The Role of Helminth and Microbiome-Derived Metabolites in the Healing of Inflammatory Bowel Diseases Related Wounds *in vitro*

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ABSTRACT:

In recent decades North America and Europe have been faced with a new epidemic: inflammatory bowel diseases (IBD). Approximately 1 out of 150 Canadian suffers from this incurable autoinflammatory disease. IBDs include conditions such as Crohn's disease and ulcerative colitis, which create inflammation in parts of the gastrointestinal tract. The treatments available today are occasionally ineffective and can be extremely expensive. However, new types of therapies are emerging based on the observation made on the helminth-microbiota-IBD patient relationship. Helminth and microbiome therapies are new research areas focused on how helminth infections and microbiome-derived metabolites could be used as possible treatments for IBDs. Many studies have shown improvements in IBD mice and patients' conditions after a helminth infection or the administration of specific microbiome-derived metabolites. No studies have looked into the capacity of helminths and microbiome-derived metabolites to heal IBD-related wounds. We aim to assess which species of helminths and which microbiome-derived metabolites have the most potent metabolites in healing these wounds using an *in vitro* Caco-2 intestinal model scratch assay. In our research, we are screening the metabolites of three different helminths species and microbiota chemical metabolites. We found that *Ascaris suum* metabolites (50% ACN) at a 150 mg/mL concentration, 300 µMolar m-tolyacetic-acid and 1000µMolar urocanic acid showed increased growth compared to our controls and other substances tested. We also developed a new *in vitro* platform to screen metabolites and other compounds.

ABRÉGÉ :

Durant les dernières décennies, l'Amérique du Nord et l'Europe ont été confrontées à une nouvelle épidémie : les maladies inflammatoires chroniques de l'intestin (MICI). Approximativement 1 Canadien sur 150 souffre d'une maladie auto inflammatoire incurable. Les MICI comprennent des maladies telles que la maladie de Crohn et la colite ulcérative, lesquelles provoquent une inflammation de certaines parties du tube digestif. Aujourd'hui, les traitements disponibles sont parfois ineffectifs et peuvent être très chers. Cependant, de nouveaux types de thérapies sont en train d'émerger grâce aux découvertes faites sur la relation entre les patients souffrant de MICI et les helminthes. La thérapie helminthique et la thérapie microbiologiques sont de nouvelles disciplines de recherche qui se concentrent sur l'utilisation des infections helminthiques et des métabolites microbiologique comme possibles traitements pour les MICI. Aucune étude n'a encore examiné les capacités des métabolites helminthiques et microbiologiques dans la guérison des blessures liées aux MICI. En utilisant un test de rayure sur un modèle intestinal *in vitro* composé de la ligne cellulaire Caco-2 nous visons à évaluer quelle espèce d'helminthes ou quelle métabolite dérivée du microbiome a la meilleure capacité de guérison. Notre dépistage a montré que le métabolite d*'Ascaris suum* (50% ACN) à une concentration de 150mg/ml, le métabolite d'acide m-tolyacetique à une concentration de 300µM et le métabolite d'acide urocanique à 10000 µM ont guéri les blessures plus rapidement que le reste des métabolites helminthiques et microbiologiques testées. Nous avons aussi développé une nouvelle plateforme *in vitro* qui peut être utilisée pour le dépistage des aptitudes de guérison cellulaire de différentes métabolites et composés chimiques.

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LIST OF ABBREVIATIONS

IBD: Inflammatory bowel disease

UC: Ulcerative colitis

CD: Crohn's disease

5-ASA: Aminosalicylic acids

DNBS: Dinitrobenzene sulfonic acid

NACSI: Piroxicam

T. spiralis: Trichinella spiralis

H. poly: Heligmosomoides polygyrus

T. suis: Trichuris suis

A. suum: Ascaris suum

TSO: *T. suis* ova

ESP: Excretory-secretory products

TNBS: Trinitrobenzene sulfonic acid

DSS: Dextran sodium sulphate

PBS: Phosphate-buffered saline

FBS: Fetal bovine serum

DMEM: Dulbecco's Modified Eagle Medium

ACN: Acetonitrile

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CHAPTER I

INTRODUCTION

INTRODUCTION

In recent decades North America and Europe have been faced with a new epidemic: inflammatory bowel diseases (IBD). The two most prominent forms of IBDs are ulcerative colitis (UC) and Crohn's disease (CD), chronic autoinflammatory conditions that disrupt parts of the intestines. Although UC and CD's origin remains uncertain, studies indicate that these disorders are triggered by environmental factors, genetic predispositions, immune system abnormalities, and microbiota changes. IBDs affect approximately 233,000 Canadians; in other words, 1 out of 150 individuals (Crohn's and Colitis Foundation of Canada 2012). There is no cure available for IBDs, but treatments exist to relieve the symptoms. Popular treatments such as corticosteroids, aminosalicylates and immunosuppressive agents are widely used but are accompanied by various, possibly life-threatening, side effects (Fakhoury et al. 2014). Also, the treatments and eventual surgeries are expensive. Consequently, there is an urgent need for diverse, affordable and safe novel treatments to treat broad IBD patient cohorts.

New kinds of therapies have immerged in the field of autoimmune disorders: helminth therapy and microbiome therapy. Based on the observations that third-world and developing countries are more susceptible to helminth infections as acceptable hygiene practices are not extensively performed compared to developed countries such as Canada, the USA, and Western Europe countries where auto-inflammatory diseases are prevalent. Many third-world populations still do not have access to running water nor accessible health care, which raises their chances to contract a parasitic infection. In contrast, their chances of developing an autoimmune disease are slim (Okada et al. 2010). However, the big question remained: how do these parasitic infections protect these populations from conditions such as IBDs? How could we develop a commercially and ethically accepted potent treatment for patients using helminth therapy? Additionally, could microbiome therapy be an effective treatment?

1.1 Crohn's Disease and Ulcerative colitis:

Crohn's disease (CD) and Ulcerative Colitis (UC) are part of IBDs. Crohn's disease causes a relapsing transmural inflammation of the GI tract, anywhere from the mouth to the anus, traversing the entire intestinal wall, from luminal mucosa to serosa. In contrast, Ulcerative Colitis causes relapsing inflammations in the colon (Figure 1).

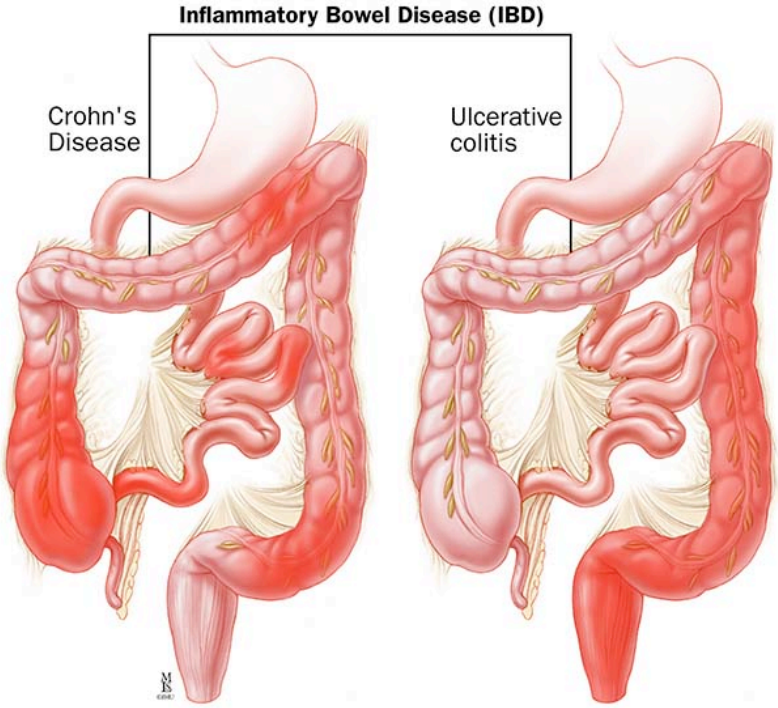


Figure 1: Location of the gastro-intestinal inflammation (in red) in CD and UC. (Jin 2014)

Both diseases exhibit similar symptoms. The villi of the intestines scare affecting the absorption of nutrients and therefore possibly causing extreme weight loss in CD. Moreover, UC and CD patients' appetite was observed to decrease during flares, leading to weight loss (Baumgart and Sandborn 2012). Ulceration of the mucosal wall is observed in both UC and CD, leading to extended bleeding in the GI tracks causing anemia (Trost and McDonnell 2005). Additionally, the

disappearance of transporter proteins from the epithelium responsible for the reabsorption of Na+ increases the sodium retained in the GI tract, which causes severe diarrhea in both conditions, leading to weight loss (Ghishan and Kiela 2014). Micro perforations of the intestinal wall might lead to anal fistulas and anal abscesses, which are also tremendously painful for UC and CD patients (Parray et al., 2011). The symptoms of these diseases are excruciating and significantly affect the lifestyle of their victims. Nevertheless, the causation is still unclear; serval factors are thought to be behind IBDs pathogenesis: genetics, the environment, the immune system and the microbiota.

Genes and genetic loci contributing to IBDs susceptibly have been identified in the literature; a meta-analysis of the genome of both UC and CD patients showed 99 non-overlapping genetic risk loci, 29 of them were shared among UC and CD patients(Franke et al. 2012). The genes and loci implicated in IBDs were investigated, and it was shown that these genes were involved in serval pathways “crucial” for intestinal homeostasis, barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species stress and metabolic pathways associated with cellular homeostasis (Khor, Gardet, and Xavier 2011). It was also found, in a family study, that if both parents of a child developed IBD by the age of 30, there was a one in three chances that the child would develop an IBD as well, demonstrating the importance and significance of genetics in the pathogenesis of IBD (Halme et al. 2006).

Several environmental factors are linked to IBD, such as smoking and diet. It has been found, via a population study, that CD is associated with smokers, whereas UC is mainly observed in former and non-smokers. It was also found that smoking exasperated the course of CD (Karban and Eliakim 2007). Other factors such as oral contraception, appendectomy, infectious agents, drugs and stress are also linked to an increased incidence of IBDs (Ananthakrishnan 2013).

The relationship between the gastrointestinal tract and its microbiota is an essential element in the development of IBDs. Indeed, through DSS mice models, it has been found that in the absence of microbiota (germ-free mice), the colonic inflammation was reduced compared to conventional mice, reinforcing the importance of the gut flora in the pathogenesis of IBDs (Hernández-Chirlaque et al. 2016). Serval studies have investigated the difference in the microbiota composition of IBD patients vs. healthy individuals, most notably the microbial diversity and the abundance of specific bacterial taxa. CD patients have been shown to have a microflora reduced by 50% and UC patients by 30% compared to healthy individuals (Ott et al. 2004). A reduction in *Bacteroides* and *Firmicutes*, families thought to have anti-inflammatory properties, is observed in IBD patients. In contrast, members of the Proteobacteria phylum, such as Enterobacteriaceae, known for *Escherichia coli* and *Salmonella enterica,* are increased compared to healthy individuals (Figure 2) (Ni et al., 2017) (Frank et al. 2007). An increase in mucosal bacteria, the bacteria on the mucus layer, is linked to a rise in IBD's severity, both in non-inflamed and inflamed colons. It was shown that bacteria of fecal origins were found near the lamina propria, implying that healthy individuals can hold back fecal bacteria and that this ability is disturbed in patients suffering from IBD (Swidsinski et al., 2002).

These studies emphasize the importance of the microbiota and the need for more research to understand better how and why these dysregulations occur. A more profound knowledge could be used to prevent the development and to worsen of IBDs. These studies also imply that individual families of bacteria offer protection to their host, leading to the thought that maybe members of these protective families could be reintroduced to IBD patients to ease their symptoms. This protection is an idea that we will also explore in this thesis.

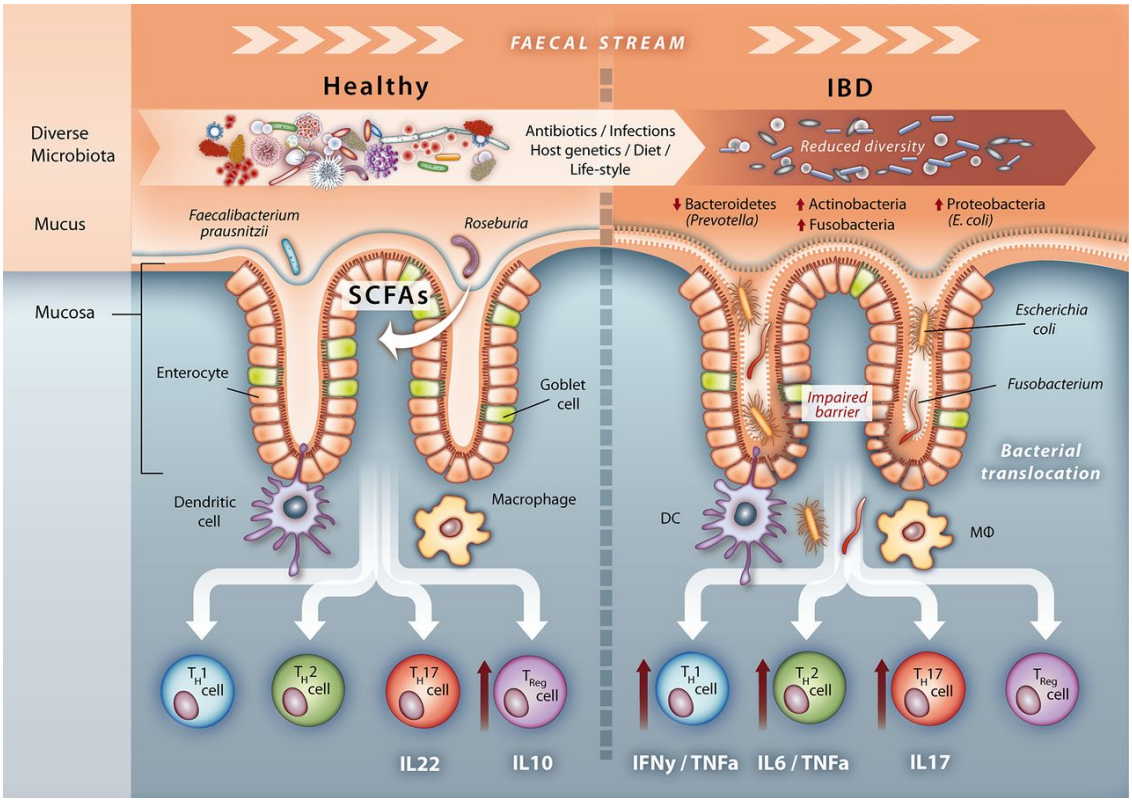


Figure 2: Diagram of a cross view of the mucosa of a healthy and IBD individual (Sommer et al. 2017)

