimicrobial effects of hBD-2 on oral Streptococci. Curr Microbiol. 48:85-87. https://pubmed.ncbi.nlm.nih.gov/15057473/

43. Nutramigen with Enflora LGG infant formula. 2020. Retrieved May 4, 2021 from https://www.enfamil.com/products/nutramigen-powder-infant-formula/

44. Petrova M.I., Imholz N.C., Verhoeven T.L., Balzarini, J., Van Damme, E., Schols, D., Vanderleyden, J. & Lebeer, S. 2016. Lectin-like molecules of *Lactobacillus rhamnosus* GG inhibit pathogenic *Escherichia coli* and Salmonella biofilm formation. PLoS One. 11:0161337. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0161337

45. Sadan, H., Shanthala, BM., Zareena, MA., Babu, G., Vijayan, V. 2020. In vitro evaluation of milk-based, soy-based, and amino acid-based infant formulas on *Streptococcus mutans* biofilm formation.

46. Sender, R., Fuchs, S., & Milo, R. 2016. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. Cell. 164:337-340. https://pubmed.ncbi.nlm.nih.gov/26824647/

47. Sheng, J., Baldeck, J. D., Nguyen, P. T., Quivey, R. G., Jr, & Marquis, R. E. 2010. Alkali production associated with malolactic fermentation by oral streptococci and protection against acid, oxidative, or starvation damage. Can J Microbiol. 56:539-547. https://pubmed.ncbi.nlm.nih.gov/20651853/

48. Sherman, N. & Cappuccino J. 2002. Microbiology: a laboratory manual. Pearson Education, Inc. as Benjamin Cummings.

49. Similac Soy Isomil infant formula. 2021. Retrieved May 4, 2021 from https://www.similac.com/products/baby-formula/soy-isomil-powder/30-8oz-can-4pack.html

50. Song, S., Lauber, C., Costello, E., Lozupone, C., Humphrey, G., Berg-Lyons, D., Caporaso, J., Knights, D., Clemente, J., Nakielny, S., Gordon, J., Fierer, N. & Knight, R. 2013. Cohabiting family members share microbiota with one another and with their dogs. eLife. 2:e00458. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3628085/

51. Sonnleitner, B. 2006. Basic Biotechnology. Third edition. Cambridge Uni. Press. Pg 254.

52. Sulyanto, R.M., Thompson, Z.A., Beall, C.J., Leys, E.J. & Griffen, A.L. 2019. The predominant oral microbiota is acquired early in an organized pattern. Sci Rep. 9:10550. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6646312/

53. Thomas A.M., Gleber-Netto F.O., Fernandes G.R., *et al*. 2014. Alcohol and tobacco consumption affects bacterial richness in oral cavity mucosa biofilms. BMC Microbiol. 14:1-12. https://pubmed.ncbi.nlm.nih.gov/25278091/

54. Timby, N., Domellof, M., Holgerson, P.L., West, C.E., Lonnerdal, B., Hernell, O., & Johansson, I. 2017. Oral microbiota in infants fed a formula supplemented with bovine milk fat globule membranes- a randomized controlled trial. PLoS ONE. 12:e0169831. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0169831

55. Turnbaugh P., Ley, R., Hamady, M., Fraser-Liggett, C., Knight, R. & Gordon, J. 2007. The human microbiome project. Nature, 449, 804-810. https://www.nature.com/articles/nature06244

56. Verma, D., Garg, P.K., & Dubey, A.K. 2018. Insights into the human oral microbiome. Arch. Microbiol. 200:525-540. https://pubmed.ncbi.nlm.nih.gov/29572583/

57. Wade, W.G. 2013. The oral microbiome in health and disease. Pharm Res. 69:137-143. https://pubmed.ncbi.nlm.nih.gov/23201354

58. Wang, Q., Chen, X., Hu, H., Wei, X., Wang, X., Peng, Z., Ma, R., Zhao, Q., Zhao, J., Liu, J. & Deng, F. 2021. Structural changes in the oral microbiome of the adolescent patients with moderate or severe dental fluorosis. Sci Rep. 11:2897. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7859183/

59. Xiao, J., Fiscella, K.A., & Gill, S.R. 2020. Oral microbiome: possible harbinger for children's health. Internat J Sci. 12:12. https://www.nature.com/articles/s41368-020-0082-x

60. Ying, S., Zeng, D., Chi, L., Tan, Y., Galzote, C., Cardona, C., Lax, S., Gilbert, J. & Quan, Z. 2015. The influence of age and gender on skin-associated microbial communities in urban and rural human populations. PLoS One. 10:e0141842. https://pubmed.ncbi.nlm.nih.gov/26510185/

61. Zhang, G., Chen, R. & Rudney, J.D. 2008. *Streptococcus* cristatus attenuates Fusobacterium nucleatum-induced interleukin-8 expression in oral epithelial cells. J Periodontal Res. 43:408416. https://pubmed.ncbi.nlm.nih.gov/18942189/

Tables

Table 1: **Composition of infant formulae.** The nutritional composition (per 100 kcal) of the non-probiotic (Enfamil NeuroPro) and probiotic (Nutramigen Enflora) infant formulae.

	CARBOHYDRATE	FAT	PROTEIN	IRON
ENFAMIL NEUROPRO	Lactose: 11.3 g	Palm olein, coconut, soy, and high oleic sunflower oils: 5.3 g	Nonfat milk, Whey protein: 2 g	1.8 mg
NUTRAMIGEN ENFLORA	Corn syrup solids: 10.3 g	Palm olein, coconut, soy, and high oleic sunflower oils: 5.3 g	Casein hydrolysate: 2.8 g	1.8 mg

Table 2: **Mean CFUs of *S. mutans* and *S. mitis* over time.** Data points without standard deviation had insufficient replicates to calculate standard deviation.

Media	Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
TSB	$4.31x10^6 \pm 6.58x10^6$	$2.91x10^6$	$3.50x10^6$	$1.69x10^7$	$4.23x10^7$
	$5.07x10^6 \pm 1.80x10^6$	$5.37x10^6 \pm 4.03x10^6$	$4.87 x10^6 \pm 2.51x10^6$	$1.52 x10^7$	$3.00x10^5 \pm 6.05x10^4$
NeuroPro	$2.40x10^5$	$4.02x10^5 \pm 4.37x10^5$	$1.16x10^6 \pm 1.59x10^6$	$9.15x10^4$	$2.03x10^5 \pm 1.02x10^5$
	$2.59x10^7 \pm 2.80x10^7$	$1.71x10^7 \pm 1.82x10^6$	$4.25x10^7 \pm 6.72x10^7$	$7.89x10^7 \pm 2.60x10^7$	$1.04x10^8 \pm 1.57x10^7$
Enflora	$3.90x10^5$	$1.82x10^5 \pm 1.80x10^5$	$9.30x10^4 \pm 1.31x10^4$	$6.80x10^4$	$5.03x10^4 \pm 4.81x10^4$
	$2.40x10^7 \pm 5.70x10^7$	$2.77x10^7 \pm 2.91x10^7$	$4.42x10^7 \pm 2.14x10^7$	$3.35x10^7 \pm 3.35x10^7$	$4.99x10^7 \pm 3.50x10^7$

Table 3: **Mean pH values of *S. mutans* and *S. mitis* over time.** Reported error is standard deviation of the mean (n = 3).

Media	Hour 0	Hour 1	Hour 2	Hour 3	Hour 4	Hour 5	Hour 6	Hour 7	Hour 8
TSB	7.28 ± 0.03	7.18 ± 0.03	7.18 ± 0.03	7.15 ± 0.01	7.11 ± 0.02	7.03 ± 0.02	6.88 ± 0.02	6.70 ± 0.04	6.32 ± 0.03
	7.15 ± 0.01	7.10 ± 0.01	7.10 ± 0.03	7.07 ± 0.01	7.08 ± 0.02	7.08 ± 0.02	7.05 ± 0.01	7.01 ± 0.01	6.96 ± 0.02
NeuroPro	6.6 ± 0.04	6.61 ± 0.01	6.60 ± 0.01	6.60 ± 0.02	6.61 ± 0.01	6.59 ± 0.01	6.62 ± 0.03	6.62 ± 0.03	6.62 ± 0.03
	6.69 ± 0.01	6.65 ± 0.02	6.63 ± 0.03	6.64 ± 0.03	6.64 ± 0.02	6.62 ± 0.02	6.63 ± 0.02	6.59 ± 0.03	6.56 ± 0.02
Enflora	6.69 ± 0.01	6.69 ± 0.01	6.68 ± 0.01	6.68 ± 0.01	6.70 ± 0.01	6.69 ± 0.01	6.72 ± 0.00	6.73 ± 0.01	6.72 ± 0.01
	6.74 ± 0.01	6.73 ± 0.00	6.73 ± 0.01	6.74 ± 0.02	6.72 ± 0.01	6.72 ± 0.00	6.72 ± 0.01	6.68 ± 0.02	6.66 ± 0.02

Table 4: **P-values from the independent t-tests comparing the pH of media between cultures inoculated with *S. mutans* and *S. mitis* at different intervals throughout the incubation.** Non-normally distributed data were not included (denoted by nn). pH was the same for each replicate in Enflora at hour 6 (denoted by var= 0).

Media	Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
TSB	nn nn	0.026 nn	nn 0.029	nn 0.315	<0.001 0.033
NeuroPro	nn	nn	nn	var=0	0.003
Enflora					

Supplemental Tables

Table S1: **P-values from Mann-Whitney test for non-normally distributed data comparing the pH of media between cultures inoculated with *S. mutans* and *S. mitis* at different intervals throughout the incubation.** Normally distributed data were not included (denoted by nd).

Media	Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
TSB	0.077	nd	0.164	0.077	nd
NeuroPro	0.077	0.184	nd	nd	nd
Enflora	0.072	0.077	0.077	nd	nd

Table S2: **Post-hoc p-values of an ANOVA comparing the pH of TSB between time points throughout the incubation.** Reported post-hoc p-values from an ANOVA comparing the pH of *S. mutans* and *S. mitis* in TSB over time indicate if the difference in pH between time points is significant for a given species.

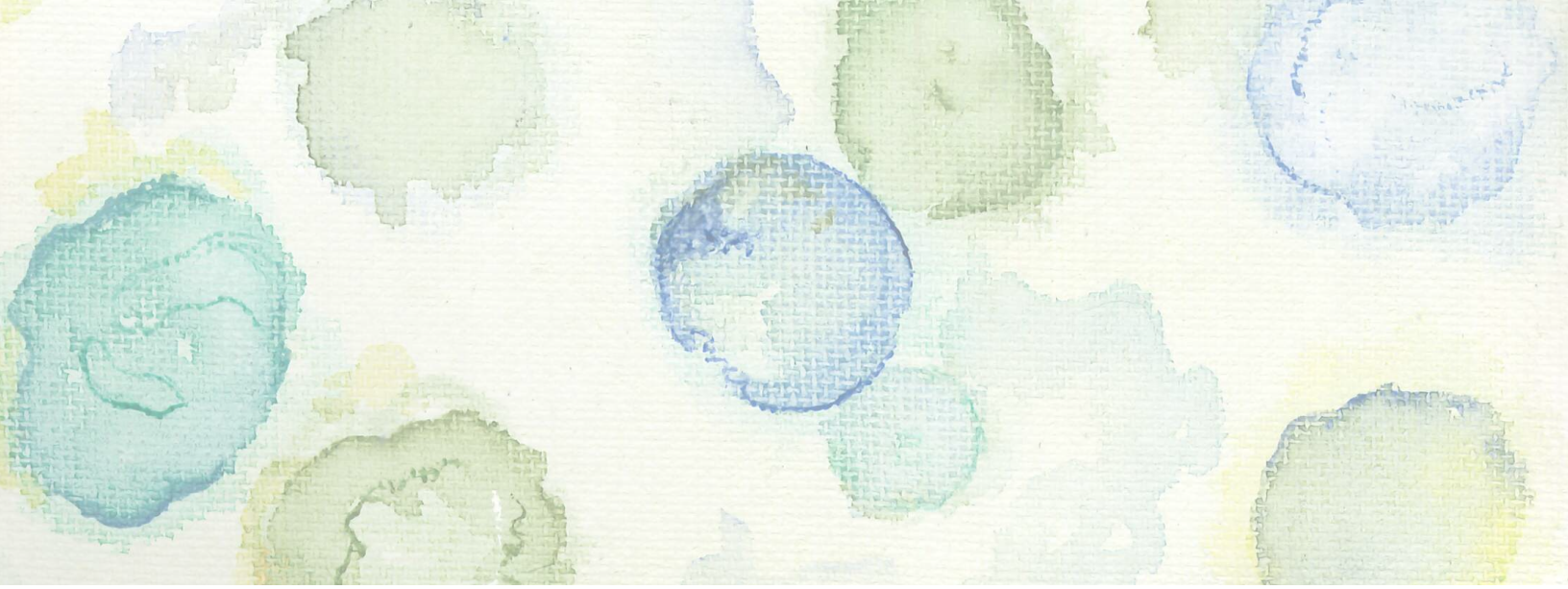
Hour	0	1	2	3	4	5	6	7	8
0		0.015	0.026	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
1	0.004		1	0.45	0.895	0.771	0.015	<0.001	<0.001
2	0.004	1		0.31	0.771	0.612	0.008	<0.001	<0.001
3	<0.001	0.925	0.925		0.995	1	0.612	0.005	<0.001
4	<0.001	0.049	0.049	0.423		1	0.202	<0.001	<0.001
5	<0.001	<0.001	<0.001	<0.001	0.049		0.31	0.002	<0.001
6	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		0.202	<0.001
7	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		0.005
8	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Table S3: **Post-hoc p-values of an ANOVA comparing the pH of NeuroPro between time points throughout the incubation.** Reported post-hoc p-values from an ANOVA comparing the pH of *S. mutans* and *S. mitis* in NeuroPro over time indicate if the difference in pH between time points is significant for a given species.

