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An Arms Race Against Disinfectant Resistance
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Abstract
Antimicrobials, compounds which inhibit or destroy microorganism growth, have revolutionized modern medicine. However, the overuse and misuse of these antimicrobials has led to the emergence of antibiotic and disinfectant resistance through intrinsic and population resistance mechanisms. Single-cell resistance mechanisms include target alteration, inactivation, efflux pumps, and impermeability, while bacterial populations employ biofilms. New technology, such as quantitative polymerase chain reaction, allows for the quantification of this resistance, which is superior to the minimum inhibitory concentration method. Through quantification, prevention strategies, and further research, full comprehension of combating disinfectant resistance can be acquired.

Introduction
With exponentially advancing technology and scientific discovery, successful treatment of human pathogens and enhanced clinical outcomes have become prominent in today’s society. The discovery of penicillin, in 1928, revolutionized twentieth century medicine; it became the first commercialized antibiotic to fight bacterial infections. However, due to the wide range of microorganism pathogenicity, several types of antimicrobials have been developed. Antimicrobials, which are able to destroy or inhibit microorganism growth, are classified into two main groups (Table 1). Anti-infectives are used to treat infectious diseases and include antibiotics. In contrast, biocides are chemical agents that act outside the human body. However, with the wide range of antimicrobial availability, the evolution of resistant bacteria has become a worldwide health concern.

Table 1: Types of antimicrobials. Anti-infectives treat various types of infections in the body. Biocides include treatment of consumer products, inanimate objects, clinical instruments, and surface wounds.

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocides</td>
<td>Disinfectant</td>
</tr>
<tr>
<td></td>
<td>Preservative</td>
</tr>
<tr>
<td></td>
<td>Antiseptic</td>
</tr>
<tr>
<td>Anti-Infectives</td>
<td>Antibacterial</td>
</tr>
<tr>
<td></td>
<td>Antiviral</td>
</tr>
<tr>
<td></td>
<td>Antifungal</td>
</tr>
<tr>
<td></td>
<td>Antiparasitic</td>
</tr>
</tbody>
</table>

Antimicrobial Resistance
The widespread use of antimicrobials, especially biocides, has been prevalent for centuries. One of the most common antiseptics, alcohol, has been used since ancient civilizations. Moreover, the introduction of antibiotics in clinical settings during the 1940’s revolutionized modern medicine. However, the overuse and misuse of antibiotics has led to antibiotic resistance through excessively or incorrectly prescribing antibiotics. This has made antibiotic resistance one of the most prevalent and costly health concerns in medicine. The race to discover novel antibiotics is additionally hindered by regulations such as prolonged approval times placed on pharmaceutical companies and lack of economic motivation. In the United States, antibiotic resistance results in an additional $55 billion per year of excess healthcare costs. Thus, the antibiotic resistance pandemic has prompted the scientific community to extensively study this concern. However, one area with limited research is disinfectant resistance, which will be the focus of this review. Challenges of previous research conducted includes different mechanisms of antimicrobial activity and the lack of specificity of disinfectants. Disinfectants, compared to antibiotics, have a wider range of targets and lower antimicrobial activity, which hinders the determination of resistance mechanisms. Thus, by not being able to identify the target of resistance, it poses a challenge to discover novel antimicrobials.

Disinfectant Resistance
Disinfectants are used to kill or hinder microbial growth on inanimate surfaces. They are characterized into three main types based on their effectiveness and type of contact: high-level, intermediate, and low-level (Table 2). High-level disinfectants, which kill microorganisms through liquid immersion, are used in surgical settings on instruments such as endoscopes. Intermediate and low-level disinfectants are used in non-surgical disinfection, such as patient care items, and they kill through liquid contact. Although used in everyday life, the most prevalent disinfectant usage is in healthcare settings. Similar to antibiotics, the misuse of disinfectants such as over-dilution, incorrect duration time, and lack of contact can lead...
to disinfectant resistance\textsuperscript{10}.

**Table 2: Types of disinfectants and their targets.**

High-level disinfectants involve immersion in disinfectant with heat-sensitive items where sterilization is not possible. Intermediate and low-level disinfectants suffice with liquid contact and heat sterilization but cannot kill spores\textsuperscript{10}.

<table>
<thead>
<tr>
<th>Type</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-level</td>
<td>All microorganisms. Exception: high levels of spores.</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Microorganisms, most fungi and viruses. Exception: spores.</td>
</tr>
<tr>
<td>Low-level</td>
<td>Microorganisms, some fungi and viruses. Exception: spores, mycobacteria.</td>
</tr>
</tbody>
</table>

**Disinfectant Resistance Mechanisms**

Research on bacterial resistance mechanisms against disinfectants was previously limited due to the broad range of targets and low antimicrobial activity\textsuperscript{4}. However, considerable progress has been made in the last 20 years. Resistance mechanisms incorporate multifaceted complexity and are grouped into single-cell or population resistance\textsuperscript{11-15}. Single-cell resistance mechanisms involve efflux pumps, impermeability, inactivation, and target alteration\textsuperscript{11-16}. These four main mechanisms are considered intrinsic resistance mechanisms\textsuperscript{12,14}. In addition, research shows that there are species-specific acquired resistance mechanisms such as bypassing of certain steps in a metabolic pathway\textsuperscript{12,16}. However, these are outside the scope of this review.

**Single-Cell Resistance**

**Efflux Pumps**

A common way that bacteria acquire disinfectant resistance involves the use of bacterial efflux pumps\textsuperscript{16}. These active transporters, located in the cytoplasmic membrane, are responsible for removing toxins from the bacterial cell and contribute to bacterial pathogenicity\textsuperscript{16,17}. Encoded by either genomic DNA or DNA from plasmids, there are several types of efflux pumps categorized as multidrug resistance (MDR) efflux pumps\textsuperscript{17}. These include the major facilitator superfamily (MFS), the ATP binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, the multidrug and toxic-compound extrusion (MATE) family, and the resistance nodulation division (RND) family\textsuperscript{17}. These efflux pumps are able to export the disinfectants out of the cell (Figure 1A). A notable example involves the qacA pump which is responsible for resistance to chlorhexidine, a disinfectant and antiseptic used to sterilize surgical instruments and patients’ skin\textsuperscript{18}. In *Staphylococcus aureus*, the qacA efflux pumps use the proton gradient to export chlorhexidine out of the cell, rendering the disinfectant ineffective\textsuperscript{12}.

**Impermeability**

Furthermore, impermeability of bacterial membranes to biocides leads to disinfectant resistance (Figure 1B)\textsuperscript{14,16}. However, research in this area is controversial. Bacteria are classified into (i) gram-negative bacteria, which possess an outer membrane and a thin layer of peptidoglycan, and (ii) gram-positive bacteria, containing a thicker layer of peptidoglycan\textsuperscript{15}. Some research indicates that gram-positive bacteria are less susceptible to biocides due to several factors, such as genetic composition or higher tolerance for oxidizing agents\textsuperscript{1,22}. However, most studies conclude that the outer membrane of gram-negative bacteria allows for higher biocide resistance\textsuperscript{21,22}. Additionally, this outer membrane can change its hydrophobicity, ultrastructure, protein composition, and fatty acid composition to prevent biocide entry\textsuperscript{23-27}. One study showed that chlorhexidine resistant *Pseudomonas aeruginosa* changed their membrane ultrastructure by becoming rough and amorphous. Nonetheless, the mechanisms of changing the outer membrane composition without genetic change are still unknown\textsuperscript{16,23-27}. Given the limited research on this topic, such a gap in knowledge may be a possible research direction.

**Inactivation**

Bacteria can acquire disinfectant resistance through the inactivation of Triclosan by *Pseudomonas putida* (*P. putida*)\textsuperscript{28-30}. *Triclosan*, a common household and medical disinfectant, disables fatty acid synthesis\textsuperscript{28-32}. It interacts with the enoyl-acyl carrier protein reductase (ENR) encoded by the fabI gene where NAD+ affinity is increased\textsuperscript{31,32}. By forming a NAD+-Triclosan complex, fatty acid synthesis is unable to occur\textsuperscript{31,32}. It was confirmed that *P. putida* resistant bacteria have a homolog to fabl, a fabK gene, which enables the degradation of Triclosan and its use as a carbon source rather than fatty acid synthesis inhibition\textsuperscript{28-32}. Although *P. putida* inactivated Triclosan, this is not a common intrinsic resistance mechanism found in other bacteria\textsuperscript{16,28-30}. Overall, more research is necessary to comprehensively understand the degradation mechanism\textsuperscript{28-30}.

**Target Alteration**

Most antibiotics have target specificity, so bacterial mutations to modify the target is a common way to confer antibiotic resistance\textsuperscript{4,9}. To combat this, disinfectants have a wide range of targets, making it...
possible to alter one or a few disinfectant targets through mutations to decrease or eliminate susceptibility (Figure 1D)\textsuperscript{11,14,16}. One prominent example involves \textit{Escherichia coli} exhibiting Triclosan resistance\textsuperscript{31,32}. Triclosan, as mentioned previously, interacts with the ENR's active site needed for fatty acid biosynthesis\textsuperscript{31,32}. Through a missense mutation of \textit{fabl} – the gene encoding the reductase, affinity for Triclosan is decreased and the inactivating NAD\textsuperscript{+}-Triclosan complex is not formed\textsuperscript{31,32}. Thus, fatty acid biosynthesis is able to occur, rendering \textit{E. coli} Triclosan-resistant\textsuperscript{31,32}.

**Figure 1: Mechanisms of intrinsic disinfectant resistance.** Disinfectant indicated in yellow. (A) Disinfectant exported by efflux pump. (B) Impermeability to disinfectant. (C) Inactivation of disinfectant. (D) Target alteration of disinfectant. Alterations indicated by asterisks. Adapted from Poole \textit{et al.} (2002) and Gnanadhas \textit{et al.} (2013)\textsuperscript{14,16}.