The current treatments for IBDs vastly vary and often change throughout the progression or regression of the disease. They also come with diverse, sometimes harmful side effects (Figure 3). According to the mayo clinic, the first step of treatment is the prescription of anti-inflammatory drugs, 5-ASA, such as Balsalazide, Mesalamine, Olsalazine and Sulfasalazine. The common side-effects of this treatment are headaches and diarrhea. Moreover, 5-ASA has been found to significantly lower the prevalence of *Helicobacter pylori*, a bacterium that has been found to have a protective role against IBDs (Yu et al., 2018). If the previous drugs did not work or stopped working, the next step is the prescription of corticosteroids, such as budesonide. Prolonged, above 12 weeks, use of corticosteroids may lead to osteoporosis, osteonecrosis of the femoral head and susceptibility to infection. Withdrawal effects may include adrenal insufficiency, myalgia syndrome, malaise and intracranial pressure. The next step of treatment, if the corticosteroids fail, is administering immunomodulators comprised of thiopurines (Azathioprine and mercaptopurine)

and methotrexate. Thiopurines are effective for maintaining remission and treating active diseases. Up to 20% of the patients experience side effects from flu-like symptoms to more rarely leucopenia, chemically driven liver damage, and pancreatitis. Methotrexate is effective for remission or relapse prevention of CD. Nevertheless, 10-18% of the patients discontinue the treatment due to the severe side effects (hepatotoxicity and pneumonitis) (Carter, Lobo, and Travis 2004). Biologics are next in the treatment line; they have shown high effectiveness in patients with a corticosteroid-dependent, refractory tendency. Whatsoever, the cost and side effects cannot be overlooked. The worst side effect linked to biologics is the possible development of progressive multifocal leukoencephalopathy, a devasting neurologic illness and neoplasia. This treatment price can go from 6023 USD to 16,258 USD (Stallmach, Hagel, and Bruns 2010). When medical therapy fails, surgery can be the last resort for patients. Approximately 47% of CD and 16% of UC will require surgery in their lifetime ("Inflammatory Bowel Disease: Medical Therapy What Are the Goals for the Treatment of IBD?" n.d.). Different types of surgeries can be performed for UC and CD. Proctocolectomy (removal of the rectum and parts of the colon), ileoanal pouch anastomosis (creating a J-pouch) and colectomies are the surgical treatment for UC. The usual surgeries for CD are laparoscopic ileocecal resection (the removal of the cecum and the terminal ileum), stricturoplasty (the opening up of the narrowed parts of the intestine) as well as resection (the removal of the damaged and diseased parts of the intestines while sewing together the ends of the remaining healthy sections) (Larson and Pemberton 2004).

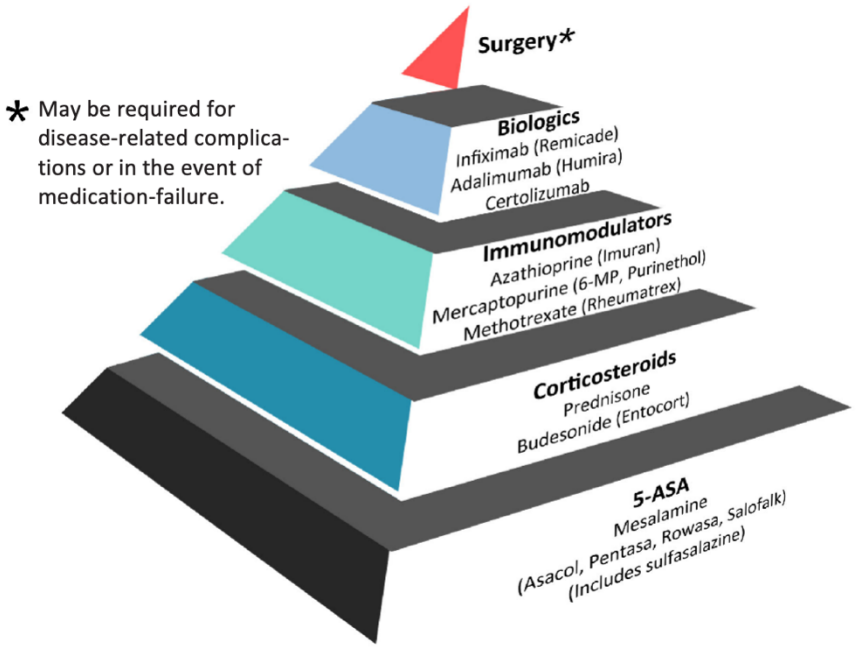


Figure 3: IBD treatment pyramid, the higher on the pyramid, the more invasive the treatment (NSCIBD, 2017)

Although surgical interventions are effective and might improve certain patients' quality of life, they are highly costly, up to 46.354 USD and extremely invasive (Petryszyn and Witczak 2016) (D'Ugo et al. 2020). It is crucial to develop new treatments with lesser side effects and lower costs to treat IBDs.

1.2 The Hygiene Hypothesis

As society evolves, populations in developed countries are mostly living in urbanized environments and are less exposed to pathogens and parasites; primarily due to the advance in science and education. Indeed, parasite infections are extremely low in North America and western Europe, meat is checked for parasites, and populations are taught the importance of cooking foods at high temperatures to kill the possible pathogens. Handwashing, clean water and access to the

clean and modern restroom are everyday privileges that significantly reduce the chances of contracting a parasite. Moreover, many parasites have been eradicated from circulation thanks to easy and broad access to health care. Parasites have co-evolved with humans since the beginning of time and became proficient modulators of the immune system to promote their survival. Nevertheless, could the subtraction of these microorganisms from our organisms also diminish our immunity?

Dr. Strachan initially formulated the hygiene hypothesis in 1989 in his publication "Hay fever, hygiene and household size." In this study, he observed an inverse relationship between family size and atopic diseases such as asthma and eczema; children that had unhygienic contacts with their older siblings showed a lower incidence of atopic diseases as the young children would be exposed to dust, mud, pollen and other outside pathogens brought in by their older brothers or sisters. Immunologists further investigated this concept, and it was added that a decline in early childhood microbial exposure was a major factor in the incidence of atopy (Bloomfield et al. 2006). This hypothesis has since been broadened to include other types of auto-immune diseases such as IBDs and rheumatoid arthritis.

For instance, it has been observed that the geographical distribution of auto-immune disorders in the world is inversed to the prevalence of helminth infections (Figure 4). This observation leads to the rise of a new field of research: helminth therapy. Helminth therapy is defined by the use of parasitic helminth as a novel treatment against auto-inflammatory diseases such as IBD, arthritis, allergies, etc.

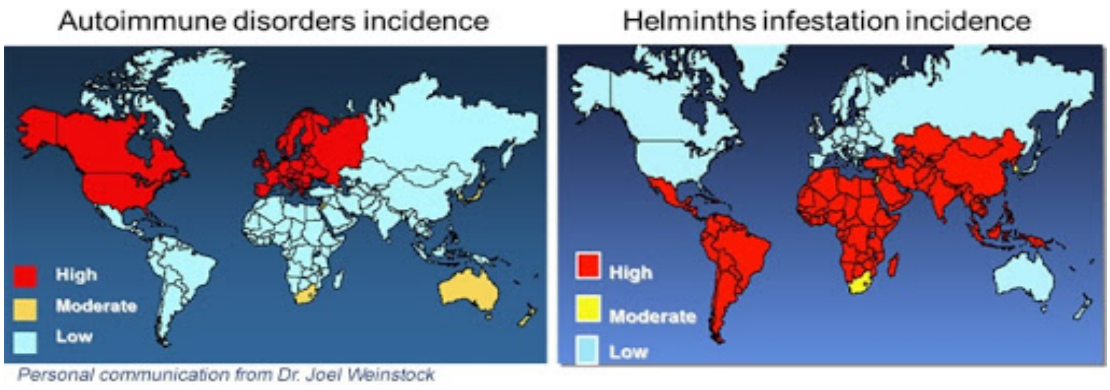


Figure 4: World map of auto-inflammatory incidence vs. helminth infestation incidence. Their incidence is geographically inversed (Joel Weinstock personal correspondence, Coronado Bioscience (n.d.))

1.3 Helminth Therapy:

To assess the efficacity of helminths as an anti-inflammatory therapy, many studies have been performed primarily to evaluate the side effects and possible adverse complications and identify which species of helminths show the most efficacity. These parameters were investigated through mice models and clinical trials.

In his study "Induction of Th2 response Inhibition of Th1 response," Dr. Khan and his team investigated the effects of shifting the immune system of mice toward a Th2 response using *Trichinella spiralis*, a pig roundworm. Th1 type immune response is usually associated with CD, whereas a Th2 type immune response is associated with helminth infections, reducing the effects of Th1 mediated diseases. In this study, 375 *Trichinella spiralis* infective larvae were orally administered to the experimental mice 21 days before colitis induction. On day 21, colitis was induced using dinitrobenzene sulfonic acid (DNBS), whereas control mice were intercolonially injected with 50% ethanol. The mice were euthanized, and their colons were removed to assess the severity of colitis, macroscopically and histologically. The macroscopic score (calculated by evaluating a scale to mucosal damage, thickening of the colonic wall, presence of adhesion between the colon and other organs, the constituency of feces and the presence of hyperemia) was

7.5 for the DNBS group and 5 for the DNBS+*T. spiralis* group on day 3 versus 2.8 for the control group. The same pattern was observed on day 7, with a score of 3 for the DNBS group and 2.6 for the DNBS+ *T. spiralis* group. The mice that were treated with *T. spiralis* show lesser macroscopic damage compared to the control group. The histology score (a measurement that allows experts to assess the severity of colitis by assigning a score to the degree of mucosal architectural changes, cellular infiltrations, goblet cell depletion and the presence of crypts abscesses) illustrated the same observation as the macroscopic score. On day 3 of the induced colitis, the DNBS group had a score of 8.5, whereas the DNBS+*T. spiralis* group had a score of 5.5, and on day 7, the DNBS group had a score of 5.2 and the DNBS+*T. spiralis* had a score of 3. This study shows that mice infected with *T. spiralis* larvae before the induction of colitis show lesser colonic damages than the colitis-only group. This study suggests that a pre-infection of *T. spiralis* protected the mice from developing the worst symptoms (Khan et al. 2002). However, what would occur to the inflammation if the helminths were administered after the inducing of colitis? Which would recreate a setting more similar to IBD patients trying to treat their symptoms after contracting the disease.

This is a question that Dr. Elliott and his team decided to answer. In this study, IL-/- mice were given piroxicam (NSAID) for two weeks to induce severe colitis. Two days after the end of the NSAID administration, the experimental group of mice was colonized with 200 third stage *Heligmosoides polygyrus* (*H. poly*, rodent's roundworm) larvae via gastric gavage and the control was not. The mice's colons were removed and histologically assessed and looked at the inflammatory score (blindly scored on a scale from 0-4 by trained pathologists). They found that the control group (NSAID alone) had a score of 3.5, whereas the experimental group (NSAID+ *H. poly*) had a score of 0.5 (Elliott et al. 2004). This study shows that post-colitis induction *H. poly*

colonization improves the severity of the inflammation. Therefore, they could be used as a treatment to improve the symptoms of IBD patients.

Several clinical trials have been performed since; one of the most notable clinical trials was conducted in 2003 by Dr. Summers in which he repeatedly infected CD patients with a CD activity index (CDAI) above 220 (a score determining the severity of the CD, under 150 being in remission and above 220 being moderate to severe), with 2500 *T. suis* ova (TSO) every three weeks for 24 weeks. He found that on the 24th week of treatment, 72.4% of the patients were remitted by displaying a CDAI score of under 150. Nevertheless, those ameliorations were temporary with a single dose, but maintenance with three-weeks of re-inoculations fixed this problem. This shows that continuous infection is needed for patients to keep those benefits (R. W. Summers et al. 2005). Controversially, it should be noted that a similar double-blind clinical trial was performed in 2017, with 252 CD patients treated with either 250, 2500 and 7000 TSO or a placebo. Although they found that a range from 250-7500 TSO is safe as a treatment, however, it did not show a clinically relevant effect over the placebo treatment (Schölmerich et al. 2017). Dr. Summers, soon after his first clinical trial, tested TSO on UC patients. He chose 54 patients with a UC index of less than 4. This index is established by giving a score from 0 to 3 to stool frequency, rectal bleeding, mucosal appearance, and UC's rating by a physician. Thirty patients received 2500 *T. suis* ova, and 24 patients received a placebo. The patients were inoculated every two weeks for 12 weeks. It was found that 43.3 % of the patients treated with *T. suis* ova treatment showed an improvement in their UC index and that 16.7% of the placebo group showed an improvement (Robert W. Summers et al. 2005). Surprisingly, a human hookworm, *Nector americanus*, was also tested in a CD pilot clinical trial. Nine patients with active CD were inoculated with stage 3 infective larvae and re-inoculated from week 27 to week 30; their CDAI were monitored and used to illustrate the treatment's efficacity. The CDAI did lower with a mean of 165 vs 75 initially, although four

patients dropped out of the trial, and some side-effects such as itching were recorded (Croese et al. 2006). For apparent reasons, *N. americanus* is not the best option as a novel IBD treatment as there is a need for a human reservoir host, which would be highly unethical. Other similar clinical trials were performed and listed in addition to the mentioned trial in the table below (Table 1).

Nonetheless, it is important to consider the likelihood that patients would not accept to be treated with live worms; indeed, in a modern society like ours, many patients will refuse a live worm treatment due to the "gross" factor as well as the potential but rare side effects and risks such as accidental migration. Therefore, finding a way to deliver the healing and protective abilities of the helminths without the actual colonization is crucial. More recently, a study decided to look at the effect of helminth excretory-secretory proteins (ESP) in the colitis animal model. ESPs are small proteins secreted by a helminth in the gut or culture media in a laboratory that can then be harvested in the laboratory to use in models by recovering the culture media of helminths and using columns to fractionate the ESPs based on their polarity. It was shown that *Ascaris suum* (a pig roundworm) metabolites have positive outcomes in a DSS-induced colitis animal model. They found that the mice treated with the *A. suum* ESPs suffered from lesser weight loss than the control group and that their colon did not shorten as much as the control group (Siciliani et al. 2020). Colon shortening is one of UC's characteristics; the observation of a shorter colon is associated with more severe colitis. In contrast, a longer colon would be associated with less severe colitis. The study of these specific proteins is the future of helminth therapy.

