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The Effects of Human Milk Oligosaccharides on Neglected Infectious Diseases

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Shams A. Al-Azzam

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2018

Table of Contents

Signature Page	iii
Table of Contents.....	iv
List of Abbreviations	vi
List of Figures.....	vii
List of Tables	viii
Abstract of the Thesis.....	xi
1 Introduction	1
1.1 Structural Diversity and Complexity of HMOs.....	2
1.2 Prevention Against Microbial, Viral, and Parasitic Pathogenesis	5
1.3 Anti-inflammatory and immune system modulators.....	6
1.4 The Diseases Background	8
1.4.1 Primary amoebic meningoencephalitis (PAM).....	8
1.4.2 Schistosomiasis.....	11
1.4.3 Chagas Diseases	12
1.4.4 African Sleeping Sickness	14
1.4.5 Giardiasis	16
2 Methodology.....	18
2.1 Sample Preparation- Human Milk Oligosaccharides	18
2.1.1 Donor Samples	18
2.1.2 Synthetic samples.....	19
2.2 Cell based assays.....	19
2.2.1 Naegleria fowleri.....	19
2.2.2 Schistosoma mansoni	20
2.2.3 Trypanosoma cruzi.....	21
2.2.4 Trypanosoma brucei.....	22
2.2.5 Giardia lamblia.....	22
3 Results	24
3.1 Naegleria fowleri:.....	24
3.2 Schistosoma mansoni	25
3.3 Trypanosoma cruzi.....	26
3.4 Trypanosoma brucei.....	30

3.5 Giardia lamblia.....	31
4 Discussion.....	36
4.1 Naegleria fowleri:.....	36
4.2 Schistosoma mansoni	38
4.3 Trypanosoma cruzi.....	39
4.4 Trypanosoma brucei.....	40
4.5 Giardia lamblia.....	41
5 Conclusion	44
6 References	47

List of Abbreviations

HMO	Human milk oligosaccharide
2'FL	2'-fucosyllactose
3'SL	3'-sialyllactose
6'SL	6'-sialyllactose
LNT	Lacto-N-tetraose

List of Figures

Figure 1. HMO blueprint and selected HMO structures	4
Figure 2. The effect of HMOs on <i>N. fowleri</i>	25
Figure 3. Effect of disaccharide HMOs on <i>T. cruzi</i>	28
Figure 4. Effect of disaccharide HMOs and monosaccharides on <i>T. cruzi</i>	28
Figure 5. <i>T. cruzi</i> fluorescence images.....	29
Figure 6. Effect of HMOs on <i>T. cruzi</i>	29
Figure 7. Inhibition of <i>T. cruzi</i> Pathogenesis against pHMOs and sHMOs.....	30
Figure 8. Effect of HMOs <i>T. brucei</i>	31
Figure 9. Effect of HMOs <i>T. brucei</i> through cell count approach.....	31
Figure 10. Effect of HMOs on <i>G. lamblia</i>	32
Figure 11. Effect of disaccharides HMOs and monosaccharides on <i>G. lamblia</i>	38
Figure 12. Unfiltered pHMOs against <i>G. lamblia</i>	34
Figure 13. Filtered vs unfiltered pHMOs against <i>G. lamblia</i>	34
Figure 14. Co-incubation of Giardia with pHMO or HT-29 cells	35
Figure 15. Co-incubation of pHMOs and sHMOs with Giardia and HT-29 cells over 6 hours.....	36

List of Tables

Table 1. Qualitative measure of the effect of HMOs on schistosomula.....	26
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ABSTRACT OF THE THESIS

The Effect of Human Milk Oligosaccharides on Neglected Infectious Diseases

by

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Master of Science in Biology

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Neglected tropical diseases (NTDs) cause over one million deaths each year; the majority of which are caused by diseases such as Chagas disease, African trypanosomiasis, intestinal and nematode infections, such as giardiasis, Schistosomiasis, and many others. The treatment options are not effective in treating all stages of the parasitic life cycle or the phase of the disease. Therefore, a novel approach was used to investigate human milk oligosaccharides (HMOs), which are complex carbohydrate found in breast milk, on parasitic organisms because of their role as anti-adhesive antimicrobials that have proven to lower the risk of pathogenic

infections in infants. This study was designed to gain insight on the effect of structurally diverse HMOs on different parasites including *Naegleria fowleri*, *Giardia lamblia*, *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Schistosoma*. Through whole organism screenings, purified pooled HMOs from donor samples and synthetic individual HMOs were tested against each of the parasites. The results obtained did not demonstrate a reproducible inhibitory or facilitatory effect on *T. brucei*, *T. cruzi*, *G. lamblia* and *N. fowleri*. However, a mild phenotypic change in *Schistosoma mansoni* was observed post incubation with the pooled HMO. In conclusion, the results obtained demonstrate that HMOs (pooled and individual) did not inhibit or enhance the growth of any of the protozoa. Potentially, an alternative experimental approach will be used to investigate the antiadhesive property of HMOs on the pathogenesis of *G. lamblia* in a co-culture experiment with gastrointestinal tract epithelial cells.

1 Introduction

Infectious diseases affect millions of people each year. The Global Burden of Disease Study 2013, found that over one million deaths were caused by neglected tropical diseases (NTDs). The majority of which are parasitic diseases such as Chagas disease, African trypanosomiasis, intestinal and nematode infections, such as Giardiasis, Schistosomiasis and many others (1). The responsible pathogens are the vector-borne protozoa *Trypanosoma cruzi* and *Trypanosoma brucei*, intestinal *Giardia lamblia*, and nematode *Schistosoma*, respectively. These diseases affect people of all ages due to their prevalence of transmission through water, food, insects, and human body fluids. These parasitic infections can be endemic to certain regions but can also be transmitted to other regions of the world through human migration and climate change.

These diseases are called neglected tropical disease because they occur in the poorest regions of the world. As the name indicates, these diseases are found mainly in rural, underserved communities, and urban slums in low-income countries. Around 13 parasitic tropical infections are considered to be the biggest impediment to these countries where the person can be infected with more than one parasite (3,4). These diseases face over a billion people in developing countries. These parasitic diseases have been overlooked because the research focus has been on other diseases such as Malaria and HIV/AIDS. The World Health Organization (WHO) has directed its efforts to control and eliminate NTDs as part of a 10-year plan that started in 2006 (2). In 2013, during the World Health Assembly, NTDs were recognized as a major burden in underserved tropical and sub-tropical countries.

In order to solve a global health, and the economic and social problem, there need to be more efforts towards basic research to find a medical cure or therapy to prevent or cure these infections. In this respect, the WHO has directed some of its efforts towards basic research on infectious disease in the aim of developing new treatments and potentially reduce some of the burdens infectious disease has intensified, such as poverty (5,6).

In support of finding new therapeutics to treat or control parasitic infections, a novel approach was used to study the effect of Human Milk Oligosaccharides (HMOs) on neglected parasitic infections. These structurally complex carbohydrates are the third most abundant active component in human milk. They have shown to play an important role in preventing pathogenic bacterial, viral, and parasitic infections in-vivo and in-vitro. These compounds are naturally produced in the mammary glands and have a diverse structural complexity that makes it unique and essential for the growth and development of infants.

1.1 Structural Diversity and Complexity of HMOs

There are over 150 structurally complex free HMOs that differ in abundance, charge, and size in mothers' milk. The composition of these complex carbohydrates varies from mother to mother and over the course of the lactation (9). HMOs concentration ranges from 5-20 g/L and exceeds the amount of protein found in breast milk. All of the human milk oligosaccharides have a basic core building block of glucose and galactose linked in β 1-4 linkage at the reducing end, or commonly known as lactose, which is the most abundant sugar. Lactose is further elongated and modified by glycotransferases making up the diverse pool of four types natural terminal

fucose linkages and three types of acidic sialic acid linkages (7). Some natural oligosaccharides are polymerized with one to five fucose groups and make up 50-80% of the total HMOs. The most abundant natural oligosaccharides found in human milk are monofucosylactoses (FL), difucosyllactose (2'FL), Lactos-N-tetraoses (LNT), monofucosylactos-N-tetraoses (LNT-F), and difucosyllacto-N-tetraoses (LNT-2F) (8). For all of these oligosaccharides, there is a successive transfer of a lactosamine group which is a galactose linked to an N-acetylglucosamine (GlcNAc) in a β 1-3 or β 1-4 linkage. This addition is repeated few or multiple time to elongate or branch the structures followed by a modification with either Sialic acid or fucose (See Figure1, 12). LNT is considered to be the one of the major components of complex oligosaccharides and can be found at a concentration of 0.5-1.5 g/L(9). The acidic oligosaccharides are all the sialylated oligosaccharides which are negatively charged and include about 20% of all HMOs including 3'-sialyllactose (3'SL), 6'-sialyllactose, and other complex glycans (10). This terminal modification with fucose and sialic acid makes the oligosaccharides highly resistance to enzymatic digestion in the stomach which allows them to reach the gut fully intact.