**Population Resistance**

**Biofilms**

In addition to the intrinsic resistance mechanisms of bacteria mentioned above, the presence of biofilms enables bacteria to participate in population resistance\textsuperscript{11,33}. Biofilms are composed of bacteria surrounded by an extracellular polysaccharide matrix preventing disinfectants from reaching internal layers through steric hindrance (Figure 2A)\textsuperscript{33}. A study conducted by Jang \textit{et al.} (2006) showed that chlorine only penetrated 100 μm in a 150-200 μm dairy biofilm\textsuperscript{34}. This gives internally placed bacteria a higher chance of survival. Furthermore, electrostatic forces play a role in disinfectant resistance in biofilms\textsuperscript{33,35}. Some biofilms may have positively or negatively charged extracellular environment for cell-to-cell communications\textsuperscript{33,35}. It was shown that cationic cetlypyridinium chloride, a disinfectant, was hindered when encountering negatively charged biofilms\textsuperscript{36}. The attractive forces of the ions prevented the disinfectant from reaching internal layers. In addition to electrostatic and steric forces, bacteria in biofilms are considered heterogeneous due to the different physiological features\textsuperscript{33}. Internal bacteria have less access to nutrients and oxygen, accumulating more waste products and thus inducing metabolic modifications\textsuperscript{33}. This physiological heterogeneity increases bacterial chances to acquire resistance against disinfectants in biofilms\textsuperscript{33}. Overall, many studies confirm the resistance of biofilms to disinfectants mainly through physicochemical interactions rather than steric hindrance\textsuperscript{33}. Combined with intrinsic resistance mechanisms of individual bacteria, disinfectant resistance is an ever-growing concern.

**Figure 2: Population resistance using biofilms.** Disinfectant indicated in yellow. (A) Steric hindrance of disinfectant. (B) Hindrance due to electrostatic forces. (C) Resistance due to physiological heterogeneity. Modifications indicated by an asterisk. Adapted from Bridier \textit{et al.} (2011) Ganeshnarayan \textit{et al.} (2009)\textsuperscript{33-35}.

**Detection of Disinfectant Resistance**

**Current: MIC**

The most widespread method of disinfectant resistance detection involves calculating the minimum inhibitory concentration (MIC)\textsuperscript{36,37}. It is the lowest concentration of disinfectant or antibiotic needed to prevent growth\textsuperscript{36}. This efficient and easy method allows for screening of many strains and disinfectants at one time\textsuperscript{36,37}. Not only is the MIC used as a diagnostic tool for resistance, but it is also utilized as a measure of activity of disinfectants\textsuperscript{37}. For example, the MIC of chlorohexidine diacetate is 1 μg/mL in \textit{S. aureus}. In contrast, an MIC of 0.025 μg/mL is needed for Triclosan in \textit{S. aureus}\textsuperscript{38}. However, the MIC is limited to disinfectants that inhibit colony growth rather than destroy. In addition, MIC quantifies the amount of disinfectant needed rather than the bacterial viability of the sample\textsuperscript{36}.

**Future: qPCR**

Currently, research methodologies are being developed and perfected to quantify gene expression and bacterial viability in microbial resistance. Quantitative polymerase chain reaction (qPCR) uses florescence and PCR principles to measure amplification signals in thermocyclers\textsuperscript{39,40}. By using photo-reactive DNA-binding dye such as propidium monoazide (PMA), the viability of bacterial
cells is able to be detected and quantified through the use of qPCR. When bacterial samples are exposed to disinfectants, cells are lysed due to the dead cells' inability to maintain the cell membrane. PMA binds to the exposed DNA of dead cells and inhibits florescence, which enables quantification between live and dead cells. However, assays vary across bacterial species where no current standardized method exists.

Cross-Resistance
The alarming, increasing rates of disinfectant and antibiotic resistance increases the potential for cross-resistance to occur and leads to the evolution of superbugs. Cross-resistance prevails when bacteria resistant to one antimicrobial, such as a disinfectant, become resistant to another antimicrobial. This is seen when both antimicrobials share a target, a pathway, or route of access. Many studies have supported this claim, as seen in Table 3. In P. aeruginosa, biocide resistance of Triclosan enabled ciprofloxacin resistance through a mutation in the nfxG gene. In Salmonella, Triclosan facilitated chloramphenicol, erythromycin, imipenem, tetracycline cross-resistance through the increased activity of efflux pumps. The proposed mechanism of this resistance is through an active site mutation enabling these pumps to become MDR and sensitive to other antibiotics. These intrinsic single-cell based resistance mechanisms are promoted by the misuse of disinfectants such as contamination, over-dilution, incorrect duration, and lack of contact.

Table 3: Common cross-resistant organisms. Triclosan is a common household and medical disinfectant. Resistance mechanisms involve intrinsic single-cell based resistance.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Biocide and Antibiotic Resistance</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>Triclosan, ciprofloxacin</td>
<td>nfxG mutation</td>
</tr>
<tr>
<td>Mycobacterium</td>
<td>Triclosan, isoniazid</td>
<td>inhA mutation</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Triclosan, chloramphenicol, erythromycin</td>
<td>Active efflux pumps</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Triclosan, ciprofloxacin</td>
<td>Cell membrane alteration</td>
</tr>
</tbody>
</table>

Prevention
In the era of disinfectant and antibiotic resistance, prevention of resistance is not only feasible but should be put into practice in households and hospitals. A crucial step before disinfection involves prior standard cleaning methods, such as removing debris through washing, sterilization if feasible, and following manufacture labels. By cleaning surfaces, biofilms and accumulation of other debris that may hinder disinfectant efficacy are removed. For example, insufficient bacterial inactivation is the result of failure to properly clean medical instruments. In addition, proper usage of disinfectants should be practiced. Disinfectant resistance develops rapidly through human error mentioned previously such as over dilution, outdated products, and so forth. Following newer, more efficient guidelines and using CDC and FDA-cleared disinfectants will further prevent disinfectant resistance.

Conclusion
The ability of bacteria to obtain antibiotic and disinfectant resistance has been a growing concern in modern medicine. The overuse and misuse of these antimicrobials has led to intrinsic and population resistance mechanisms. Single-cell resistance mechanisms include target alteration, inactivation, efflux pumps, and impermeability while bacterial populations employ biofilms leading to increased disinfectant resistance. These strategies allow for the evolution of superbugs which are resistance to many types of antimicrobials through cross-resistance. Without working antimicrobials against these superbugs, new epidemics may arise in the future. The detection methods of these resistant strains involve calculating the MIC and newer assay developments such as qPCR. However, disinfectant resistance is deficient in research in comparison to antibiotic resistance. More research is required to understand the underlying mechanisms. In combination with prevention strategies, full comprehension of disinfectant resistance would provide an advantage in this arms race against disinfectant resistance.

Acknowledgments
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References


Probiotics and Obesity: The Therapeutic Potential of *Lactobacillus gasseri* SBT2055

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**Abstract**

Obesity is an ever-growing problem in today’s world. This disease serves as a significant public health burden in Canada, costing billions of dollars annually. However, more troubling is the impact this disease has on individual health and mortality. Diet and exercise have fallen short at combatting the growing prevalence of obesity, emphasizing the need for alternative treatment options. Recent research highlights the potential therapeutic effect of probiotics. Specifically, there is an abundance of research supporting the anti-obesity effects of the probiotic *Lactobacillus gasseri* SBT2055 (LG2055). Here, we aimed to critically evaluate two of the studies supporting these findings. We conclude that LG2055 holds therapeutic potential for obesity, however more research is needed to substantiate this claim.

**Introduction**

Obesity is a metabolic disorder which has recently begun to plague the lives of millions around the globe. Specifically, in Canada, the percentage of adults with obesity has more than doubled since 1978-1979. Currently, 64% of Canadian adults and about 14% of Canadian youth are obese². This is concerning because obesity is associated with an increased risk of type 2 diabetes, heart disease, certain cancers and premature death³. It is estimated that excess weight causes 1 in 10 premature deaths among Canadians between 20 and 64 years of age⁴. Additionally, obesity costs Canada around $11.1 billion annually – accounting for both direct healthcare costs which are estimated to be between $4.6 to 7.1 billion and indirect impacts (i.e. loss of productivity)⁵. Considering the significant burden this disease puts on health and economy, finding an effective cure becomes of paramount importance.

The multifactorial and complex nature of obesity makes it difficult to treat. Obesity often develops as a result of a sustained positive energy imbalance which can be caused by an increase in energy intake or a decrease in energy expenditure (i.e. decreased activity levels)⁶. The excess energy leads to weight gain and excess adiposity which is likely a result of increased lipid absorption at the level of the small intestine⁷. The cornerstone of obesity treatment has long been lifestyle modification (i.e. exercise and diet), however these methods have been unable to combat the rapid spread of this disease⁸. A growing body of evidence suggests that the biological adaptations associated with obesity persist through remission, preventing patients from ever truly recovering (e.g. neuronal changes which cause individuals to feel a reward deficit while dieting, thereby promoting overeating)⁹. Behaviorally induced weight loss strategies are therefore not sufficient for the treatment of this disorder – putting an emphasis on the need for more biologically based interventions⁸. Several biological based treatment routes have been proposed, however more research is needed to evaluate their efficacy.

One proposed treatment for obesity involves the administration of probiotics or prebiotics aimed at altering the composition of the gut microbiota. The gut microbiota has been implicated in obesity as it is an important player in metabolism (i.e. digestion and absorption of nutrients) and immune system function. Research findings indicate that there are obesity-associated changes in the gut microbiota which allow for an increase in energy absorption⁹. Additionally, evidence suggests that this trait is transferable as transplanting obese mouse microbiota leads to obesity in germ-free mice⁹. In light of this information, there is a reasonable basis to target the gut microbiota in obesity treatment.

If used in conjunction with changes in diet and exercise, the administration of probiotics or prebiotics may provide a more effective method for obesity treatment. A recent finding by Scheiman *et al.* (2019) helps push this narrative as they demonstrated the ability of probiotic intervention to effectively boost exercise capacity in mice¹⁰. Probiotics are living microorganisms, commonly referred to as the ‘good’ bacteria (i.e. those involved in conferring health benefits to the host). Ingestion of probiotics can transmit positive health effects if given in adequate quantities¹¹. Specifically, probiotics have been shown to improve lactose intolerance, prevent diarrhea, and have anti-tumour and anti-obesity effects¹²-¹⁵. Alternatively, prebiotics refer to indigestible parts of food such as fibre which is not digested by the gut and instead enters the colon to
be fermented by the bacteria there. Prebiotics serve as a food source for the good bacteria within the gut, and they have been shown to reduce hypercholesteremia\textsuperscript{16} and have anti-obesity effects\textsuperscript{17}. Both can be ingested as part of common foods (e.g. yogurt) or incorporated into supplements. In short, the ingestion of probiotics or prebiotics can have numerous beneficial effects for host metabolic health.