Hour	0	1	2	3	4	5	6	7	8
0		0.285	0.029	0.098	0.204	0.008	0.029	<0.001	<0.001
1	0.996		0.931	0.999	1	0.628	0.931	0.29	0.001
2	1	0.985		0.999	0.975	0.999	1	0.285	0.019
3	1	1	1		1	0.931	0.999	0.098	0.005
4	1	1	1	1		0.75	0.975	0.044	0.002
5	1	0.957	1	1	0.996		0.999	0.628	0.066
6	1	1	0.996	1	1	0.985		0.285	0.019
7	0.957	1	0.904	0.985	0.996	0.825	1		0.855
8	0.957	1	0.904	0.985	0.996	0.825	1	1	

S4: **Post-hoc p-values of an ANOVA comparing the pH of Enfamil between time points throughout the incubation.** Reported post-hoc p-values from an ANOVA comparing the pH of *S. mutans* and *S. mitis* in Enflora over time indicate if the difference in pH between time points is significant for a given species.

Hour	0	1	2	3	4	5	6	7	8
0		0.791	0.559	0.995	0.337	0.18	0.088	<0.001	<0.001
1	1		1	0.995	0.995	0.946	0.791	<0.001	<0.001
2	0.977	0.821		0.946	1	0.995	0.946	0.002	<0.001
3	1	0.977	1		0.791	0.559	0.337	<0.001	<0.001
4	0.527	0.821	0.11	0.263		1	0.995	0.003	<0.001
5	0.977	1	0.527	0.821	0.977		1	0.008	<0.001
6	0.002	0.005	<0.001	<0.001	0.11	0.015		0.018	<0.001
7	<0.001	<0.001	<0.001	<0.001	0.015	0.002	0.977		0.337
8	<0.001	0.002	<0.001	<0.001	0.041	0.005	1	1	



Antibacterial Effects of Bitter Melon Extract in Combination With Commonly Prescribed Antibiotics

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Abstract:

Antibiotics are commonly overprescribed or taken incorrectly, which has resulted in an alarming increase of antibiotic-resistant bacteria. One potential solution to combat this problem is administering multiple antibiotics together to achieve antibiotic synergy; when two or more antibiotics work together to increase antibacterial efficacy. When considering potential synergistic combinations of antibiotics, one possibility is to utilize antibacterial plant extracts in addition to common antibiotics. The goal of our research was to compare the antibacterial properties of the Chinese medicinal plant bitter melon (*Momordica charantia*) and four common antibiotics alone or in combination with bitter melon against *Micrococcus luteus*, *Pseudomonas putida*, and *Escherichia coli*. We hypothesized that combining the antibiotics with bitter melon extract would result in increased antibacterial effects against one or more bacterial strains. Oil from dried bitter melon was prepared using the Soxhlet extraction method. Antibacterial properties of bitter melon extract and carbenicillin, streptomycin, colistin, and tetracycline alone or in combination with the extract were determined by performing disk diffusion assays. Diameters of the resulting zones of inhibition for the two treatments were measured and analyzed for statistical significance by performing a two-tailed, paired sample t-test using *Rguroo*. We found that bitter melon extract individually had little to no antibacterial effect against any of the organisms tested. Interestingly however, combining bitter melon extract with common antibiotics resulted in synergistic effects in some cases, as well as one example of antibiotic antagonism. These results demonstrate that plant-derived extracts can enhance the antibacterial effects of commonly prescribed antibiotics if paired correctly.

Introduction

The injudicious prescription and use of antibiotics creates selective pressure for bacteria to evolve resistance. According to the Centers for Disease Control and Prevention, each year over 2.8 million people are infected with antibiotic-resistant bacteria (4). In the United States alone, a person acquires an antibiotic- resistant infection every 11 seconds, while every 15 minutes someone dies from such an infection (4). This startling statistic can be attributed in part to the fact that nearly one-third of the antibiotics prescribed are not appropriate for the conditions being treated. The continuous misuse of readily available conventional antibiotics is proving to be a catalyst for the persistence, evolution and spread of antibiotic resistance. This severely limits the efficacy of antibiotics that were once used to treat life threatening bacterial infections and is now resulting in increased hospitalization rates and even deaths due to the rise in resistance. In turn, this leads to the requirement for heavier dosages and new antibiotic drugs to treat infections that were once readily treatable (4). Health care professionals, scientists and the public must do their part in combating this growing problem as antibiotic resistance is evolving into a serious threat to the human healthcare field globally.

One potential solution to combat the rising number of antibiotic-resistant strains is administering multiple antibiotics together to achieve antibacterial synergy. If an antibiotic demonstrates stronger antibacterial effects in combination with another antibiotic rather than when used alone, the combination treatment can be deemed synergistic. Alternatively, antibiotic antagonism occurs when the overall antibiotic efficacy of two or more antibiotics administered together is decreased relative to the effects of each when used alone (1). Combining extracts from plants with commonly prescribed antibiotics is one possible way to achieve antibacterial synergy. Plant-derived compounds can exhibit a direct antibacterial activity as well as indirect activity as antibiotic resistance modifying compounds that, when combined with antibiotics, can potentially increase their efficacy (13). The organic compounds in plants are referred to as biologically active substances and include phenolic compounds, terpenes, and alkaloids. These substances can be isolated into crude extracts, some of which have prominent antibacterial activity (13).

In this study, we tested the antibacterial properties of an organic extract prepared from the Chinese medicinal plant bitter melon (*Momordica charantia*) alone and in combination with commonly prescribed antibiotics. While this is not the first investigation of the antibacterial properties of bitter melon, to our knowledge this is the first study in which antibiotic synergy was tested between bitter melon and antibiotics. We hypothesized that bitter melon would itself have antibacterial properties against one or more organisms, as well as a synergistic effect with at least some of the common antibiotics tested.

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli, Micrococcus luteus, and *Pseudomonas putida* were tested in order to compare antibiotic efficacy against a variety of organisms. All of these organisms meet the current biosafety guidelines for Curry College in that all are designated BSL-1, in addition to being clinically relevant organisms. Bacterial cells were suspended in Luria-Bertani (LB, Miller) or Mueller-Hinton broth and incubated with continuous shaking at 225 rpm, or grown on solid agar plates of the same media. *E. coli* and *M. luteus* were incubated at 37 ºC, while *P. putida* was grown at a temperature of 30 ºC.

Media preparation

LB (Miller) broth and agar (Fisher) used for routine growth of bacterial strains was prepared according to the manufacturer's instructions. Mueller-Hinton broth and agar (Fisher) used for the antibacterial susceptibility assays was prepared according to the manufacturer's instructions. Broth was stored at room temperature (68-72 ºF), while plates were stored at 4 ºC.

Preparation of Antibiotic Solutions

The following common antibiotics were tested against each organism: carbenicillin, streptomycin, colistin, and tetracycline. Antibiotics were obtained from Midwest

Scientific (MidSci) and prepared by dissolving the antibiotic powder in molecular grade water (Invitrogen) or 70% ethanol (Fisher) for tetracycline, and then aseptically passing the sample through a 0.22 μm filter (Whatman) using a 10 mL syringe (Fisher). Antibiotic solutions were prepared in the following stock concentrations: carbenicillin (100 μg/μL), streptomycin (10 μg/μL), colistin (30 μg/μL), and tetracycline (30 μg/μL).

Preparation of Bitter Melon Extract

Oil from bitter melon *(Momordica charantia*) was extracted using the Soxhlet method, following a previously developed protocol (19). The bitter melon was purchased at Kam Man Supermarket (Quincy, MA), cut up, then dried in the oven at 200 ºF overnight. 12.45 g of bitter melon was extracted in 250 mL of 80% ethanol and subsequently concentrated by boiling off the ethanol to a final volume of 9 mL.

Disk Diffusion Assays

Disk diffusion assays were performed according to a previously published protocol (7). Briefly, bacteria were grown in an overnight culture in the appropriate conditions for the bacteria as described. The next morning, cultures were diluted to an OD_{600} of 0.05 and swabbed as a lawn on a Mueller-Hinton agar plate, swabbing the entire surface area of each plate three times to ensure complete coverage. Then each antibiotic, bitter melon extract, or combination was tested by gently placing 6mm paper disks (ThermoScientific Oxoid blank disks) containing the appropriate antibiotic solution in the center of each plate using sterilized forceps.

To prepare the disks, 20 μL of antibiotic stock solution, extract, or control (molecular grade water, 70% ethanol, or 80% ethanol) was aseptically pipetted onto each disk tested, and then allowed to dry in sterile conditions for approximately one hour. For disks with both common antibiotic and bitter melon extract, 20 μL of the common antibiotic solution (or water/70% ethanol as control) was first pipetted onto the disk, and then allowed to dry for ~1 hour. Then, 20 μL of bitter melon extract (or 80% ethanol as control) was pipetted onto the disk, and the disk was dried for an additional ~1 hour. Plates were wrapped individually in parafilm, and incubated overnight (~16-24 hours) at the preferred growth temperatures for each organism as described. Four to six replicates were performed for each control, antibiotic, bitter melon extract, or combination tested against all three organisms.

Data Analysis

After overnight growth, the diameter of the zone of inhibition (ZoI), or area resulting in no observable bacterial growth, was measured in millimeters using a ruler and recorded. Average (mean) ZoI diameters were calculated for all replicates along with standard error of the mean to analyze variance in the data.

Data were then categorized by each different organism and further by each common antibiotic tested. The ZoI measurements for each replicate of the common antibiotic alone was included, along with the measurements for the same common antibiotic tested in combination with bitter melon extract. Each spreadsheet was uploaded into the statistical analysis program *Rguroo* (22). A mean inference analysis between two populations (with population 1 as the common antibiotic ZoI diameters and population 2 as the same common antibiotic tested with bitter melon extract) was selected in order to perform a two-tailed, paired sample t-test evaluating the difference between each population mean and generate p-values. Unequal variance was assumed when performing all t-tests. Tests for normality of each dataset were also performed.

Results:

Bitter melon extract alone has little to no effect at preventing bacterial growth

Since our research aimed to test whether or not bitter melon extract enhances the antibacterial properties of commonly prescribed antibiotics, we first tested the efficacy of four different common antibiotics (carbenicillin, streptomycin, colistin, and tetracycline) at preventing growth of *E. coli, P. putida*, and *M. luteus* by setting up disk diffusion assays. For each organism tested, four to six replicates were performed. Controls were also tested for all three organisms by soaking disks in either molecular grade water or 70% ethanol. None of the control disks produced a zone of inhibition. Table 1 shows the average ZoI diameter

measurements for all antibiotics tested for *E. coli, P. putida*, and *M. luteus*, respectively. Unsurprisingly, while different antibiotics demonstrated varying degrees of efficacy against each organism, all were at least somewhat effective at preventing growth and resulted in a ZoI surrounding the antibiotic-soaked paper disk.

Next, we performed disk diffusion assays on the same three organisms using disks soaked with bitter melon extract or control disks soaked in 80% ethanol, the solvent used to prepare the bitter melon extract. Bitter melon extract had no effect at preventing growth of either *E. coli* or *P. putida*, since no ZoI was found around the paper disks (Reported as "0 mm" in Table 1). Bitter melon had only a small effect at preventing the growth of *M. luteus,* resulting in an average ZoI diameter of 5 mm (Table 1). All commonly prescribed antibiotics tested against *M. luteus* were found to produce a substantially greater average ZoI diameter than bitter melon, ranging from 22.1 mm for colistin to 47.25 mm for tetracycline (Table 1). As before, none of the control disks soaked in 80% ethanol as the control for bitter melon produced a ZoI.

Antibiotics:	***E. coli***		***P. putida***		***M. luteus***	
	Average Zol diameter (mm):	Standard Error of the Mean:	Average Zol diameter (mm):	Standard Error of the Mean:	Average Zol diameter (mm):	Standard Error of the Mean:
Carbenicillin	31.25	1.4930	25.2	1.5979	41.45	1.3525
Streptomycin	18.77	1.2925	18.15	0.2872	33.45	1.8715
Colistin	21.4	1.7709	19.6	0.9055	22.1	0.1291
Tetracycline	40	2.1909	23.35	1.0874	47.25	2.2867
Bitter melon	0	0	0	0	5	2.8868

Table 1. **Zone of inhibition measurements for antibiotics and bitter melon extract tested alone against *E. coli, P. putida,* and *M. luteus*. The zone of inhibition (ZoI) diameter measurements (in mm) for each common antibiotic tested (carbenicillin, streptomycin, colistin, and tetracycline, respectively) or for bitter melon extract are reported here. For each antimicrobial substance tested, a total of four to six replicates were performed. The average zone of inhibition diameter was calculated from all replicates performed, and the standard error of the mean was calculated as a relative measure of how consistent the measurements were.**

Bitter melon extract used in combination with commonly prescribed antibiotics influences their antibacterial efficacy

Although bitter melon extract itself had minimal to no effect against the three bacterial strains tested, we decided to test whether bitter melon extract could enhance the antibacterial properties of any of the four commonly prescribed antibiotics when used in combination. Disks for the combination disk diffusion assays were prepared by first soaking with 20 µL of the common antibiotic solution (carbenicillin, streptomycin, colistin, and tetracycline). Once dry, the same disk was then soaked in an additional 20 µL of bitter melon extract and set out to dry completely in a sterile environment. Control disks were also prepared and consistently resulted in no ZoI.