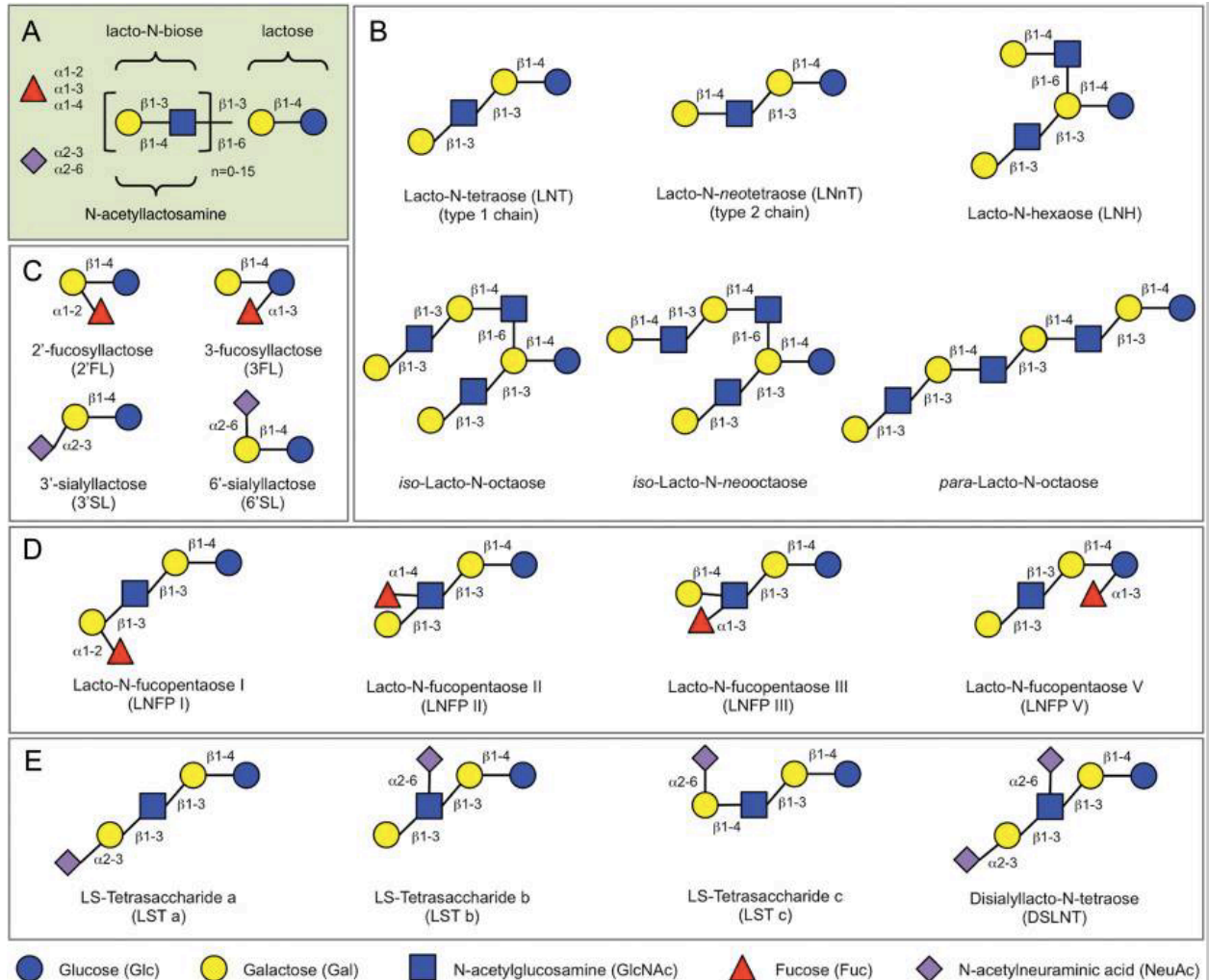


Figure 1. HMO blueprint and selected HMO structures. (A) HMOs follow a basic structural blueprint. (B) Lactose can be fucosylated or sialylated in different linkages to generate trisaccharides. (C) Lactose can be elongated by addition of either lacto-N-biose (type I) or N-acetylglucosamine (type II) disaccharides. Addition of disaccharides to each other in the $\beta 1-3$ linkage leads to linear chain elongation (para-HMO); a $\beta 1-6$ linkage between two disaccharides introduces chain branching (iso-HMO). (D) Elongated type I or II chains can be fucosylated in different linkages to form a variety of structural isomers, some of which have Le blood group specificity. (E) The elongated chains can also be sialylated in different linkages to form structural isomers.

The HMOs composition varies in the breast milk in-between groups and are characterized by secretors and non- secretors. This characterization is based on the addition of the Fucose group by Fructosyltransferases which are gene dependent. In women who are considered secretors, their oligosaccharides possess the $\text{Fuc}\alpha 1-2\text{Gal}\beta 1-3\text{GlcNAc}$ residues where the $\text{Fuc}\alpha 1-$

2 is added by the α 1-2-fructosyltransferase (FUT2). The non-secretor group is Lewis gene dependent and where the major Fructosyltransferases forms an α 1- 3/4 linkage of Fuc and GlcNAC through the expression of FUT 3/4 (9,18, 52).

1.2 Prevention Against Microbial, Viral, and Parasitic Pathogenesis

Human Milk Oligosaccharides are one of the largest and third most abundant bioactive components found in breast milk. Bioactive components are elements that serve a biological function in the body and affect the person's health (11). HMOs have shown to serve various biological functions such as prebiotics, antiadhesive antimicrobials, and anti-inflammatory (12). The HMOs were first discovered as prebiotics that promotes certain microorganisms, such as *Bifidobacterium bifidum*, and helps lower infections caused by harmful bacteria, therefore, increasing the survival rate of breast-fed infants when compared to formula-fed infants. HMOs have been more than just prebiotic that build the microflora of infants. Further, they have shown to protect against infectious diseases caused by bacterial and viral pathogens through their prebiotic property or by directly interfering with the pathogenesis (7, 12). Part of many bacterial and viral pathogenesis is adherence to cell surfaces through glycan-lectin recognition.

Since HMOs are synthesized by the same glycotransferases as the glycans on the surface of cells, it is hypothesized that they share similar structural moieties (7). HMOs can act as antiadhesives by mimicking the cell surface glycans and competitively binding to the pathogens, thereby, preventing the pathogens from binding to the surface receptors. Further, in the case of *Campylobacter jejuni*, the bacterium cell surface recognizes and binds to different fucosylated

epitopes found on H(O) antigen of epithelial cell, including, the most prevalent HMO, 2-fucosyllactose. In a *in-vitro* experiment, when soluble 1-2-fucosyllactose (2'FL) was added to *C.jejuni*, the fHMO acts as receptor analogs reduced the infection of the bacteria on the intestinal mucosal surface (14). HMOs that have a similar structure to cell surface glycans can act as soluble receptors for the pathogens that require cell surface adherence for pathogenesis. For example, immunodeficiency virus (HIV) expresses envelope glycoproteins, gp120, coated with mannose glycans that recognize and bind to a dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) expressed on a dendritic cells mucosal surface. Further, HMOs can competitively bind to DC-SIGN and thereby act as decoys (51). The role of HMOs as anti-adhesive antimicrobials has been shown to prevent the parasitic infection caused by *Entamoeba histolytica*. Structure-specific unmodified natural HMOs with a Galactose terminal can mimic the structures of glycan found on the surface of *E. histolytica* and prevent its adhesion by 80% to cells surface lectins (20).

1.3 Anti-inflammatory and immune system modulators

Human milk oligosaccharides play an important role in the innate immune system of infants within the gut, which is the main site of infection for many enteric pathogens, and in the circulatory system. The oligosaccharides from human milk reach pass the digestion in the stomach to the small and large intestine fully intact. The concentration of three different profiles of oligosaccharides was measured in the original milk, urine, and feces. About 1% of the concentration found in the original milk sample was found in the urine, thus, indicating that the human milk oligosaccharides get observed into the bloodstream and circulate in the body where

they finally get excreted through the urine. In the feces, the concentration of HMOs was ten-fold higher than the original concentration in milk (15).

The higher concentration of HMOs in the gut indicates that these oligosaccharides are essential in protecting against pathogens in breast-fed infants. Many of the evidence have shown that the physiological concentration of breast milk helps prevent against pathogens. This could be through modulating the immune system directly or indirectly to prevent against pathogenic infections. Structural specific HMOs with fucose moiety such as 2'FL, have shown to reduce inflammation due to an elevated production of inflammatory mediators such as IL-8 induced by lipopolysaccharide found on gram-negative bacteria such as type 1 pili *E. coli* (16). Lacto- N-fucosyltransferase III (LNFP-III) have shown to modulate the B-Cells to produce IL-10 and thereby reduce the Th1-type T-cells (19). In addition to fucosylated HMOs, sialylated HMOs have shown to play a role as an anti-inflammatory in one sialylated HMOs, 3'SL, to significantly reduce IL-8, -12 and TNF- α gene in intestinal epithelial cell lines, Caco2 (17).

HMOs can modulate cell signaling through binding to lectins on the surface and mediate the immune response. Sialylated and fucosylated HMOs can be recognized by various lectins including siglecs, elaectins, and selectins expressed on the cell surface of different cells such as immune cells, endothelial, and epithelial cells to contribute for various physiological and pathological functions and pathways (18). Additionally, HMOs have shown to recognize and bind to Toll-like receptors and trigger anti-inflammatory signaling pathways. The pooled natural fucosylated fractions of HMOs have shown to inhibit stable toxins secreted by *Escherichia coli* through glycan-lectin interaction and therefore lower mortality rate of mice due

to ST-induced diarrhea (13). These structure specific HMOs bind to lectins found on the surface receptors of T84 immune cells and stops the induction of cyclic GMP and therefore preventing activation of the pathway related to diarrhea symptoms.

As shown from previous studies, HMOs' biological function in health and disease can lower the risk of infection caused by bacteria, viruses, and parasites. We hypothesize that HMOs can play a role in the pathogenesis of parasitic infections caused by neglected and subtropical organism including *Trypanosoma cruzi*, *Trypanosoma brucei*, *Giardia lamblia*, *Naegleria fowleri*, and the nematode *Schistosoma* that can be quantitatively and qualitatively measured to give better predictions of the effects. This hypothesis can be tested in two folds: firstly, investigate the effect of individual and pooled HMOs on the growth of the parasites that causes each of these diseases. Secondly, investigate whether the HMOs can affect the adherence of parasites to epithelial cells such as *Giardia lamblia*. Each of the parasites studied against the HMOs is neglected in the sense that there is no effective drug or treatment that is specific for the disease. In the cases where treatment is available, it is most effective for the acute phase of the diseases where many cases go misdiagnosed making the disease life threatening as it develops into the chronic phase. Each of the infection lifecycle, treatment options, epidemiology, and pathogenesis are further discussed.

1.4 The Diseases Background

1.4.1 Primary amoebic meningoencephalitis (PAM)

PAM is caused by *Naegleria fowleri*, commonly known as ‘brain-eating amoeba’, which is transmitted through contaminated waters including, hot springs, lakes, streams, and swimming pools in tropical and subtropical areas. The cases that have been documented have had a 90% fatality listing PAM on category B priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) (23,21).

Since the first cases reported in 1965 in southern Australia, there have been approximately 300 cases reported (24,25). The most reported cases were due to recent contact with contaminated waters. Others have been related to ablutions practices in religious countries such as Pakistan (25). The majority of the case study reported were based on clinical and research epidemiology reports. It is believed that there are other missing reported cases in other countries and the numbers of infected cases might be higher.

This amoeba has various life stages that are determined by nutrient availability in the environment. There is a total of three life forms which include the flagellated, cyst, and trophozoite forms. The trophozoite stage is the only infectious and reproductively-active stage. When nutrients are scarce in the environment, the trophozoite stage transforms into the flagellated form in search of nearby food or the cyst form which is non-feeding and non-productive (23). This eukaryotic single-celled protists infection is accompanied by non-specific symptoms including headache, fever, changes in mental state, seizures, and can eventually end in a coma. The symptoms are triggered by inflammation that appears few hours post infection and causes fatality within 3-7 days (22).