One probiotic showing promise as a potential therapeutic for obesity is \textit{Lactobacillus gasseri} SBT2055 (LG2055). LG2055 is thought to colonize, proliferate and subsequently inhibit lipid absorption at the level of the small intestine\textsuperscript{18, 15}. Ogawa \textit{et al.} (2015) found LG2055 supplementation to be associated with increased lipid levels in human feces, indicating decreased lipid absorption\textsuperscript{19}. Decreased lipid absorption as a result of LG2055 likely occurs through suppression of pancreatic lipase-mediated fat hydrolysis\textsuperscript{19}. The proposed mechanism of action does not involve direct inhibition of the lipase, instead LG2055 likely plays a role in increasing fat emulsion size which is linked to decreased specific surface area. Consequently, the lipase has less area on which it can act – leading to suppression of its activity\textsuperscript{19}. Through this mechanism, LG2055 has the capacity to alter the energy balance ratio by decreasing fat absorption and increasing energy output – positioning it as a prime candidate for obesity treatment.

LG2055 also has anti-inflammatory properties which could help combat the chronic-low grade inflammation that often presents itself alongside obesity\textsuperscript{20}. Targeting this inflammation is central to helping improve the prognosis of obesity as chronic low-grade inflammation is a known cause of insulin resistance, a major risk factor for type 2 diabetes\textsuperscript{21}. Kawano \textit{et al.} (2016) highlighted LG2055’s ability to attenuate several key hallmarks of obesity-related inflammation including high-fat diet (HFD)-mediated increases in intestinal permeability and adipose tissue inflammation\textsuperscript{22}. Additionally, Miyoshi \textit{et al.} (2014) provided evidence for a link between LG2055 treatment and decreased expression of pro-inflammatory genes in the adipose tissue of obese mice\textsuperscript{20}. Hence, the anti-inflammatory properties of LG2055 provide another avenue for its anti-obesity effects.

Although inflammation is a key player in obesity, the increase in number or enlargement of adipocytes are also important features of this disease\textsuperscript{23}. Sato \textit{et al.} (2008) linked LG2055 supplementation to a decrease in adipocyte size after reporting a significant reduction in the size of mesenteric and retroperitoneal adipocytes in LG2055-treated rats fed an HFD\textsuperscript{24}. Considering the close relationship between adipocyte size and excess fat mass, this finding provides support for LG2055 as a candidate for obesity treatment. Additionally, many studies have reported decreased liver triglyceride (TG) levels in animal models post-LG2055 treatment, thereby offering further support for LG2055 as a therapeutic for obesity\textsuperscript{20, 24}.

LG2055 was initially isolated from human feces and it is part of the larger group of lactic acid bacteria\textsuperscript{25}. It is often incorporated into dairy products where it functions to increase bio-preservation and digestibility\textsuperscript{25}. This probiotic can also be found in dietary supplements as its consumption has been associated with several beneficial health effects. Such effects of LG2055 consumption are further enhanced by its safety profile. \textit{Lactobacillus gasseri} strains are considered safe, but they may cause some unwanted side-effects such as gas or bloating. Nonetheless, their therapeutic potential seems to outweigh these minor forms of discomfort. Along with this low risk factor, proposing LG2055 as a therapeutic for obesity is also feasible as this probiotic is already widely available in capsule form. However, it is important to note that this probiotic would not be recommended to everyone – namely those with compromised immune systems or those taking immunosuppressive medications as they are more prone to infection of cardiac tissue\textsuperscript{26}.

Both animal studies and human trials have demonstrated the ability of LG2055 to alleviate obesity symptoms. The purpose of this report is to critique these studies in order to take an informed stance on the suitability of LG2055 as a treatment for obesity.

\textbf{Critical Review}

Many studies have reported on the anti-obesity effects of LG2055. A study by Kadooka \textit{et al.} (2013) suggests that LG2055 reduces abdominal adiposity and that constant intake of this probiotic may be needed to maintain its anti-obesity effects\textsuperscript{27}. The study found LG2055 intake to be associated with lowered abdominal adiposity in individuals with obese-like tendencies. They conducted a randomised, controlled trial involving 210 Japanese adults who had large visceral fat stores but were otherwise considered healthy. The individuals in the study were randomly divided into three groups: those who received fermented milk (FM) containing 10\textsuperscript{7} CFU of LG2055/g; those who received FM containing 10\textsuperscript{6} CFU of LG2055/g; or a control group...
Table 1. Percentage changes and corresponding measures from baseline in abdominal fat areas. Table modified from Kadooka et al. (2013)\textsuperscript{27}. (Mean values and 95% confidence intervals)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Week 8 Mean (95% CI)</th>
<th>Week 12 Mean (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral‡</td>
<td>10⁷ dose</td>
<td>-4.8** - 7.6, - 1.9</td>
<td>-8.5***† - 11.9, - 5.1</td>
</tr>
<tr>
<td>%</td>
<td>-5.3</td>
<td>-8.4, - 2.2</td>
<td>-9.6</td>
</tr>
<tr>
<td>cm²</td>
<td>-5.6**</td>
<td>-8.1, - 3.1</td>
<td>-8.2**†</td>
</tr>
<tr>
<td>10⁶ dose</td>
<td>%</td>
<td>-5.8</td>
<td>-8.3, - 3.2</td>
</tr>
<tr>
<td>cm²</td>
<td>-1.2</td>
<td>-4.1, 1.7</td>
<td>-0.7</td>
</tr>
<tr>
<td>Control</td>
<td>%</td>
<td>-1.3</td>
<td>-4.5, 2.0</td>
</tr>
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</table>

Mean values were significantly different for within-group comparisons from baseline: * P<0.05, ** P<0.01.
†Mean values were significantly different for between-group comparisons from control (P<0.05).
‡ There was a significant group x time interaction effect (P<0.05).

who received FM containing 0 CFU of LG2055/g. The participants consumed 200 g of FM per day for 12 weeks. By the end of the 12 weeks, the researchers observed a decrease in visceral abdominal fat area in both the 10⁷ and 10⁶ groups (Table 1). Additionally, individuals in the 10⁷ and 10⁶ groups had reductions in BMI, waist and hip circumference, and body fat mass (Table 2). The researchers also found that four weeks after ending the treatment, these effects were reduced (Table 2).

This study bears a near-perfect resemblance to a previous report on the effect of FM containing 10⁶ CFU of LG2055/g on individuals with obese-like tendencies; the fundamental difference is in the administered dose of LG2055. All other variables remained nearly constant, including the formation of the FM, the study schedule and protocol\textsuperscript{27,28}. The calibre of participants was also similar as both trials recruited healthy Japanese males with large areas of visceral fat\textsuperscript{27,28}. This approach of changing one variable from a previous experiment and keeping all else constant is an effective way to extrapolate accurate information. As a result of employing this approach, the researchers were able to indicate that even at lower doses of LG2055 (at the recommended minimum and intermediate levels), the probiotic was equally potent in its ability to reduce abdominal visceral fat area\textsuperscript{27}. These results have an added layer of credibility due to the authors’ strict adherence to the methods used by the previous study, however this characteristic may also serve to undermine the generalisability of this research.

The participants within this study were of a specific cohort, and so the results may not be extrapolative of the wider population. Specifically, all participants were of Japanese descent and their average age was around 47 years\textsuperscript{27}. This is troublesome because the population demographics of those who could benefit from this probiotic are variable in terms of age and race. This study aims to describe LG2055 as an effective way to lower abdominal adiposity in adults, however this conclusion is based on results from a specific subsection of the population. Future studies should include individuals from a wide range of backgrounds and ages, as this would better support the view of LG2055 as a probiotic capable of lowering abdominal adiposity in humans.

Additionally, the authors made an apparent generalisation in the discussion when mentioning the trial’s ability to demonstrate LG2055’s anti-obesity effects. The central issue traces back to the fact that the subjects within the study were not obese\textsuperscript{27}. The subjects were healthy despite having large visceral fat stores. Therefore, although the participants had obese-like tendencies, they did not serve as a true model for human obesity. A more accurate statement would be to say that LG2055 helped reverse abdominal adiposity in healthy individuals, however more research is needed to confirm the same effect in obese patients.