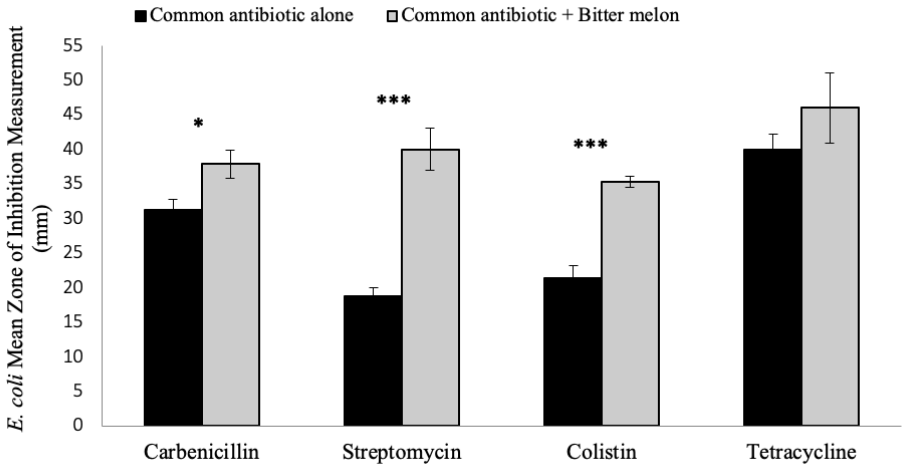

Figure 1. E. coli **average zone of inhibition statistical analysis between common antibiotics alone and in combination with bitter melon extract. The average diameter (in mm) of the zones of inhibition produced in the disk diffusion assays by each common antibiotic or common antibiotic combined with bitter melon extract are represented by the black and grey columns, respectively. Average diameters were calculated from the four to six replicates performed for each assay. Error bars represent the + and - values for standard error of the mean. A t-test was performed (with an assumption of unequal variance) to calculate the difference between the mean values for common antibiotic tested alone or in combination with bitter melon extract; those resulting in statistically significant p-values are indicated with asterisks (* indicates a p-value of <0.05, and *** indicates a p-value of <0.001). The t-test for carbenicillin resulted in a p-value of 0.0295; for streptomycin, 0.0004; for colistin, 0.0002; and for tetracycline, 0.3106.**

For *E. coli*, all four commonly prescribed antibiotics tested in combination with bitter melon extract showed enhanced ability to prevent bacterial growth. The most striking difference was for streptomycin, as the ZoI diameter more than doubled with the combination resulting in an average ZoI of 40 mm relative to the 18.76 mm observed for streptomycin alone (Figure 1).

Interestingly, bitter melon extract used in combination with commonly prescribed antibiotics did not always result in enhanced antibacterial properties. For *P. putida*, bitter melon extract actually decreased the average ZoI diameter from 25.2 mm to 19.55 mm when tested in combination with carbenicillin (Figure 2). The antibiotic effects of the other three antibiotics against *P. putida,* however, were enhanced when combined with bitter melon extract. The most striking enhancement was for colistin, which increased from an average ZoI diameter of 19.6 mm for colistin alone compared to an average of 33.65 mm for the combination (Figure 2). When tested for efficacy at inhibiting *M. luteus* growth, bitter melon extract combined with carbenicillin showed only a modest increase in the average ZoI diameter from 41.45 mm to 49 mm (Figure 3). All other combinations resulted in either the same effect against *M. luteus* growth or a decreased effect against *M. luteus* growth (Figure 3).

Antibacterial synergy between bitter melon extract and some commonly prescribed antibiotics is statistically significant

Since our data suggested that bitter melon extract can influence the antibacterial properties of the commonly prescribed antibiotics tested, our final goal was to determine whether the differences observed between the average ZoI for the common antibiotics alone compared to those for the combinations with bitter melon extract were statistically significant. To determine this, the raw data derived from the disk diffusion assay ZoI measurements was compiled in an excel spreadsheet and categorized by each bacterial species and common antibiotic. The data for each replicate was included, and each spreadsheet was uploaded into the

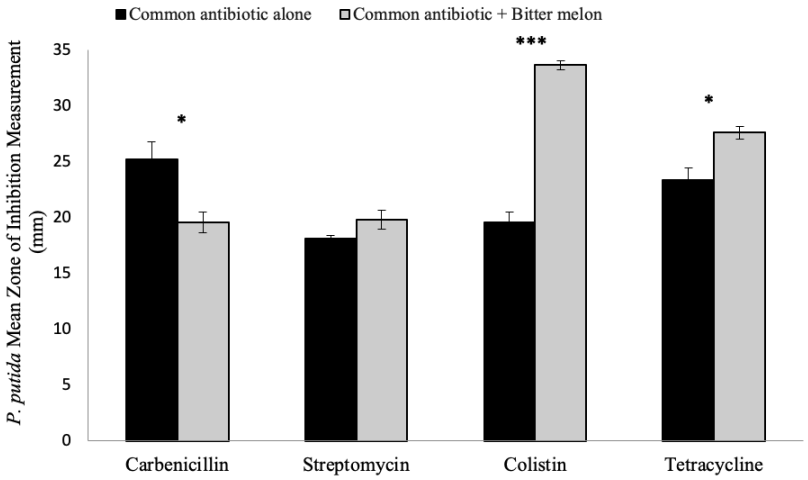

Figure 2. P. putida average zone of inhibition statistical analysis between common antibiotics alone and in combination with bitter melon extract. | The average diameter (in mm) of the zones of inhibition produced in the disk diffusion assays by each common antibiotic or common antibiotic combined with bitter melon extract are represented by the black and grey columns, respectively. Average diameters were calculated from the four to six replicates performed for each assay. Error bars represent the + and - values for standard error of the mean. A t-test was performed (with an assumption of unequal variance) to calculate the difference between the mean values for common antibiotic tested alone or in combination with bitter melon extract; those resulting in statistically significant p-values are indicated with asterisks (* indicates a p-value of <0.05, and *** indicates a p-value of <0.001). The t-test for carbenicillin resulted in a p-value of 0.0296; for streptomycin, 0.1386; for colistin, 0.0001; and for tetracycline, 0.0212.

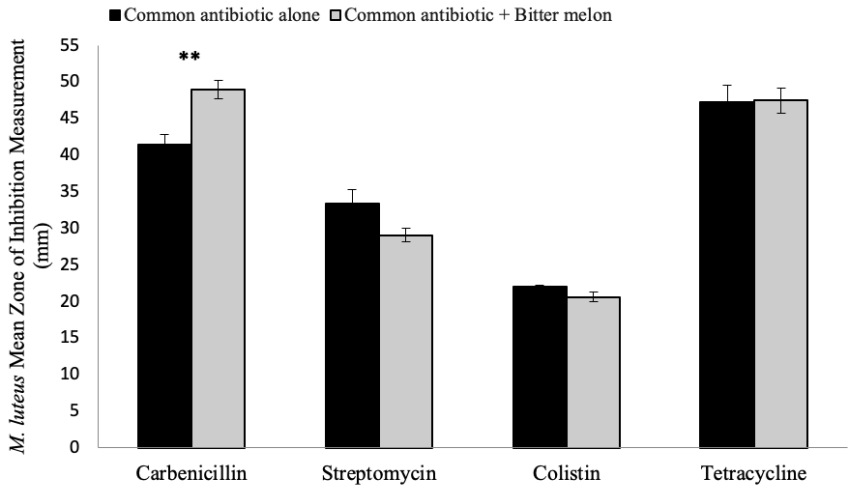

Figure 3. M. luteus average zone of inhibition statistical analysis between common antibiotics alone and in combination with bitter melon extract. The average diameter (in mm) of the zones of inhibition produced in the disk diffusion assays by each common antibiotic or common antibiotic combined with bitter melon extract are represented by the black and grey columns, respectively. Average diameters were calculated from the four to six replicates performed for each assay. Error bars represent the + and - values for standard error of the mean. A t-test was performed (with an assumption of unequal variance) to calculate the difference between the mean values for common antibiotic tested alone or in combination with bitter melon extract; those resulting in statistically significant p-values are indicated with asterisks (** indicates a p-value of <0.01). The t-test for carbenicillin resulted in a p-value of 0.0068; for streptomycin, 0.1002; for colistin, 0.1284; and for tetracycline, 0.9333.

statistical analysis program *Rguroo* (22). A mean inference analysis between two populations (average common antibiotic ZoI diameter relative to the average diameter for each tested with bitter melon extract) was used to perform a t-test evaluating the difference between each population mean and generate p-values (Figures 1, 2, and 3).

For all organisms and antibiotics tested, data was highly repeatable with relatively low variance as measured by standard error of the mean (Table 1, Figures 1, 2, and 3). Despite the low observed variance, t-tests were performed with an assumption of unequal variance, which allowed for a more rigorous comparison. A test for normality of each dataset was also performed in *Rguroo,* with each set of replicates found to be normally distributed (22).

The data for *E. coli* show that there is a statistically significant difference between the mean ZoI diameter for the common antibiotics carbenicillin, streptomycin, and colistin alone relative to the mean ZoI diameter for each of these combined with bitter melon extract, with p-values of 0.0295, 0.0004, and 0.0001, respectively (Figure 1). For *P. putida,* bitter melon extract was found to significantly increase the mean ZoI diameter of colistin and tetracycline relative to the common antibiotics alone, with p-values of 0.0001 and 0.0212, respectively (Figure 2). On the other hand, bitter melon extract combined with carbenicillin when tested against *P. putida* resulted in a significantly smaller average ZoI diameter compared to carbenicillin alone, with a p-value of 0.0296 (Figure 2). For *M. luteus*, bitter melon extract only significantly increased the mean ZoI diameter when combined carbenicillin, with a p-value of 0.0068 (Figure 3).

Discussion:

The extensive use of antibiotics in healthcare has led to a dramatic rise in antibiotic resistance and requires immediate action. The development and discovery of new drugs to treat such resistant infections has risen to be the top priority of the World Health Organization (WHO) (23, 25). It is critical to test new antibiotic combinations in order to treat a wider range of pathogens. The goal of our research was to test the antibacterial properties of bitter melon extract alone and in combination with four commonly prescribed antibiotics (carbenicillin, streptomycin, colistin, and tetracycline) against three different bacterial species: *E. coli, P. putida,* and *M. luteus.* The disk diffusion data show that bitter melon alone had little to no antibacterial properties when tested against the bacteria individually (Table 1). However, when tested in combination with the common antibiotics, there were in some cases significant effects on the efficacy of the antibacterial properties (Figures 1, 2, and 3). The data primarily showed a statistically significant enhancement in the antibacterial activity of the common antibiotics (i.e. antibacterial synergy), but also one example of antibacterial antagonism.

In this study, we aimed to test a broad range of organisms to explore differences in the potential synergistic or antagonistic effects of bitter melon extract. Although the three organisms selected are all Biosafety Level 1 organisms, all have important clinical relevance. *E. coli* is a Gram-negative bacterium that is resistant to many prescribed antibiotics (18). One study found that the rate of adaptive mutations in *E. coli* is about 10^{-5} per genome per generation, which is 1000 times higher than previous estimates (18). These antibiotic-resistant *E. coli* strains are passed on, often carrying multiple drug resistant plasmids that can easily transfer to other organisms (20). *M. luteus* is a Gram-positive organism that is detected as a commensal organism in the mucous membrane as well as in soil and water (27). Although *M. luteus* is described as low virulence, the bacterium can become pathogenic under certain conditions (5). *P. putida* is found in soil and water and has been reported as an opportunistic pathogen that can occasionally cause hospital-acquired infections (11, 12). Strains of this species are known to exhibit resistance to many antibiotics through the presence of plasmids that encode antibiotic resistance factors (12).

Carbenicillin, streptomycin, colistin, and tetracycline were the common antibiotics chosen to test in our study. Carbenicillin is an antibiotic among the semisynthetic penicillin group and is used to treat illnesses such as bladder infections. It has Gram-negative coverage but limited Gram-positive coverage (17). Streptomycin is an antibiotic that is commonly used to treat aerobic Gram-negative bacterial infections such as tuberculosis. The original wide spectrum of activity exhibited by streptomycin against both Gram-negative and Gram-positive bacteria has severely decreased due to the rise of antibiotic resistance; commonly

to Enterobacteriaceae such as *E. coli* (24). Colistin is a polymyxin "last line" antibiotic used to treat infections caused by multi-drug resistant Gram-negative bacteria such as *P. aeruginosa*. Being a "last line" antibiotic, this means there are no other novel antibiotics to treat such infections and research for an alternative treatment is crucial (16). Tetracycline is a protein synthesis inhibitor antibiotic that is used to manage and treat bacterial infections. Originally tetracycline showed antibacterial activity against most medically relevant aerobic and anaerobic bacteria, both Gram-positive and Gram-negative (21).

Many plants used in Chinese traditional medicine have been shown to have antibacterial properties. In one study, essential oils prepared from 21 different plants were all found to have at least some degree of antibacterial activity against 10 *Pseudomonas* species tested, with oil from *Cinnamomum zeylanicum* displaying the highest inhibitory effect (9). In another study, berberine, an alkaloid derived from goldenseal (*Hydrastis canadensis)* was itself found to have antibacterial activity against *Staphylococcus aureus.* Flavonoids isolated from a different part of the plant were found to have a synergistic effect on berberine efficacy despite having no inherent antibacterial effect when used individually (8). Previous studies have explored the various biological activities of bitter melon such as antiviral, antioxidant, and antibacterial components (3, 6, 15, 26). For example, one study determined that essential oils of bitter melon have significant inhibitory effects on *S. aureus* growth, as well as significant antibacterial activity against several other bacteria including *Pseudomonas multocida, Salmonella typhi,* and *Staphylococcus epidermidis*(6). Bitter melon pulp extract also was proven to have a broader spectrum of antibacterial activity against *E. coli, Staphylococcus*, and *Pseudomonas* (6). In addition to plant-derived compounds themselves exhibiting antibacterial properties, many plant extracts have been shown to have synergistic effects with antibiotics, thus warranting our research (8, 13).