This free-living ameba infects the human body through the nasal cavity where it travels up into the brain via the olfactory neuroepithelia infecting the central nervous system, causing acute inflammation and lethality within a few days. Adhesion is the first thing that occurs during this parasitic invasion. In fact, adhesion to nasal cavity epithelium is considered to be a very important factor for cytopathogenicity and diseases progression. When the parasites adhere to the cell surface they cause major cell damage and death leading to triggering inflammation. An in-vitro experiment showed that *N. fowleri* trophozoites have glycoconjugates that contained α -D-mannose, α -D-glucose, N-acetylneuraminic acid, Fucose, and α -L-glucose residues on the cell surface (26). An in vivo study showed that when structure specific lectins were introduced prior to infection, the parasites have difficulty adhering to the epithelium surfaces leading to higher survival rate in mice infected with pretreated trophozoites with lectins than those there were not (27).

The counter stone treatment continues to be antifungal amphotericin B. This current drug is nonspecific and had adverse side effects due to its toxicity and in turn, has its own limitation use and restriction (23). Further, the numbers of PAM cases continues to increase, and the current drug of choice treatment has shown weak recovery of 5% of those infected. Until this date, there has not been one treatment that was shown to be both effective and safe. Moreover, there is a desperate need for medication that can specifically treat the infection. This cause has pushed more scientific research to further understand the basic biology behind the infection and develop better therapeutic drugs. Moreover, until a cure is developed, *N. fowleri* will still be considered a neglected parasitic infection.

1.4.2 Schistosomiasis

Schistosomiasis, or commonly known as bilharzia, is the most neglected parasitic infections that have the second most reported cases per year after Malaria. The *Schistosoma* trematode is responsible for causing this infectious disease. The most responsible strain for the majority of reported cases in Brazil is *S. mansoni* (30).

The parasitic infection is transmitted through exposure to polluted waters with cercaria. This is the free-swimming, infectious stage of the Schistosomiasis that sheds from an intermediate host, snails, species of *Biomphalaria*. The cercariae infect the human host by penetrating through the skin to get access to the bloodstream losing its tail end to transform into schistosomula, where the sexual cycle occurs. They get access to the systemic circulation and migrate into organs of the body such as the liver and bladder where they mature into adults. The male and female adults pair up and migrate to the mesenteric arteries of the bowel and rectum to lay eggs that circulate to the liver or bladder and shed in the stool or urine. The eggs hatch in water bodies releasing the miracidium which goes on to infect the snail host, where they reproduce asexually and the cycle continues (29).

Treatment for parasitic trematode worms becomes more difficult when individuals are infected with more than one genus. There is a total of seven classified *Schistosoma* species and only three of them are pathogenic to human, which include *S. japonicum*, *S. mansoni*, that reside in the portal system of the liver and mesenteric arteries, and *S. haematobium*, that resides in the bladder and genital tract. The infection can start as asymptomatic in the acute stage of the infection. Once, the infection becomes chronic, the symptoms worsen leading to death in some

cases. Many of the symptoms are due to the eggs rather than the adult worms which include inflammation, and fibrosis. When the adult female worm lays eggs inside the mesenteric venules, the body response by inflammation that can cause a rupture of the venule wall, leading to the release of the eggs into the intestinal lumen (29). This causes manifestations such as fever, vomiting, diarrhea, headaches, and weight loss. The chronic stage of the infection can cause anemia, impaired growth and cognition, hepatosplenomegaly, physical difficulties, and neurological complications (31). The most vulnerable groups are pregnant women and children between the ages of 10-14 years old as they are more prone to reinfection (29,31). The most common treatment option is praziquantel which is available through the Center for Disease Control.

The majority of reported cases have been in sub-Saharan Africa make up 90% of the 252 million reported cases of Schistosomiasis (28). The presence of this infection has spread to Latin America, where many of the cases reported are from Brazil (31). Further, this infectious disease remains classified as one of the neglected tropical diseases as the rest of the world profoundly relies on one drug to treat this disease since the 1970s. Until this day, praziquantel (PZQ) is the most effective and affordable drug of use to treat and cure 60-90% of patients, however, relying on one drug to treat millions of people raises a major concern of drug resistance. Therefore, there need to be more research efforts to find new therapeutics drugs that are as effective as PZQ and hinders drug resistance development.

1.4.3 Chagas Diseases

Chagas disease continues to be the leading vectorborne disease caused by the protozoa *Trypanosoma cruzi*. As oppose to *T. brucei*, where the parasites are transmitted through the salivary glands of the tsetse fly, *T. cruzi* is a staercorarian transmission. When the triatomine vector takes a blood meal, it inoculates its feces next to the bite site. In responses to the bit irritation, the host rubs the bite site and contaminates the bite site with the vector's feces that contains the trypomastigotes, the infectious stage of the parasite life cycle (36)

This parasitic infection is endemic to South America and Central America as the vector thrives in tropical conditions. However, with large-scale migration, this parasitic infection has been reported in other parts of the world such as North America and Europe. There have been different of initiatives in control disease that took extreme measure in to reduce the number of cases reported in 1991 form 18 million to 5.7 million in 2010 (33). Nonetheless, the disease is still one the leading parasitic infections that are causing both social and economic burden that out weight those caused by Malaria by a 7.5 fold. Therefore, it is important to eliminate the spread of the infection through measures that include disease control and prevention, and proper treatment.

Chagas disease has two ways of transmission, either through a vector, or human fluids, such as blood. The vector born transmission involves the insect, triatomine, commonly known as the kissing bug. When it takes a blood meal it transmits the trypomastigotes through their feces and from there gain access into the bloodstream of the host. The trypomastigotes go to invade the host muscle cells and invade its nucleuse. They differentiate into the amastigotes and replicate in the host cells leading to a rupture in the host cells where they get access to the bloodstream to further infect other cells. The disease can be transmitted from the infected individual through

blood transfusions and congenital transmission (32). During pregnancy, infected women are at high risk of transmitting Chagas disease to their infant (33). Another way the disease can be transmitted is through contaminated food and drinks with vector feces (34).

The hallmarks of the disease can appear as asymptomatic or symptomatic, which range from acute and later progress into chronic phase shortly after the infection or over the lifetime of the infected person. In the acute phase, symptoms are mild and include non-specific symptoms such as fever, discomfort to symptoms caused by inflammation such as hepatosplenomegaly and atypical lymphocytosis (32). In many cases, the acute phase is misdiagnosed or diagnosed once it becomes chronic making the disease life threatening by causing meningoencephalitis and cardiomyopathy.

The Chagas disease continues to be one of the major neglected tropical diseases in terms of new drug discovery. There is no currently approved drug by the FDA to treat the *T. cruzi* infection. The only drugs that have shown efficacy against the infection are Nifurtimox and benznidazole which can only be obtained through the CDC. Other drugs such as Benznidazole, a nitroimidazole derivatives have been used as a line of treatment but have shown mild side effects (35). The current drug treatments are only effective in the acute phase of the disease (37). As the disease continues to progress it develop into the chronic phase where there is no current approved drug to treat it. Additionally, drug resistance has been emerging in pushing the urgent need for discovering new drugs that are both effective and safe (38). Therefore, there needs to be more research towards finding new drug regimens that prove to be effective in the chronic phase of the disease in clinical trials.

1.4.4 African Sleeping Sickness

Human African sleeping sickness or Human African trypanosomiasis (HAT) is a parasitic infection that is caused by the *Trypanosoma brucei* and transmitted to humans through the tsetse flies. It is considered one of the neglected diseases that effects endemic regions such as sub-Saharan Africa. There are two subspecies of the protozoa, *Trypanosoma brucei gambiense*, the most prevalence in western and central Africa, and *Trypanosoma brucei rhodesiense* which is endemic to eastern and southern Africa (42).

The parasites undergo many life stages in the two different hosts. The infective stage of the parasites is a non-dividing metacyclic stage which is inoculated into the bloodstream of the host by the tsetse fly. The metacyclic stages undergo varies morphological changes and proliferates in the bloodstream of the host during parasitemia. When the vector takes it blood meal, it ingests the metacyclic stages that transforms into the procyclic stage in the midgut of the tsetse fly (43). They later migrate into the salivary glands to develop into the infective form (44).

Trypanosoma brucei gambiense is found about 97% of the HAT reported cases and has very similar symptoms to *Trypanosoma brucei rhodesiense* (38,39). As the name indicates, the disease disturbs the sleeping pattern of the person infects as one of the early sign of the acute stage. The first stage of the disease is the acute stage, where the parasites are found circling in the blood and have clinical features such as fever, lymph node swelling, and pain. However, as the disease progress, it becomes chronic as it infects the nervous central system. This is when minor neurological and endocrine futures become more apparent by symptoms such as sever

headache and pain, motor and sensory abnormalities, depression, mood disorders, dementia, and can eventually lead to coma and death (40).

Treatment in the acute phase of the disease is most successful with pentamidine for *Trypanosoma brucei gambiense* HAT while suramin is used to treat *Trypanosoma brucei rhodesiense* HAT. However, treatment becomes more difficult in the second stage of the disease as these drugs can poorly cross the blood-brain barrier and more toxic drugs are used as an alternative to treating the second stage such as eflornithine or a combination therapy of nifurtimox- eflornithine (41).

Due to the lack of treatment options of HAT, it is considered to be of the most neglected diseases. In the last 50 years, the only registered drug to use against HAT is eflornithine. This demonstrates the lack of new initiatives to develop more potent drugs that can be accessible to more than half a million infected cases.

1.4.5 Giardiasis

Acute diarrhea is considered one of the major causes of childhood morbidity and mortality in developing countries. The majority of diarrhea cases reported have been linked to parasitic infections, such as giardiasis, in developing countries like Ghana (45). Giardiasis continues to be one of the leading cause of gastrointestinal infections worldwide (48). The World Health Organization included Giardiasis in its ‘Neglected Diseases Initiative’ as one of the neglected tropical diseases that affect over-crowded areas and low-income countries resulting in

socioeconomic burden (47). However, *Giardia* prevalence is not only among low-income countries but also in high-income countries.