Furthermore, the study did not control for diet, as participants were allowed to continue with their normal eating habits\textsuperscript{27}. The over-arching influence of diet on the composition of the gut microbiota has been widely reported\textsuperscript{29,30}. Therefore, it is difficult to accept the results from this study as they suggest a direct link between LG2055 treatment and reduced abdominal adiposity, without accounting for variability in participants’ diets. Future studies should include more strict diet restrictions or record daily consumption habits as this would more accurately elucidate LG2055’s effect on obesity symptoms.
### Table 2. Percentage changes and corresponding measures from baseline in BMI, waist, hip and fat mass in 10⁷, 10⁸ and control mice. Table modified from Kadooka et al. (2013)²⁷.
(Mean values and 95% confidence intervals)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Week 8</th>
<th></th>
<th>Week 12</th>
<th></th>
<th>4 weeks after finishing consumption</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>95% CI</td>
<td>Mean</td>
<td>95% CI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI ‡‡</td>
<td>10⁷ dose</td>
<td>%</td>
<td>−0.6**†</td>
<td>−1.0, −0.2</td>
<td>−1.1***‡‡</td>
<td>−1.6, −0.6</td>
<td>−0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kg/m²</td>
<td>−0.2</td>
<td>−0.3, −0.1</td>
<td>−0.3</td>
<td>−0.4, −0.2</td>
<td>−0.1</td>
</tr>
<tr>
<td></td>
<td>10⁸ dose</td>
<td>%</td>
<td>−1.0***‡‡</td>
<td>−1.4, −0.6</td>
<td>−1.6***‡‡</td>
<td>−2.1, −1.1</td>
<td>−0.6†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kg/m²</td>
<td>−0.3</td>
<td>−0.4, −0.2</td>
<td>−0.4</td>
<td>−0.5, −0.3</td>
<td>−0.2</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>%</td>
<td>0.3</td>
<td>0.0, 0.6</td>
<td>0.3</td>
<td>−0.1, 0.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kg/m²</td>
<td>0.1</td>
<td>0.0, 0.2</td>
<td>0.1</td>
<td>−0.1, 0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Waist ‡‡</td>
<td>10⁷ dose</td>
<td>%</td>
<td>−0.9***†</td>
<td>−1.3, −0.6</td>
<td>−1.4***‡‡</td>
<td>−1.8, −1.0</td>
<td>−0.8**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>−0.9</td>
<td>−1.2, −0.5</td>
<td>−1.3</td>
<td>−1.7, −0.9</td>
<td>−0.8</td>
</tr>
<tr>
<td></td>
<td>10⁸ dose</td>
<td>%</td>
<td>−0.8**</td>
<td>−1.2, −0.4</td>
<td>−1.2***‡‡</td>
<td>−1.7, −0.7</td>
<td>−0.7**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>−0.7</td>
<td>−1.1, −0.4</td>
<td>−1.1</td>
<td>−1.5, −0.7</td>
<td>−0.7</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>%</td>
<td>0.0</td>
<td>−0.4, 0.3</td>
<td>0.1</td>
<td>−0.4, 0.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>−0.1</td>
<td>−0.4, 0.3</td>
<td>0.1</td>
<td>−0.5, 0.3</td>
<td>0.2</td>
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<tr>
<td>Hip ‡‡</td>
<td>10⁷ dose</td>
<td>%</td>
<td>−0.8**†</td>
<td>−1.0, −0.5</td>
<td>−1.2***‡‡</td>
<td>−1.5, −0.9</td>
<td>−0.5**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>−0.8</td>
<td>−1.0, −0.5</td>
<td>−1.2</td>
<td>−1.5, −0.9</td>
<td>−0.5</td>
</tr>
<tr>
<td></td>
<td>10⁸ dose</td>
<td>%</td>
<td>−0.5**</td>
<td>−0.8, −0.3</td>
<td>−0.9***‡‡</td>
<td>−1.1, −0.6</td>
<td>−0.3*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>−0.6</td>
<td>−0.8, −0.3</td>
<td>−0.9</td>
<td>−1.1, −0.6</td>
<td>−0.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>%</td>
<td>−0.2</td>
<td>−0.4, 0.0</td>
<td>0.2</td>
<td>−0.4, 0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cm</td>
<td>−0.2</td>
<td>−0.5, 0.1</td>
<td>−0.2</td>
<td>−0.5, 0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Fat mass ‡‡</td>
<td>Value (%)</td>
<td>0.4</td>
<td>0.1, 0.6</td>
<td>0.6</td>
<td>0.3, 0.9</td>
<td>0.9</td>
<td>0.5, 1.2</td>
</tr>
<tr>
<td></td>
<td>10⁷ dose</td>
<td>%</td>
<td>−0.9†</td>
<td>−2.1, 0.4</td>
<td>−2.4***‡‡</td>
<td>−3.8, −0.9</td>
<td>−0.2††</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kg</td>
<td>−0.3</td>
<td>−0.6, 0.0</td>
<td>−0.6</td>
<td>−1.0, −0.3</td>
<td>−0.1</td>
</tr>
<tr>
<td></td>
<td>10⁸ dose</td>
<td>%</td>
<td>−0.8†</td>
<td>−1.9, 0.2</td>
<td>−2.2***‡‡</td>
<td>−3.4, −1.0</td>
<td>−0.3††</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kg</td>
<td>−0.2</td>
<td>−0.4, 0.0</td>
<td>−0.5</td>
<td>−0.7, −0.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>%</td>
<td>1.5**</td>
<td>0.7, 2.3</td>
<td>2.2**</td>
<td>1.2, 3.3</td>
<td>3.3**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kg</td>
<td>0.4</td>
<td>0.1, 0.6</td>
<td>0.5</td>
<td>0.2, 0.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Mean values were significantly different for within-group comparisons from baseline: * P<0.05, ** P<0.01.
Mean values were significantly different for between-group comparisons from control: † P<0.05, †† P<0.01.
‡‡ There was a significant group × time interaction effect (P<0.01)
A study by Miyoshi et al. (2014) also highlights LG2055’s ability to attenuate obesity-related symptoms. Researchers observed a relationship between LG2055 treatment, and decreased weight and adipose tissue mass (retroperitoneal/perineal and epididymal) in diet-induced obese mice. The mice were divided into three groups: 5% fat group (5% fat); 10% fat group (10% fat); and 10% fat group with LG2055 (10% fat-LG). The group names indicate the proportion of fat the mice received from their diet. The mice were fed these diets for a period of 24 weeks in order to induce obesity. At the end of the 24 weeks, the mice were anesthetized, and body weight, adipose tissue mass, liver TG levels, expression of inflammatory genes in fat tissue and the expression of lipogenic and lipolytic genes in the liver were quantified. The researchers found LG2055 intake to be associated with decreased liver TG levels (Table 3), decreased expression of pro-inflammatory genes (e.g. CCL2) in epididymal adipose tissue (Table 4), and decreased expression of lipogenic genes (e.g. ACC1) in the liver (Table 5).

A distinct feature of this study centres around the route of administration for LG2055. Miyoshi et al. (2014) did not use FM containing LG2055 as the delivery method for this probiotic. Previously, many studies have reported anti-obesity effects in animal models upon administration of FM containing LG2055. This study suggests that LG2055 alone is the source of these effects – not the metabolites present in FM. This has implications for determining the most effective delivery route for this probiotic.

Although the route of administration provides more validity to the results, the method used to create obese mice does not resemble the development of obesity in humans. The researchers fed the mice a 5% or 10% fat diet for 24 weeks in order to induce obesity. However, it could be more valuable to induce obesity through administration of an HFD (i.e. 60% fat, 20% protein and 20% carbohydrates) as this would allow for a model of obesity that better resembles the human condition. The results from such an experiment would provide more insight on the potential therapeutic role of LG2055 in human obesity.

Moreover, having a group of mice on a normal chow diet (NCD) could serve as a useful addition to this study. This trial included mice on a 5% or 10% fat diet. An NCD and a low-fat diet (e.g. 5% fat) are not equivalent as they elicit different phenotypes in mice. For example, mice on a low-fat diet have significantly higher total serum cholesterol levels than NCD mice. Obesity is often characterised by high LDL cholesterol levels in the blood, and so a low-fat diet would not serve as an accurate depiction of healthy mouse physiology. Therefore, the researchers failed to demonstrate the effects of LG2055 on healthy mice when doing so could allow for post-treatment comparisons between healthy and obese mice. This in turn, could allow us to make inferences on whether LG2055 serves a preventative role against obesity in healthy individuals.

### Table 3. Morphometric and biochemical parameters between 5% fat, 10% fat and 10% fat-LG mice. Table modified from Miyoshi et al. (2014).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% fat (n = 10)</td>
</tr>
<tr>
<td>Average diet intake (g/day)</td>
<td>3.62 ± 0.17</td>
</tr>
<tr>
<td>Average energy intake (kJ/day)</td>
<td>58.28 ± 2.73</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>21.78 ± 1.24</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>31.41 ± 2.25a</td>
</tr>
<tr>
<td>Adipose tissue weight (% of final body weight)</td>
<td></td>
</tr>
<tr>
<td>Mesenteric</td>
<td>2.24 ± 0.27a</td>
</tr>
<tr>
<td>Epididymal</td>
<td>3.14 ± 1.14a</td>
</tr>
<tr>
<td>Perirenal/Retroperitoneal</td>
<td>1.22 ± 0.46a</td>
</tr>
<tr>
<td>Total</td>
<td>6.60 ± 1.75a</td>
</tr>
<tr>
<td>Liver TG (mg/g tissue weight)</td>
<td>14.92 ± 8.52a</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD. Values with different superscript letters (a–c) within the same row differ significantly from each other (P<0.05, Games–Howell test).
Table 4. Expression of pro-inflammatory genes in epididymal adipose tissue. Table modified from Miyoshi et al. (2014)\textsuperscript{20}.

<table>
<thead>
<tr>
<th>Pro-inflammatory genes</th>
<th>5% fat</th>
<th>10% fat</th>
<th>10% fat-LG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>1.00</td>
<td>1.87</td>
<td>1.20</td>
</tr>
<tr>
<td>CCR2</td>
<td>1.00</td>
<td>1.40</td>
<td>0.65</td>
</tr>
<tr>
<td>ICAM1</td>
<td>1.00</td>
<td>1.59</td>
<td>1.55</td>
</tr>
<tr>
<td>IL6</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBP4</td>
<td>1.00</td>
<td>0.69</td>
<td>0.88</td>
</tr>
<tr>
<td>RETN</td>
<td>1.00</td>
<td>0.59</td>
<td>0.84</td>
</tr>
<tr>
<td>PAI-1</td>
<td>1.00</td>
<td>1.41</td>
<td>1.06</td>
</tr>
<tr>
<td>TNFα</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are represented as a fold change relative to levels in the 5 % fat group. ND not determined.

Table 5. Expression of lipogenic genes in liver. Table modified from Miyoshi et al. (2014)\textsuperscript{20}.

<table>
<thead>
<tr>
<th>Lipogenic genes</th>
<th>5% fat'</th>
<th>10% fat</th>
<th>10% fat-LG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC1</td>
<td>1.00</td>
<td>1.24</td>
<td>1.03</td>
</tr>
<tr>
<td>FAS</td>
<td>1.00</td>
<td>1.36</td>
<td>0.90</td>
</tr>
<tr>
<td>SREBP1</td>
<td>1.00</td>
<td>1.24</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Data are represented as a fold change relative to levels in the 5 % fat group.