As we expected, all commonly prescribed antibiotics tested had at least some effect at inhibiting the growth of each bacterial species tested (Table 1). It was also unsurprising that the efficacy of each antibiotic tested varied depending on the organism. For example, ZoIs for *P. putid*a were typically smaller for all antibiotics tested. *Pseudomonas* species are known for their high degree of intrinsic antibacterial resistance, which can account for this observation (14). Bitter melon extract tested alone had no effect against either *E. coli* or *P. putida,* and only a very minimal effect against *M. luteus* (Table 1). We found this result surprising given that previous research has shown antibacterial activity of bitter melon against some microorganisms, including *E. coli* and *Pseudomonas* species (6). Differences in bitter melon's antibacterial activity however, have been observed depending on which part of the plant was tested (i.e. seeds, leaves, fruit), and whether mature or immature plants were tested (15, 26). It is thus possible that by using the entire plant, antibacterial properties were diluted out and not detected in our assays. It is also possible that differences in the methodology and solvent used for extraction of oils as well as the amount of plant used and extent to which the oils were concentrated account for the lack of antibacterial activity we observed.

Although bitter melon extract had little to no antibacterial activity against the strains tested when applied individually to paper disks, we nonetheless decided to test whether bitter melon could enhance the antibacterial activity of any of the four commonly prescribed antibiotics tested (Figures 1, 2, and 3). While most studies that assay for synergistic combinations of antibacterials test two or more compounds that have antibacterial activity individually, a study by Junio *et al*. (2011) found that some compounds can have synergistic effects on other antibacterials without themselves having any direct antibacterial properties (8). Interestingly, our results do suggest that bitter melon extract can have indirect effects on some of the antibiotics tested, when used against specific organisms. For *E. coli,* bitter melon extract significantly increased the ZoI size when used in combination with carbenicillin, streptomycin, and colistin, compared to each of these antibiotics administered alone (Figure 1). For *P. putida*, statistically significant enhancement in ZoI size for antibiotics used in combination with bitter melon extract was seen for colistin and tetracycline (Figure 2). For *M. luteus,* significant enhancement of growth inhibition was seen when bitter melon extract was used in combination with carbenicillin, compared to carbenicillin alone (Figure 3). This suggests that bitter melon extract has bioactive components that work synergistically in combination with

commonly prescribed antibiotics, when used against certain microorganisms. Of note, we did find one potentially antagonistic combination, as carbenicillin combined with bitter melon extract resulted in less inhibition of *P. putida* growth than carbenicillin alone (Figure 2).

Our finding of significant antibiotic synergy between colistin and bitter melon extract when used against *P. putida* is of particular interest. Colistin is commonly used as a "last line" antibiotic to treat multidrug- resistant *Pseudomonas aeruginosa* infections, but resistance to colistin has been steadily increasing over the years (10). If our results are found to be repeatable when tested against *P. aeruginosa,* this synergistic combination could provide another potential avenue for treatment of these deadly infections. Testing for synergistic antibiotic combinations to treat *P. aeruginosa* is already underway. For example, the combination of streptomycin and cefadroxil were reported to be synergistic against *Pseudomonas aeruginosa* isolates in vitro when compared to the effects of each of these antibiotics alone (2). Further testing will be the key to determining which combinations work best in vivo, when treating patients.

On the other hand, our result of an antagonistic effect of bitter melon extract when used with carbenicillin relative to carbenicillin used alone against *P. putida* is also noteworthy. Since carbenicillin is a commonly prescribed antibiotic for Gram-negative pathogens such as *Pseudomonas* species, it is important to be aware of the limitations of combination treatments and have a thorough understanding of both synergistic and antagonistic combinations in order to optimize treatment outcomes (1, 17). Antibiotic antagonism is not unexpected when combining antibiotics. For example, Murray and colleagues found that treating *P. aeruginosa* first with low concentrations of polymyxin B resulted in its decreased antibiotic susceptibility when later exposed to gentamicin, neomycin, tobramycin, or ciprofloxacin (14). It is therefore critical that combinations of antibiotics are tested prior to use either combinatorially or subsequently to avoid the possibility of antagonistic effects such as this.

Our research presents a possible solution to combat the growing problem of antibiotic resistance. Although the results of our study are preliminary, they contribute to the foundational understanding necessary for future advancements in furthering the discovery of synergistic antibiotic combinations. Future directions of this work include repeating assays using isolated and purified components of bitter melon. Bitter melon has many phytochemicals with antioxidant, anti-inflammatory, and antibacterial properties (3, 15). Further research on the efficacy of bitter melon's antibacterial properties when used either alone or combinatorially should thus be done through fractionation and chromatography assays, as well as minimum inhibitory concentration analysis (MIC). Ultimately, our findings will need to be tested using in vivo models and with physiologically relevant concentrations of antibiotics and purified bitter melon components. In conclusion, the findings of this research give one potential solution to help combat the drastic increase in antibiotic-resistant pathogens that are threatening our global healthcare.

Acknowledgements

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References

1. Acar, J. F. (2000). Antibiotic synergy and antagonism. Medical Clinics of North America, 84(6), 1391–1406. https://doi.org/10.1016/s0025-7125(05)70294-7

2. Ahmed, Z., Saeed Khan, S., & Khan, M. (2013). In vitro trials of some antimicrobial combinations against *Staphylococcus aureus* and Pseudomonas aeruginosa. Saudi journal of biological sciences, 20(1), 79–83. https://doi.org/10.1016/j.sjbs.2012.10.005

3. Arshad, M.S. & Ahmad, M.H. (Eds). (2021). Functional food–Phytochemicals and health promoting potential. IntechOpen. https://www.intechopen.com/chapters/77462

4. Centers for Disease Control and Prevention. (2021, December 13). About antibiotic resistance. Centers for Disease Control and Prevention. https://www.cdc.gov/drugresistance/about.html

5. Dürst U.N., Bruder E., Egloff L., Wüst J., Schneider J., Hirzel H.O. (n.d.). *Micrococcus luteus*: A rare pathogen of valve prosthesis endocarditis. Zeitschrift fur Kardiologie. https://pubmed.ncbi.nlm.nih.gov/1862670/

6. Jia, S., Shen, M., Zhang, F., & Xie, J. (2017). Recent advances in *Momordica charantia*: Functional components and biological activities. International Journal of Molecular Sciences, 18(12), 2555. https://doi.org/10.3390/ijms18122555

7. Johnson, T. R. & Case, J. (2015). Laboratory experiments in microbiology. Pearson.

8. Junio, H.A., Sy-Cordero, A.A., Ettefagh, K.A., Burns, J.T., Micko, K.T., Graf, T.N., Richter, S.J., Cannon, R.E., Oberlies, N.H., & Cech, N.B. (2011). Synergy-directed fractionation of botanical medicines: A case study with goldenseal (Hydrastis canadensis). Journal of Natural Products, 74(7), 1621-1629. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3142294/

9. Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., Klūga, A., Tokár, M., Kluz, M., & Ivanišová, E. (2017). The antioxidant and antimicrobial activity of essential oils against Pseudomonas spp. isolated from fish. Saudi Pharmaceutical Journal, 25(8), 1108–1116. https://doi.org/10.1016/j.jsps.2017.07.005

10. Loho, T., & Dharmayanti, A. (2015). Colistin: An antibiotic and its role in multiresistant Gram-negative infections. Acta Medica Indonesiana, 47(2), 157-168. https://pubmed.ncbi.nlm.nih.gov/26260559/

11. Martino, R., Martínez, C., Pericas, R., Salazar, R., Solá, C., Brunet, S., Sureda, A., & Domingo-Albós, A. (1996.). Bacteremia due to glucose non-fermenting Gram-negative bacilli in patients with hematological neoplasias and solid tumors. European Journal of Clinical Microbiology and Infectious Diseases, 15, 610-615. https://link.springer.com/article/10.1007%2FBF01709374

12. Molina, L., Udaondo, Z., Duque, E., Fernández, M., Molina-Santiago, C., Roca, A., Porcel, M., de la Torre, J., Segura, A., Plesiat, P., Jeannot, K., & Ramos, J.-L. (2014). Antibiotic resistance determinants in a *Pseudomonas putida* strain isolated from a hospital. PLoS ONE, 9(1), e81604. https://doi.org/10.1371/journal.pone.0081604

13. Mundy, L., Pendry, B., & Rahman, M. (2016). Antimicrobial resistance and synergy in herbal medicine. Journal of Herbal Medicine, 6(2), 53-58. https://doi.org/10.1016/j.hermed.2016.03.001

14. Murray, J. L., Kwon, T., Marcotte, E. M., & Whiteley, M. (2015). Intrinsic antimicrobial resistance determinants in the superbug Pseudomonas aeruginosa. MBio, 6(6). https://doi.org/10.1128/mbio.01603-15

15. Naqvi, S. A., Ali, S., Sherazi, T. A., Haq, A.-U., Saeed, M., Sulman, M., Rizwan, M., Alkahtani, S., & Abdel-Daim, M. M. (2020). Antioxidant, antibacterial, and anticancer activities of bitter gourd fruit extracts at three different cultivation stages. Journal of Chemistry, 2020, 1–10. https://doi.org/10.1155/2020/7394751

16. Nation, R. L., & Li, J. (2009). Colistin in the 21st century. Current Opinion in Infectious Diseases, 22(6), 535–543. https://doi.org/10.1097/qco.0b013e328332e672

17. National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 20824, Carbenicillin. https://pubchem.ncbi.nlm.nih.gov/compound/Carbenicillin.

18. Perfeito, L., Fernandes, L., Mota, C., & Gordo, I. (2007). Adaptive mutations in bacteria: High rate and small effects. Science, 317(5839), 813–815. https://doi.org/10.1126/science.1142284

19. Redfern, J., Kinninmonth, M., Burdass, D., & Verran, J. (2014). Using Soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. Journal of Microbiology and Biology Education, 15(1), 45-46. https://journals.asm.org/doi/10.1128/jmbe.v15i1.656

20. Salyers, A., Gupta, A., & Wang, Y. (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology, 12(9), 412–416. https://doi.org/10.1016/j.tim.2004.07.004

21. Shutter, M.C., Akhondi, H. (2022, January 19). Tetracycline. StatPearls [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK549905/

22. Soflytics Corp. (2021). *Rguroo* (Version March 2021). https://www.rguroo.com/userguide_03_2021.pdf

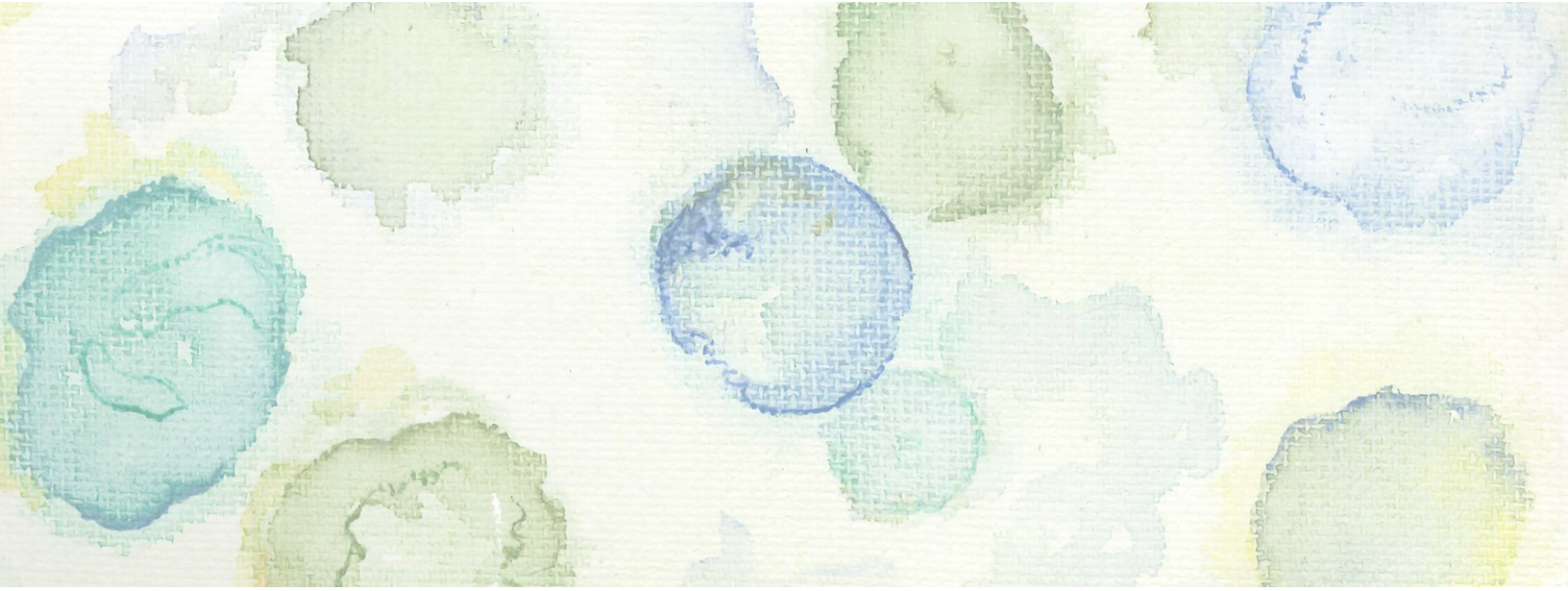
23. The Lancet Infectious Diseases. (2017). Antibiotic research priorities: Ready, set, now go. The Lancet Infectious Diseases, 17(4), 349. https://doi.org/10.1016/s1473-3099(17)30140-8

24. Waters, M., Tadi, P. (2021, September 29). Streptomycin. StatPearls [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK555886/

25. World Health Organization. (2017, February 27). WHO publishes a list of bacteria for which new antibiotics are urgently needed. World Health Organization. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

26. Yaldız, G., Sekeroglu, N., Kulak, M., & Demirkol, G. (2014). Antimicrobial activity and agricultural properties of bitter melon (*Momordica charantia* L.) grown in northern parts of Turkey: A case study for adaptation. Natural Product Research, 29(6), 543–545. https://doi.org/10.1080/14786419.2014.949706

27. Yang, S., Sugawara, S., Monodane, T., Nishijima, M., Adachi, Y., Akashi, S., Miyake, K., Hase, S., & Takada, H. (2001). *Micrococcus luteus* teichuronic acids activate human and murine monocytic cells in a CD14- and toll-like receptor 4-dependent manner. Infection and Immunity, 69(4), 2025–2030. https://doi.org/10.1128/iai.69.4.2025-2030.2001



The Impact of PETase's Active Site Disulfide Bond on PET Biodegradation

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ABSTRACT

Plastic pollution is one of the largest problems globally, with polyethylene terephthalate (PET) plastic as one of the main sources. Effective depolymerization of PET to its monomers for upcycling is a challenge. PETase is reported to be an effective enzyme for biodegradation of PET via C-O bond cleavage of ester linkage. The role of the disulfide bond, present in PETase's active site sequence, is unknown in the cleavage of PET's ester linkage. To understand the role of this bond, two separate versions of PETase – one containing the disulfide bond, and the other without the disulfide bond - were modeled using PyMol™, synthesized, and tested for degradation of PET surrogate compound, bis (2-hydroxyethyl) terephthalate (BHET). Several experiments were performed in the presence and absence of phenylmethylsulfonyl fluoride (PMSF), a serine protease. The results reveal that the role of the disulfide bond in the degradation of BHET's ester linkage is insignificant and the variation in the results (ethylene glycol yields, BHET degradation per microgram of enzyme) are within the experimental uncertainty. This finding is a stepping-stone to further modifying PETase and improving its activity towards commercial adaption of this technology for PET upcycling and creating a circular carbon economy, improving the world's carbon footprint, and mitigating ocean and environmental plastic pollution.