There are an estimated 200 million people infected with Giardiasis over three continents including Asia, Africa, and Latin America (46). Giardiasis is caused by *Giardia lamblia* or other strains such as *G.doudenalis* or *G.intestinalis*. *G. lamblia* is flagellated protozoan that is transmitted through ingestion where it travels into the gastrointestinal system to replicate and infect. The life cycle stage that is ingested is the cyst stage that sheds from fecal matter that cross contaminates with water and food and becomes the medium for disease transmission.

Contaminated foods and water contain the cyst form, which is the infective stages of the life cycle of the parasite. When the cyst is ingested, it migrates to the gastrointestinal tract where it hatches into trophozoites that multiply and colonize epithelial tissues. During the colonialization phase of giardiasis, they invade surrounding epithelium cells to acquire nutrients. As a result, this causes an apoptotic cascade of the cells and the epithelium barrier function is lost. This results in the activation of the immune system where the infection cause progress from acute to chronic (49). The symptoms of giardiasis varies from acute to chronic. However, it can also be asymptomatic causing an increase in disease prevalence as go undiagnosed. The most common symptoms in adult and children is diarrhea in the acute phase of the infection. However, children can have more severe consequence that affect growth, development, and cognition. This parasitic infection is not deadly and treatment is available for both the acute and chronic phase with six different classes of drugs including 5-nitroimidazole compounds that has the first choice of treatment, metronidazole (50).

This intestinal protozoan remains the major cause of diarrheal infections worldwide with high morbidity and mortality in children and adults. There is a need to examine the disease through its biology, epidemiology, and interaction with the host. Regardless to say, the current treatment remains to be metronidazole which is poorly therapeutic drug in terms of its efficacy, low patient compliance and varies side effects. There is a major hope that exploring biology and the pathogenesis through molecular methods will give a better insight into the disease and finding other alternatives to treat it.

2 Methodology

2.1 Sample Preparation- Human Milk Oligosaccharides

2.1.1 Donor Samples

Pooled HMOs were collected from donor samples through a multi-step purification process as previously described ([7](#)). Firstly, lipids were removed from the samples through centrifugation and proteins were precipitated out using ice-cold ethanol and subsequent centrifugation. Followed by roto-evaporation to evaporate the ethanol from the sample. The lactose and salts were removed through size exclusion chromatography, leaving behind, the HMOs. The HMOs were then labeled at the reducing end with the fluorescent tag 2-aminobenzamide (2AB) by heating at 65°C for 2 hours ([5](#)). Later, the 2AB labeled

oligosaccharides were analyzed on the HPLC for lactose concentration (4). The oligosaccharides that had less than 2% lactose were used to test against the parasites. An additional purification step was used for blood borne parasites. The pooled HMOs were run twice on a Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher 20344) to remove Lipopolysaccharide (LPS) from the HMO sample. The samples were tested for LPS concentration using Endpoint Chromogenic LAL Assay (Lonza 50-648U).

2.1.2 Synthetic samples

The individual oligosaccharides were purchased. These oligosaccharides were further purified from LPS using Detoxi-Gel Endotoxin Removing Gel. Synthetic oligosaccharides were purified once on the columns because they had less LPS present than the pooled HMOs. The samples were diluted in water for the desired concentration for cell based assay.

2.2 Cell based assays

2.2.1 *Naegleria fowleri*

The pathogenic strain *N. fowleri* KUL (ATCC 30808) was used for compound screening experiments. The trophozoites were cultured in Nelson's media supplemented with 10% FBS and incubated at 37 °C. The trophozoites were harvested during the logarithmic growth phase to conduct the cell based assay experiments to screen against the HMOs. For the primary screen, the positive control used was 46 µg/mL amphotericin B diluted in DMSO. The HMOs samples

concentration tests were 100 µg/mL in water. A volume of 0.5 µL of amphotericin B and DMSO control were transferred to white, solid bottom tissue culture 96-well plates (E&K Scientific) followed by addition of 99.5 µL of 10,000 N. *fowleri* in Nelson's medium. In addition, 2 µL of HMOs and water control were added to the wells and 98 µL of 10,000 N. *fowleri* trophozoites in Nelson's medium were added. Assay plates were incubated for 48 h at 37°C. Screening was performed in duplicates and the assay plates were equilibrated to room temperature for 30 min before lysing the cells with 50 µL of CellTiter-Glo, a luminescent cell viability assay reagent (Promega). The plates were then placed on an orbital shaker at room temperature for 10 min to induce cell lysis followed by 10 min equilibration period at room temperature to stabilize the luminescent signal (3). The luminescence was measured at room temperature using an EnVision plate reader (PerkinElmer) (2).

2.2.2 *Schistosoma mansoni*

A Puerto Rican *S. mansoni* is maintained in the laboratory using the intermediate snail host, *Biomphalaria glabrata* and the Golden Syrian hamster *Mesocricetus auratus* (5–6 weeks old; Simonsen labs) as the definitive host. The cercariae (infectious stage) were harvested from the snails using a shedding method. Upon light exposure, the cercariae shed from the snails into the surrounding water. The cercariae are cleaned over a series of washes using sieves and water and transferred to a 50 mL tube that was left on ice for an hour. The icing method helps concentrate the cercariae as they tend to settle to the bottom of the tube and clump and stick to the surface. The water is then removed and replaced with 9 mL of cold 'Incomplete' Medium 169 that contains 1× penicillin-streptomycin. The cercariae are transformed into schistosomula by mechanical

passing the cercariae through a two 10 mL syringes attached via a 22-gauge double-headed needle to detach the tail from the head. The heads (schistosomula) were separated from the tails through careful aspiration that leaves the heads behind and removes the majority of the tails. The schistosomula are washed three times with Incomplete Medium 169 under sterile conditions. The parasites are left to settle in a 1.5 mL tube on ice for 2 hours before screening on the HMOs. About 20-40 schistosomula were plated with the 100 ug/mL final concentration of HMO in a complete media in a clear 96-well plate. Phenotypic observations were recorded daily for up to 10 days (8).

2.2.3 *Trypanosoma cruzi*

For the assay, *T.cruzi* trypomastigotes were incubated with mouse myoblast cell line C2C12 (ATCC#CRL-1772) and were harvested after 4-7 days of infection to carry on the cell assay. The C2C12 cell line was cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS), 25 mM HEPES, 2mM L-glutamin, 100 U/ml penicillin and 100 ug/mL streptomycin. The cultures were incubated at 37°C with 5% CO₂. For the infection assay, 1 uL of HMOs were plated in a 384-well plate and seeded with 500 of C2C12 suspended in 40uL of DEME media. Additionally, 10 uL of 2,500 parasites were added to each well totaling a final volume of 51 uL in each well. The plates were incubated for 72 hours at 37°C with 5% CO₂. After the incubation period, the cell was fixed with 50 uL of 8% paraformaldehyde solution and washed with PBS. The cells were stained with 0.5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) and left to incubate for at least 4 hours. The parasitic infection was calculated using Image express were analyzed based on the nucleus parameter of the host cell and the parasite

quantification. The analysis is based on the ratio of the parasitic amastigotes compared to the number of host cells to determine the growth inhibition of the compound post infection. The results were normalized against the negative DMSO control, positive controls treated with 50 μ M benznidazole, and the untreated infected cells (6).

2.2.4 *Trypanosoma brucei*

The strain *T.brucei brucei* 221 blood stream form was maintained in HMI-9 medium supplemented with 10% fetal bovine serum (FBS). HMOs were seeded in a 96 well-plate to each well and suspended with 100 μ L of media followed by the addition of 25,000 cells/mL in 100 μ L totaling a final volume of 200 μ L and final HMOs concentration of 100 μ g/mL. Plates were incubated for 48 hours at 37°C and 5% CO₂. Assay optimization were carried through to determine incubation period and parasite concentration (supplemental data). After incubation period, each well was lysed using a lysis buffer that contains 30mM Tris HCL, 7.5 mM EDTA, 0.012% saponin, 0.12% Triton X-100, and 3x Sybr green (Sigma Aldrich S9430). Plates were covered with a parafilm sticker and shook vigorously for 90 seconds on plate vortex to lysis the cells. The plates were left at room temperature for an hour incubation before measuring the Sybr green read out on the plate reader (Envision). The fluorescent reading was measured at (excitation and emission) (supplemental data).

2.2.5 *Giardia lamblia*

G. lamblia strain was maintained in TYI-S-33 medium which consisted of nutrient broth TYI (Trypticase, yeast extract, iron-serum), vitamin-Tween, and bovine serum (1). The medium is supplemented with penicillin (100 U/ml) and streptomycin (100 ug/ml). The trophozoites were harvested during the logarithmic growth phase to conduct the cell based assay experiments to screen against the HMOs. For the primary screen, the positive control was compound 8881, which showed a 100% inhibition in previous experiment, at a final concentration of 100 uM. The HMOs were diluted to stock concentration of 100 mg/mL. Followed by the addition of 0.5 µL of diluted positive control and DMSO to white, solid bottom tissue culture 96-well plates (E&K Scientific) followed by addition of 99.5 µL of 50,000 cells/mL of G. lamblia. A 10 uL of HMOs (individual and pooled) were added to the wells, followed by the addition of 90 uL of 50,000 cells/mL of G. lamblia. The final concentration of the HMOs plated was 10 mg/mL and of the 100 uM for the positive control. The negative control, DMSO, which is the vehicle control, was shown previously that it has no effect on the growth on G. lamblia (1). The vehicle control for the HMOs was water, which had no effect on the growth of the parasites. Assay plates were maintained in anaerobic condition by placing them in GasPak EZ Anaerobe Gas Generating Pouch System (VWR) throughout the incubation period. The plates were incubated for 48 hours at 37°C. The screens preformed in triplicates. Cell- Titer Glo was used to lysis the parasites and luminesces was measured on plate reader (EnVision ,PerkinElmer).

2.3 Co- Incubation Assay

HT-29 cells were grown in 75 cm² asks to 80% confluence in DMEM media with 10% FBS and 1% Pen-Strep prior to platting into a 24 well plate. The HT-29 cells were plated in 24

well plate over night at 1.0×10^6 over night in 1 mL DMEM media with 10% FBS. The medium was aspirated and the cells were washed with 1X PBS twice. *G. lamblia* was cultured and passaged for the experiment as described previously. Prior to incubating with HT-29 with *G. lamblia*, trophozoites were washed twice with 37 °C PBS to remove media and serum traces. A 1:2 ratio of HT-29 to *G. lamblia* were incubated for 3 hours or 6 hours using serum free DMEM in 5% CO₂ at 37 °C. HT-29 Cell viability was measured after the incubation period using trypan blue. Percent adhesion of the *G. lamblia* was measured through counting suspended and adherent cells to the HT-29 monolayer.