Conclusion

In summary, LG2055 shows promise as a treatment for obesity. The anti-obesity effects of LG2055 have been widely reported. Kadooka et al. (2013) showed that LG2055 was effective in attenuating several obesity-related symptoms in humans\textsuperscript{27}. The researchers associated LG2055 intake (at the recommended minimum or intermediate level) to decreased visceral abdominal fat area (Table 1), reductions in BMI, waist and hip circumference, and body fat mass (Table 2)\textsuperscript{27}. However, it is difficult to make generalisations from these data as they focused on a specific subsection of the population (Japanese adults around 47 years of age) who were not obese. Nonetheless, the results from this study are strengthened through the employed methodology. The methods used in this study were nearly identical to those from a previous study\textsuperscript{27,28}, the only difference being in the administered dose of LG2055. This allows for less variability in the results, while also validating the anti-obesity effects of LG2055 observed in the previous study\textsuperscript{27,28}.

In addition, a study by Miyoshi et al. (2014) advanced a similar hypothesis as they linked LG2055 treatment with decreased weight and adipose tissue mass in diet-induced obese mice\textsuperscript{20}. The researchers found LG2055 intake to be associated with decreased liver TG levels (Table 3), decreased expression of pro-inflammatory genes in epididymal adipose tissue (Table 4) and decreased expression of lipogenic genes in the liver (Table 5). The diet-induced obese mice used within this study were not ideal for modelling obesity in humans. However, the results from this study provide essential information in relation to LG2055’s ability to attenuate obesity-related symptoms\textsuperscript{20}. The data also indicates that the consumption of LG2055 alone is sufficient to elicit its anti-obesity effects.

These data highlight LG2055’s ability to improve obesity symptoms, further promoting it as a potential therapeutic for obesity. LG2055 reduced visceral abdominal fat area and fat mass in humans\textsuperscript{27}, and it caused a decrease in adipose tissue mass in mice\textsuperscript{20}. These events indicate a shift away from the obese phenotype and they are likely caused by LG2055’s ability to decrease fat absorption at the level of the small intestine through suppressing pancreatic-lipase mediated fat hydrolysis\textsuperscript{18}. This leads to an increase in energy output and a decrease in energy intake, thereby working to help rescue the sustained positive energy imbalance in obesity\textsuperscript{6}. In light of this knowledge, LG2055 presents itself as a prime
candidate for obesity treatment, however more research should be done to determine its effectiveness, particularly in conjunction with behaviorally induced weight loss strategies.

Acknowledgements
I would like to thank Patrycja Jazwiec, a PhD candidate in the Slobo Lab, for her support and guidance through this project. This work was completed as part of the requirements for BIOCHEM 4M03, Winter 2019, at McMaster University.

References


The role of activins in TGFβ profibrotic signaling in chronic kidney disease
Snow Wangding
1Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Canada

Abstract
Chronic kidney disease (CKD) is a global health condition affecting upwards of 14% of individuals worldwide. Characterized by stages of decreased kidney function and increased albuminuria, dialysis and kidney transplantation are the only existing therapies. Current research has established the role of TGFβ profibrotic signaling in a dysregulated wound healing response, resulting in the excessive accumulation of extracellular matrix components in kidney glomeruli and interstitium. This accumulation detrimentally and often irreversibly affects kidney function. While efforts have been made to investigate the efficacy and feasibility of blocking TGFβ as a therapeutic target, the consequences of inhibition result in many adverse side effects. Recent studies have identified a potential role for activins in profibrotic signaling. As cytokines in the TGFβ superfamily, activins play an important role in cell growth and differentiation and are shown to fine-tune fibrotic pathways. Here, we review the mechanisms of action involving activins and renal disease.

Introduction
Chronic kidney disease (CKD) is a rising health problem characterized by kidney damage and reduced glomerular filtration rate persisting for over 3 months. The global increase in the incidence and prevalence of CKD is driven by corresponding surges in diabetes mellitus, hypertension, obesity, and aging. CKD is often asymptomatic in its early stages but can be clinically identified by abnormalities in kidney structure and increasing albuminuria as the disease progresses. Pathogenically, the excessive accumulation of extracellular matrix (ECM) components in the glomeruli and tubular interstitium results in renal fibrosis and interstitial scarring which alter and affect renal structures. This accumulation is primarily accomplished by the conversion of quiescent fibroblasts to active myofibroblasts which deposit ECM. The resultant nephron loss and scarring ultimately lead to compromised function and may eventually lead to end-stage renal failure, for which there is no current cure. Dialysis and kidney transplantation are the only treatments for end-stage kidney disease, yet these involve complicated measures with varying levels of success. The increasing disparities between organ supply and demand further highlight the need for a method of reversing renal fibrosis.

Transforming growth factor beta (TGFβ) has been identified as an important mediator of the renal fibrotic process, particularly in the conversion of quiescent to active fibroblasts. Despite its role as a major profibrotic cytokine, TGFβ cannot be targeted clinically in light of its well-documented pleiotropic nature, specifically the potential abrogation of its anti-inflammatory and anti-tumorigenesis properties which would result in adverse effects. In fact, in studies using TGFβ1-deficient mice, massive inflammation resulted in mortality. Therefore, investigation into alternative TGFβ superfamily ligands is crucial in determining potential mediators of renal fibrosis and more feasible therapeutics. More recently, activins have been found to contribute to TGFβ profibrotic effects; however, the mechanism and magnitude by which they do so remains unclear. Thus, this review aims to better understand how activins contribute to renal fibrosis and elucidate their potential as a therapeutic target for CKD.

Activins as Profibrotic Cytokines
Activins belong to the TGFβ superfamily of cytokines and are important regulators of cell growth and differentiation. Their structure consists of homo- or heterodimerized inhibin β subunits linked by disulphide bonds. Four β subunits have been identified in mammals: βA, βB, βC, and βE. Transcripts for βA and βB are expressed in nearly all tissue, whereas the latter two are predominantly found in the liver. Activin A and Activin B, homodimers composed of inhibin βA and βB subunits respectively, have been studied more widely. Activin A associates with activated type I receptor ALK4 while Activin B binds to ALK3. Interestingly, Activin A is not detected in normal adult kidneys, but is upregulated in renal tubular cells following ischemia and reperfusion injury. Studies have shown that Activin A promotes cell proliferation and enhances type I collagen mRNA expression in primary cultured renal interstitial fibroblasts, essentially identifying Activin A as a profibrotic factor. Together, these findings provide insight into the role of Activin A in renal fibrosis and suggest therapeutic potential for CKD treatment.
Activins transduce their signals through complexes consisting of heteromeric type I and II receptors and downstream effectors. The N-terminal domains of type I and II receptors engage in ligand binding while their C-terminal domains exhibit serine/threonine kinase activity. Activins initially bind to type II receptors (ACVR2A/B or ActRIIA/B) which phosphorylate the GS domains of recruited type I receptors, thereby activating them. Examining the interactions of activin receptors has introduced a novel approach to understanding the mechanism behind profibrotic activin action.

**Mechanism of Activins in TGFβ Signaling**

The contribution of activins to TGFβ fibrotic signaling has yet to be clearly elucidated; however, some studies have shown a link between the regulation of activins and TGFβ pathway components. An early study used an activin receptor-inducible cell line, KAR6, and induced receptor expression to determine how activin receptors were interacting with TGFβ signal transduction. Through transfections of TGFβ pathway specific signal transducers Smad2/3, Smad4, and Smad7, it was found that ALK4 associated with Smad2/3, but not Smad4\(^\text{11}\). These results established that Activin A receptors mirror the role of TGFβ receptors in the canonical fibrotic response by activating the Smad signal transduction pathway (Figure 1). Upon phosphorylation, Smad2/3 multimerizes with Smad4 and the complex translocates to the nucleus to affect transcription of target genes\(^\text{11}\).

Few studies have focused on the role of Activin B in TGFβ profibrotic signaling. In polycystic kidney disease (PKD), the inhibition of Activin receptor type-2B (ActRIIB) shows similar effects in inhibiting cyst progression. PKD is a type of CKD in which cyst growth prevents kidney function and can lead to
renal failure. PKD phenotype is associated with increased proliferation and expression of Smad2/3 target genes, relating its development to the activation of TGFβ fibrosis. In a study examining ActRIIB-inhibited mice, the inhibited groups showed reduced expression of type I and α1 collagen, as well as decreased total Smad2 levels. In another study examining the inhibition of ActRIIB in acute kidney injury, it was found that Activin A receptor inhibition resulted in decreased accumulation of fibroblasts, which are an indicator of developing fibrosis. These findings suggest that Activin A may play a role in contributing to Smad2/3-dependent signaling and fibrosis.

Between Activin A and Activin B, Activin A is the more elucidated cytokine in reference to the contribution of activins to TGFβ-induced renal fibrosis. Our group found that there was a notable 18-fold increase in Activin A secretion in cells treated with TGFβ1. Comparatively, there was only a 0.5-fold increase in Activin B secretion. Additionally, we found that inhibition of Activin B using an anti-Activin B antibody had no effect on the transcriptional activity of an established marker of myofibroblast differentiation, αSMA. In current literature, these findings suggest that while Activin B may play a role in the TGFβ profibrotic response, Activin A is much more relevant in investigating the potential downstream effects of activins in fibrotic signaling. Interestingly, our group found that when Activin A was added to TGFβ1-treated cells, there was no significant increase in the transcriptional activity of Smad3. This suggests that activins may also be contributing to renal fibrosis in a non-canonical (non-Smad3 dependent) manner. In the complexity and layered mechanisms of TGFβ profibrotic signaling, developing a more comprehensive understanding of activin-induced downstream effects and transcriptional interactions is crucial. This will be further explored below.

Transcription Factors and αSMA

Mentioned previously, TGFβ is critical in the activation of myofibroblasts, cells which deposit ECM components in pathogenic renal fibrosis. In kidney glomeruli, activated mesangial cells exhibit myofibroblast cell characteristics pertaining to their ECM production and contractility. Thus, activated mesangial cells are important models for understanding the conversion of inactive fibroblasts to active myofibroblasts. A distinct and well-documented marker of glomerular disease in activated mesangial cells is alpha smooth muscle actin (αSMA). While normally expressed in adult smooth muscle and transiently in cardiac and skeletal muscle, expression of αSMA is also a prominent feature of differentiated myofibroblasts. In mice, pulmonary and renal fibrosis were significantly associated with the accumulation of αSMA-producing myofibroblasts. Together, this makes αSMA an appropriate marker for renal fibrosis.