Introduction

Polyethylene terephthalate (PET) is one of the most common consumer plastics, which has a variety of uses ranging from plastic water bottles to polyester clothing items.(Paydar & Olfati, 2018) In 2013, the worldwide production of plastic was 299 million metric tons.(Chen *et al*., 2018) By 2015, the worldwide production had reached 322 million metric tons per year, and this amount will continue to increase. Despite plastic's versatile uses, it is also an environmentally hazardous substance because it does not biodegrade in natural ecosystems.(Chen *et al*., 2018)

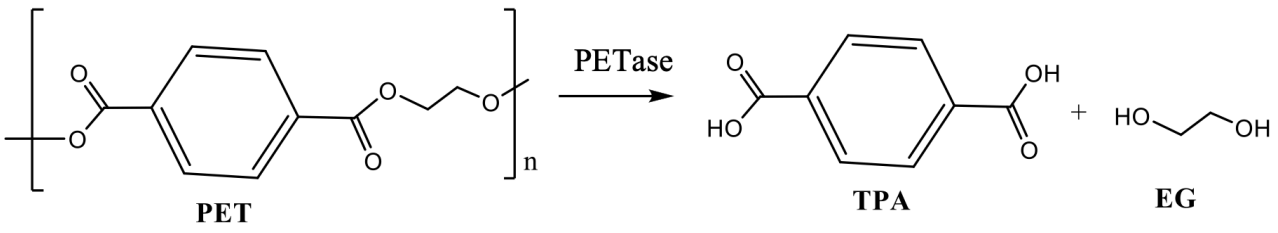
A small fraction of post-consumed plastics is currently recycled, mostly by physical recycling. Physical recycling refers to the process of melting and then re-shaping the plastic into new consumer products. For example, waste PET plastic bottles can be melted to make polyester carpeting.(Tullo, 2019) This method of recycling introduces impurities, ultimately reducing its quality and value. As a result of these challenges, only 18.4% of PET is recycled annually. (Tullo, 2019) All large-scale PET recycling today is physical recycling, and due to the lower quality end product, most manufacturers produce new PET from monomers, derived from crude oil, to meet the market demand.

Chemical recycling of PET plastic is now being explored as an alternative. It involves degradation of PET into its constituent monomers, ethylene glycol (EG) and terephthalic acid (TPA) (Scheme 1). The produced monomers can be chemically re-bonded to form PET plastic of the same caliber as new PET. This advantage makes chemical recycling preferable to physical recycling. Chemical recycling is not used in large-scale facilities due to the lack of an effective technology as well as use of corrosive chemicals such as sodium hydroxide, and harsh reaction conditions (e.g., high pressure requiring expensive reactors).(Khoonkari *et al*., 2015)

Glycolysis is the most efficient method of depolymerizing PET,(Khoonkari *et al*., 2015) which is done at high temperature (180°C to 240 °C).(Khoonkari *et al*., 2015) Ionic liquids such as 1-butyl-3-methylimidazolium bromide ([bmim] Br) have been used as catalysts for PET glycolysis. Wang *et al*. used [bmim] Br to degrade 100% of PET in 8 hours at 180 °C.(Khoonkari *et al*., 2015) This reaction depolymerized long ester chain of PET into short chain ester intermediates and did not fully degrade to produce desired EG and TPA.

Despite glycolysis showing high depolymerization yields, because it does not totally degrade PET, and necessitates high energy (up to 240 °C), more energy efficient and effective alternative methods have been explored for chemical recycling of PET.

One such method is enzymatic degradation of PET.(Kawai *et al*., 2020; Taniguchi *et al*., 2019) This method reduces the activation energy of the reaction, requires no pressure, and involves equipment which is inexpensive and commonly available. Enzymatic degradation of PET film was first accomplished by Muller in 2005.(Kawai *et al*., 2019; Müller *et al*., 2005) Their method depolymerized two kinds of PET films by approximately 40–50% at 55 °C in 3 weeks using cutinase obtained from the bacterium Thermobifida fusca. (Kawai *et al*., 2019; Müller *et al*., 2005) Since then, several thermostable cutinases have been discovered with an ability



Scheme 1. **PET breakdown to terephthalic acid (TPA) and ethylene glycol (EG).**

to break down PET film,(Furukawa *et al*., 2019; Ribitsch *et al*., 2012) but their degradation rates for PET are lower than that with cutin.

Recently, a Gram-negative bacterium, Ideonella sakaiensis 201-F6 was isolated that can consume PET as an energy and carbon source to survive.(Yoshida *et al*., 2016) Research revealed that this bacterium contains the enzyme, PETase, which can biodegrade PET into mono(ethylene terephthalate (MHET) and (bis(2-hydroxyethyl) terephthalate) (BHET). MHET and BHET are then hydrolyzed into EG and TPA. (Yoshida *et al*., 2016) 200 nM of wild type PETase enzyme was found to degrade 3.7 mg/L of PET at 30 °C over the course of 72 hours.(Seo *et al*., 2019) When compared with PET degradation activity of all reported enzymes, PETase demonstrated the best performance (Table 1).

PETase's active site is composed of a serine-histidine-asparagine (Ser-His-Asp) triad, which is one of the most commonly studied catalytic triads, and is often found in α/β hydrolases.(Jones & Solomon, 2015; Rauwerdink & Kazlauskas, 2015) It catalyzes a redox reaction to break the ester bond in PET.(Yoshida *et al*., 2016) The redox process is initiated when asparagine removes a hydrogen atom from histidine; thus, effectively removing a proton and an electron. The histidine then replaces its hydrogen by taking a proton and an electron from serine, creating a nucleophile.(Yoshida *et al*., 2016) The Ser-His-Asp triad removes an electron from the oxygen atom of PET's ester linkage and passes the electron to serine.(Yoshida *et al*., 2016) Then this oxygen atom takes an electron from its neighboring atom, making a free radical on the carbon atom, and weakens the C-O bond of the ester linkage. This results in cleavage of the ester linkage and complete the redox cycle. The extra electron gained by asparagine at the beginning of the reaction is given to O2, an electron acceptor, resulting in formation of two molecules of H2O.

PETase does not degrade PET film at a rate high enough to be utilized in recycling facilities. Commercial adaptation of this process would need much higher degradation rate. (O'Brien, 2019) In 2018, Austin *et al*. modified wild type PETase and improved the activity of the modified PETase to 2.5 times the wild type.(Ma *et al*., 2018) In the modification process, width of the active site cleft of the modified PETase was lowered through mutagenesis of two amino acids (phenylalanine and serine). The results indicated that specificity of the active site caused by a narrower cleft allows the substrate (PET) to better interact with the enzyme. (Austin *et al*., 2018) While other enzymes (e.g., cutinases) have already been perfected by nature because of their long existence, PETase, a recently developed enzyme, can be further modified through appropriate protein design.

Ma *et al*. also modified wild type PETase. In their modification, hydrophobicity was increased near the active site of the enzyme through mutagenesis under the hypothesis that hydrophobicity would increase PET degradation. This modification led to a 15-fold increase in degradation of PET as compared to the wild-type PETase.(Ma *et al*., 2018)

Although significant achievements have been made by researchers in improving wild type PETase's activity, more research is necessary in developing enzymes with higher thermostability, and better degradation capability, enabling their utilization for PET recycling by waste management facilities and chemical industries. To develop PETase for effective degradation of ester linkages, the following section summarizes desirable features, e.g. Isoelectric point, amino acid sequence, thermostability etc. of modified PETase.

PETase shares 52% sequence identity with T. fusca cutinase, its closest homolog.(Austin *et al*., 2018; Yoshida *et al*., 2016) This sequence can be used to identify features of PETase which make it an effective degrader of PET. These features include 3-fold wider active site cleft and higher Isoelectric point (caused by dipole; Isoelectric point of PETase is 9.6 while this value of T. fusca cutinase is 6.3) of PETase as compared to T. fusca cutinase.(Austin *et al*., 2018; Yoshida *et al*., 2016) In addition, PETase contains many basic amino acids such as lysine and arginine, which are charged and form salt bridges to make PETase stable.(Yoshida *et al*., 2016) (Austin *et al*., 2018)

Another important feature is thermostability. For example, Leaf Branch Compost cutinase (LCC), another cutinase with similar sequence to PETase, has high thermostability at

70 °C.(Shirke *et al*., 2018) Because glass transition temperature of PET plastic is around 70 °C, and PET becomes more pliable and its bonds weaken, LCC can degrade PET effectively at this temperature. In contrast, PETase is stable and active at much lower temperature (~37 °C) at which PET is not pliable and its bonds are rigid. Modified PETase with high thermostability would be beneficial.

PETase contains a disulfide bond in its active site. LCC and T. fusca cutinases do not have this disulfide bond.(Fecker *et al*., 2018) Simulations predicted that this disulfide bond increases flexibility around the active site.(Fecker *et al*., 2018) Austin *et al*. (Austin *et al*., 2018) have shown that a narrower binding cleft of the active site, arising from flexibility of enzyme, can improve enzyme's affinity to substrates, e.g., PET or BHET.

Table 1

Entry	Enzymes	Reaction Conditions	Degradation amount (methods used)	Ref
1	Modified PETase (mutant I179F)	Modified PETase expressed in *E. coli*, 5 μg PETase reacted with PET (1.5x1 cm2 size pieces) in bicine buffer; pH 8.5, 48 hours, 30°C	Mutant I179F has 2.5 times more degradation than wild-type PET [22.5 mg μmol-1·L−1 PETase per day biodegraded by mutant] (SEM)	(Austin *et al*., 2018)
2	PET-G/TfH*	20-25 mg PET (12 mm diameter disks) reacted with enzyme of concentration 0.1 mg/mL in buffer; 5 mL buffer; pH 7.0; 21 days; 55 °C	49.7 ± 1.0 % mass loss (mass weighed)	(Müller *et al*., 2005)
3	PET-G/rTfH	20-25 mg PET (12 mm diameter disks) reacted with enzyme of concentration 0.1 mg/mL in buffer; 5 mL buffer; pH 7.0; 21 days; 55 °C	54.2%% mass loss (mass weighed)	(Müller *et al*., 2005)
4	AB300432, AB298783, AB300774	7 × 7 cm2 Biomax® films in compost; 55-60 °C; 70-100cm below surface; 3 weeks and 4 weeks	All films fragmented in 4 weeks	(Hu *et al*., 2008)
5	Est119	1 × 1 cm2 PET film reacted with sufficient amount enzyme in the presence of 300 mM Ca2+;50°C; pH 7; 3 h	No weight loss or visible surface change (mass weighed and SEM)	(Thumarat *et al*., 2015)
6	Est1	1 × 1 cm2 PET film reacted with sufficient amount enzyme in the presence of 300 mM Ca2+;50°C; pH 7; 3 h	No weight loss or visible surface change (mass weighed and SEM)	(Thumarat *et al*., 2015)

Entry	Enzymes	Reaction Conditions	Degradation amount (methods used)	Ref
7	Thc_Cut1	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50 °C; pH 7.0; 2 hours	WCA decreased from 74.2° ± 1.6° to 66.3° ± 2.7° (WCA analyzer)	(Herrero Acero *et al*., 2011)
8	Thc_Cut2*	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50 °C; pH 7.0; 2 hours	WCA decreased from 74.2° ± 1.6° to 71.2° ± 0.9° (WCA analyzer)	(Herrero Acero *et al*., 2011)
9	Thf42_Cut1	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50 °C; pH 7.0; 120 hours	Crystallinity loss (FTIR-ATR)	(Herrero Acero *et al*., 2011)
10	Tha_Cut1	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50°C; pH 7.0; 2 hours	WCA decreased from 87.7° ± 4.8° to 45.0° ± 6.0° (WCA analyzer)	(Ribitsch *et al*., 2012)

*Tfu = T. Fusca; **TfCu = T. Fusca Cutinase; SEM = Scanning Electron Microscopy; FTRI = Fourier-transform Infrared spectroscopy; WCA = Water contact angle

It is hypothesized that the flexibility provided by the disulfide bond attributed to PETase's degradation performance.