3 Results

3.1 *Naegleria fowleri*:

Through using a cell-based assay, the effect of the HMOs on *N. fowleri* was determined (Fig.2). The individual and pooled HMOs were tested against the trophozoites at 100 ug/mL. The inhibition percent was calculated using the water as the negative control and amphotericin B, the common drug PAM, as the positive control. The result of the experiment shows no inhibition in any of the individual or pooled HMOs.

Further, an invitro run was run using a higher concentration of LPS free HMOs at 10 mg/mL. The result obtained showed that higher concentrations of the HMOs tend to be cytotoxic to the cell (data not shown).

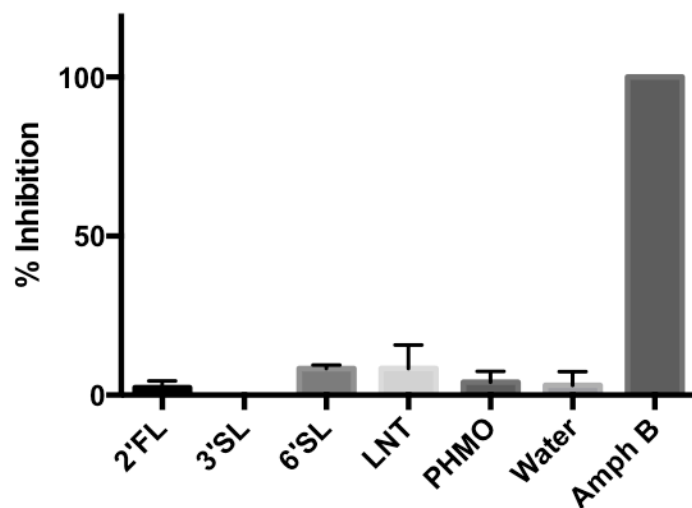


Figure 2. The effect of HMOs on *N. fowleri*. Trophozoites were incubated with the LPS free HMOs (individual and pooled) in triplicates. Water was used as a negative control and the amphotericin B as the positive control. The percent inhibition was calculated using the positive and negative controls.

3.2 *Schistosoma mansoni*

The results in Table 1 demonstrate the phenotypic observation recorded in numeric values to rate the effectiveness of the compounds on the schistosomula. The matrices is developed to classified the phenotype of the parasites using a number range 0-4 that include 6 different types of phenotypes that rang from ‘dead’, ‘overactive’, ‘round’, ‘dark’, ‘and ‘slow’ relative to the negative controls, DMSO and water.

In the first 24 hours of incubation time, all the schistosomula look healthy and had the same phenotypic morphology as the controls. After 48 hours, the pooled HMOs showed some phenotypic difference when compared to the controls and the individual HMOs. The schistosomula that were incubated with the pHMO at 100 ug/mL looked a darker and had a rounder shape than the control wells. These two phenotypic classifications were quantified with the number two. Across the 10-day incubation period, phenotypic changes remained somewhat

consistent. The mobility of the parasites was not compromised, and they appeared to be normally mobile. There was some background death in the wells, however, this was not significant when compared to the number of the parasites in the conditioned wells and the controls.

Table 1. Qualitative measure of the effect of HMOs on schistosomula. A qualitative scoring system was used to record the phenotypic changes over 10 days period incubation of the HMOs with the schistosomula. ‘0’ indicates that there were no phenotypic changes. ‘1’ indicates that one phenotypic changes occurred such as a change in color, mobility, or shape of the schistosomula. Two and three indicates that there are more than two or three phenotypic changes recorded respectively. Finally, ‘4’ indicates that the schistosomula are dead. The pooled HMO showed two mild phenotypic changes which include dark and rounded shape schistosomula but no significant death.

Name	SEVERITY SCORE							
	24 h	2 d	3 d	4 d	7 d	8 d	9 d	10 d
2'FL	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
3'SL	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
6'SL	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
LNT	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
PHMO	0	2	2	1	2	2	2	2
	0	2	2	1	2	2	2	2

3.3 *Trypanosoma cruzi*

The HMOs were tested on the protozoa *T. cruzi* in the cell-based assay showed that pooled HMO inhibit the pathogenies of the protozoa by 47% (Fig. 3). The individual HMOs did not show a significant inhibition when compared to the water control. Further, the experiment was repeated with the addition of the monosaccharides, glucose, and lactose (Fig 4). The pooled HMOs showed a reproducible inhibition; however, it was lower than the previous results and

was approximately 39%. The lactose control did not show a significant inhibition while the pooled HMOs with a lactose building core did across two different experiments. Additionally, the monosaccharide, glucose, did not inhibit against the protozoa infection.

Images of both the control, water, and the pooled HMO are shown side by side for a qualitative analysis of the inhibition (Fig. 5). In the water control image, almost every mouse cell nuclei are surrounded with the amastigotes while the positive control, benznidazole, had almost a 100% inhibition of the infection (Fig 5, A&B). Comparatively, the pooled HMOs wells had some cells that were not infected with the protozoa and the amastigotes were not apparent inside the cells (Fig 5, c). To confirm result reproducibility, the experiment was repeated a third time (Fig. 6). Unfortunately, the results initially obtained were not reproduced in the experiments that followed as the pooled HMOs did not show an inhibition against the parasites.

Since the previous results were not reproducible in demonstrating the inhibitory effect observed in the initial experiments, an alternative experimental approach was used. In this approach the C2C12 cells were incubated with sHMOs and pHMOs 4 hours prior to introducing the *T. cruzi* (Fig 7). By letting the HMOs interact with the mammalian cells, they more likely will have less of competition with the protozoa and might interact with the cell receptors and binding more effectively. This approach allows us to look at the pathogenesis of *T. cruzi* from a different angle. The results obtained showed that the HMOs do not inhibit the pathogenesis of *T.cruzi*.

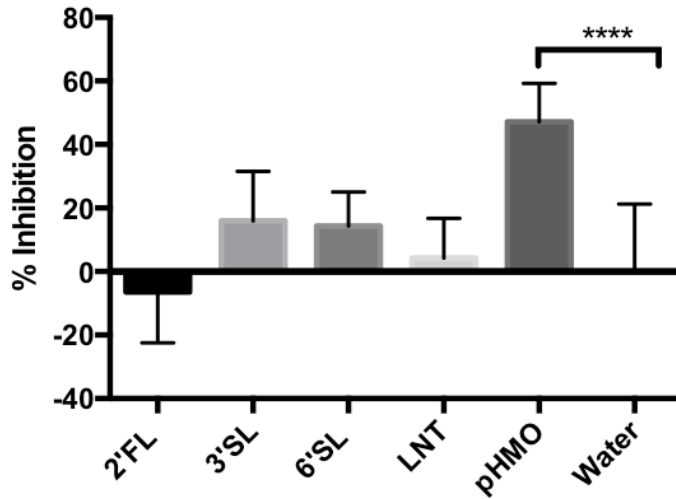


Figure 3. Effect of Disaccharide HMOs on *T. cruzi*. % Inhibition of different structures of 100 ug/mL HMOs after 72 hours incubation. Bars represent standard errors of the means of 10 replicates. Two tailed t-test, $P < 0.0001$.

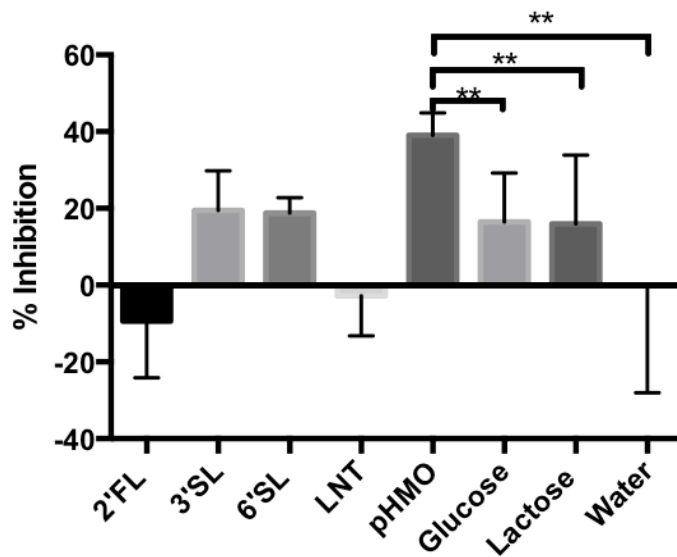


Figure 4. Effect of Disaccharide HMOs and Monosaccharides on *T. cruzi*. % Inhibition of different structures of 100 ug/mL HMOs and the monosaccharides after 72 hours incubation. Bars represent standard errors of the means of 10 replicates. Two tailed t-test, $P < 0.05$.

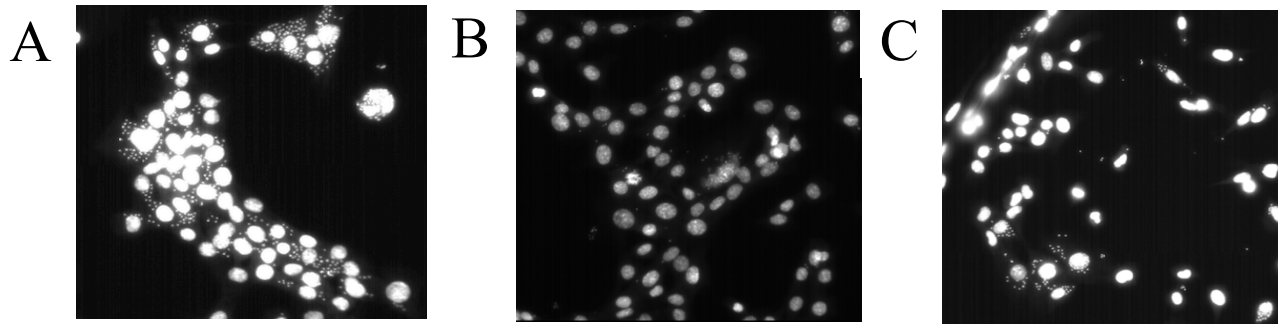


Figure 5. *T. cruzi* fluorescence images. (A) Negative control image of host cells infected with *T. cruzi*. (B) Positive control image of infected *T. cruzi* post treatment with benznidazole. (C) Image of cells infected with *T. cruzi* and treated with pHMO.