Table 1: Major clinical trial using several different helminth species in IBD and other auto-inflammatory diseases (Adapted from Poles et al., 2016, Maruszewska-Cheruiyot, Donskow-Lysoniewska, and Doligalska 2018)

Author	**Trial**	**Results**
Summers et al., 2003	A single or repeated dose of 2500 live eggs of *T. suis* was administered every three weeks for 28 weeks to 3 UC and 4 CD patients.	No side effects Remission was noticed on every patient administered with repeated dose.
Summers et al., 2005	A repeated dose of 2500 live eggs of *T. suis* was administered every three weeks for 24 weeks to 29 CD patients.	No side effects. Remission was noticed on 72.4% of patients.
Summers et al., 2005	A repeated dose of 2500 live eggs of *T. suis* was administered every two weeks for 12 weeks to 54 UC patients.	No side effects. Remission was noticed on 43.3% of patients.
Sandborn et al., 2013	A single dose of 500, 2500 or 7500 live eggs of *T. suis* administered to 36 CD patients.	Every dose is very well tolerated. Quantity of dose does not influence gastrointestinal tract response.
Coroesee et al., 2006	A single or repeated dose of 25-50 L3 larvae of *N. americanus* was administered to 9 CD patients.	Side effects: itching, enteropathy, eosinophilia. The condition of the majority of the patients improved.

NYU Langone Health, 2012	2,500 *T. suis* eggs by mouth every two weeks for 12 weeks in 4 UC patients.	Change from Baseline of the Simple Clinical Colitis Activity Index at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 Weeks

Microbiome derived metabolites:

As mentioned earlier, the microbiome plays a critical role in the health of the guts. Microbiome-derived metabolites are products originating from the gut microbiota and the host. They behave like messengers between the gut microbiome and the host and take part in many different physiological processes such as energy metabolism, cell-to-cell communication and host immunity (Lavelle and Sokol., 2020). These metabolites are currently being isolated thru serum, urine and fecal samples, and their chemical structures being identified via mass spectrometry. These new findings allow researchers to find analogs to specific metabolites of interest. These analogs might be commercially available, helping to have access to large quantities of those analogous metabolites. These metabolites can be used to test their properties and abilities in different contexts. In an unpublished study by Dr. Xia from McGill's Institute of parasitology, the microbiome of healthy vs unhealthy patients was investigated, and the metabolites secreted were identified. They noticed which kind of metabolites were low in IBD patients but higher in healthy individuals. These specific metabolites were then catalogued, and their chemical analogs were identified. They found that hydroxycinnamic acid, 3, 4 dimethyl benzoic acid and 3-methylphenylacetic acid, urocanic acid and N-Acetyl-L-phenylalanine were lowered in the microbiota of IBD patients. As mentioned earlier, microbiota alterations are commonly seen in

IBD patients. Microbiome-derived metabolites become new tools to target certain pathological bacteria families such as the Enterobacteriaceae family are in high prevalence in the gut, such as *Salmonella enterica.* Indeed, it has been found that *Salmonella* promotes the onset of IBDs by permanently changing the intestinal microbiota, disturbing the epithelial barrier and alternating the immune response after an infection (Schultz et al., 2017). *Salmonella typhimurium* owes its host cell invasion success to its ability to modulate its gene expression. A study showed that certain aromatic (cyclic compounds) microbiome-derived metabolites could repress the expression of the gene encoding the master regulator of the invasion gene in *Salmonella (hiiA).* They found that cells treated with hydroxycinnamic acid, 3, 4 dimethyl benzoic acid and 3-methylphenylacetic acid showed a significantly lower expression of *hiiA* by flow cytometry (Peixoto et al. 2017). These two studies suggest that, perhaps, these specific microbiomes derived metabolites and their analogs play an essential role in the protection of the guts, and their decrease can be linked to inflammation,

To summarize, IBD prevalence has increased alarmingly. The current treatment, besides, to be costly, can come with severe side effects and might lead to essential surgeries such as the removal of parts of the large intestine and colon. Finding safer, efficient and affordable solutions is crucial—the hygiene hypothesis undercover the possibility to use helminth and microbiome metabolites as capable and safe treatments. Many studies have been performed to assess these metabolites' efficacy in animal models using these metabolites in mice models and other models. Still, the healing capacities of these metabolites *in-vitro* on human tissue have never been explored. My master thesis aims to assess and understand the healing abilities of several helminthic and microbiome-derived metabolites at different concentrations using a scratch assay. To recreate a colon-like environment, we decided to use the caco-2 cell line, human colon epithelial cells from colorectal adenocarcinoma. This cell line forms a tissue that adheres to the plates' bottom and is an adequate *in vitro* model to replicate the wounds IBD patients suffer. We

decided to test three different helminth species: *Ascaris suum (A. suum), Trichuris suis (T. suis)* and *Heligmosomoides polygyrus (H. poly)*, all of these species showed efficacity in animal models and clinical trials as illustrated in the table below (Table 2).

Table 2: Helminth metabolites and their relevance.

Species	**Study**
A.suum	It was found that *A. suum* positively modulates allergic and asthma inflammation in mice (Araújo et al. 2008) (Itami et al. 2005).
T. suis	The mice model and the clinical trial showed that a *T. suis* infection improved CD and CD patient condition.
H. poly	Mice model showed that *H. poly* efficacity in decreasing the inflammation during induced colitis.

We will also test urocanic acid, as it has been found by Dr. Jardim laboratory (not publish at the moment of the submission of this thesis) to be an analog for *T. suis*MP686 (Figure 5), a *T. suis* metabolite that showed protective abilities in a mice model. This specific metabolite was identified via mass spectrometry. It was found that urocanic acid was the closest related compound to Urocanic acid, which, has also been shown to be lowered in IBD patients'

microbiome and has shown protection ability in a DSS induced colitis study (Unpublished Dr. Xia) (Kammeyer et al. 2012).

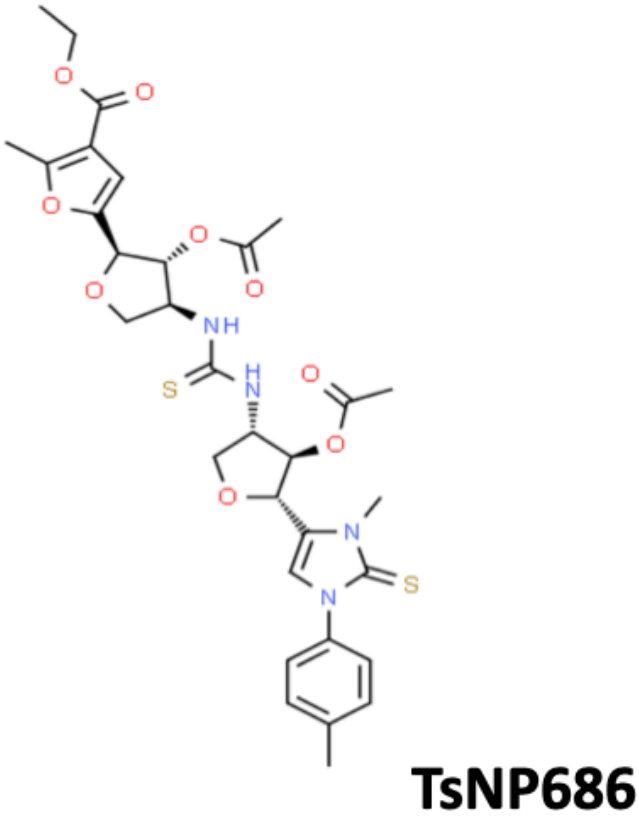
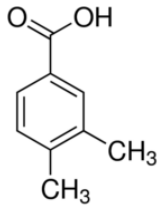
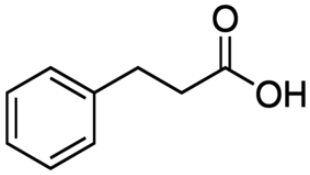
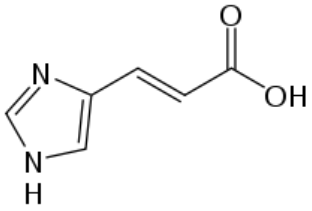
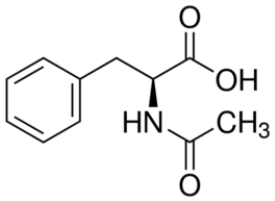
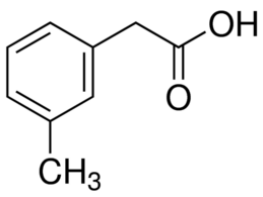


Figure 5: Chemical structure of an effective *T. suis* metabolite (Jardim, unpublished)

These helminth metabolites were extracted based on their polarity, allowing us to test a wide range of different concentration of different polarity of metabolites to assess their healing capacities. Furthermore, several microbiome-derived metabolites were also tested: Urocanic Acid, 3,4 dimethyl benzoic acid, 2-hydroxycinnamic acid, N-acetyl-L-phenylalanine and m-tolyacetic acid mentioned in the paragraph above and listed with their relevant activities and chemical structures in Table 3 below.

Table 3: Microbiome-derived metabolites, their chemical structure and their benefits.

Chemical	**Chemical structure**	**Relevance**
3,4 dimethyl benzoic acid		-Showed strong inhibition of the gene encoding the regulation of invasion genes in *salmonella*, a bacterium linked to IBDs (Peixoto et al. 2017). - Lowed in the fecal matter of IBD patients in Dr. Xia's study.
2-hydroxycinnamic acid		-Showed strong inhibition of the gene encoding the regulation of invasion genes in *salmonella,* a bacterium linked to IBDs (Peixoto et al. 2017). - Lowed in the fecal matter of IBD patients in Dr. Xia's study.
Urocanic acid		-*Ex- vivo* and *in vivo* IBD models showed that Urocanic acid has an anti-inflammatory effect (Kammeyer et al. 2012)

		- Lowed in the fecal matter of IBD patients in Dr. Xia's study
N-Acetyl-L-phenylalanine		- Lowed in the fecal matter of IBD patients in Dr. Xia's study.
m-tolyacetic acid		-Showed strong inhibition of the gene encoding the regulation of invasion genes in *salmonella*, a bacterium linked to (Peixoto et al. 2017. - Lowed in the fecal matter of IBD patients in Dr. Xia's study.

HYPOTHESIS

We hypothesize that helminth and microbiome-derived metabolites accelerate the healing of gastrointestinal epithelial cells in an *in vitro* IBD model.

OBJECTIVES

1. Create a platform to screen metabolites for the ability to heal epithelial wounds.
2. Identify which helminth metabolites has a greater healing capacity on epithelial wounds and at which concentrations.
3. Identify which microbiome-derived metabolites has a greater healing capacity on epithelial wounds and at which concentrations.

CHAPTER II

METHODS

METHODS:

2.1 Helminth metabolites extraction

The metabolites were extracted by Dr. Armando Jardim's laboratory from the Institute of parasitology, McGill University. The helminths were set in culture media for 24 hours to allow them to excrete their excretory-secretory proteins (ESP). The culture media was sequentially centrifuged and filtered through a 3kDa filter to remove larger molecules. The supernatant was passed through a C18 column; the column was then washed with acetonitrile (ACN) to isolate the different fractions of non-polar metabolites (100%, 50% and 25%). The C18 column's flow-through was passed through an activated charcoal column, which was then washed with ACN to isolate the different fractions of non-polar metabolites (10%, 50% and 25%). The ACN was removed via vacuum filtration.

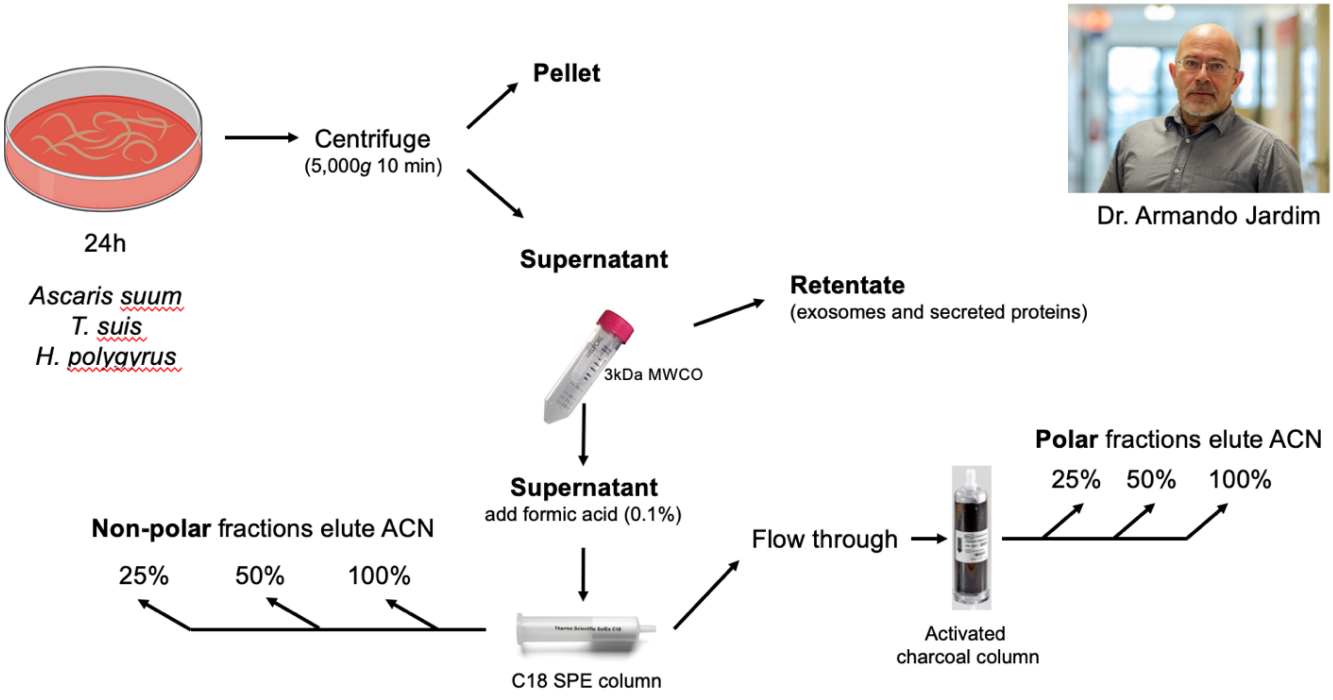


Figure 6: Helminth metabolites extraction process. The proteins were separated based on their polarity to recover the metabolites of interest (polar).