The role of the disulfide bond, which is unique to PETase, has not been experimentally tested in the degradation of PET, despite it predicted positive traits from simulation. The present work experimentally demonstrates the effect of disulfide bond of PETase to the degradation of ester linkage of PET. First, amino acid sequence of a PETase protein without disulfide bond was modeled. Then PETases with and without disulfide bond were synthesized following their sequences and their activities in the degradation of ester linkage of PET have been experimentally tested using BHET as a model compound. BHET, a building block oligomeric unit of PET which contains EG and TPA monomeric units in 2:1 molar ratio, is used as a surrogate substate in this study because (1) it is expected to have faster degradation rate than PET and (2) it can completely degrade to EG and TPA, which can be quantitatively measured to accurately determine the role of the disulfide bond. Controlled experiments were performed without PETase and in the presence of phenylmethylsulfonyl fluoride (PMSF) to evaluate if PMSF disrupts PETase's activity by interacting with their catalytic triad serine. The results indicate that the disulfide bond does not significantly influence the degradation of BHET's ester linkage.

Experimental Section

Materials

Luria Bertani (LB) broth, calcium chloride, ampicillin, lysogeny broth, Tris buffer, HCl, phenylmethylsulfonyl fluoride (PMSF), Hi-Trap columns, and NaCl were procured from Sigma-Aldrich. Isopropyl β- d-1-thiogalactopyranoside (IPTG) and bichionic acid assay were obtained from Fisher Scientific. A refractometer was purchased from Hanna

Instruments. BL21 *E. coli* cells were obtained from Dr. Clark Gedney's lab in Biological Science department at Purdue University. PETase recombinant plasmid containing the gene to produce PETase with a disulfide bond (referred hereto as A2) was purchased from Integrated DNA Technologies (IDT). Amino acid sequence of the modified PETase without a disulfide bond (referred here to as A1) was modeled using PyMol™ software (Schrödinger, Inc. Version 3).
The sequence was sent to IDT to synthesize the modified PETase. Both the organism containing/expressing the plasmid were cultured for extracting proteins by following similar procedures as described below.

PETase Sequence Modification

The software Pymol™ was utilized in order to model A1 and A2. A2, which contains a disulfide bond, was produced by isolating the active site of PETase. Its sequence is shown in Figure 1c. To produce the sequence of A1 (Figure 1b), disulfide bond (red colored portion) of the sequence in Figure 1c was removed. Rather than using the entire PETase sequence, the active site was isolated to enable a focused investigation into the disulfide bond's effect on PETase's active site.

Transformation of *E. coli* cells

Frozen BL21 cells were grown on petri dishes of Luria-Bertani broth media until 20 colonies were visible on each plate. Approximately 20 colonies of *E. coli* were transferred to 250 µl of 0.1M calcium chloride solution in a sterile microcentrifuge tube and mixed. To another microcentrifuge tube, 20 colonies of *E. coli* were added to 250 µl of calcium chloride solution and mixed. 10 µl of PETase plasmid (e.g. A1) was added to one of these two tubes (Tube 1) and vortexed for 5 seconds. The second tube without plasmids is referred here to as Tube-2. Then both tubes were placed in an ice bath for 10 minutes, followed by 90 seconds in a heating block preset at 42 °C, and finally submerged in an ice bath for an additional 2 minutes. 500 µl of Super Optimal (SO) broth was added to both tubes and vortexed, and then the tubes were placed in a heating block at 37 °C for 30 minutes.

The contents of Tube-1 and Tube-2 were added to two separate flasks, each of which contained 1 L of lysogeny broth medium and 200 mg ampicillin, and they were cultured in a shaker at 37 °C until an optical density of 0.6 at 600 nm was reached. To confirm transformation, the cells from flask-1 were checked against the cells from flask-2. Flask-1 was cloudy due to the addition of the plasmids, whereas flask 2 was the negative control and remained clear. After this control test, the content of flask 2 was discarded. 0.1 mM IPTG was added to flask-1 at this stage, and the cells of flask-1 were further incubated for 24 h at 37 °C in the shaker at rpm of 250.(Yoshida *et al*., 2016)

The content of flask-1 was transferred to several 15 mL centrifuge tubes and they were centrifuged at 4000 rpm for 20 minutes on an Allegra centrifuge instrument. Supernatant from all of the centrifuged tubes was stored in a glass bottle. The cell pellets from all centrifuged tubes were collected and resuspended in 50 mL of 40 mM pH 7.4 Tris-HCl buffer containing 0.5M NaCl (Buffer 1). The resuspended cells were vortexed and divided equally into two centrifuge tubes of size 30 mL. To one of the tubes, 0.1M PMSF was added to test the effect of this protease inhibitor on enzyme activity. It is used for protein purification during cell lysis to prevent the proteases from degrading the protein.

Lysis

The cells from both 30 mL size tubes were separately added to a mortar and pestle containing liquid nitrogen in it to allow the content of each tube to solidify and ground until fine powders were formed, which were then stored at -20 °C. 5 grams of these lysed cells were resuspended in 20 mL of 20 mM phosphate buffer at pH 7.4 (Buffer 2) containing 20 mM imidazole. They were centrifuged at 4300 rpm for 30 minutes. The pellet was discarded and the supernatant, which contained protein expressed from the transformed cells, was syringe-filtered through a 0.45 micrometer syringe.

Protein Purification

A histidine column (1-mL HiTrap syringe) was used to purify the protein in the supernatant. The elution buffer consisted of 500 mM imidazole in Buffer 1, and the binding buffer consisted of 20 mM imidazole in Buffer 1. The Hi-Trap column was first prepared by eluting the binding buffer twice through the column at a rate of approximately 1 mL/min.

The supernatant containing the expressed protein was then eluted through the column. The elution buffer was run through the column in batches of 4 mL each time to elute the expressed protein which was collected separately in 15 mL centrifuge tubes. A total of 5 samples of each containing 4 mL eluant was collected. These 5 aliquots or samples were used for studying degradation experiments of PET model compound written below. Plasmid containing PETase enzyme with disulfide bond (A2) was similarly processed to obtain expressed protein for degradation study written below.

A bichionic (BCA) assay of each aliquot was done to measure total protein concentration. For this assay, each sample was mixed with bichionic acid obtained from Sigma and the mixture was kept on a microplate for 30 minute at 37 °C before measuring its color change at 526 nm using a Tecan microplate reader.

BHET Degradation

1M of Bis(2-Hydroxyethyl) terephthalate (BHET) was added to each eluted protein samples and the mixtures were allowed to continuously shake at 250 rpm and 37 °C for 72 hours. After 72 hours of reaction, the tubes were centrifuged, and 1 mL supernatant from each tube was collected for analysis by a refractometer and a UPLC instrument. The reaction was terminated by heating the solution in tubes at 85 °C for 15 minutes .(Austin *et al*., 2018) A total of 5 A1 samples, 5 A2 samples and 2 buffer solutions (control) were tested for BHET degradation.

Product Analysis.

Analysis of EG production in each sample collected at 72 hours was done using a UPLC equipped with a C18 column. A mixture of water and acetonitrile (45/55 wt/wt) was used as a mobile phase at a flow rate of 50 μL/min. The sample injection volume was 1 μL. The yield of EG was calculated from UPLC peak area using a pre-calibrated plot of peak area versus EG concentrations ranging between 10 μg/mL to 1000 μg/mL.

A refractometer was also used to measure EG production. For refractometer, a standard calibration plot for EG solutions of known concentrations was prepared to assess the accuracy in measurements of EG percentage for known solutions. Then, the percentage of EG yields in the BHET degraded solutions were measured. A good correlation in the yield of EG from UPLC and refractometry techniques was observed. The conversion of BHET from the yield of EG was calculated using the following equation:

BHET conversion

BHET (g) = ((% EG)(1097 g/L EG)(254.238 g/mol BHET)(0.5))/((100)(62.07 g/mol EG))

The yield of terephthalic acid was not measured because it precipitates in water.

Results and Discussion

Modeling modified PETase plasmid sequence

It has been reported that *E. coli* is a compatible host of PETase.(Seo *et al*., 2019) BL21 *E. coli* cells were utilized as vectors of the plasmids. Using PyMol™ software (Schrödinger, Inc. Version 3), amino acid sequence of PETase was modeled. Figure 1a shows the complete PETase structure, with the active site highlighted in yellow and red. In Figure 1b, the active site of PETase was isolated and modified, and the region which contains disulfide bond sequence was removed. This was done to test the effect of this disulfide bond on the activity of PETase. Removal of the disulfide bond sequence produced a much shorter protein with two helices and three beta strands (Figure 1b). The sequence of the modified active site (A1) was GVMGWSMGGGGSLISAANNPSLKAA APQAPWDSSTNFSSVTVPTLIFACENDSIAPVN. The sequence of the modified PETase was sent to IDT to synthesize it. The unmodified PETase active site sequence (A2) consisted of three alpha helices and four beta strands (Figure 1c). The portion highlighted in red displays the section of protein which was not included in A1. The sequence of A2 was GVMGWSMGGGG SLISAANNPSLKAAAPQAPWDSSTNFSSVTVPT LIFACENDSIAPVNSSALPIYDSMSRNAKQFLEI

NGGSHSC. BUSCA software,(Savojardo *et al*., 2018) which is a server through which a protein sequence can be analyzed, was used to predict expression of the PETase active site. This program determined that unmodified PETase

is an extracellular protein while the modified PETase is intracellularly expressed in the cytoplasm. 6-histidine tags were attached to the ends of the modified PETase to enable purification with a histidine syringe column.

BHET degradation efficiency using A1 and A2 PETase active sites

Figure 2 shows protein concentrations, which is equivalent to enzyme concentrations of A1 and A2 PETase, in the absence and presence of PMSF. The error bars show standard deviation from five measurements. Protein concentrations of A1 PETase in the absence and presence of PMSF are 31.57 ± 3.09 μg/mL and 37.52 ±2. 59 μg/mL, respectively, while these values for A2 PETase are 33.82 ± 3.52 μg/mL and 33.82 ± 3.48 μg/mL respectively.

The results indicate that protein concentration of A2 PETase, which contains disulfide bond, did not change significantly in the presence of PMSF. There is a significant increase in protein concentration of A1 PETase, which does not contain disulfide bond, in the presence of PMSF. PMSF is a serine protease inhibitor, which blocks the activity of proteases such as elastase that degrade PETase. A1 PETase is less stable in comparison to the A2 due to its lack of the disulfide bond. The disulfide bond makes proteins more globular, less likely to denature, and increases protein durability. Removal of this disulfide bond from A1 made PETase more susceptible to degradation by protease inhibitors, and the addition of PMSF blocked proteases from degrading A1. Total protein concentration of A1 PETase increases in the presence of PMSF. A2 is more stable due to its disulfide bond, so it was not impacted by proteases to the same extent as A1, and the addition of PMSF did not create a significant difference in protein concentration in it. BCA assay of a control experiment without addition of PETase shows no protein.

BHET degradation by A1 and A2 PETase demonstrate that both can efficiently degrade BHET to EG and TPA within 72 hours (Figure 3). A control experiment containing only buffer solution showed no BHET degradation in terms of the yield of EG. A1 and A2 PETase produced similar amount of EG (14 – 15%). The error bars in Figure 3 represent standard deviation from 5 replicates. The presence of PMSF with A1 or A2 PETase showed insignificant changes in the yield of EG.

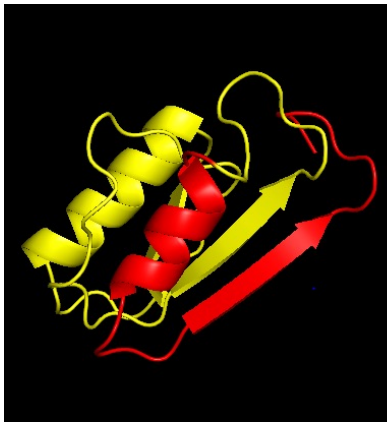


Figure 1a. **Structure of complete PETase.**

Figure 1b. **Modified PETase sequence without a disulfide bond (A1).**

Figure 1c. **Unmodified PETase sequence with a disulfide bond (A2).**

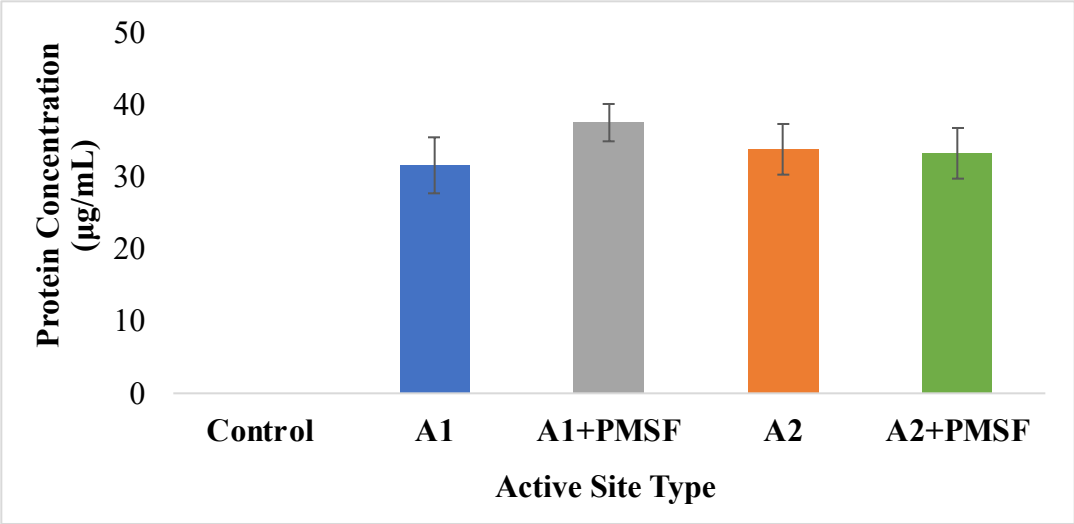


Figure 2. **Experimentally measured protein concentration of A1 and A2 PETase in the presence and absence of PMSF.**