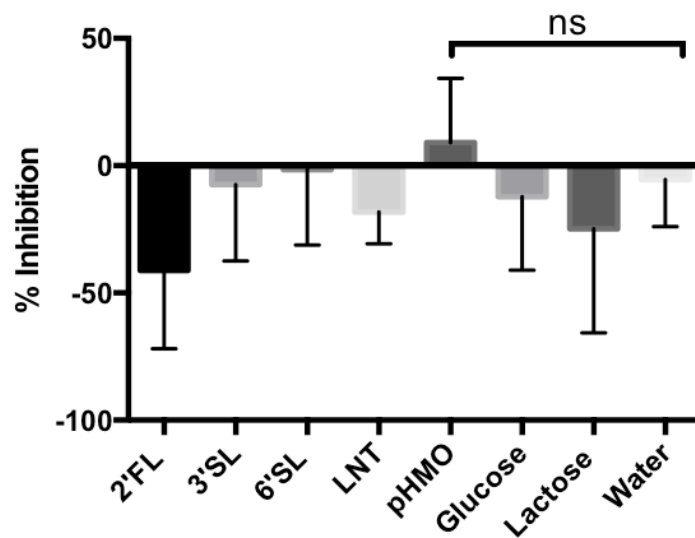


Figure 6. Effect of HMOs on *T. cruzi*. % Inhibition of different structures of 100 ug/mL HMOs and the monosaccharides after 72 hours incubation. Bars represent standard errors of the means of 10 replicates.

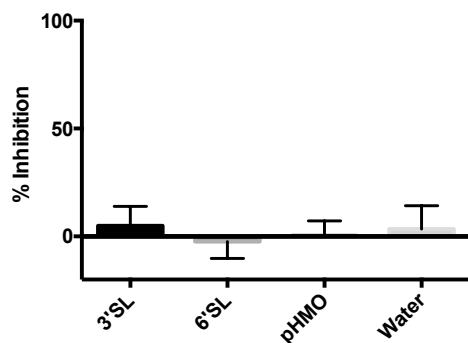


Figure 7. Inhibition of *T. cruzi* Pathogenesis against pHMOs and sHMOs. Structures of 100 ug/mL HMOs were preincubated with C2C12 for 4 hours prior to introducing the protozoa. % Inhibition was measured after 72 hours incubation. Bars represent standard errors of the means of 10 replicates.

3.4 *Trypanosoma brucei*

The HMOs were tested against the strain *T. brucei* to measure if there is an effect on the growth of the parasites. Predictably, the HMOs showed an enhancement in the growth of the parasites across two different experiments (Fig. 8). The results were normalized against the positive and negative controls to equal a value of 1. Any results above the normalization value indicate a growth enhancement of the parasites and anything below the 1 value indicates an inhibition. The individual oligosaccharides, 3'SL, and 6'SL showed a growth enhancement of 14% and 16% respectively. Additionally, the pooled HMO showed an enhancement in parasitic growth of approximately 20%.

Due to lack of reproducibility after the initial runs and the small significant percentage in the effect of the HMOs on the parasitic growth, further assay optimization was needed. After few data optimization methods indicated in the supplementary data, the data showed that the Sybr green readings were not a better measure of the enhancement, therefore, cell count assay was

used instead. The result from the cell count assay did not show a significant difference in the growth of the parasites in any of the individual or pooled HMOs (Fig. 9).

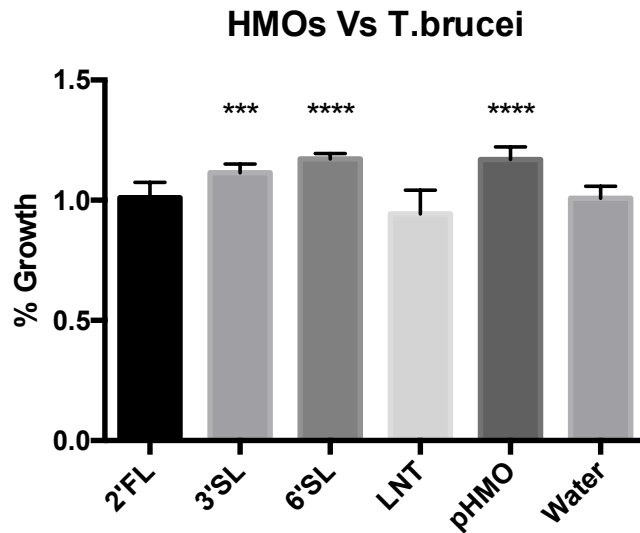


Figure 8. Effect of HMOs *T. brucei*. The %growth enhancement of *T. brucei* is referenced to the normal threshold of 1. Bars represents the standard errors of the means derived from two experimental runs.

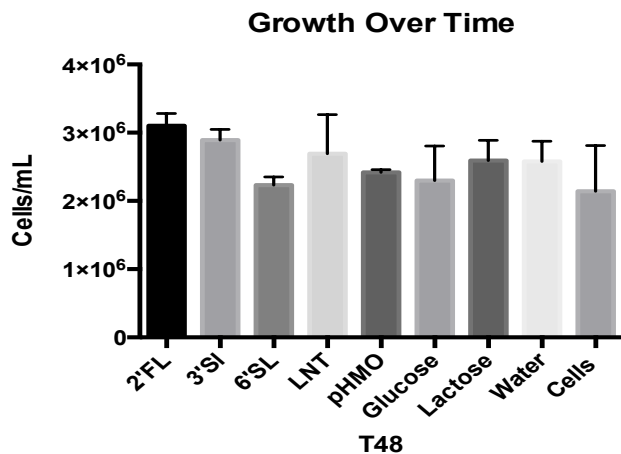


Figure 9. Effect of HMOs on *T. brucei* through cell count approach. The concentration of *T. brucei* was measure by counting the parasites using a hemocytometry. Bars represents the standard errors of the means derived from triplicates.

3.5 *Giardia lamblia*

Investigating the effect of HMOs on *Giardia* can give us a better understanding of both the direct role of HMOs in the growth of parasites and how its biological functions affect the pathogenesis of Giardiasis. The effect of individual and pooled HMOs was determined through an incubation assay with the flagellated form of *Giardia* and the ATP was measured to quantify the results. The natural and acidic individual oligosaccharides did not inhibit the growth of the parasites over the incubation period. However, the pooled HMOs had a less RLU (Relative Luminacins Unite) values than the individual oligosaccharides and the positive and negative controls (Fig. 10). This experiment was repeated with the addition of the monosaccharide glucose and the disaccharide lactose but the RLU values indicate that these controls did not inhibit the growth of the parasite (Fig 11).

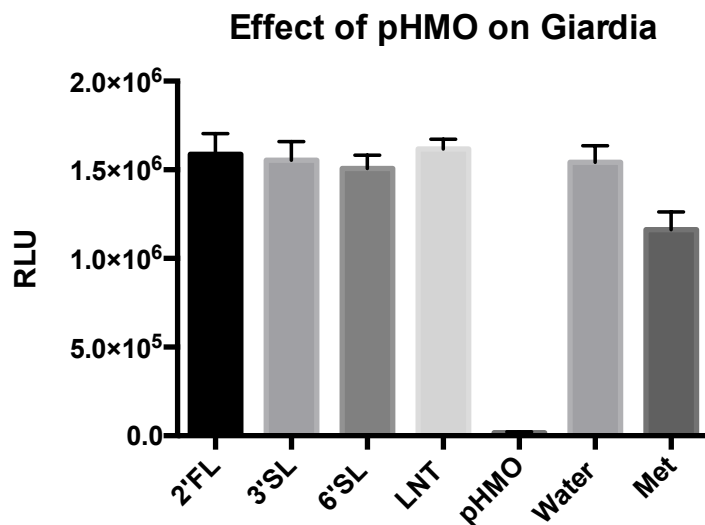


Figure 10. Effect of HMOs on *G. lamblia*. Measured inhibition by different structures of HMOs in triplicates after 48 hours incubation period at a final concentration of 10 mg/mL. Bars represent standard errors of the means derived from triplicates.

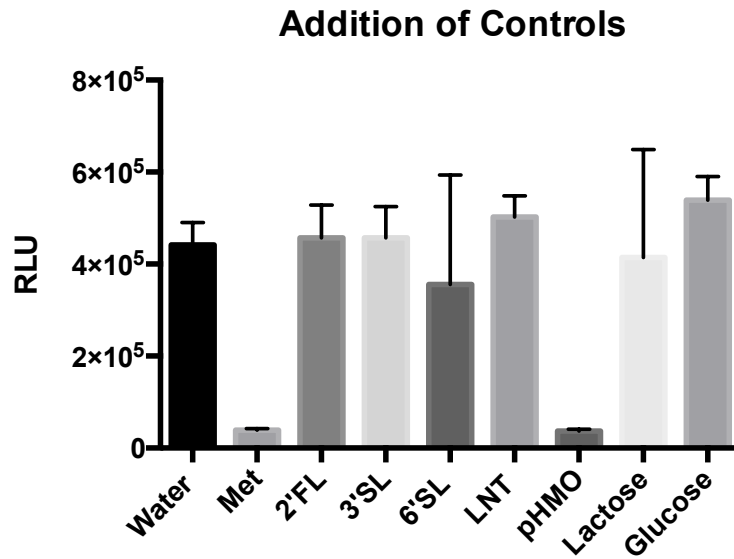


Figure 11: Effect of disaccharides HMOs and monosaccharides on *G. lamblia*. Measured inhibition of mono and disaccharides after 48 hours incubation period at a final concentration of 10 mg/m. Bars represent standard errors of the means derived from triplicates.

Few additional experiments were repeated for reproducible measures and the results confirmed that the pHMOs were causing an inhibition in parasitic growth based on the raw values obtained (Fig. 12). However, when the pooled HMOs were run in a dose-response curve, the RLU values did not decrease relative to the decreasing concentration of HMOs (results not shown). Therefore, phenotypic observations over time were needed to understand the correlation of cell count to RLU measurements. Unfortunately, it was determined that bacteria growth was present only in the pooled HMOs wells within the first 4 hours of incubation. This contamination was not in any of the other wells, including the controls. Therefore, it was critical to filter all the pooled HMOs to prevent any potential bacterial contamination that might have been carried over during the filtration process from the donor samples. The samples were either filtered once or twice and microscopic observation was recorded to detect any bacterial contamination that might have emerged over the course of the experiment. The results from the filtered samples were recorded by ATP measurement where it showed the unfiltered samples were contaminated with

bacteria and caused an inhibition in the parasite growth, whereas, the filtered samples did not inhibit the growth of the parasites over the incubation period (Fig. 13).