2.2 Caco-2 culture:

The original caco-2 cells were provided to us by Dr. Gruenheid's laboratory from the department of microbiology at McGill University. The Caco-2 cell line is immortalized from the cells of 72 years old male cancer patient suffering from colorectal adenocarcinoma ("Caco-2 [Caco2] ATCC ® HTB-37TM Homo Sapiens Colon Colorectal" n.d.). The cells were thawed and placed in a T25culture flask with media (5% FBS DMEM media + Penicillin/Streptomycin + non-essential amino acids). When the cells were confluent, they were washed twice with PBS to remove the FBS, which, if not removed, would not allow the trypsin to dislocate the cells in the next step. The cells were dislocated from the plastic using 3mL trypsin; the flask was incubated for 40 minutes. Once the incubation period over, the trypsin was neutralized using 6 mL of media. The trypsin-media liquid was then added to a 10mL test tube and centrifuged (1500 RPM, 4ºC, 5min). The supernatant was discarded, and 5mL of media was added. The cells and media were mixed using a 10mL pastor pipette. The 5mL of cell-media mix was added to a T75 culture flask. The cells were maintained and passed approximately once every week. Some cells were also frozen in liquid nitrogen to keep a stock of low passage cells.

2.3 Scratch experiment- 6 wells plate.

As the cells were passed, 10µL were transferred to a 1.5mL microtube. 90µL of media was added to the microtube and mixed. 10µL of the mix was mixed with 10µL of trypan dye. 10µL of this mix was then added to a cell count microscope slide, and a cell count was performed. The amount of cell-media mix consisting of a million cells was added to each well of 6 wells plates and 2mL of media. The six wells plates were monitored every day under the microscope. Once it was observed that the cells were confluent, the previous media was discarded, and 1mL of starving

media (0.05% FBS DMEM media + Penicillin/Streptomycin + non-essential amino acids) was added to each well.

Starving media is used in order to observe the migration of the cells and not cell proliferation. Twenty-four hours after the addition of the staving media, the cells were wounded using a razor blade. The razor blade was positioned perpendicular to the cells and dragged for a couple of centimetres. The media was then removed, and the cells were washed with PBS before the addition of 2mL of 5% DMEM media and the treatment (helminth metabolites or microbiome metabolites or control). The six wells plates were then incubated for 48 hours. After 48 hours, the plates were removed from the incubator, and the media was removed and saved for further analysis. The cells were washed with PBS, and 2mL of cold methanol was added to each well. The plates were incubated at room temperature for 15 minutes and transferred to the fridge for 30 minutes. After 30 minutes of fridge incubation, the methanol was removed, and the cells were washed with PBS. Methylene blue (1:4 in ddH2O, 2mL) was added to each well, and the plates were incubated in the fridge for 30 minutes. After the 30 minutes incubation period, the cells were washed with PBS and consequently with ddH2O. The plates were then taken to the microscope.

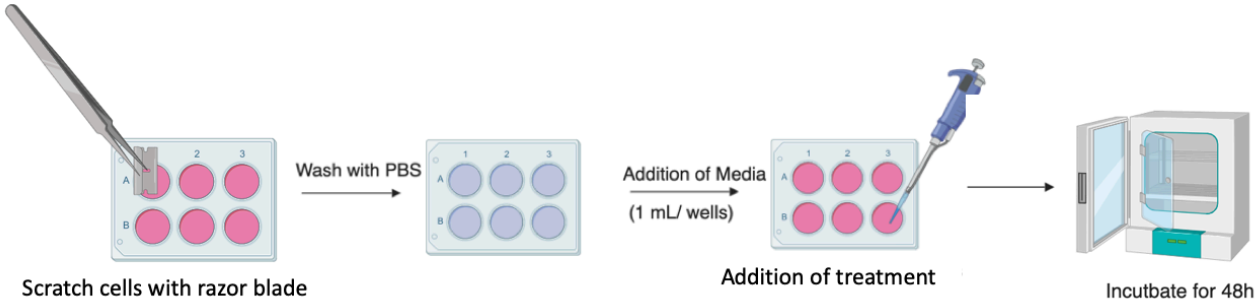
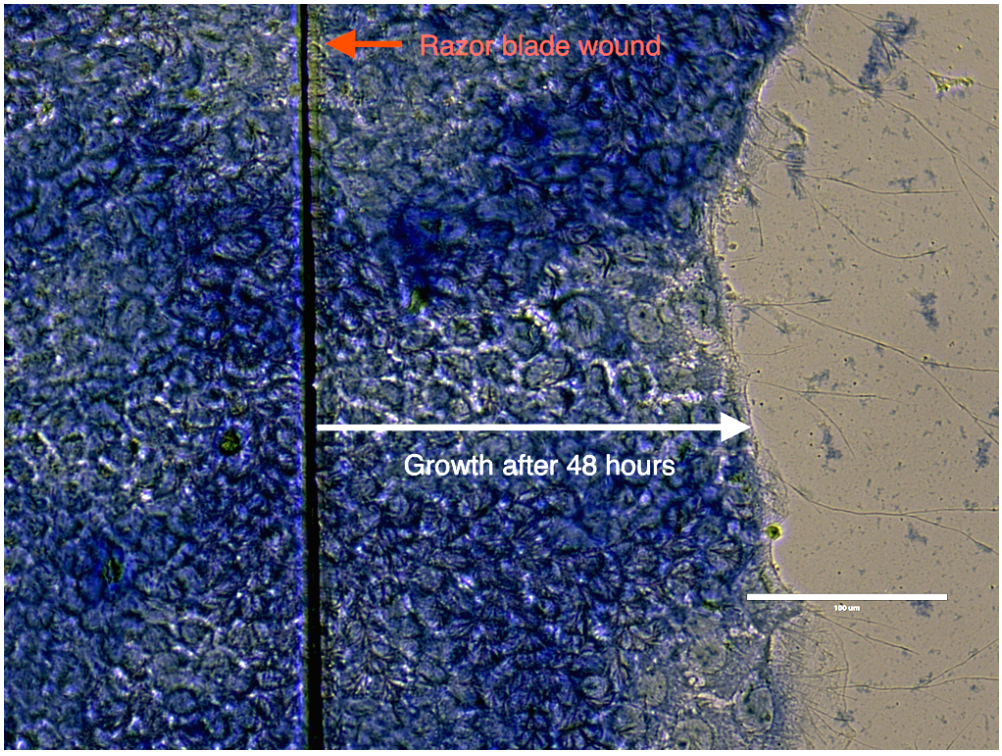


Figure 7: Scratching process for the 6 wells protocol. A wound is created using a razor blade, the cells are then washed with PBS, the respective treatment and 1mL of media is added to each well. The plate is then incubated for 48h.

2.4 6 wells plate analysis.

The fixed and stained six wells plates were taken to the microscope. The magnification was set at 10. Three pictures were taken along the dark line left by the razor blade in each well. Each picture was saved on a USB key and then uploaded to a computer (Figure 7). To analyze pictures, the software ImageJ was used. The polygon selection highlighting feature was used to outline the cells' growth area after the dark razor blade line (Figure 9). The growth areas are quantified in squared pixel. Each image's growth area was saved and entered into the software Prism 6, in which each wells' results were averaged. The growth for each treatment was compared to the control's growth.

A



B

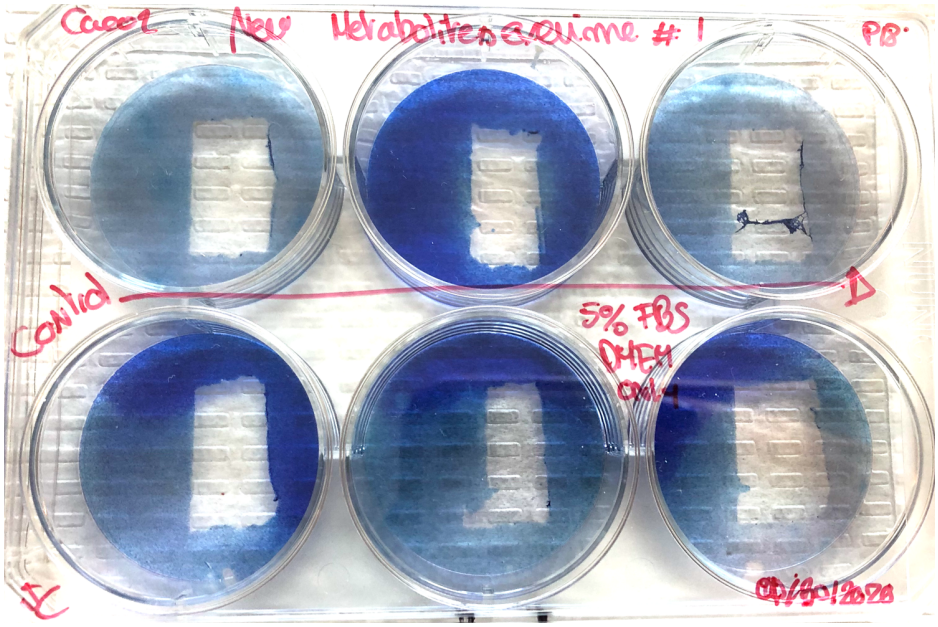


Figure 8: Photograph of the razor wounds on the stained Caco-2 cells, showing the initial razor wounds taken at a magnification of x10 with a light microscope (A). The 6 wells plate pictured above is a control plate for one of our experiments, the wounds and the starting line of the razor blade can clearly be observed (B).

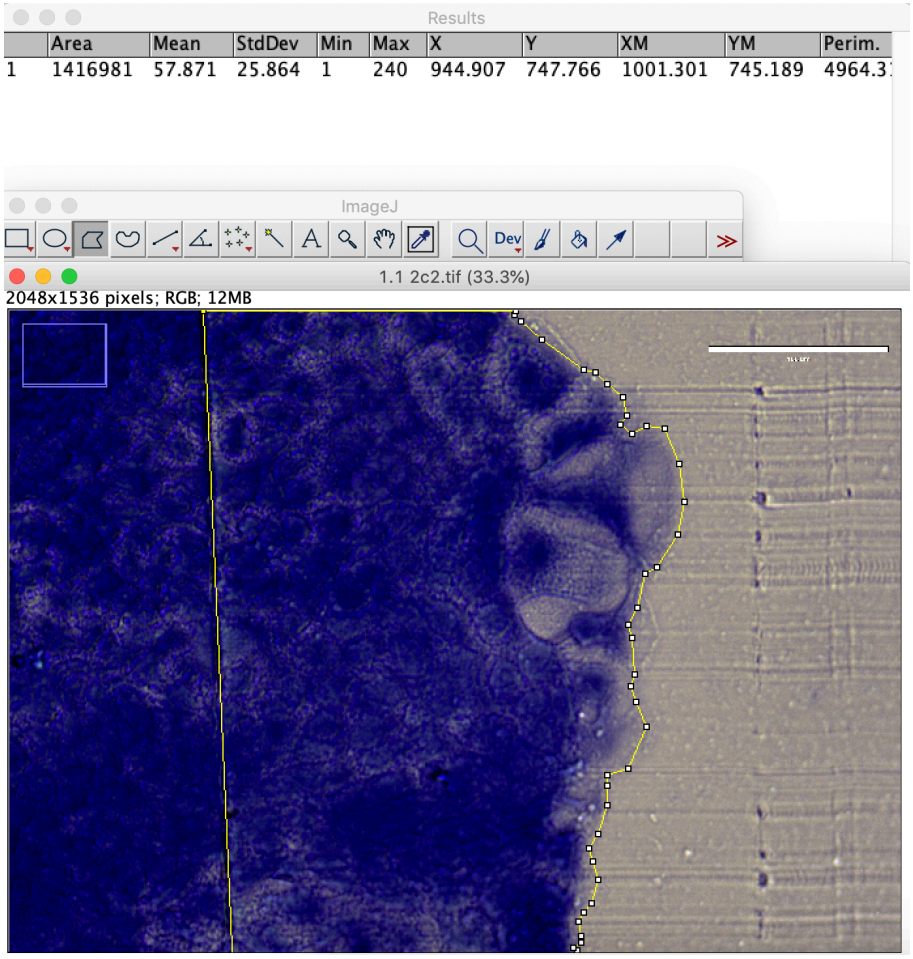


Figure 9: ImageJ process in analyzing the pictures from wound assays, the photograph shown above was taken at x10 magnification with a light microscope. The yellow line showing the outline of the area, created using the polygon option. The area is displayed in the chart at the top of the image.

2.5 Scratch experiment-96 wells plate

As the cells were passed, 10µL were transferred to a 1.5mL microtube. 90µL of media was added to the microtube and vortexed. 10µL of the mix was mixed with 10µL of trypan dye. 10µL of this mix was then added to a cell count microscope slide, and a cell count was performed. The amount of cell-media mix consisting of 250 000 cells was added to each well of a 96 wells plate,

as well as the remaining volume of media needed to have a final volume of 200µL. The 96 wells plate was monitored every day under the microscope. Once it was observed that the cells were confluent, the previous media was discarded, and 1mL of starving media (0.05% FBS DMEM media + Penicillin/Streptomycin + non-essential amino acids) was added to each well. Twenty-four hours after the addition of the staving media, the cells were wounded using a 20 µL micropipette tip connected to a tube connected to an air pomp to aspire the cells in the middle of each well by suction (Figure 10). The suction left a circular wound at the center of the well. The treatment or control was then added to each well with media to accomplish a final volume of 200µL.

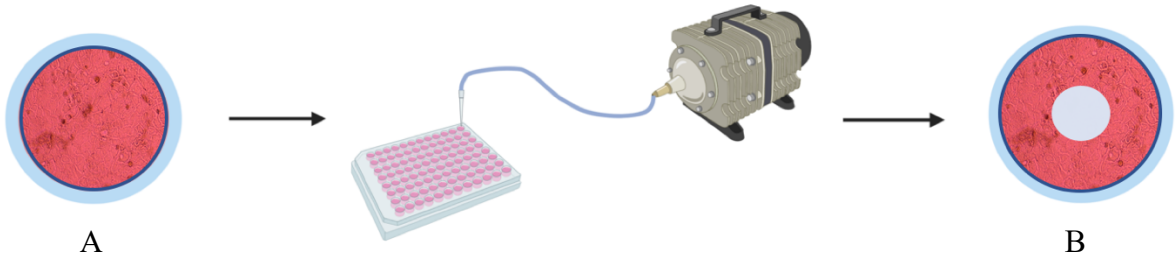


Figure 10: The process used to create the circular wound using an air pump. The circle "A" represents a well in the 96 wells plate prior to the suction created by the air pump and the circle "B" represent the same well after the suction.