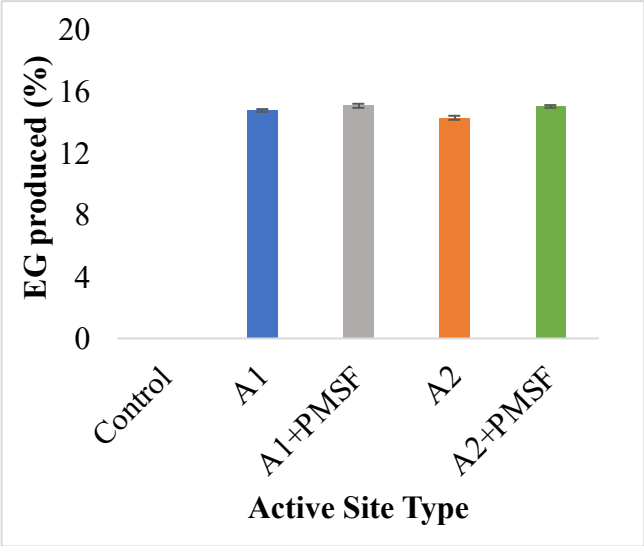


Figure 3. **Percentage of ethylene glycol produced by A1 and A2 PETase with and without PMSF in 72 hours.**

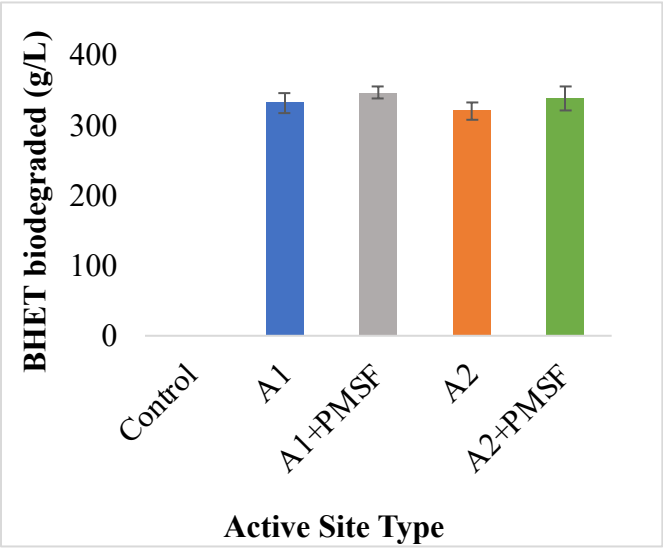


Figure 4. **Total BHET degraded by A1 and A2 PETase with and without PMSF in 72 hours.**

Figure 4 shows total amount of BHET degraded in 72 hours. A1 and A2 PETase resulted in 332.05 ± 14.44 g/L and 320.85 ± 12.58 g/L BHET conversion, respectively, in the absence of PMSF, which is quite high. BHET conversion by A1 PETase is a little higher in the presence of PMSF, which is likely because of a slightly higher amount of protein in the solution as seen in Figure 2.

The activity of both proteins was further determined in terms of BHET degradation per unit (microgram) of each protein (Figure 5). The error bars in Figure 5 display standard deviation from 5 replicates. It shows that the activity of A1 PETase (0.1073 ± 0.0168 g/µg enzyme) is slightly higher than that of A2 (0.0962 ± 0.0113 g/µg). The activity of A1 in the presence of PMSF is slightly lower (0.0843 ± 0.0191 g/µg) because of total measured protein concentration in the presence of PMSF was higher as discussed above. Upon consideration of standard deviation, the activity of A1 and A2 PETase appears to be similar, which ranges between

0.08-0.1 g BHET degradation/μg of protein. While absence of the disulfide bond in A1 structural sequence makes it less stable, owing to the lack of globular structure, and its activity was expected to be lower with reference to the activity of A2, the similar activity of A1 and A2 indicates that the active site disulfide bond does not have an impact on PETase's activity.

The results shown in Figure 5 demonstrate that the activity of A1 PETase is significantly affected by the presence of PMSF. It indicates that the short structure of A1 is unstable in the presence of PMSF as the serine is blocked in the catalytic triad. In contrast, A2 exhibits a slight increase in activity upon the addition of PMSF because of the higher stability given by the disulfide bond, but it could be within the experimental uncertainty. Thus, PMSF did not inhibit the activity of A2 PETase's active site as hypothesized from the fact that PMSF would block the serine at the catalytic triad and disrupt enzyme activity. The addition of PMSF prevents protease activity throughout the reaction, resulting in preserving the activity of enzyme A2.

This work advances the understanding on the role of the disulfide bond of PETase in the degradation of BHET as a model compound, which will enable future development of more active PETase without this bond for degradation of PET to EG and TPA.

Conclusions

This work described the cleavage of ester linkages, present in PET plastic, with PETase enzymes in the presence and absence of a disulfide bond to elucidate the role of the disulfide bond on the enzyme's activity. A modified PETase enzyme without disulfide bond sequence was first modeled and synthesized. Then, PETase enzymes with and without the disulfide bond were cultured, purified, assayed, and used for cleavage of ester linkages of a PET surrogate substrate, BHET. Typically the reaction between PET and PETase must be run for several days, and the results are analyzed with cumbersome microscopic techniques to measure crystallinity loss of PET, which does not give a direct measurement of ester linkage cleavage. In contrast, BHET degradation forms EG and TPA monomers to quantitively measure the degree of ester linkage degradation and allows evaluation of enzyme's activity more effectively. Controlled experiments were also conducted in the presence and absence of PMSF as well as without an enzyme. The results showed that the total BHET degradation to EG and TPA was not significantly influenced by the disulfide bond of PETase, which strongly conflicted prior works that predicted the disulfide bond increased protein activity.

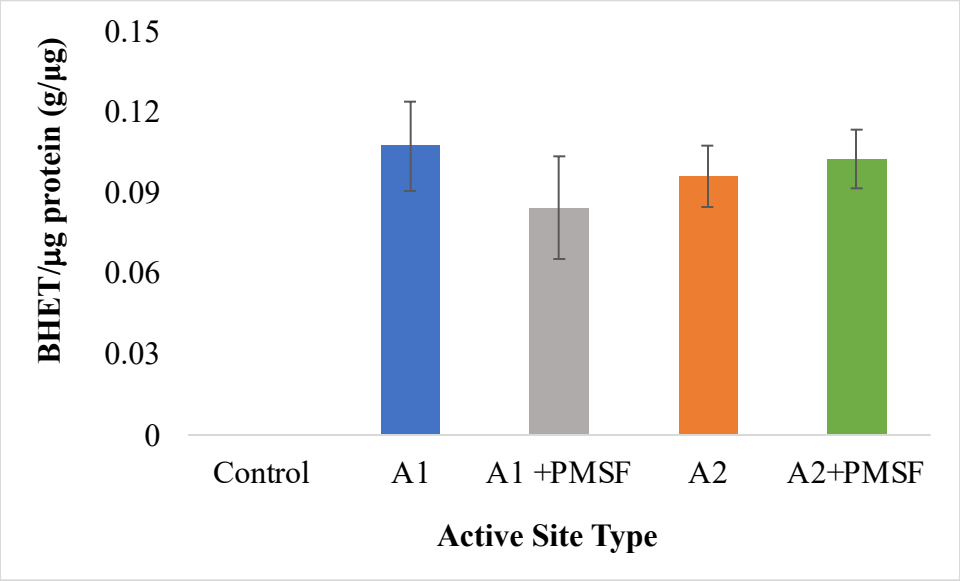


Figure 5. **Comparison of BHET degradation per microgram of enzymes in the presence and absence of PMSF. Control experiment showed no degradation of BHET.**

Further research is necessary to determine the cause of this phenomenon. It is likely that A1's short structure facilitates easy accessibility of its active site to BHET in the degradation process. Although A2 may be more stable, A1 has the ability to degrade a greater amount of BHET in the same time period. The finding of this work - that removal of the active site disulfide bond in PETase does not have an impact on its activity - is a stepping stone in designing a more effective version of the PETase enzyme.

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References

1. Austin, H. P., Allen, M. D., Donohoe, B. S., Rorrer, N. A., Kearns, F. L., Silveira, R. L., Pollard, B. C., Dominick, G., Duman, R., & El Omari, K. (2018). Characterization and engineering of a plastic-degrading aromatic polyesterase. Proceedings of the National Academy of Sciences, 115(19), E4350-E4357.

2. Chen, C. C., Han, X., Ko, T. P., Liu, W., & Guo, R. T. (2018). Structural studies reveal the molecular mechanism of PETase. The FEBS Journal, 285(20), 3717-3723.

3. Fecker, T., Galaz-Davison, P., Engelberger, F., Narui, Y., Sotomayor, M., Parra, L. P., & Ramírez-Sarmiento, C. A. (2018). Active site flexibility as a hallmark for efficient PET degradation by I. sakaiensis PETase. Biophysical journal, 114(6), 1302-1312.

4. Furukawa, M., Kawakami, N., Tomizawa, A., & Miyamoto, K. (2019). Efficient degradation of poly (ethylene terephthalate) with Thermobifida fusca cutinase exhibiting improved catalytic activity generated using mutagenesis and additive-based approaches. Scientific reports, 9(1), 1-9.

5. Herrero Acero, E., Ribitsch, D., Steinkellner, G., Gruber, K., Greimel, K., Eiteljoerg, I., Trotscha, E., Wei, R., Zimmermann, W., & Zinn, M. (2011). Enzymatic surface hydrolysis of PET: effect of structural diversity on kinetic properties of cutinases from Thermobifida. Macromolecules, 44(12), 4632-4640.

6. Hu, X., Osaki, S., Hayashi, M., Kaku, M., Katuen, S., Kobayashi, H., & Kawai, F. (2008). Degradation of a terephthalate-containing polyester by thermophilic actinomycetes and *Bacillus* species derived from composts. Journal of Polymers and the Environment, 16(2), 103-108.

7. Jones, S. M., & Solomon, E. I. (2015). Electron transfer and reaction mechanism of laccases. Cellular and Molecular Life Sciences, 72(5), 869-883.

8. Kawai, F., Kawabata, T., & Oda, M. (2019). Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. Applied microbiology and biotechnology, 103(11), 4253-4268.

9. Kawai, F., Kawabata, T., & Oda, M. (2020). Current State and Perspectives Related to the Polyethylene Terephthalate Hydrolases Available for Biorecycling. ACS Sustainable Chemistry & Engineering, 8(24), 8894-8908. https://doi.org/10.1021/acssuschemeng.0c01638

10. Khoonkari, M., Haghighi, A. H., Sefidbakht, Y., Shekoohi, K., & Ghaderian, A. (2015). Chemical recycling of PET wastes with different catalysts. International Journal of Polymer Science, 2015.

11. Ma, Y., Yao, M., Li, B., Ding, M., He, B., Chen, S., Zhou, X., & Yuan, Y. (2018). Enhanced poly (ethylene terephthalate) hydrolase activity by protein engineering. Engineering, 4(6), 888-893.

12. Müller, R. J., Schrader, H., Profe, J., Dresler, K., & Deckwer, W. D. (2005). Enzymatic degradation of poly (ethylene terephthalate): rapid hydrolyse using a hydrolase from T. fusca. Macromolecular rapid communications, 26(17), 1400-1405.

13. O'Brien, K. (2019, May 31, 2019). Biodegradation of Plastic Waste. https://www.advancedsciencenews.com/biodegradation-of-plastic-waste/. Advanced Science News.

14. Paydar, M. M., & Olfati, M. (2018). Designing and solving a reverse logistics network for polyethylene terephthalate bottles. Journal of Cleaner Production, 195, 605-617.

15. Rauwerdink, A., & Kazlauskas, R. J. (2015). How the same core catalytic machinery catalyzes 17 different reactions: the serine-histidine-aspartate catalytic triad of α/β-hydrolase fold enzymes. ACS catalysis, 5(10), 6153-6176.

16. Ribitsch, D., Acero, E. H., Greimel, K., Eiteljoerg, I., Trotscha, E., Freddi, G., Schwab, H., & Guebitz, G. M. (2012). Characterization of a new cutinase from Thermobifida alba for PET-surface hydrolysis. Biocatalysis and Biotransformation, 30(1), 2-9.

17. Savojardo, C., Martelli, P. L., Fariselli, P., Profiti, G., & Casadio, R. (2018). BUSCA: an integrative web server to predict subcellular localization of proteins. Nucleic acids research, 46(W1), W459-W466.

18. Seo, H., Kim, S., Son, H. F., Sagong, H.-Y., Joo, S., & Kim, K.-J. (2019). Production of extracellular PETase from Ideonella sakaiensis using sec-dependent signal peptides in *E. coli*. Biochemical and biophysical research communications, 508(1), 250-255.

19. Shirke, A. N., White, C., Englaender, J. A., Zwarycz, A., Butterfoss, G. L., Linhardt, R. J., & Gross, R. A. (2018). Stabilizing leaf and branch compost cutinase (LCC) with glycosylation: mechanism and effect on PET hydrolysis. Biochemistry, 57(7), 1190-1200.

20. Taniguchi, I., Yoshida, S., Hiraga, K., Miyamoto, K., Kimura, Y., & Oda, K. (2019). Biodegradation of PET: current status and application aspects. ACS Catalysis, 9(5), 4089-4105.

21. Thumarat, U., Kawabata, T., Nakajima, M., Nakajima, H., Sugiyama, A., Yazaki, K., Tada, T., Waku, T., Tanaka, N., & Kawai, F. (2015). Comparison of genetic structures and biochemical properties of tandem cutinase-type polyesterases from Thermobifida alba AHK119. Journal of bioscience and bioengineering, 120(5), 491-497.

22. Tullo, A. H. (2019, October 6, 2019). Plastic has a problem; is chemical recycling the solution? . C&EN, 97(39).

23. Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., & Oda, K. (2016). A bacterium that degrades and assimilates poly (ethylene terephthalate). Science, 351(6278), 1196-1199.