In the canonical profibrotic response, stimulated TGFβ receptors phosphorylate Smad2/3 and enable the multimerization with Smad4. Smad4 is crucial for signal transduction, as loss of Smad4 in mesangial cells inhibits TGFβ-induced ECM component accumulation and deposition. The activated complex consisting of Smad2/3 and cofactor Smad4 then translocates to the nucleus and acts as a transcriptional factor in binding to Smad-specific DNA binding motifs. Notably, Smad3 directly binds to regions within the αSMA promoter, called Smad-binding elements (SBE). In addition to Smad3, other binding factors impact the transcriptional activity of αSMA. In fact, the yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are transcriptional cofactors that have been shown to contribute to fibrosis. YAP has been shown to associate with Smad7, an inhibitory Smad in the TGFβ signaling pathway, and also Smad3 in mesothelioma cells. In addition, YAP has been shown to be induced by TGFβ treatment, implying a role in fibrosis.

YAP/TAZ in Fibrosis

YAP and TAZ are important regulators of organ size, stem cell fate, and solid tumour growth, yet little is known regarding their mechanism of action. Studied primarily in the context of the Hippo signaling cascade, YAP/TAZ have been shown to localize to the nucleus when dephosphorylated. YAP and TAZ transcription factors may bind to different DNA-binding proteins, but both associate with TEA DNA-binding domain (TEAD) elements in the αSMA promoter region. This implicates their role in renal fibrosis. In addition to phosphorylation, the regulation of YAP/TAZ activity is mechanosensitive. Recently studied, ECM stiffness has been found to modulate the localization of YAP/TAZ based on cytoskeletal tension. In healthy tissue, cytoskeletal tension is reduced and YAP/TAZ localize to the cytoplasm. However, YAP/TAZ have been found to localize to the nucleus in cells grown on stiff and injured surfaces. This is seen in studies examining idiopathic pulmonary fibrosis, mammary epithelial cells, HeLa cells, and kidney fibroblasts. Stiff tissue is characteristic of fibrosis, as the accumulation of ECM components causes the ECM to stiffen. In a particular study using stiff substrates...
to model injured fibrotic kidneys, immunostaining revealed the localization of YAP/TAZ to the nucleus. This suggests that the presentation of fibrosis involves the activation and thus nuclear localization of YAP and TAZ. Other studies have clearly demonstrated this effect. In pulmonary fibrosis, YAP and TAZ levels have been shown to be elevated. Knockdown studies have also found reduced pro-collagen, αSMA, and lowered levels of proteins responsible for myofibroblast differentiation. Together, these findings strongly imply that YAP and TAZ induce renal fibrosis through activation of myofibroblasts in a mechano-sensitive manner.

**YAP/TAZ Interaction with Smads**

Recent studies with YAP/TAZ inhibitors have demonstrated a relationship between ECM stiffness and TGFβ-induced SMAD localization mediated by YAP/TAZ. TGFβ treatment induced equivalent Smad2/3 phosphorylation in fibronectin substrates modelling healthy and damaged tissue. Soft fibronectin substrates were used to model healthy tissue, and stiff substrates for damaged tissue. However, localization of Smad2/3 to the nucleus was diminished in cells grown on soft substrates, where Smad-induced transcriptional activity was also reduced. These findings indicate that ECM stiffness mediates Smad2/3 localization without affecting TGFβ-induced phosphorylation. This effectively implies that a positive feedback loop drives pathological fibrosis. YAP/TAZ are implicated in driving this mechanism. Smad2/3 TGFβ-stimulated accumulation in the nucleus was shown to be decreased upon inhibition of YAP/TAZ using verteporfin treatment. Verteporfin had no effect on TGFβ-induced phosphorylation of Smads. In addition, the inhibition of YAP/TAZ resulted in attenuation of Smad3 transcriptional activity and endogenous expression of Smad-inducible collagen genes. These findings suggest possible crosstalk between YAP/TAZ and Smad-dependent pathways modulating TGFβ fibrosis.

Our group found that activin inhibition with follistatin prevented TGFβ1-induced YAP activation (Figure 2). As follistatin does not inhibit TGFβ, this response suggests that activins indeed play a substantial role in TGFβ profibrotic signalling by perhaps regulating YAP/TAZ activation. A study examining TAZ expression in human kidney cells showed that TAZ silencing neutralized TGFβ1-induced expression of target genes without affecting Smad3 phosphorylation. This suggests that TAZ is a non-Smad downstream effector of renal fibrosis, implying that non-canonical pathways may be significantly involved in the regulation of TGFβ profibrotic events. Another study also supports the Smad3-independent transcriptional activity of TAZ.

In this study, fibroblast cells were treated with a Smad3 inhibitor, SIS3, before being treated with TGFβ. In addition to the Smad3 inhibitor, a separate experiment was conducted using Smad3 siRNA prior to TGFβ treatment. Both groups failed to reduce TAZ expression, suggesting that TAZ is mediated by non-canonical pathways. In contrast, a study using pig kidney cells demonstrated that TAZ confers Smad3 sensitivity to the αSMA promoter by associating with SBE. Where Smad3 alone was not sufficient to induce activation of the αSMA promoter, the presence of TAZ allowed for a synergistic effect. Taken together, these findings further support the implication that activins could be mediating YAP/TAZ activation in both canonical and non-canonical mechanisms of action.

**Conclusion**

CKD is a health problem characterized by renal fibrosis and prolonged kidney dysfunction affecting filtration and waste clearance. CKD progresses rapidly and irreversibly, eventually causing death if left untreated. The predominant cytokine upregulating fibrosis, TGFβ, transduces its signal through a Smad-dependent signalling cascade; however, TGFβ cannot be targeted clinically due to adverse side effects. Major advancements have been made in understanding the role of activins in TGFβ-induced renal fibrosis. With recent studies suggesting that activins may contribute significantly to the canonical and non-canonical processes of fibrosis, more investigation is required into the complexities of how activins interact with YAP/TAZ transcription factors to develop feasible targets to reverse CKD renal fibrosis.

![Figure 2: Activin inhibition with follistatin (Fst) prevents TGFβ-induced nuclear YAP activation.](image)
Examined from western blot data; mouse mesangial cells were treated with TGFβ-1 and TGFβ-1 with Fst over 18h. Significantly reduced nuclear YAP expression with Fst observed, n=9, t-test *p < 0.05. Adapted from Soomro et al. (2019).

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Exploring Misconceptions Surrounding Vaccinations: A Health Advocacy Project
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Abstract
Despite a consistent effort by the scientific community to inform individuals about the efficacy and safety of vaccinations, harmful misconceptions still persist which have prevented their widespread acceptance within the general public. According to the most recent survey from the Public Health Agency of Canada (2017), childhood vaccination rates (children aged two years or less) were approximately 70-90% depending on the type of vaccine, and in each province, were below the national goal1. Additionally, the Canadian childhood vaccination rates reported for the common vaccines diphtheria, tetanus, pertussis, and measles were reported to be some of the lowest in the developed world2. To better understand these disparities, this video explores common misconceptions individuals may have toward vaccines. Specifically, this project takes the form of a skit which explores the perspectives of a female high school student, a mother of young children, and a male university student to understand their skepticism towards the HPV, MMR, and flu vaccines respectively. Overall, the aim of this project is to better inform the public about the benefits of vaccines in order to increase vaccination rates in Canada and improve health care outcomes for infectious diseases that are preventable by vaccines.

For more information regarding the safety, efficiency, and accessibility of vaccines, please contact your local health-care provider, as well as refer to these online resources: Immunize Canada (https://immunize.ca/resources); Infection Prevention and Control Canada (https://ipac-canada.org/immunization-resources-2.php); and The Canadian Vaccine Catalogue (https://cvc.canimunize.ca/en/home).

An Interview with Vaccine Skeptics Video: https://youtu.be/CtWcj_13dZ8

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Abstract References

Video References
Malaria: An Overview of Past and Current Strategies to Attain Attenuation and Eradication

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Abstract
As antimicrobial resistance continues to rise, infectious diseases that were once thought to be easily treated are now re-emerging with greater strength. Malaria, a deadly vector-borne disease, continues to be prevalent in predominantly developing countries, despite the numerous attempts that have been undertaken to develop new strategies for elimination and eradication. This literature review will highlight the past and current approaches that have been developed for malaria eradication purposes, and future strategies that emphasize the importance of the mosquito gut microbiota in discovering novel small molecules to attenuate transmission.

Introduction
The staggering proliferation of antibiotic resistance over the past few decades has led to the re-emergence of devastating infectious diseases, such as malaria. It is estimated that approximately half of the world’s population is at risk of contracting malaria, with 219 million cases and 435,000 deaths reported in 2017. While cases have been documented from 87 countries, 92% of these instances originate from the WHO African region. In Africa, 99.7% of cases are caused by the deadliest and most common form of the six plasmodial species that infect humans, known as Plasmodium falciparum1-3. Although this parasite begins as a mild febrile disease, it often becomes severe in individuals with an underdeveloped or compromised immune system, as in young children and pregnant women4,5. These individuals may develop cerebral malaria and severe anemia, which can potentially cause death2,3,6. Therefore, there is an urgent need for new therapeutic strategies to combat resistance towards current drugs to reduce the transmission of malaria in highly affected areas. This review will introduce the pathway of malaria transmission and pathogenesis by outlining the life cycle of P. falciparum and illustrate the potential intervention sites. Once the molecular mechanisms of the disease have been discussed, past and current approaches for attenuating the transmission of malaria will be explored, along with future directions. This literature review will additionally highlight the potential of manipulating the mosquito gut microbiota as a means to reduce vector competence, and therefore attenuate malarial transmission in this manner7,8.

Malaria Transmission and Pathogenesis
Malaria is a vector-borne disease with three main stages: the pre-erythrocytic liver stage and asexual blood stage, which occur in the vertebral host. The third stage, known as the sexual cycle, occurs in Anopheles mosquitoes. During a blood meal, female Anopheles release sporozoites from their salivary glands into the dermis of the victim3,10,11. The motile sporozoites will penetrate nearby blood vessels and enter the bloodstream, quickly accessing the liver via the process of traversal. This involves sporozoites crossing the sinusoidal barrier, which is composed of fenestrated endothelial cells and Kupfer cells. Transient vacuoles are then formed in association with pore-forming proteins: SPECT (sporozoites microneme protein essential for traversal), PLP1 (perforin-like protein 1) and CelTOs (cell-traversal protein for okinetes and sporozoites). This leads to the penetration of hepatocytes and the initiation of the liver stage2,3. During this stage, sporozoites proliferate into merozoites, replicating asexually for seven days. Shortly thereafter, 40,000 merozoites per hepatocyte are released into the bloodstream, resulting in the invasion of erythrocytes and establishment of the asexual blood stage (Figure 1)2,12.