2.5.a Incucyte: The 96 wells plates were incubated for 24 hours in the Incucyte, a live-cell imaging and analysis platform that quantifies cell growth over time by automatically gathering and analyzing images during the length of an experiment's incubation period. The Incucyte has a structure in which the 96 wells plate is securely placed. Using the software IncuCyte Zoom, the position of the 96 wells plate was indicated to the machine (Figure 11). Using the same software, the time lapse between each photograph was set (for example, a photograph each 30 min or hour)

and the start and endpoint of the incubation period. The cell growth was also calculated with the IncuCyte ZOOM software.

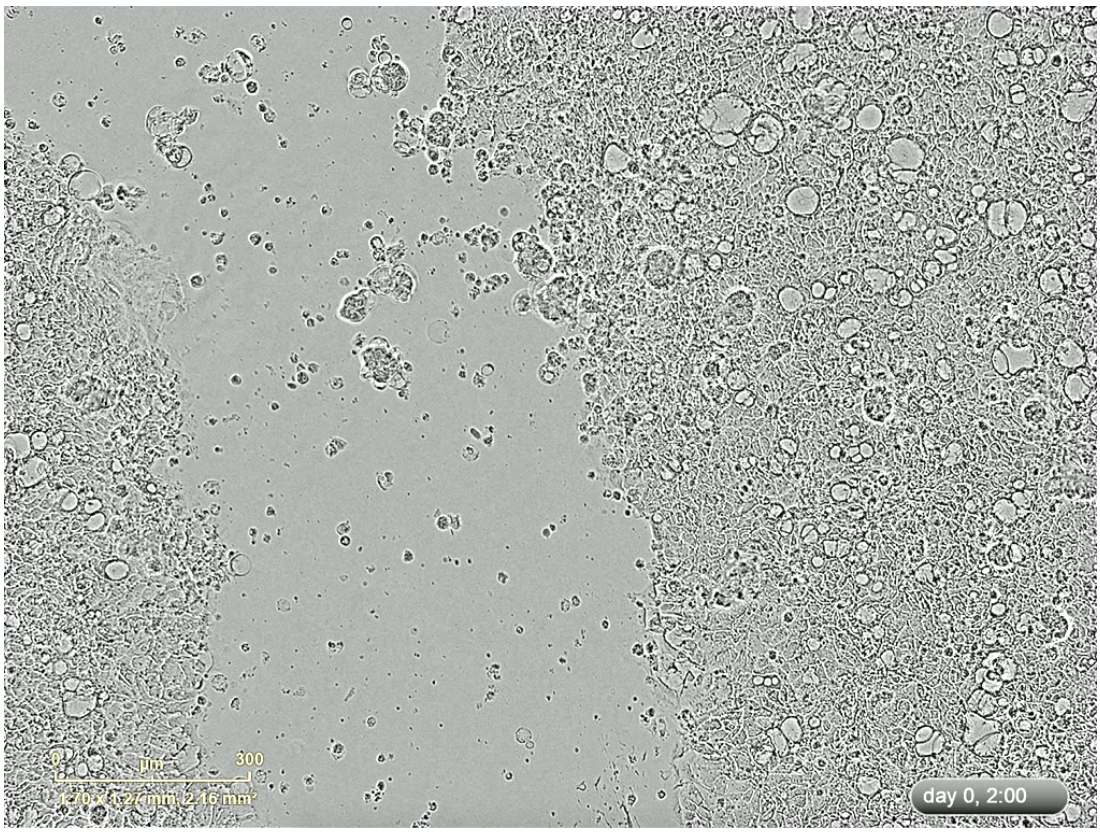


Figure 11: Photograph of a vertical wound created in the well of a 96 wells plate taken by the IncuCyte on day 0 at hour 2.

2.5.b ImageJ: A picture of each circular wounds was taken with the microscope and saved before adding the treatment or control. After 24 hours, a picture of each circular wounds was taken. Using ImageJ, the final area was subtracted from the initial area to give the growth area (Figure 12). The growth areas were recorded in Prism 6. The growths of each treatment were compared to the control growth.

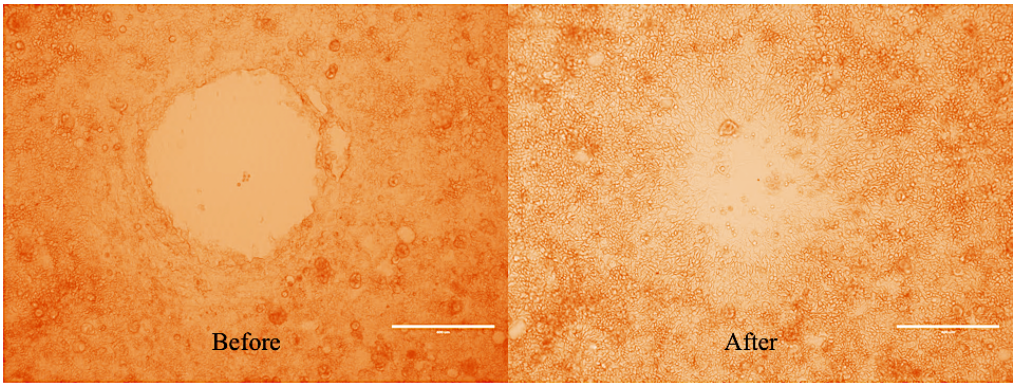


Figure 12: Before and post-treatment photographs of the circular wound in a 96 wells plate. The photographs were taken at a magnification of x40 with a light microscope.

CHAPTER III

RESULTS

RESULTS

3.1 Helminth metabolites

3.1.1 *A. suum* metabolites

A million cells were seeded into each well of a six wells plate. The cells were wounded using a razor blade (as described in the method section above) and treated with a treatment (either control or *A. suum* metabolites). The different *A. suum* metabolites tested were from the 50% and 25% ACN fractions as well as *A. suum* ESPs. Different concentrations of these treatments were tested. Their growth was quantified via fixation, staining and microscopy as described in the methods section above.

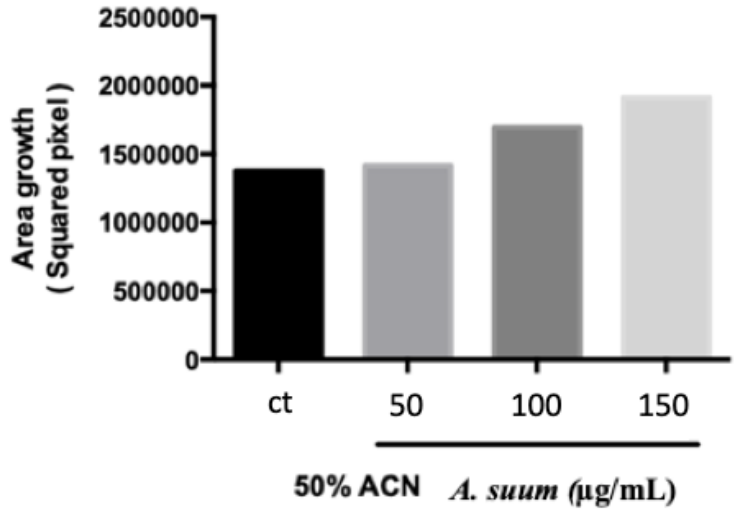


Figure 13: Averaged growth in squared pixel with 50% ACN *A. suum* (50, 100, 150 µg/mL) treatments (n=1).

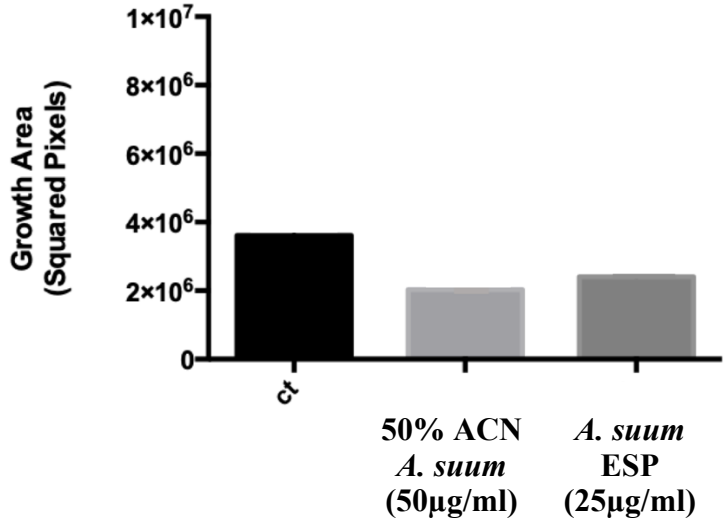


Figure 14: Averaged growth in squared pixels with a 50 % ACN *A. suum* and *A. suum* ESP treatments (n=1).

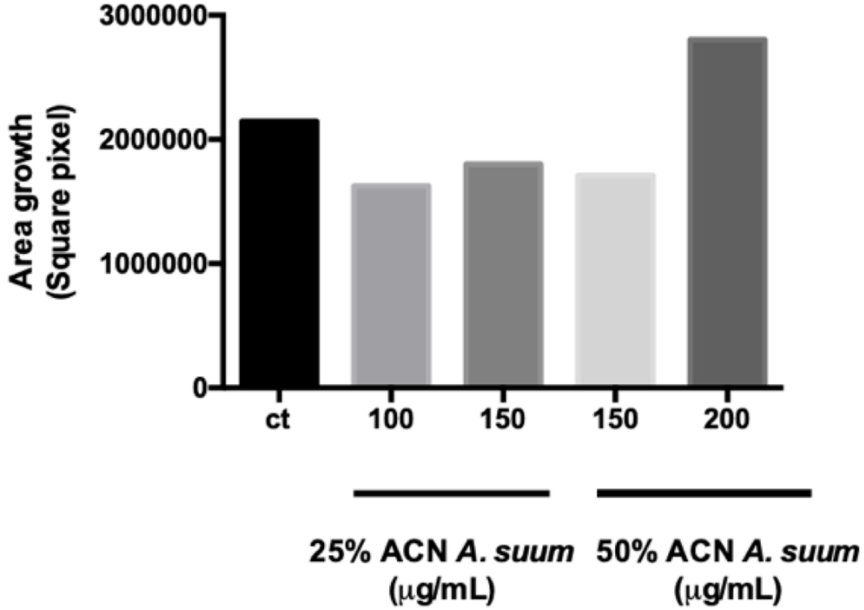


Figure 15: Averaged growth in squared pixels with 50 % ACN *A. suum* (150 and 200 µg/mL) and 25% ACN *A. suum* (100 and 150 µg/mL) treatments (n=1).

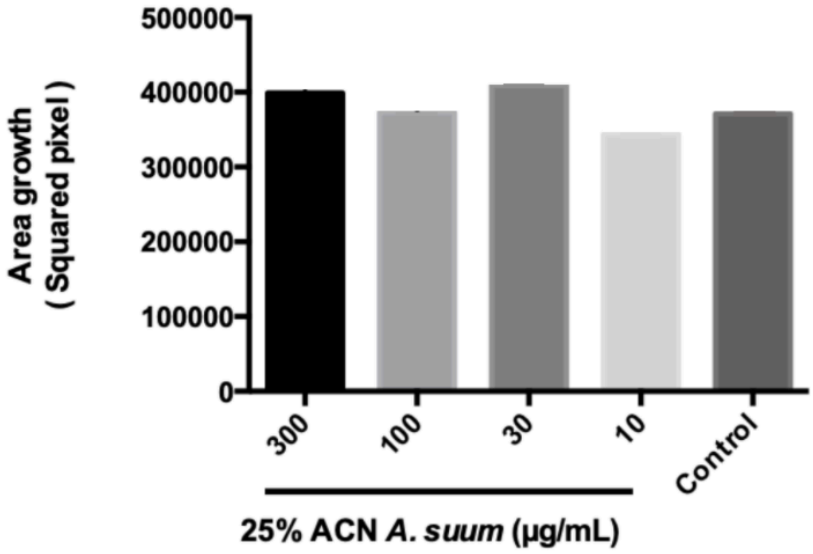


Figure 16: Average growth in squared pixels of the cells treated with 25% ACN *A. suum* (300,100,30,10 µg/mL) treatments (n=1).

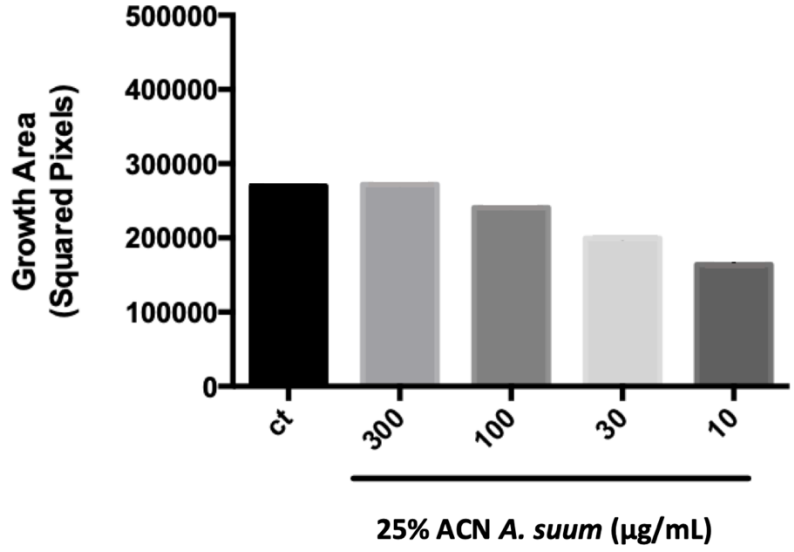


Figure 17: Average growth in squared pixels of the cells treated with 25% ACN *A. suum* (300,100,30,10 µg/mL) treatments (n=1).