Bacteriophage: An Underutilized Bacterial Combatant

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Introduction

Viral infections are commonly known to occur in humans, but what most people do not know is that viral infections are just as common in bacteria [1]. Bacterial viruses are termed "bacteriophage" or "phage" for short and can be found in any environment: soil, water, snow, food products, and even sediments. Viruses are generally 1000X smaller than their bacterial hosts and can possess a wide variety of different genotypic and phenotypic characteristics. Such diversity includes morphology (size, shape, and structure), the number and type of open reading frames (translational portions of the genome), host range, and mode of infection. Phage differ from animal viruses in a few distinct aspects. Phage attach directly to the bacterial cell wall, inject their viral nucleic acids into the cytoplasm, and become synthesized by bacterial components. In contrast, most animal viruses have to bind to a cell membrane protein, become engulfed by the mammalian cell, outer protective layer digested by enzymes, and genetic material finally synthesized in order to cause infection [2]. Bacteriophage were first discovered in 1915 by F. W. Twort and have yet to be utilized to their full potential over 100 years later [3].

Bacteriophage Characteristics and their Detection

Bacteriophage harbor a few unique physical and genetic characteristics. Their physical characteristics (phage morphology) can be separated into 5 categories: icosahedral, hexagonal, spherical, tailed, and filamentous [1]. The length of the phage tail varies among phage types as some have no tail, some possess a short tail, and others have a tail twice the length of the capsid head. Phage morphology and tail formation dictate their functional capabilities. Genetically, genome types are variable and may consist of either double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), or single-stranded RNA (ssRNA) which ultimately determine how the phage will function in the host, and what particular mode of infection they harbor. In contrast, viruses that infect humans are typically single-stranded RNA (ssRNA) that function as messenger RNA (mRNA) and can be directly translated into a polyprotein [4].

Another unique characteristic is that some phage can infect multiple species of bacteria while others are more species-specific [1]. This is termed 'host range' and can be of great importance when trying to find a way to combat multi-drug resistant infections. Due to the ease with which phage can acquire genes from bacterial hosts, phage can evolve to infect a wide variety of bacteria; advantageous to the phage, disadvantageous to the bacterial hosts. The mode of infection differs among phage types as they can be either lytic, lysogenic, or latent [1]. Before entering either the lytic cycle or lysogenic cycle, all phage must first exhibit the latent stage in which the phage attaches to the cell surface, penetrates the membrane, and incorporates its DNA into the host genome. Lytic phage replicate inside of the bacterial host before lysing (breaking open) the cell, releasing more phage into the environment. Lysogenic phage incorporate their DNA into the bacterial genome in order to have the bacteria, unknowingly, replicate the phage genetical material numerous times. This allows the phage to have its genome carried within the bacterial progeny, an advantage to the phage itself. However, lysogenic phages can, at times, become virulent due to the protection they obtain from "hiding" inside the bacterial cells and going undetected. This could deem problematic as this is a similar mechanism to how MRSA (multi-resistant *Staphylococcus aureus*) infects: *S. aureus* "hides out" inside immune cells, going undetected by the immune system, and ultimately rendering antibiotics useless. This gives MRSA time to replicate before the immune cell undergoes autophagy, releasing the MRSA into the bloodstream.

One method used by scientists for detecting phage is by observing plaque formations within agar [5]. Plaques are formed when phage lyse bacterial cells, spread to neighboring bacterial cells, and lyse again [6]. The lysing of the neighboring cells forms a circular, clear "lawn" that is visible to the human eye and is known as a plaque. Plaques can be counted to determine how infectious a phage is to the host and to help determine host range. Plaque formation is dictated and influenced by the phage genome, rate of

adsorption (attachment), time spent inside of the host cells, and phage morphology [5]. Depending on the phage genome, different genes could be present that ultimately affect the size of the plaque. Adsorption rate impacts plaque size as well because as the phage is slowly adsorbed into the bacteria, the phage have more time to reproduce before lysing the cell. This phenomenon leads to a higher concentration of phage within the plaque formations. The density of phage inside the host cell dictates the formation of the plaque. In a study done by Gallet, Kannoly, and Wang, it was found that "the higher the adsorption rate then the lower the phage concentration within the plaques."

The timing of the lysis stage determines how long the phage has to reproduce inside the cell beforehand [5]. Gallet et. al. found that there was a linear relationship between lysis time and burst size (plaque formation). Phage with a low adsorption rate spend more time externally to the bacterial cells, therefore, by the time the phage DNA is inserted into the cell, there is less time for the virus to cause infection. The physiology of the bacterial cells themselves could have changed by the time the phage infects, resulting in a smaller plaque size. The less time the phage have to diffuse out of the lysed cell, the smaller the plaque. Phage morphology and architecture also determine the plaque size and formation after lysis. The longer the sheath and legs of the phage, the smaller the plaque size. This is due to the ability of the virus to diffuse through the agar layer when forming a plaque. A smaller virion would lead to a larger plaque due to the ease at which it can diffuse through the matrix of the agar.

Phage Classification

As of 2011, approximately 750 different phage types have been successfully isolated and fully sequenced, with about 5,000 phage types yet to be elucidated or identified [7]. There are two types of comparative analyses that allow for a better understanding of a specific phage: viral metagenomics and prophage mining. Viral metagenomics is the process by which phage are harvested in large numbers and sequenced at random. This allows for large numbers of phage samples to be examined together from a specific environment and compared. Utilizing the mining method entails comparing phage genomic sequences against portions of a complete bacterial genome that harbor areas of prophage sequences. To attempt to isolate an individual phage genome, it is best to extract DNA from individual plaques following host-range experiments.

Most interestingly, bacteriophage can possess either DNA or RNA genomes, however, the vast majority possess linear double-stranded DNA genomes (dsDNA) and a tail (sheath): Order *Caudovirales* [7, Figure 1]. The dsDNA of this phage order is generally comprised of 55%-70% G+C. Most of the order *Caudovirales* fit into the *Siphoviridae* family (55%) possessing dsDNA and flexible non-contractile sheaths, while others make up either the *Myoviridae* (25%) family who possess contractile sheaths, or the *Podoviridae* (20%) family that exhibit short, stubby sheaths. The structure of the phage sheath has been found to reflect the phage genome, mode of infection, assembly, and maturation [8]. It has been determined that the long non-contractile tails of the *Siphoviridae* were assembled first then added to the head, whereas the short non-contractile tails were assembled after the phage heads. The similarity of the phage types in this order is currently being described as a result of horizontal gene transfer among phage. Other, less common phage families include *Tectiviridae* (lipid-containing), *Corticoviridae* (lipid-containing, circular genome), *Plasmaviridae* (enveloped), *Lipothrixviridae* (rod-shaped), *Rudiviridae* (non-enveloped), and *Fuselloviridae* (non-enveloped, lemon-shaped) [9]. These are the current, known and classified phage families but more remain undiscovered.

The *Myoviridae* family is comprised of many species of phage: T4, T2, P1, P2, Mu, *Bacillus* phage SP01, and *Halobacterium* phage ϕH [10]. Phage in this family are characterized as having an elongated head (90-110nm in diameter) and a long tail to match (100-120nm in length). The species share about 50-70% of their genomes to one another, making characterization of unknown phages difficult. Meanwhile, the *Siphoviridae* family is comprised of phage T1, T5, *Mycobacterium* L5, *Lactococcus* c2, λ, and ΨM1 [9]. These phages are characterized by having a smaller head (60nm in diameter) and longer sheath (70-90nm in length). What is most interesting is that the *Myoviridae* and *Siphoviridae* families are the only phage known to infect both bacteria and archaea. It is hypothesized that this is due to their contractile tails and the evolution of phages from

prokaryotes [11,12]. The lesser known family, *Podoviridae*, is comprised of phage T7, enterobacteria phage P22, and *Bacillus* phage ϕ29 [9]. *Podoviridae* are characterized as having a head of about 60nm in diameter and a short tail with maximum length of about 20nm.

Conclusions

Bacteriophage have been studied for years but have yet to be utilized to their full potential. Phage can be beneficial to both the food industry and pharmaceutical industry. Phage with a wide host range can be used to combat multiple bacterial infections, while phage who are selective to a certain bacterial host can be used against one particular infection. Additionally, when antibiotics are rendered useless, phage could be the answer. Phage can more easily bind to and attack bacterial cells. Phage could also be used in combination with antibiotics to fully rid a patient of an infection. Phage do not target mammalian cells, therefore, they may also be a safer therapeutic.

In the food industry, bacteriophage could be used when pasteurization and antibiotics fail to rid the product of bacteria. Bacteria can form a protective outer layer (biofilm) that antibiotics and high temperatures fail to penetrate. When the environment is right for bacteria to disperse from the biofilm, phage can be used as a final protective measure for food products. With Science and Technology rapidly advancing, now is the time to begin adding phage to the list of more commonly used biological control agents.

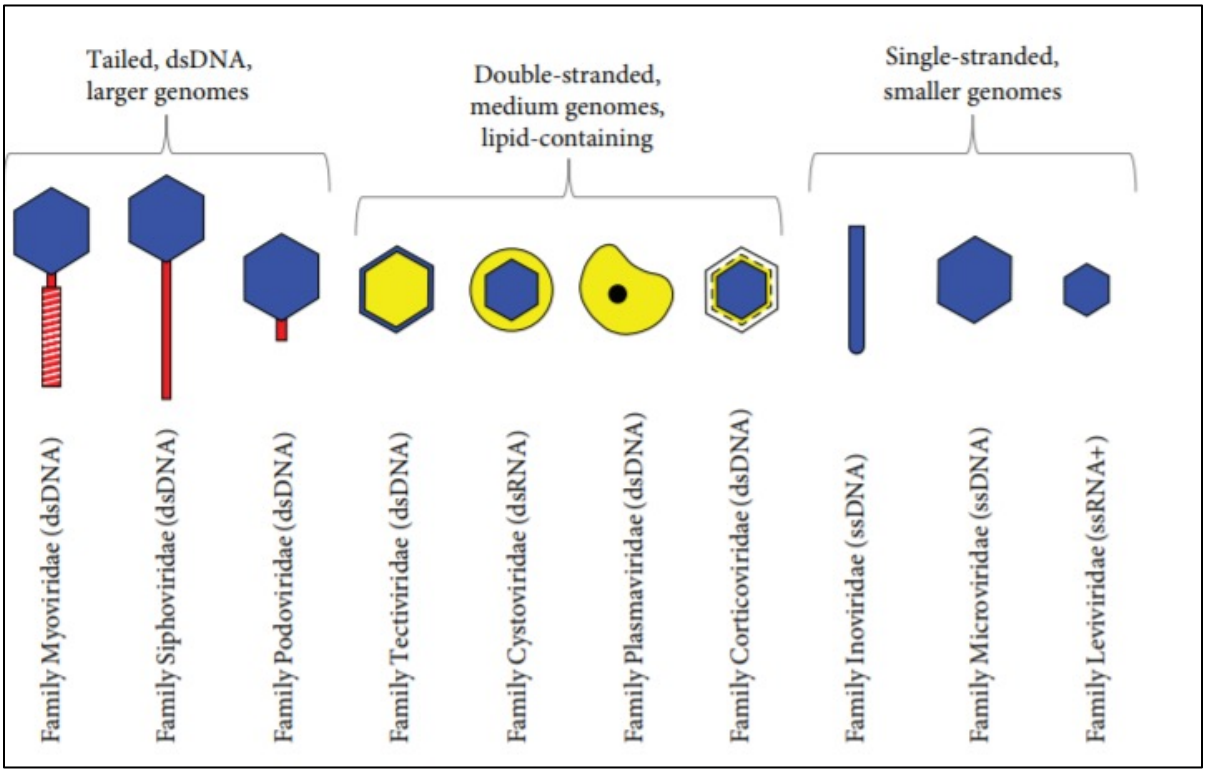

Figure 1. **Bacteriophage Familial Identification [7] Phage are classified into 13 distinct families, 3 of which have been grouped together into the Order *Caudovirales* [7]. *Caudovirales* make up the vast majority of phages (roughly 96%) that have been reported [13]. They are comprised of the families *Myoviridae*, *Siphoviridae*, and *Podoviridae*. The other 10 phage families make up only 4% of reported and classified phage.**

References

1. Hyman, P. and S.T. Abedon, Smaller fleas: viruses of microorganisms. Scientifica (Cairo), 2012. 2012: p. 734023. (4)
2. Cohen, F. H., How Viruses Invade Cells. Biophysical Journal: NCBI, 2016. 110: p. 1028-1032.
3. Duckworth, D.H., Who discovered bacteriophage? Bacteriol, 1976. Rev. 40, p. 793-802.
4. Pellett P.E., Mitra S., Holland T.C. Basics of virology. Handb. Clin. Neurol. 2014;123:45–66.
5. Ács, N., Gambino, M., and Brønsted, L., Bacteriophage enumeration and Detection Methods. Frontiers in Microbiology, 2020. 11.
6. Gallet, R., S. Kannoly, and I.N. Wang, Effects of bacteriophage traits on plaque formation. BMC Microbiol, 2011. 11: p. 181.
7. Hatfull, G.F. and R.W. Hendrix, Bacteriophages and their genomes. Curr Opin Virol, 2011. 1(4): p. 298-303.
8. Shao Y, W., Bacteriophage adsorption rate and optimal lysis time. Genetics, 2008. 180: p. 471-482.
9. Maniloff, J.a.A., H.W., Taxonomy of bacterial viruses: establishment of tailed virus genera and the order *Caudovirales*. Arch Virol, 1998. 143(10): p. 2051-2063.
10. Ackermann, H.W., Frequency of morphological phage description in 1995. Arch Virol, 1996. 141: p. 209-218.
11. Ackermann, H.W.a.D., MS, Viruses of prokaryotes. CRC Press, 1987.
12. Zilling, W., Prangishvilli, D., Schleper, C., Elferink, M., Holz, I., Albers, S., Janekovic, D., and Gotz, D., Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic Archaea. FEMS Microbiol Rev, 1996. 18: p. 225-236.
13. Matsuzaki, S., *et al*., Bacteriophage therapy: A revitalized therapy against bacterial infectious diseases. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy, 2005. 11: p. 211-9.

Editorial Board

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