The intracellular proliferation of the parasite during the asexual blood stage confers parasite protection against host immune responses. It is also responsible for causing indicative malarial symptoms, such as fever and tremors2,11. During this stage, the parasite creates significant changes to erythrocytes. Infected erythrocytes develop digestive vacuoles that house vital amino acids and assist in the digestion of hemoglobin to support parasite growth and development9. Subsequently, infected erythrocytes are remodeled through the surface presentation of the P. falciparum erythrocyte membrane protein 1 (PfEMP1). The presentation of this surface protein allows infected erythrocytes to bind to endothelial cells in order to avoid spleen clearance2,6. During the blood stage, merozoites develop through three stages and asexually replicate within the infected erythrocytes. Schizonts are ultimately formed, rupturing erythrocytes and...
resulting in anemia and the spread of the parasitic infection\textsuperscript{3}. Throughout this proliferative process, a small proportion of parasites differentiate into male and female gametocytes. These gametocytes undergo five stages of development in the extravascular space of the bone marrow\textsuperscript{12}. The victim becomes infectious once male and female gametocytes (Stage V) re-enter the peripheral circulation\textsuperscript{12}.

The propagation of \textit{P. falciparum} relies on the transmission of gametocytes from an infected vertebral host to non-infected mosquitoes through blood-feeding\textsuperscript{12}. As gametocytes from the blood meal enter the mosquito gut lumen, environmental factors, such as the presence of xanthuric acid, trigger gametogenesis. The result is the formation of male microgametes and female macrogametes, which undergo fertilization to form zygotes\textsuperscript{10,12}. Within 24 hours, the zygotes differentiate into motile ookinete that invade the mosquito midgut epithelium. After crossing the epithelium, ookinete’s develop into oocysts which reside below the basal lamina of the midgut wall\textsuperscript{10,13,14}. At this stage, the parasite experiences a severe bottleneck, where only five ookinete’s are able to traverse the gut epithelium and develop into oocysts, making the midgut an important site for intervention\textsuperscript{15,16}. It is important to note that the epithelial gut lining (peritrophic matrix), which is composed of chitin fibrils and glycoproteins, has been implicated in the bottleneck experienced in the midgut\textsuperscript{17,18}. Current research suggests that the peritrophic matrix could be targeted to attenuate ookinete traversal fully\textsuperscript{16}. Alteration of the mosquito’s midgut microbiota may assist in this initiative, as mosquitoes can be fed factors that induce biofilm formation. The creation of a biofilm would make it more difficult for the ookinete to traverse the midgut epithelium.
Nevertheless, successful oocysts undergo a maturation process to produce sporozoites, which are subsequently released into the mosquito hemocoel (body cavity). Ten to fourteen days after the initial blood meal, mosquitoes become infectious due to sporozoite invasion of the salivary glands, allowing for the efficient transfer of sporozoites into vertebrate hosts in subsequent blood meals.\textsuperscript{12,14} Due to the complex life cycle of \textit{P. falciparum}, many intervention sites exist and can be coopted to attenuate malaria transmission.

**Past and Current Approaches in Treating and Preventing Malaria**

Since the twentieth century, many approaches have been developed to attenuate malaria, either targeting the parasitic forms within the vertebrate host or coopting the mosquito vector to prevent the completion of the sexual cycle. Additionally, the development of prevention strategies, such as utilizing insecticide-treated nets and promoting indoor residual spraying (IRS), has assisted in the reduction of malaria cases in highly endemic areas\textsuperscript{19}. IRS reduces the female mosquito lifespan, thereby limiting its transmission capacity, which ultimately decreases mosquito vector density\textsuperscript{19,20}. However, concern surrounding the possibility of resistance towards insecticides and prevention strategies has driven research efforts towards elucidating novel ways to eradicate malaria.

**Malarial Drugs in the Past and Present**

Malaria drug discovery has sought to identify compounds that target the asexual blood-stage. These compounds are highly desired as they can improve host survival, disease symptoms, and reduce the risk of progression towards severe malaria\textsuperscript{2}. However, drugs have also been developed to target gametocytes formed during the blood-stage. The first compound discovered to treat malaria is known as quinine, an alkaloid isolated from the bark of the chinchona tree\textsuperscript{21}. Quinine functions by eliminating asexual schizonts during the blood-stage\textsuperscript{19}. Despite its therapeutic activity, quinine presented significant challenges involving poor tolerability and poor compliance with dosing procedures\textsuperscript{21}. As a result, there was an increased demand to find quinine substitutes in the early twentieth century, which led to the discovery of 8-aminoquinolone primaquine and 4-aminoquinolone chloroquine\textsuperscript{22}. The discovery of these compounds was partly due to previous work conducted by Ehrlich with methylene blue\textsuperscript{8}.

Chloroquine is thought to target asexual parasites in infected erythrocytes by interrupting hematin detoxification during hemoglobin digestion in the digestive vacuole, thereby resulting in parasite poisoning\textsuperscript{22,23}. Although the success of chloroquine as a first-line treatment and sulphadoxine-pyrimethamine as the second-line treatment was unparalleled when used in combination with the insecticide dichloro-diphenyl-trichloroethane (DDT) in IRS, their prolific and continued use during the
mid-twentieth century eventually lead to the emergence of malarial resistance\textsuperscript{23-25}.

Chloroquine resistance is thought to have developed due to several point mutations in the \textit{pfcrt} gene, resulting in a mutant transporter that expels chloroquine from the digestive vacuole. It is postulated that the proton motive force established by the vacuole assists in the expulsion of chloroquine, thereby rendering these strains resistant\textsuperscript{26,27}. Despite the progress made by chloroquine and DDT in reducing mortality and morbidity, the lack of an alternative, low-cost therapeutic drug at the time resulted in a relapse of malaria cases\textsuperscript{22}.

The new push for discovering novel antimalarial compounds led to the isolation of artemisinin (ART), a sesquiterpene lactone, extracted from the sweet wormwood \textit{Artemesia annua} in 1972 (Figure 2)\textsuperscript{2,22,28,29}. ART function is associated with the reductive scission of its peroxide bridge by haem iron, produced in the digestive vacuole during the digestion of hemoglobin in the asexual blood stage. Additionally, other studies have associated ART with the formation of free radicals that can alter protein and lipids in the digestive vacuole. Since ART has poor tolerability and a short-half life, it is not administered as a monotherapy, but in conjunction with derivatives such as artemether or dihydroartemisinin\textsuperscript{30}. The use of derivatives increases oral bioavailability and helps reduce selective pressure in antimicrobial resistance\textsuperscript{24}. The combination of ART with other derivatives compounds is known as artemisinin-combination therapy (ACT), which is currently used as the first-line treatment in highly endemic areas. However, partial resistance towards ART and ACT has recently been reported\textsuperscript{31}. In these partially resistant strains, the use of ACT results in delayed parasite clearance in the infected vertebral host; implicated with mutations in the K13 propeller domain\textsuperscript{31}. Additionally, ACTs are only active against asexual schizonts and cannot clear late stage gametocytes, thereby retaining a vertebral host reservoir\textsuperscript{32}. Nevertheless, due to continuous emergence of resistance in various compound-based drugs throughout history, there is an urgent need to redirect research efforts towards elucidating other methods of eradicating malaria.

\textit{The Development of a Malaria Vaccine}

Due to the continuous emergence of resistance towards compound-based drugs, scientists aim to develop vaccines that confer vertebrate host protection, thereby reducing mortality. The development of a vaccine has been proposed due to evidence showcasing human acquired immunity towards malaria when such subjects were injected with irradiated sporozoites\textsuperscript{33}. This finding has spurred further research efforts that seek to develop vaccines capable of interrupting the transmission of malaria\textsuperscript{34}. Three stages of the parasite life cycle have been identified as potent targets for the development of plausible vaccines: pre-erythrocytic, blood stage, and transmission-blocking vaccines (TBVs).

Pre-erythrocytic vaccines seek to impede the establishment of the asexual blood stage, thereby preventing clinical malaria\textsuperscript{35}. They prevent sporozoites from invading hepatocytes by utilizing neutralizing antibodies to produce an antibody-mediated response; likewise, they can also attempt to destroy sporozoites within infected hepatocytes through cytotoxic CD8+ T-cell responses\textsuperscript{19,33,35}. The leading malaria vaccine, which is pre-erythrocytic and recently completed Phase III of clinical trials, is known as RTS,S/AS01E\textsuperscript{36}. This vaccine is based on generating antibodies against the parasite circumsporozoite protein (CSP), resulting in CSP-specific CD4 T cell responses. However, no CSP-specific CD8+ T cell response has been generated\textsuperscript{36}. Further strategies are currently being tested to improve RTS,S, such as incorporating liver- or blood-stage antigens alongside CSP or utilizing an adenvirus capable of expressing CSP and RTS,S/AS01\textsuperscript{36}. While these efforts may lead to promising results, complete malaria eradication will require a vaccine with higher efficacy\textsuperscript{32}. Such a vaccine can achieve greater efficacy with the addition of adjuvants, as was recently demonstrated by the addition of adjuvants Abisco®-100 and CoVaccineHTM, in a malaria vaccine tested on a mouse model of \textit{Plasmodium berghei}. The use of these adjuvants led to a greater pool of antigen specific central memory CD8+ cells. Stimulating positive T cell responses through the use of adjuvants can increase the efficacy of a vaccine, thereby conferring individuals with full protection from a single inoculation\textsuperscript{37}.

Blood stage vaccines seek to target and inactivate merozoites within infected erythrocytes or target malaria surface antigens expressed on infected erythrocytes, with the goal of mimicking immunity\textsuperscript{38}. These vaccines intend to minimize clinical symptoms and are generally mediated by an antibody response, although T cell responses have also been reported\textsuperscript{35}.