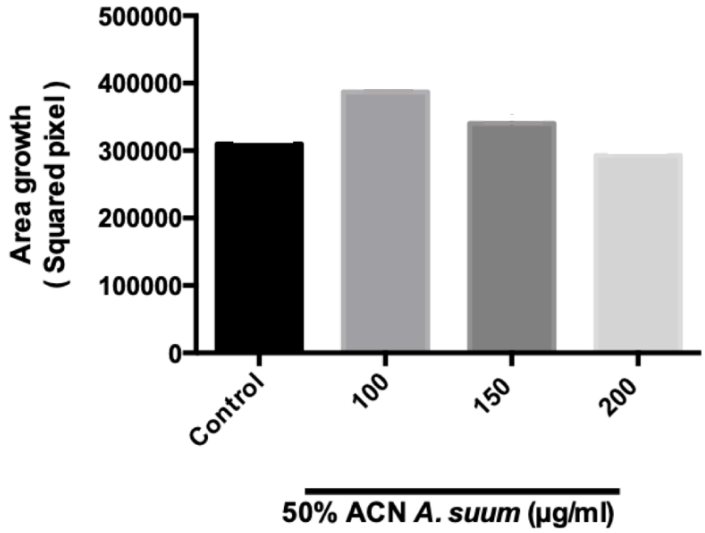


Figure 18: Averaged growth in squared pixels with 50 % ACN *A. suum* (50 and 150 and 200 µg/mL) treatments (n=1).

We found that the growth of the cells treated with 50% ACN *A. suum* at a concentration of 50 and 100 µg/mL did not show an increased growth when compared to our control, but the cells treated with 150 µg/mL 50% ACN *A. suum* showed an increase in growth (Figure 13). The average growth of the cells treated with both 50% ACN (50 µg/mL) and *A. suum* ESP (25 µg/mL) did not show bioactivity (Figure 14). Additionally, we saw that the 100 and 150µg/mL concentrations of 25% ACN *A. suum* a lower growth than the growth of the control; on the other hand, 200µg/mL of 50% ACN *A. suum* showed higher growth than the control; however, the results from that treatment come from only one technical replica (Figure 15). Different concentrations of 25% ACN *A. suum* (300,100, 30, 10 µg/mL) did not show higher growth than the control (Figure 16 and 17). Lastly, 50% ACN *A. suum* was tested again, at 100, 150 and 200 µg/mL, but the growth was not higher than the control. (Figure 18).

A. suum crude extracts were also tested, but the growth could not be quantified as the tissue was destroyed and completely dissociated from the plastic bottom of the six wells plates.

3.1.2 *T. suis* metabolites

A million cells were seeded into each well of a six wells plate. The cells were wounded using a razor blade (as described in the method section above) and treated with a treatment (either control or *T. suis* metabolites). The different *T. suis* metabolites tested were from the 100%, 50% and 25% ACN fractions as well as *T. suis* whole worm extracts. Different concentrations of these treatments were tested. Their growth was quantified via fixation, staining and microscopy as described in the methods section above.

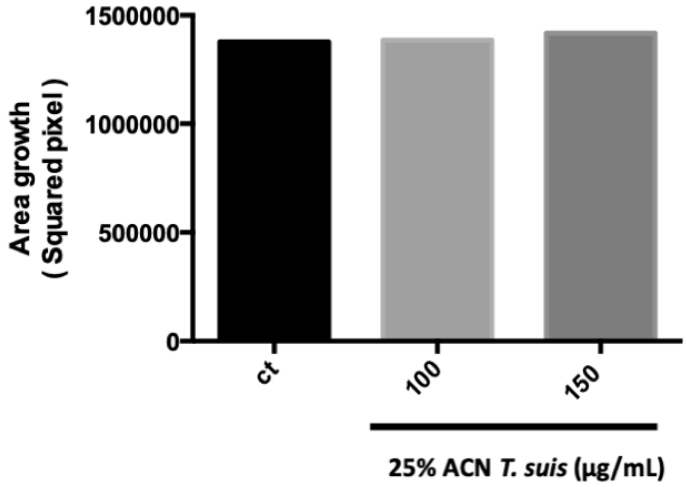


Figure 19: Averaged growth in squared pixel with 25% *T. suis* (100 and 150 µg/mL) treatments (n=1).

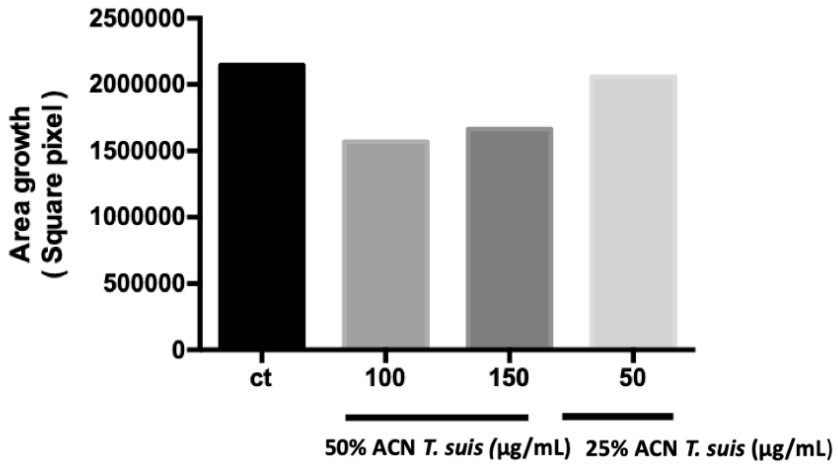


Figure 20: Averaged growth in squared pixels with 50 % *T. suis* (100 and 150 µg/mL) and 100% *T. suis* (50 µg/mL) treatments (n=1).

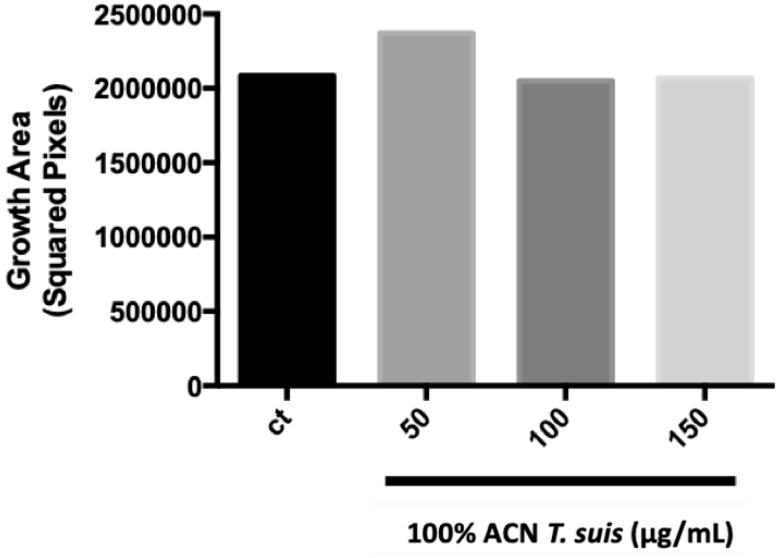


Figure 21: Averaged growth in squared pixels with 100 *% T. suis* (50 and 100 and 150µg/mL) treatments (n=1).

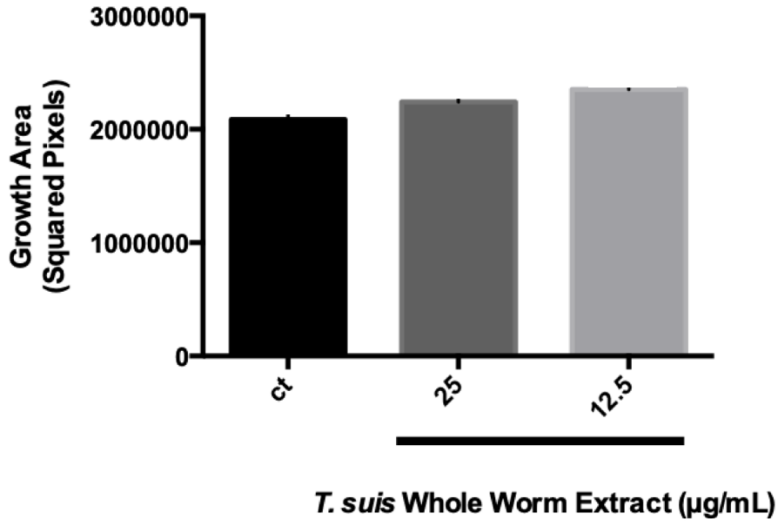


Figure 22: Averaged growth in squared pixels with *T. suis* whole worm extract (25 and 12.5 µg/mL) treatments (n=1).

None of the deferent *T. suis* fractions or ESP treatments showed higher healing abilities compared to their controls (Figure 19-22).

3.1.3 *H. polygyrus* metabolites

A million cells were seeded into each well of a six wells plate. The cells were wounded using a razor blade (as described in the method section above) and treated with a treatment (either a control or *H. polygyrus* metabolites). Different concentrations of the 50% ACN fraction were tested. Their growth was quantified via fixation, staining and microscopy as described in the methods section above.

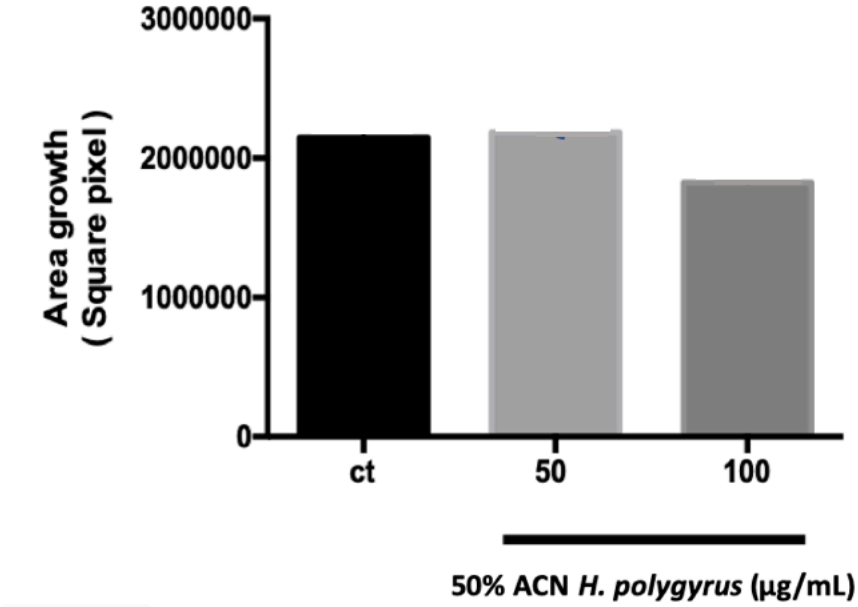


Figure 23: Averaged growth in squared pixels with 50 % *H. polygyrus* (50 and 100 µg/mL) treatments (n=1).

The cells treated with 50 and 100 µg/mL of 50% ACN *H. polygyrus* treatments did not show higher cell growth when compared to the control (Figure 23).

3.2 Microbiome derived metabolites

Approximately 250,000 cells were seeded into each well of a 96 wells plate as well as one million cells per wells of 6 wells plates depending on the experiment. The cells were wounded using a razor blade (as described in the method section above) for the 6 wells plate groups or by the 20µL micropipette tip connected to an air pump and treated with a treatment (either a control or microbiome derived metabolites). The different microbiome derived metabolites tested were 3,4-Dimethylbenzoic acid, 2-Hydroxicinnamic acid, N-Acetyl-L-phenylalanine, m-tolyacetic acid and Urocanic acid. Different concentrations of these treatments were tested. Their growth was quantified via fixation, staining and microscopy as described in the methods section above.

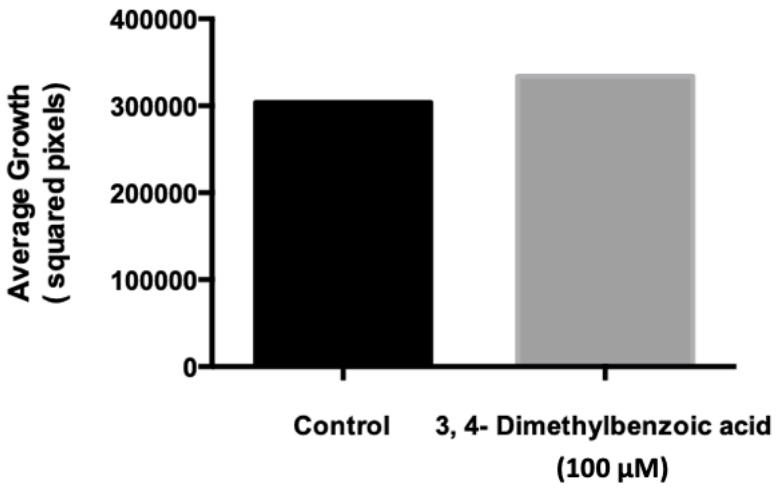


Figure 24: Average growth in squared pixels of the cells treated with 3, 4-Dimethylbenzoic acid (100µM per wells) (n=1).

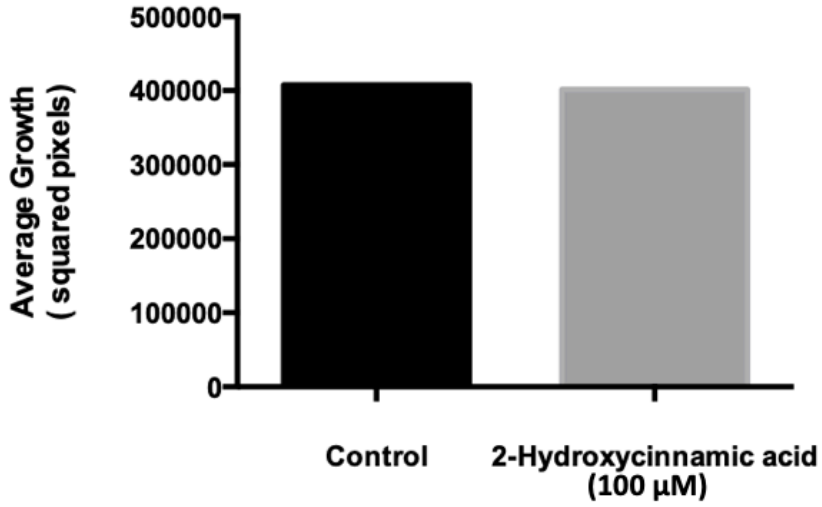


Figure 25: Average growth in squared pixels of the cells treated with 2-Hydroxycinnamic acid (100µg/mL) (n=1).

The two dose experiments were done to observe if there was any bioactivity detected, with a treatment of 3,4- dimethylbenzoic acid or 2-hydroxycinnamic acid. We did see a small increase in growth with the 3,4 dimethylbenzoic acid treatment but none with the 2-hydroxycinnamic acid treatment (Figure 24 and 25).