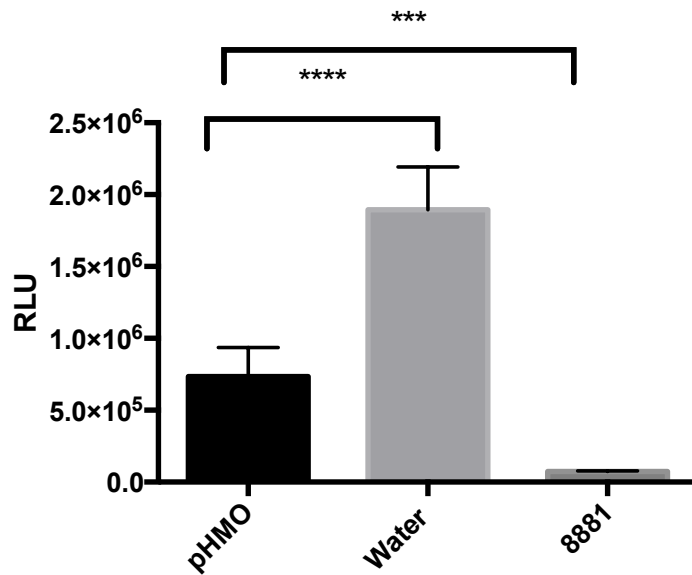


Figure 12. Unfiltered pHMOs against *G. lamblia*. The luminescent read out of three experiments were measured after 48 hours. 8881 is the drug of use to measure the 100% inhibition. Two tailed t-test, $P < 0.001$.

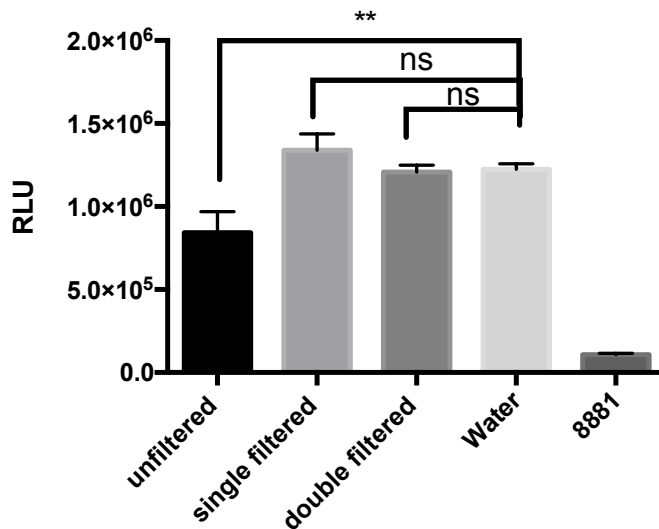


Figure 13. Filtered vs unfiltered pHMOs against *G. lamblia*. The luminesces read out of duplicates were measured after 48 hours. 8881 is the drug of use to measure the 100% inhibition. Two tailed t-test, $P < 0.05$.

The next step was to study the effect of HMOs through an co-incubation assay of the parasites and the epithelial cells (HT-29). The *G. lamblia* were incubated with the pHMOs over the course of 3 hours and the number of bound to unbound cells was measured to determine the % adhesion (Fig 14a). When the Giardia was incubated with the HMOs, it did not effect the adherence to the cell surface and the different between the Giardia only control and the Giardia incubated with the pooled HMOs was insignificant. However, when the same experiment was carried out, except with the addition of the HT-29 cells, the effect was present and there is a 10% reduction in adhesion(Fig. 14b).

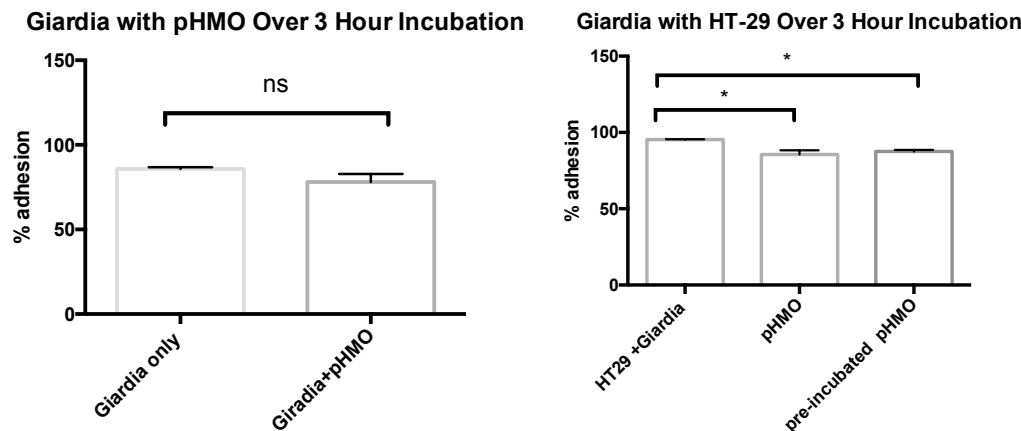


Figure 14: Co-incubation of pHMOs with Giardia or HT-29 cells, or both. (A) 2 million cells of Giardia were added alone (Giardia only) in 24 well plate or were incubated with pHMO at 10mg/ml (Giardia+pHMO). (B) HT-29 cells were plated in the wells overnight. The confluent HT-29 cells were preincubated with pHMO at 20 mg/mL for 2 hours before 2 million cells of Giardia were added (pre-incubated pHMO) or were added at the same time as the Giardia (pHMO). The control is the HT-29 and Giardia alone. The assays were left to incubate for 3 hours before the cells were collected to measure bound vs unbound and calculate % adhesion.

To further investigate if this effect is reproducible when the incubation time was increased to 6 hours, the pooled HMOs did not seem to reduce adhesion significantly (Fig. 15). Instead, when

the 3'SL and 6'SL were incubated with the Giardia and HT-29, the 6'SL showed a partial reduction in the adherence by less than 3% when compared to the Giardia and HT-29 controls.

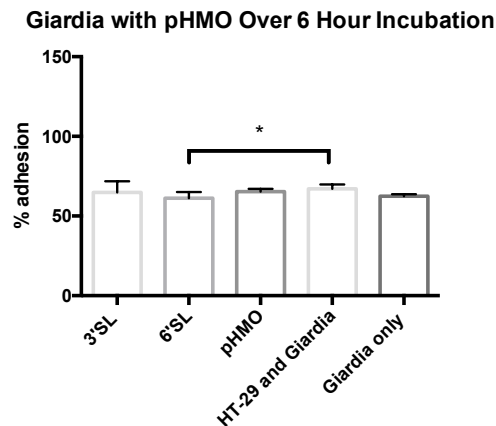


Figure 15: Co-incubation of pHMOs and sHMOs with Giardia and HT-29 cells over 6 hours. HT-29 cells were plated in the wells overnight. The confluent HT-29 cells were incubated with pHMO or 20 mg/mL with 2 million cells of Giardia. The control is the HT-29 and Giardia alone or Giardia alone. The assays were left to incubate for 6 hours before the cells were collected to measure bound vs unbound and calculate % adhesion.

4 Discussion

4.1 *Naegleria fowleri*:

The HMOs were screened against *N. fowleri* to measure if the effect is inhibitory or facilitatory showed no inhibition over the 48 hour incubation. To a certain extent, this was predictable by two folds: the first, *N. fowleri* is one of the most difficult parasites to cure effectively, and second, the drug screening approach is not the best approach to study the pathogenies of the disease and the effect of HMOs.

N. fowleri continues to be one of the fastest acting and fatal parasitic infections. The victim of this horrific disease shows symptoms within the first 48 hours of infection and later

progress within the next few days causing a fatality. Therefore, it is critical to treat the disease as soon as the symptoms appear but it remains challenging due to miss-diagnosis of the non-specific symptoms. Alternatively, treatment approaches are focused on the critical stage. That means many of the drugs discovered or repurposed, need to cross the blood-brain barrier and with fewer side effects that outweighs the damage caused to the victim. However, the drug used, amphotericin B, are very toxic at higher doses. Additionally, when the patient is treated at the chronic stage of the infection, higher doses of amphotericin B are administered either orally or directly into the spinal fluid. At this stage, this is considered a desperate measure to save the life to the patient as there are no other alternatives that are better and more effective. Until today, only 5% of the of PAM patients have been treated but continue to live with irreversible damage (citation).

Instead of looking at the effect of HMOs using a drug discovery approach, this could be looked at from a prevention angle. As has been indicated by previous studies, pathogenesis and disease progression of *N. fowleri* depends on cell surface adherence. It has also been shown that HMOs act as adhesives. This approach could be proven experimentally through investigating the effect of HMOs on parasitic adherence. HMOs could not have been directly affecting the growth of *N. fowleri* in the cell-based assay approach but they could play a role in the adherence of the parasites to epithelial surface either by acting as decoys, antiadhesives, or cell surface modulators. Further, *N. fowleri* has many cell surface glycans such as mannose and glucose that recognize certain lectins. HMOs might play a role in other recognition factors found on their surface and interfere with the pathogenesis. After all, the pathogenesis of the *N. fowleri* is still not completely

understood and that is what is it important to further investigate both a drug screen approach against the HMOs and co-incubation approach of the HMOs with the parasites.

4.2 *Schistosoma mansoni*

The nematode schistosomula has a very complex sexual and asexual life cycles which make it harder to find a drug that works for multiple life stages in the human body. Many of the drugs screen on *Schistosoma* has to show potency against both the larva and adult stage and therefore it makes it a challenging and long process to find the right drug. It also makes it critical to find an alternative drug that is as effective as PZQ in the case of drug resistance emergence.

The individual and pooled HMOs were tested on the schistosomula stage and a mild effect was present. There were two phenotypic changes that were reproducible across duplicate were the schistosomula looked dark and round across 10 days. Even the effect seem mild from a drug discovery approach, from an HMO study perspective is significant as the role of HMOs against the infectious organism has not been studied before.

The color and shape changes induced by the HMOs can be caused by metabolism or cell surface binding. It has been shown that schistosomula are covered with cell surface glycoprotein, such as glycocalyx (1). In an in-vitro study, they show that schistosomula of *Schistosoma mansoni* have specific cell surface glycoproteins that recognize only concanavalin-A-binding glycoproteins through a lectin-affinity chromatography (2). This indicates that the cell surface glycoproteins must contain α -D-mannosyl and α -D-glucosyl groups

on the non-reducing ends. It is also hypothetically possible to assume that HMOs interact with cell surface receptors and antigens and alternatively coat the cell surface causing the phenotypic observations mentioned. However, in order to confirm this, there needs to be further investigation and experimental design to draw these conclusions.