However, it can be difficult to develop such vaccines as \textit{P. falciparum} evades the immune system through
antigenic variation of surface antigens such as PIEMP1. Effective blood stage vaccines may need to employ various versions of the antigens to ensure the host attains some immunity towards the parasite.

Finally, TBVs aim to provide an antibody or complement that is ingested alongside the parasite during the mosquito blood meal, preventing the formation of a zygote in the *Anopheles* midgut. Previous studies have associated TBV efficacy with the ability of ingested antibodies to bind to parasite surface antigens during the sexual cycle. Important TBV targets include Pfs25, Pfs48/45, and Pfs230. Although TBVs are promising in the pursuit of malaria eradication, employing a multi-faceted approach that simultaneously targets multiple stages of the parasite life cycle may be more effective in blocking the transmission of *Plasmodium*.

**Mosquito Vector Control – Genetic Approaches**

Many past and current studies have postulated the importance of rendering mosquitoes incapable of supporting gametocyte development to attenuate the transmission of malaria. One manner in which mosquitoes can be made ineffective is through transgenic modifications, whereby modified mosquitoes carry antimalarial effector genes to halt the *Plasmodium* sexual cycle. Four main antimalarial effector gene classes have been identified based on their mode of action; these classes include gene products for parasite killing, parasite interaction, gene products that interact with the mosquito midgut or salivary gland, and gene products that can manipulate the vector’s immune system. Gene products with a parasite killing function are typically peptides characteristic of the innate immune system of the mosquito, such as cecropins, defensins, and gambicin. On the other hand, molecules that interact with the parasite typically prevent its transmission across the midgut epithelium. Past studies have elucidated the importance of an enolase-plasminogen interaction peptide that binds to enolase on ookinete surfaces, thereby preventing its association with plasminogen; ultimately, prevention of the enolase-plasminogen interaction inhibits the ookinete’s ability to traverse the midgut epithelium. Previous studies have genetically engineered mosquitoes to express the salivary gland and midgut peptide 1, which is thought to interact with a putative ookinete receptor on the luminal surface of the midgut epithelium and the salivary gland membrane, inhibiting ookinete traversal.

To successfully create genetically modified mosquitoes, they must have a fitness advantage and compete for available resources with the wild vector population. For successful transgenesis, gene-drive systems must be incorporated alongside antimalarial gene effectors to assist transgenic mosquitoes in infiltrating the wild vector population, thereby ensuring the inheritance of the transgene. Gene-drive systems that have been utilized in producing genetically modified insects include the maternal-effect dominant embryonic arrest (MEDEA) and homing endonuclease gene (HEG) systems. MEDEA is based on a toxin-antidote system, whereby an effector gene is linked to a miRNA toxin that is expressed during oogenesis, inactivating an essential maternal gene required for embryogenesis; a zygotic antidote, which expresses the targeted maternal gene in early embryogenesis, rescues the system. Only progeny who have successfully received the MEDEA system and antidote from the MEDEA-bearing mother survive, whereas non-MEDEA-bearing progeny lacking the antidote perish during embryogenesis. Previous use of the MEDEA system has delineated a high initial introduction rate (25%) requirement, which can pose technical issues for certain projects. On the other hand, the HEG system takes advantage of the homing endonuclease gene, a selfish genetic element indigenous to microbes which recognizes a unique 18-30bp sequence in the genome. The gene inserts into the center of the recognition sequence and protects the chromosome from being cut. The non-HEG homologous chromosome gets cut and utilizes the HEG-chromosome as a template for repair, thereby producing a homozygous individual. HEG systems can be utilized to identify a portion of the target mosquito gene and may be inserted into the middle of its own recognition sequence. HEG can be utilized to knockout genes needed for mosquito survival, thereby reducing vector density. It can also be engineered to knockout genes that promote the development of *P. falciparum*, ultimately reducing vector competence.

**Mosquito Vector Control – Gut Microbiota**

Transgenesis typically poses significant problems, such as altering *Anopheles* fitness such that they cannot compete with the wild vector population. Therefore, current research efforts seek to identify the extent to which coopting the mosquito gut microbiota can attenuate *Plasmodium* transmission. Through the manipulation of the gut microbiota, it is still possible to identify important microbial strains, which could either indirectly or directly halt the sexual cycle.

The gut microbiota is of particular importance when elucidating pathogen-insect interactions since it is
the primary site that is exposed to the parasite. It is known to provide mosquitoes with many benefits, such as assisting in digestion to provide nutritional supplements, affecting host immune responses, and providing colonization resistance towards pathogens by competing for resources (niche competition), or secreting antimicrobial molecules. Past studies have demonstrated increased susceptibility of antibiotic-treated Anopheles towards developing Plasmodium colonization when compared to mosquitoes with a functional gut microbiota. Therefore, the gut microbiota plays a critical role in attenuating the progression of the Plasmodium sexual cycle and serves as a potent intervention site.

Mosquito exposure to microbes occurs throughout their development, but they initially acquire microbes from the mother’s genitalia and from larval and pupal breeding sites. The environmental conditions from which a mosquito develops contributes to vast differences seen amongst various Anopheles species. Throughout the mosquito’s lifespan, a core microbiota is established, which is continuously altered after a blood meal ingestion. These microbial fluctuations activate immune responses such as the immune deficiency (Imd) pathway, in order to limit the parasitic infection. The Imd pathway assists in maintaining a basal level of immunity and is activated upon the detection of peptidoglycan, a core component of the bacterial cell wall. However, bacteria can also directly affect the development of the parasite. A past study isolated Enterobacter sp. Esp_Z from the midgut of wild Anopheles arabiensis and identified its ability to impair the parasite’s sexual cycle. The production of reactive oxygen species by Esp_Z was implicated in its mode of action, which involves interrupting ookinete invasion of the midgut epithelium.

An alternative approach that researchers utilize to influence the midgut microbiota of Anopheles mosquitoes is known as paratransgenesis, whereby symbiotic bacteria are genetically engineered to express effector molecules. These engineered microbes are then fed to mosquitoes during their blood meal. Bacteria that are used for the paratransgenic approach must meet three requirements: the effector molecule must function with the desirable effect; there must be a mechanism to secrete or display the effector molecule on the bacterial surface; and the bacteria must survive for a considerable duration to maintain a high concentration of the effector molecule to achieve the desired consequence.

Current research efforts seek to bridge the link between the mosquito midgut microbiota and malaria attenuation by utilizing bioinformatics approaches to elucidate the production potential of indigenous gut microbes. An extensive literature search has been performed to identify the various genera, species and strains present in the Anopheles midgut microbiota. A particularly useful paper written by Minard et al. (2013) highlights dominant genera present in the midgut microbiota of Aedes albopictus and Anopheles stephensi (Table 1). Other papers have also identified genera predominant in the midgut microbiota. To identify production potential, two approaches have been utilized: in the first approach, full genome sequences (FASTA format) were downloaded and fed through the AntiSMASH algorithm, to identify strains encoding a high-volume of biosynthetic gene clusters (BGCs). These putative biosynthetic gene clusters were verified utilizing a pull-in data set (Quince), developed by the Magarvey lab. These talented strains are currently undergoing production media panels to stimulate BGC’s for natural product discovery.

Table 1: A curated list of select bacterial genera which can be found in Aedes albopictus and Anopheles stephensi. The identification of these genera has informed strain selection for media panels.

<table>
<thead>
<tr>
<th>Aedes albopictus Bacterial Genera</th>
<th>Anopheles stephensi Bacterial Genera</th>
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<tbody>
<tr>
<td>Staphylococcus</td>
<td>Xenorhabdus</td>
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<tr>
<td>Bacillus</td>
<td>Bacillus</td>
</tr>
<tr>
<td>Planococcus</td>
<td>Acineobacter</td>
</tr>
<tr>
<td>Lactococcus</td>
<td>Photorhabdus</td>
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<tr>
<td>Paenibacillus</td>
<td>Paenibacillus</td>
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<td>Bordetella</td>
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<tr>
<td>Terrabacter</td>
<td>Leminorella</td>
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<tr>
<td>Dermacoccus</td>
<td>Agrobacterium</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>Comamonas</td>
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<tr>
<td>Arthrobacter</td>
<td>Alcaligenes</td>
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<tr>
<td>Kocuria</td>
<td>Achromobacter</td>
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<td>Chryseobacterium</td>
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<tr>
<td>Acetobacter</td>
<td>Elizabethkingia</td>
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<tr>
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<td>Flavobacterium</td>
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<td>Pantoea</td>
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<td>Klebsiella</td>
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<td>Serratia</td>
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<tr>
<td>Aeromonas</td>
<td>Herbaspirillum</td>
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<tr>
<td>Pseudomonas</td>
<td>Myroides</td>
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Future Directions
The evolution of sequencing strategies can help identify differences amongst the gut microbiota of various Anopheles mosquitoes in a species-specific manner. This strategy can assist in elucidating certain bacterial strains that may prevent certain mosquitoes from carrying P. falciparum. These sequencing approaches can also help determine the microbiota of various mosquito regions such as the salivary gland and hemocoel in better depth, to further establish their potential as intervention sites.

An interesting area of research that is currently under investigation includes the impact of the vertebral skin microbiota in promoting the transmission of P. falciparum. Previous studies have suggested that an individual’s skin microbiota composition affects the vertebral host’s degree of attractiveness. In fact, researchers have discovered that Plasmodium-infected hosts with high gametocytic loads possess altered skin microbiota that further increases their attractiveness towards Anopheles mosquitoes, thereby allowing for the propagation of the parasite. These studies suggest host odour can be targeted to attenuate malarial transmission, and further research efforts can determine methodology needed to alter mosquito olfactory cues to prevent them from blood feeding on infected vertebral hosts.

Recently, Contreras et al. (2019) identified a neurotoxin, PMP1 (paraclostridial mosquitocidal protein 1) produced by Paraclostridium bifermentans strains that are capable of paralyzing Anopheles mosquitoes via syntaxin cleavage, resulting in the disruption SNARE-mediated exocytosis. This finding can be used in conjunction with the strategies stated above, whereby artificial blood meals containing the PMP1 neurotoxin are coated with bacteria known to attract Anopheles mosquitoes; these can be distributed across homes in high endemic areas, to be utilized as prevention strategies similar to IRS and insecticide-treated bed nets.

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