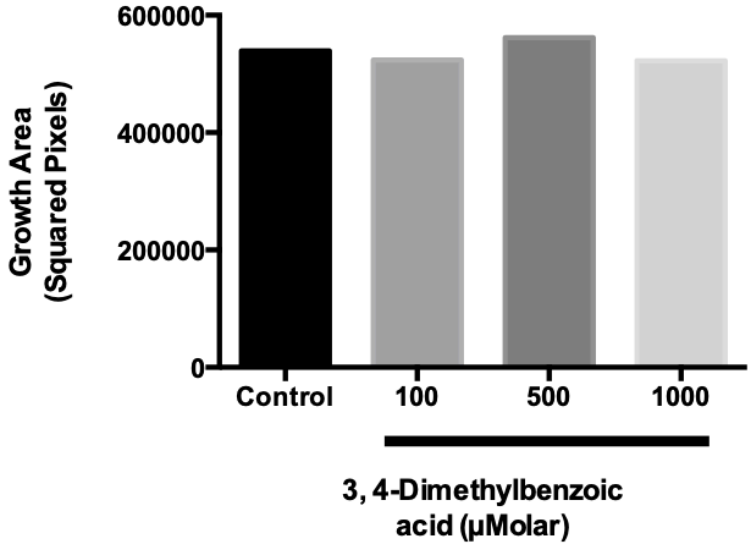


Figure 26: Average growth in squared pixels of the cells treated with a dose response of 3, 4-Dimethylbenzoic acid (100, 500 and 1000 µMolar) (n=1).

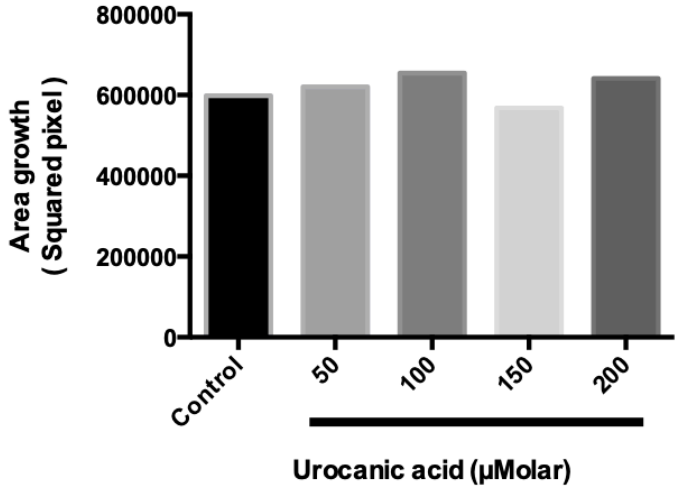


Figure 27: Average growth in squared pixels of the cells treated with Urocanic acid (50, 100, 150, 200 µMolar) treatments (n=1).

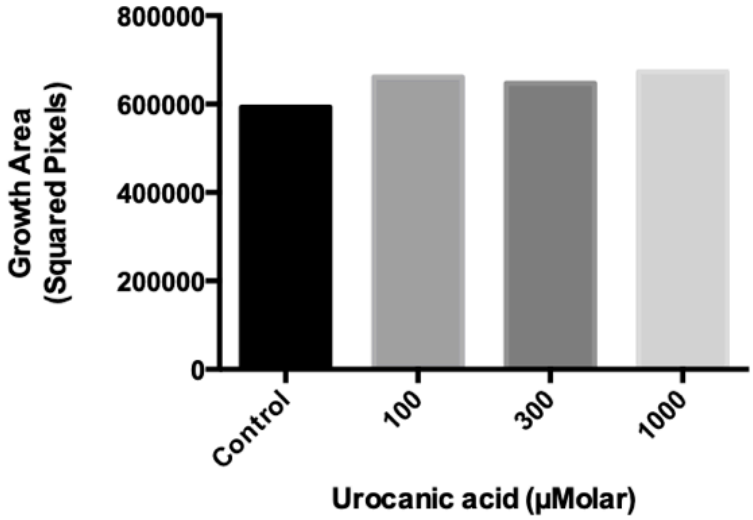


Figure 28: Average growth in squared pixels of the cells treated with Urocanic acid (100, 300, 1000 µMolar) treatments (n=1).

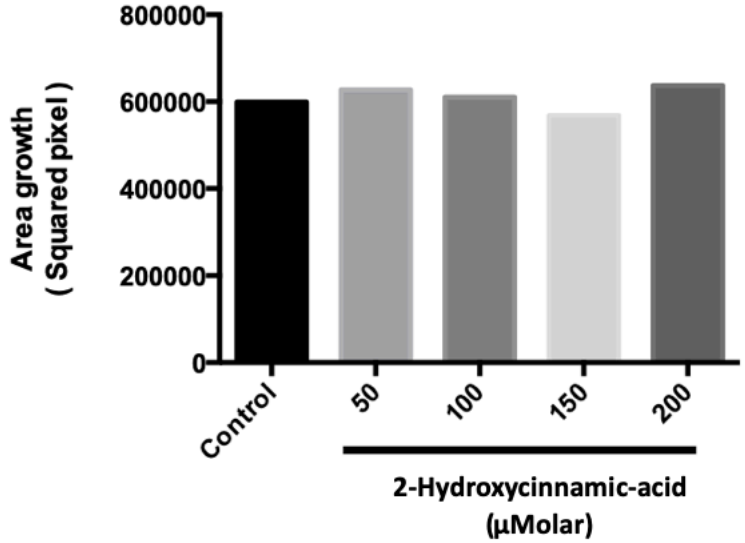


Figure 29: Average growth in squared pixels of the cells treated with 2-Hydroxycinnamic acid (50, 100, 150, 200 µMolar) treatments (n=1).

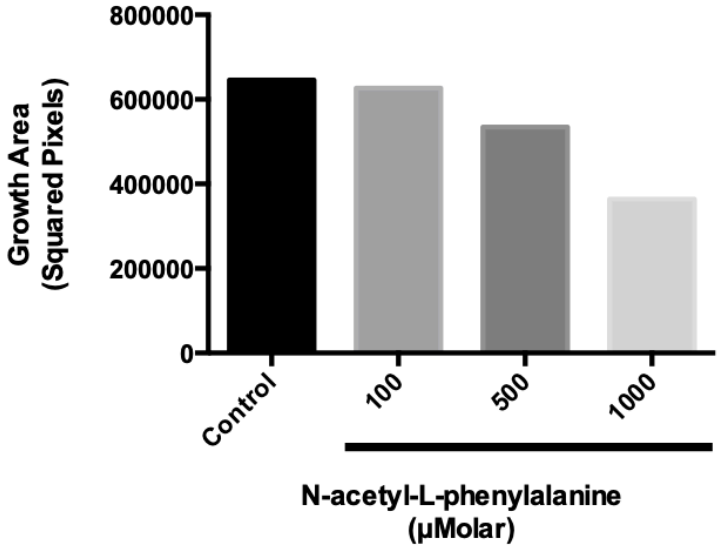


Figure 30: Average growth in squared pixels of the cells treated with N-acetyl-L-phenylalanine (100, 500 and 1000 µMolar) treatments (n=1).

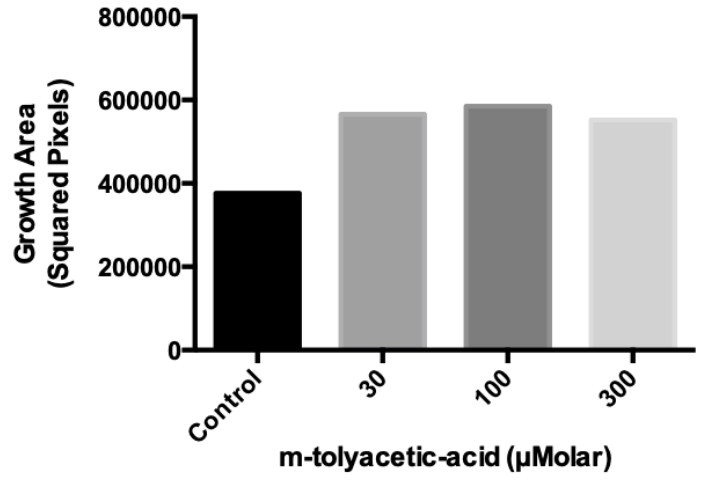


Figure 31: Average growth in squared pixels of the cells treated with m-tolyacetic-acid (30, 100, 300µMolar) treatments (n=1).

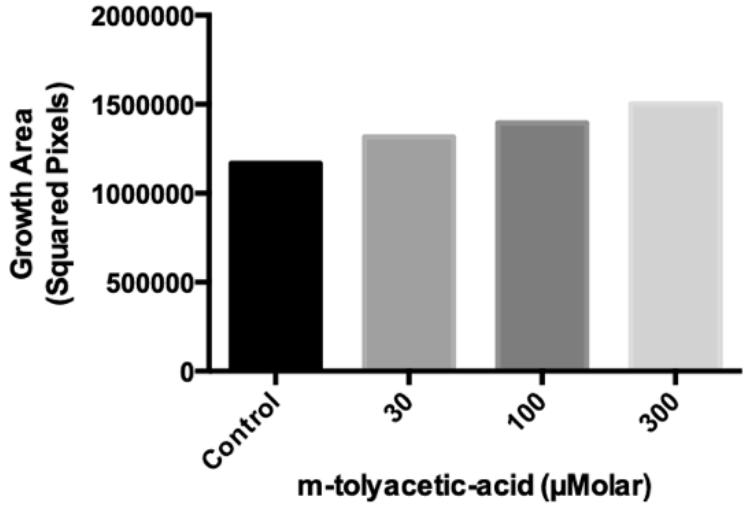


Figure 32: Average growth in squared pixels of the cells treated with m-tolyacetic-acid (30, 100, 300µMolar) treatments (n=1).

The 3,4 dimethyl benzoic acid and hydroxycinnamic acid treatment dose responses did not show higher growth than their controls (Figure 26 and 27). On the other hand, cells treated with N-acetyl-L-phenylalanine showed lower growth than the control (Figure 28). The urocanic acid experiments showed that concentrations from 50 to 200µM showed good healing abilities, but at1000µM the cells showed even higher healing capacities (Figure 29 and 30). Finally, in our m-tolyacetic acid experiment, we observed a higher growth with the treatment. We replicated this experiment. We found that a concentration of 300µM showed greater healing capacities than the control (Figure 31 and 32).

Statistics were not performed as the number of replicates for each experiment were n=1 due to the very limited amount of products. The results that were found are preliminary results that could further be investigated by the Lopes laboratory.

CHAPTER IV

DISCUSSION

Many different metabolites were screened throughout this study. We started by testing the helminth metabolites using a six wells plate starch assay. We found that the cells' growth after the 48 hours of incubation when treated with 150 and 200 µg/mL of 50%ACN *A. suum* was higher than any other fraction and concentrations of *A. suum* metabolites (Figure 13 and 15). Unfortunately, this data was retrieved from an experiment in which we were only able to have one technical replica (one well of the six wells plate). Indeed, we noticed that the six wells plate required high doses of the limited helminth metabolites available per wells. We decided to solve this problem by switching to 96 wells plates, which would allow us to use reduced amounts of helminth metabolites in each well. However, the switch from 6 wells to 96 wells did not allow us to use the same previous scratch protocol, the razor blade being too large to wound the tissue at the bottom of the 96 wells plates. Many other alternative scratches were tested, such as micropipette tip vertical scratches and sharpened pastor pipettes tips (elongated and cut above a flame). But these specific methods were not producing consistent wounds that would allow us to assess cellular growth properly. We also decided to use the Incucyte, an incubator that takes pictures at different intervals and allows for automated analysis. The different types of scratches technics were not optimal. They were not uniform or/and not in the frame of the objective. Finally, we found a technique that allows us to quantify the cells' growth by connecting a 20µL micropipette tip to a pump, producing a suction on the tissue in the bottom of the 96 wells plate, creating a circular wound. Despite being the most successful option, a problem remained; the wounds were often not in the IncuCyte's camera frame. We tried concentrating the wounds in the middle of each well, but this would still be very uneven, with success (wound in the frame of Incucyte) rate being really low, approximately 20 out of 96 wounds. Consequently, we decided to return to the light microscope while using the new 20 µL micropipette tip suction technic (Figure 33). To assess the growth, an initial picture was taken for each well's wound as well as a final

picture, 24 hours after the treatment. The outline of their areas (initial and final) was measured, and the final area was subtracted from the initial's area to obtain the growth area (Figure 12 and 10). We used this new technic to finish our study on the helminth metabolites and found this method efficient. Moreover, this consistent and useful platform can be used to screen compounds in a limited amount for their ability to heal the epithelial tissue. This assay can be used on a wide array of compounds, including but not limited to helminth metabolites, microbiome-derived metabolites, viruses, drug screenings, etc.

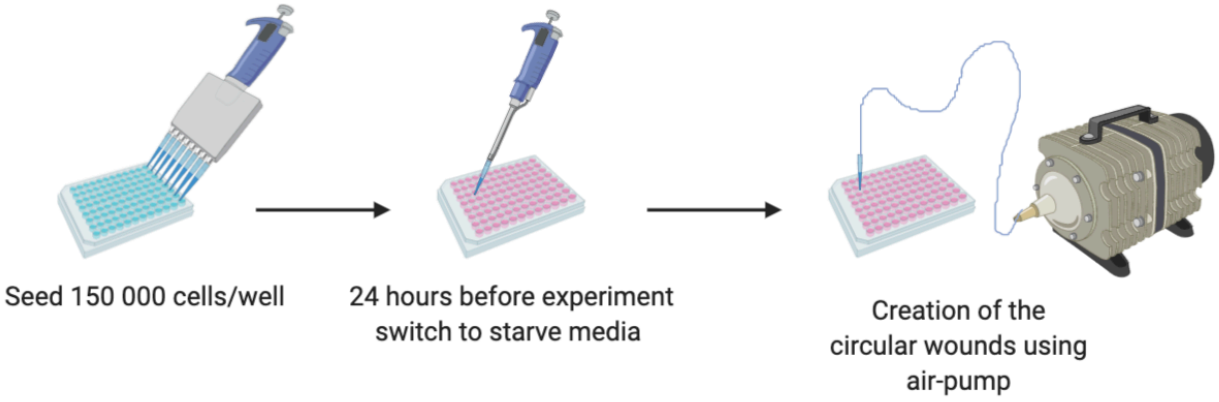


Figure 33: New air pump wound assay technic.