4.3 *Trypanosoma cruzi*

Until current date, there is still no specific drug used to treat Chagas diseases. The non-approved drugs used on infected patients are effective but are cytotoxic. Many of the symptoms that accompany this disease are life threatening. Therefore, screening and repurposing drugs are extremely important and critical. There have been major initiatives directed in every angle in drug discovery from using antimicrobials, fungi, to marine antibiotics. This is where this project has directed a new novel approach to screen oligosaccharides against the *T. cruzi*.

The results initially obtained were very promising as pooled HMOs showed on an average of 43% inhibition across two different runs. The results were further confirmed across both runs by image analysis of the pooled HMOs wells across all replicants where it showed efficacy. However, the following experiment disproved the initial results as they were not reproducible. This variation in results can be expected in high throughput compounds are not as potent and fall below a certain threshold and within the error. In other words, drugs that are considered of high potency have an inhibition of 85% or higher. These are the compounds that have reproducible results. Since the pHMOs result did not meet this standard cut off for this assay, it falls within the variation error. Additionally, the image express software contributes to the error as it is not precise when measuring the number of the parasites that infected and reproduced each

mammalian cell during the incubation period. When the parasites overlap on the nuclei of the cells, it is not able to distinguish the new parameters. This might have also contributed to the calculated inhibitions.

It is also important to realize that *T. cruzi* surface is entirely covered with a diverse pool of glycoproteins and glycoconjugates that play important role in the pathogenesis of the parasite. The major component of glycoproteins are mucins which are glycosylated proteins with O-linked glycans. Some of those O-linked glycans have the GlcNac residues which is similar to some human milk oligosaccharides. Some of those included LNT, LNnt, LNH and many other structurally complex lactosamine containing glycans. Additionally, many of these glycoproteins contain glycan structures that are modified at the terminal with sialic acid. This moiety allows the glycoproteins to recognized host surface receptors and lectins in order to infect the host system. The role of HMOs is not yet understood when it comes to the pathogenesis of *T. cruzi* but it would be worth exploring.

4.4 Trypanosoma brucei

The activity of structurally different HMOs was assessed against *T. brucei*. The results indicated that *T. brucei* growth over time is not influenced by HMOs and remains consistent when compared to the controls. However, this approach is very novel to study directly on parasitic growth. It is known that the surface of the metacyclic bloodstream form of the parasite is composed of glycosylphosphatidylinositol (GPI)-anchored glycoproteins that arranged as a densely coated monolayer containing varies surface glycoproteins (VSG). The core structure of the mature VSG GPI contains ethanolamine-HPO₄ - 6Mana1-2Mana1- 6Mana1-4GlcNa1-6PI and modified by α -galactose (3). This modification galactose at the terminal resembles the

structure of some of the HMOs, such as LNT. Based on the morphological feature of the parasite, we can hypothesize that the oligosaccharide will not interact directly with the surface of the protozoa, however, oligosaccharides directly facilitate the growth of the parasites through metabolism.

Monosaccharides, such as glucose, are needed to facilitate the growth of the parasite, but metabolism of complex sugars, such as Human milk oligosaccharides are not studied in the metacyclic bloodstream stage (4). Sialylated oligosaccharides, such as 3'SL and 6'SL, have been studied in the procyclic stage in the tsetse fly where the sialic acid is transferred onto the cell surface through trans-sialidase (5). However, this transferase is not expressed on the cell surface and sialylated terminals are shed and replaced by galactose terminals in the bloodstream from.

As the initial results indicated, there was a significant increase in parasitic growth when the incubated with 3'SL, 6'SL and pHMOs using DNA as an analytical measurement. However, we further assay optimization was carried out due to lack of reproducibility, the results were proven to be statistically insignificant when cell count data was measured. The results might not have shown an effect due to the HMOs but it gave a better understanding of the difference between procyclic from and bloodstream form in terms of utilizing sialylated oligosaccharides. It is not clear whether blood steam stage is capable of utilizing these oligosaccharides to facilitate its growth with glucose is not abundant or if it is designed to break down complex structures to use if the glycolysis cycle.

4.5 *Giardia lamblia*

Since Giardiasis continues to be the leading cause of many diarrhea infections, it was important to investigate HMOs, which have a role of protecting against enteric pathogens, against the protozoan Giardia. Structure-specific HMOs have shown to inhibit some of the toxic activity caused by certain pathogens such as E.coli and Cholera. Their direct role was by binding to glycosphingolipids found on the epithelial mucosa of the host. Thereby, preventing activation of the pathway related to causing diarrheal symptoms due to over activation of chloride channels. Instead of investigating the role of the oligosaccharides from the perspective of cell activation, the effect of the oligosaccharide was research from growth over time of the parasites. Arguably, cell surface lectins of *G.lamblia* that have and binding specifically to glucosyl and mannosyl residues (6). Since all the HMOs have a glucose residue at the non-reducing terminal, it was hypothesized that binding interaction might occur of the HMOs with the *G.lamblia* cell surface receptors. This due to the presence of the hemiacetal group on the non-reducing end of the lactose which the common building block for all oligosaccharides. The aldehyde groups makes it easier for the lectins to recognize and bind to. This binding mechanism is critical for cell surface adhesion.

This role was studied using two different experimental designs. The first was by looking at how introducing the HMOs to the giardia can have an effect on its growth in a well plate. In the case that these HMOs can bind to lectins with high avidity, it was expected to interfere with the colonization and the growth of the parasite over time. This has been observed when cysteine is not present in the media, which play a role in the adherence of the parasite to the surface (). In this case, this can affect the growth of the parasite over time and its proliferation. Therefore,

measuring the effect of HMOs using this mechanistic approach is highly reasonable. Debatably, this could be directly studied using an *in-vitro* approach with mammalian cells. This approach was taken into consideration when the effect of HMOs on giardia did not seem prominent and a co-incubation assay with epithelial cells can give a better insight into the role of HMOs in pathogenesis.

Through using an *in-vitro* approach with epithelial cells line is more applicable because it gives a better understanding of the host-parasite interaction. There are many carbohydrates linked to glycoproteins that cover the surface of the parasites and play an important role in host recognition and immune modulation. Further, a study has shown that D-glucose and mannose-6-phosphate can inhibit the binding of *G.lamblia* to epithelial cells lines, CaCo2 (8). To see if this effect is present with HMOs, pooled HMOs were incubated with either incubated with epithelial cells prior to introducing the protozoa or were introduced at the same time as the parasites. The results showed that when pre-incubating the HMOs with the epithelial cells, there is a significant reduction in the adherence of the parasites to the cells. However, since this experiment was only preformed in duplicates and the reduction in adhesion was 10%, there needs to be another experimental design that allows to determine which HMOs are responsible for the reduction and if this effect is significant when the incubation time is longer.

In addition to glucosyl and mannosyl residues, there are also sialylated residues such as Gal β 1-4GlcNAc and NeuAc α 2-6Gal β 1-4GlcNAc, which are considered one of the major non-reducing moieties of *G. intestinalis* (7). The second set of experiment tested if sialylated and pooled HMOs can reduce the adhesion and the results obtained did not show a significant reduction among the 3'SL and pHMO. In this experiment only the 6'SL significantly reduced the

adherence by less than 3%. Overall, the results obtained gave us a closer understanding of whether HMOs can play a role in adherence using morphological concentrations which has a mild effect against the protozoa and higher variability between experiments which makes it harder to draw any prediction or conclusions from the data obtained. There could be a variety of reasons why these HMOs don't completely inhibit adherence and it might be due to their binding avidity, concentration, or structural orientation. Also, the relative abundance of the different glycoproteins is not relatively understood which makes it harder to predict which glycoprotein is essential for adherence and if other glycoproteins can compensate for the parasitic adherence if the other is blocked by biosimilar glycans.

Conclusion

Studying the effect of HMOs on parasitic infections is an area of study that needs to be explored further. In this project, HMOs were tested on a total of five parasites that are the major cause of neglected infectious diseases. Overall, not all the parasites tested were affected by the HMOs. In some of the results obtained, the effect was mild in terms of changing some phenotypic morphologies in the case of *Schistosoma*. Additionally, many of the data obtained on the other parasites showed that the HMOs do have a major effect. Unfortunately, what was previously observed in the case of *Entameba* was not reproduced in this project which further explored other parasitic organisms that can be inhibited by human milk oligosaccharides.

The effect of HMOs on the parasites was not present which might be due to many reasons including the size, structural moieties, avidity, and concentration of the HMOs. As describe

earlier, there are over 150 types of structurally different HMOs which are found in different concentrations. The relative abundance changes between lactation and from mother to mother. Additionally, it is still not very well understood what each individual HMO function and role and whether individual or collective samples of HMOs are needed to serve their functions. To further explore this understudied area of Human Milk Oligosaccharides, this project took this into tested all the possible HMOs that might be present through in sample of milk by using human milk donor samples. This helps encamps the rest of the HMOs that were not tested individually. The individual synthetic oligosaccharides were tested to look at how a single structure might have a specific role measured through its effect or the growth of the parasites or adherence in the case of Giardia. For the majority of the parasites tested, neither the pooled or the individual oligosaccharides had a major effect on parasitic growth. This could be that HMOs don't interact with the parasitic on the binding or metabolomics levels that might have a direct effect on the growth and the reproduction or fusion of the parasitic organism. However, it also could be that the physiological concentration of the HMOs was not sufficient to cause a major phycological change that can be observed directly through the growth of the parasites over time. Regardless, the adherence experiment on Giardia was carried through to have a better perspective of the how might HMOs interfere with pathogen adherence, especially, on parasites that have it as critical step for pathogenesis and growth. As the results indicated, it is harder measure the effect directly and experimental variability was present making harder to rely on the results to draw precise conclusions from the results.

This project is novel in the sense that it looked at HMOs from a biological basic science research but alternatively if the HMOs had any major effect that could be used as therapeutics to treat some of these neglected diseases that are causing millions of deaths. In addition to HMOs antiadhesive antimicrobial property, they can also act as immune modulators. This role has not been investigated in these diseases and an alternative approach would be to test if HMOs might play a role in the immune system of infected patients to help them develop immunity to their parasitic infections or help compact many of the symptoms associated.

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