We continued to test *A. suum*, *T. suis* and *H. polygyrus* metabolites, but although we were using a smaller amount, the samples were exhausted. Indeed, it is tedious to extract a large amount of helminth metabolites. To start is imperative for the life cycle of the helminths to be completed, which is very difficult with helminths such as *A. suum* and *T. suis* that require pigs as their host, costly to maintain (Figure 34). Indeed, the cost of raising pigs, infecting them, recovering the helminths and conditioning them is expensive to maintain. Further, many helminths are required to produce the amount of metabolites necessary to be filtered and have minimal samples in each fraction. Our experiments also showed that using helminth metabolites would not be the most efficient way to treat a vast population of patients, as access to them is limited.

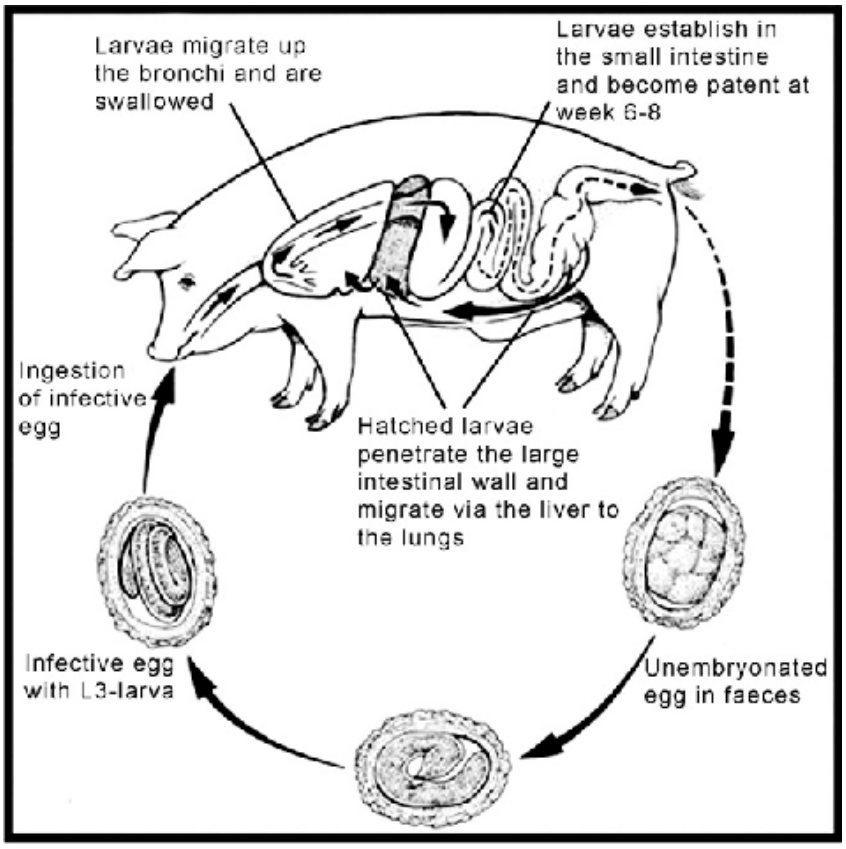


Figure 34: Life cycle of *T. suis* and *A. suum* (Loreille and Bouchet 2003).

However, a better understanding of helminth metabolites and their structure could allow us to search for their commercially available counterparts. Using mass spectrometry, we could identify, characterize and quantify these proteins by measuring the mass-to-charge ratio of ions of one or more protein and finding the amino acids that make them (Domon and Aebersold 2006). Suppose a commercially available analog corresponds or is similar enough to the helminth metabolites that show bio-activity in *in-vitro* and *in-vivo* experiments. In that case, we could use these commercial replicas and further test them. If they offer the same benefits, this could be used as novel drug treatment and be affordable and well-received by the patients as no helminthic infection or helminth by-product would part of the treatment, therefore eliminating the “gross: factor. Patients would feel more comfortable knowing that no part or products from parasites are part of their treatment, increasing the chances of the popularity of the novel possible therapies.

The other helminth metabolites tested, *T. suis*, *H. polygyrus,* and the *A. suum* ESPs did not show any growth compared to the control (Figure 14, 16, 17 and 19-23). This might be due to different factors; perhaps, the quantities tested were too small to trigger a response or too big, thus arming the cellular tissue. Moreover, it is possible that the *T. suis* and *H. polygyrus* do not work in this specific model; perhaps their role is set in the modulation of the immune system rather than the healing process, as it showed great protection abilities in the clinical trials and mouse models mentioned above.

We also tested the *A. suum* crude extracts at 25 and 50 µg/mL concentration and saw toxicity to the cells (not shown in the graphs as the treatment destroyed the tissue, and there was nothing to quantify). This might be explained by the fact that the crude extracts are metabolites that have not been filtered by the 3Kda filter, meaning that very large proteins are included in the treatment. Some of these large proteins are proteinase. Indeed, helminth produces proteinase to migrate thru the host tissue; therefore, it explains why a protein linked to tissue damage would cause such damage to our Caco-2 tissue (Donnelly, Dalton, and Loukas 2006).

The findings of *A. suum* bioactivity are exciting. It has previously been shown that pigs with a chronic infection suppressed inflammatory pathways in their intestinal mucosa while infected with *A. suum*. The same team found that dendric cells treated with *A. suum* antigenic "body fluid" secreted a minimal cytokine amount. These small proteins have a crucial role in IBD's pathogenesis and reduce IFNg production, another IBD driver (Midttun et al., 2018). *A. suum* has also shown abilities in modulating allergic and asthma inflammation in mice (Araújo et al. 2008) (Itami et al. 2005). *A. suum* metabolites should be further investigated in the context of IBDs and other autoinflammatory diseases. If future work on *A. suum* metabolites show that they decrease the inflammation of IBD in patients, it would be interesting to also test them for allergies and asthma, as this could help these patients with the management of these symptoms.

Our findings further confirm that helminths have a protective effect on our organism. This protection perhaps originates from the excretory-secretory products that they secrete while invading a host. As mentioned earlier, the lack of intestinal worms nowadays removed some protection from IBDs; these specific secreted metabolites may cause this special protection.

Once the helminth metabolites samples were exhausted, we decided to investigate urocanic acid, a *T. suis* analog, which allowed us to have unlimited access to these specific compound as it is commercially available. Thus, we were able to use larger amounts of metabolites and go back to the six wells plate protocol, as this was a consistent and technically smooth assay. We found that the cells treated with 1000µM of urocanic acid showed higher growth than the control; however, the concentrations lower than 1000µM did not (Figure 28). This observation might indicate that the urocanic has healing abilities but only at a concentration of 1000µM or above. The fact that urocanic acid showed bioactivity goes hand to hand with the different previous findings in DSS-induced colitis mice models and *ex vivo* models (Kammeyer et al., 2012). Moreover, in an unpublish study by Dr. Armando Jardim, urocanic acid has been found to be analogous to *T. suis* metabolite TsNP686 that has shown improvement in the severity of DSS-induced colitis in a mice model. Further, *T. suis* has been shown to be an efficient treatment in Dr. Summer's clinical trial and Dr. Khan and Dr. Sandborn's mouse models (Table 1). This compound has a lot more research ahead of it, but it might be a novel and affordable candidate for treating IBDs.

Furthermore, m-tolyacetic acid was the only microbiome-derived metabolite that showed bioactivity by increasing growth compared to the control, especially at a concentration of 300µM. This compound needs to be investigated further to fully understand its efficacity as it has never been tested on a scratch assay or on an *in vivo* model. As mentioned earlier, this compound is an analog to a microbiota compound that has been found to be lowered in the fecal matter of IBD

patients vs healthy patients, which means that the compound from which the analogous m-tolyacetic acid has been found might have a protective effect in healthy patients' gut. Reinforcing the importance of the microbiota in IBD's pathogenesis, this compound has also been seen to repress *Salmonella* host cell invasion. *Salmonella* is one of the bacteria linked to IBDs. The other compounds, hydroxycinnamic acid, 3,4 dimethyl benzoic acid, N-Acetyl-L-phenylalanine tested, did not show bioactivity in this specific model. Still, it would be interesting to study their bioactivity on a different model, as they might simply not have an action on the epithelial cells but perhaps on the immune cells.

FUTURE DIRECTIONS

The next step for this study would be to replicate some of our experiments, especially the *A. suum*, urocanic acid and m-tolyacetic acid experiments. The 50%ACN *A. suum* experiment should be replicated twice more with 150 and 200 µg/mL concentrations on two different cell passages to reach three biological replicates and assess this treatment's significance. The urocanic acid experiment should be replicated twice more on various cell passages. The m-tolyacetic acid experiment should be replicated once more on a different passage to assess its significance.

If these experiments suggest a significantly higher growth, it would be insightful to test them on mouse colon cells, for example, young adult mouse colonic epithelium (YAMC), which are cells that also form a monolayer (Whitehead and Robinson 2009). Our scratch assay could be used to assess their healing activity. If these compounds show similar bioactivity as on the human cells, switching to a mouse model would be the next step. A DSS-induced colitis model would allow us to assess these molecules' effects on an *in vivo* model; many different variables could be investigated, such as their histological scores, weight loss and colon length.

LIMITATION

We faced several limitations throughout our study. The most important one being the limited amount of helminth metabolites available. The entirety of the metabolites was used, and therefore we were not able to reach a biological triplicate. This led us to the inability to run statistical analysis to assess the significance of the metabolites' ability to heal our scratches in our assay. Thus, the lack of error bars on our graph and p values. The results we found in this study are preliminary to possible future investigations. Moreover, we are working with cancerous cells, caco-2, which is not the same environment as one of the non-cancerous cells of the GI tract lining of an IBD patient. Although we used starving media to prefer migration and have the cells on the same cell cycle, we could also use mitomycin c. This antitumor chemotherapy agent inhibits cell proliferation and allows us to increase the events of only observing cell migration (An et al. 2015). The reproducibility of the scratches is also an issue. It sometimes happens that the scratches either do not "work" due to human error. When scratching the tissue with the razor blade too boldly, it is possible to scratch the plastic, leaving lacerations in the wells' bottom, affecting cell migration. A scratch is not done hard enough, resulting in an inexistent dark line necessary for the analysis. These specific "unsuccessful" scratches were not taken into count when realizing our analysis. Lastly, only taking three pictures per well could lead to not having the real big picture of the growth. By choosing only three different spots, we might be missing out on higher or lower growths that could impact the final data and the experiments' results. Taking more pictures per well to cover about 90% of the wells would give us more accurate data. The Incucyte would be the best option for these experiments as the software Incucyte zoom could count the growth without the possible human eye error if we find a way to have the scratches in its view field.

CONCLUSION

This study aimed to screen different metabolites, both helminthic and microbiome-derived, for their healing capacities using a caco-2 scratch assay and develop a screening platform to screen metabolites on epithelial tissue using a 96 wells plate. We found that 200µg/mL and 150 µg/mL of 50% ACN *A. suum* had the greatest bioactivity out of all the tested concentrations of different helminth metabolites. It was also found that the treatments of 1000µM of urocanic acid and 300µM of m-tolyacetic-acid had the greatest healing ability out of all the microbiome-derived metabolites. Moreover, a successful screening assay was developed, using an air pump to create circular wounds, allowing healing assays to be conducted in the presence of a limited amount of samples. Overall, these findings indicate that 50% ACN *A. suum*, urocanic acid and m-tolyacetic-acid should be further investigated in IBD models and confirmed in *vivo* models as possible novel therapeutics for CD and UC. Indeed, if *A. suum'*s bioactivity is established in the mouse model, the specific 50% ACN fraction could be sent to mass spectrometry to identify its chemical structure. Consequently, this specific structure could be compared to a commercially available compound library such as ApxBio, to determine if a similar structure exists, as it was done with the microbiome-derived metabolites tested in this study. If such a structure does exist, it shall be tested further in a mouse model and then, if efficient, confirmed in a clinical trial. This compound could produce the same relief seen in previous helminth clinical trials to the patients without an actual helminth infection and without helminthic by-products costly and challenging to extract. Therefore, a possible affordable and efficient new treatment for IBD patients.

ANNEX

IL-35:

During this project, we also tested IL-35, a cytokine associated with the regulation of inflammation in UC. Indeed, a study by Dr. Zang and his team engineered IL-35 expressing *E. coli.* They administered this *E. coli*/IL-35 orally and empty plasmid- transformed *E. Coli* and PBS for the controls to mice in which DSS induced experimental colitis was induced. They found that the mice treated with *E. coli*/ IL-35 showed lesser colonic tissue damages than the controls (Zhang et al. 2018). Curious to see if the cytokine could have healing proprieties, we tested it on our six wells scratch assay. We found that at a concentration of 50ng, the healing of the tissue was more rapid than another concentration and control. More research should be done on this compound.

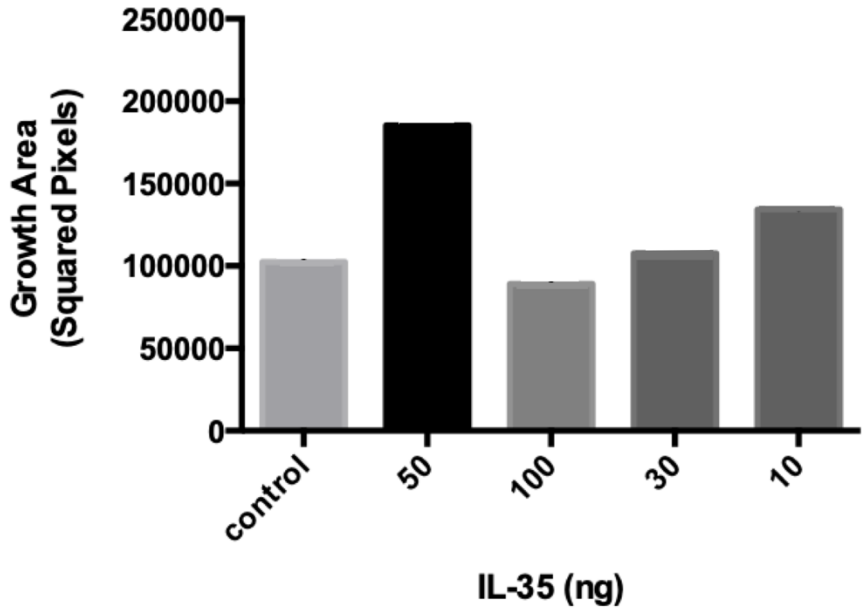


Figure 35: Averaged growth of the cells with a treatment of IL-35 (50, 100, 30 and 10 ng) (n=1).

AUTHOR CONTRIBUTION:

The laboratory work, data analysis and writing were done by Jysiane Cardot.

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