Effect of microbiota on regeneration and degrowth in *Schmidtea mediterranea*

Sree Harshitha Kanchamreddy

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Honors Advisor: Sravanti Uppaluri

Abstract

the Interaction between the microbiota, referring to all the microorganisms present in an organism and cellular processes plays an important role in the homeostasis of an organism. In humans, microbiota influences many phenotypes including the nutrition, drug disposition and behaviour. There have hardly been any studies that looked at significance of microbiota *Schimdtea mediterranea*. *S.mediterranea*, commonly known as planaria, are popular for their ability to regenerate lost body parts or complete organism from small fragments of their bodies. They also have the ability to survive up to three months in the absence of food by a process called degrowth. In this study we tried to look at the effect of change of microbiota on the processes of regeneration and degrowth. The microbiota was changed by treating the planaria with antibiotics and their size was quantified during regeneration and degrowth. The quantity relative and the diversity of microbes was observed by plating smashed planaria. From these experiments we observed that there were no drastic differences in size during regeneration and degrowth in organisms with normal and changed microbiota suggesting that microbiota may not play a role during degrowth. We also found that there was a difference in reduction in size of anterior and posterior parts during regeneration. The diversity of the microbiota varied among organisms both within and among different treatments but there were few colonies that were similar. It would be interesting to identify the colonies that are similar and different in future. is this really the last message you want to leave the reader with?

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Chapter 1: Introduction

Microorganisms and microbiota

Robert Hooke and Antoni van Leeuwenhoek, in late 1660s, discovered the existence of minute organisms that people had no idea existed. These were collectively named microorganisms. It took two centuries for people to identify their presence in the human body. In 1880s, an Austrian paediatrician named Theodor Escherich isolated a strain of E. coli from the intestines of children ("Human Microbiome" n.d.). After this discovery, many others followed identifying more microbes present in *Homo sapiens* and other organisms. There have been many estimates made by researchers about the quantity of the microbes in humans. The earliest estimate made in 1977 by Savage et al was that ratio of microbial to human cells was 10:1 (Sender, Fuchs, and Milo 2016). The latest estimate points towards equal number of microbial and human cells (Sender, Fuchs, and Milo 2016). Irrespective of whether the number of microbes is ten times more or equal to the human cells, the presence of such vast amounts (10^{13}-10^{15}) raises important questions regarding their significance in the body.

The Human genome project (HGP), started in 1990 and took around 13 years to complete led to many revelations about the human genome. One of the significant result of the project was that there were only 20,500 genes ("An Overview of the Human Genome Project" n.d.). Five years after the completion of HGP another project, a sort of extension and at as large scale as HGP, was launched called human microbiome project (HMP). The objective of this project was to make repository of sequences of 3,000 microbial genomes and find the diversity of organisms in humans by 16s and metagenomic techniques ("NIH Human Microbiome Project - About the Human Microbiome" n.d.). The HMP and other studies showed that there could be as many as 35,000 species of bacteria in the human gut and these contribute around 10 million genes to the host (Jandhyala et al. 2015). Since then, the relevance and significance of

microbiota both in humans and other organisms has come under scrutiny. We now appreciate the importance of microbiota in humans in different contexts like nutrition, drug disposition, epithelial cell formation and tolerance to pathogens (Sekirov et al. 2010).

Microbiota is not same among individuals

In an experiment conducted by Filippo et al in 2010, gut microbiota were shown to be different based on the diet followed by the individuals. The experiment compared fecal microfauna of children from Europe and from a village in Burkina Faso -The diet of the latter had significantly higher fiber content. 16s rDNA sequencing showed that amount of Bacteroidetes, which help in breaking down of fibres were higher in children Africa compared the European children(Filippo et al. 2010). This study illustrates that the microbiota could evolve and adapt to differences in food.

Microbiota helps in nutrient metabolism

It was initially assumed that the human gut is equipped with all the processes to breakdown food, but more research is showing that might not be the case. A major source of energy in humans comes from the carbohydrates that are consumed in our diet. Some of these carbohydrates can escape the process of glycogenesis in the cells can also be broken down by microbes present in the body (Jandhyala et al. 2015). Humans do not have the ability to break down oligosaccharides. It was found that around 50–100 $mmol \cdot L^{-1}$ of short chain fatty acids are made from oligosaccharides per day(Wang et al. 2017). These short chain fatty acids play a vital role in maintenance of epithelium. These microorganisms present in the body not only help indirectly in producing energy but also help in reducing harmful products of metabolism from the body such as oxalate, a by-product of carbohydrate fermentation (Jandhyala et al. 2015).

Another important macromolecule in the diet are proteins. It was seen that an enzyme encoded by a bacterial gene known as *hdcA* converts L-histadine, an amino acid, into histamine, a compound responsible in local immune responses (Jandhyala et al. 2015). This shows that microbiota is required for protein metabolism. Apart from carbohydrate and protein metabolism, microbiota also deliver vitamins such as vitamin K, folates, B_2, B_{12} and possibly other B vitamins (Wang et al. 2017).

The microbiota can vary among different individuals based primarily on environmental factors such as nutrient intake. The microbiota can regulate the metabolism of the host through different processes. It is also known that dysbiosis between host and microbiota can result in disease in the host (Isolauri 2017). Therefore, from the host's perspective, the microbiota plays an integral role in maintaining homeostasis both directly and indirectly. Is it possible then, that the host has evolved mechanisms to use this vast amount of microbiota as food source in times of low food availability.

Model system: *Schmidtea mediterranea*

To answer this question, the perfect organism in which experiments can be conducted is *Schmidtea mediterranea*, commonly known as planaria. Planaria is also a common name given to all the species in the family Planariidae. The discovery of an organism from this family is attributed to Peter Simon Pallas, a Prussian naturalist, who came across it on an expedition to Ural mountain in late 18th century (Elliott and Sánchez Alvarado 2013). *S.mediterranea* belongs to the phylum Platyhelminthes and is known for its ability to regenerate from amputated parts of its body. Unlike the other members of the phylum, *S.mediterranea* are non-parasitic worms. Theses organims are found in the fresh water of islands in the western Mediterranean like Catalan coast, Menorca, Mallorca, Corsica, Sardinia, Sicily and Tunisia(Lázaro et al. 2011).

The worm varies in length from 3mm to 30mm (as seen in Fig 1.1.a) . It has a triploblastic body plan, an organism consisting of all the three germ layers mesoderm, endoderm and ectoderm. This organism holds nervous, gastro vascular and reproductive systems within the almost 2-dimensional body (Rink, 2013). They also have two eye spots through which they can detect light(Fig 1.1.b). There are two strains of S. mediterranea, sexual and asexual. All the sexual strains are hermaphrodites and have the ability to self-fertilise or mate in the absence or presence of other individuals, respectively. The asexual strains reproduce by binary fission ("Sexual Reproduction in Schmidtea Mediterranea | Developmental Biology Interactive" n.d.).

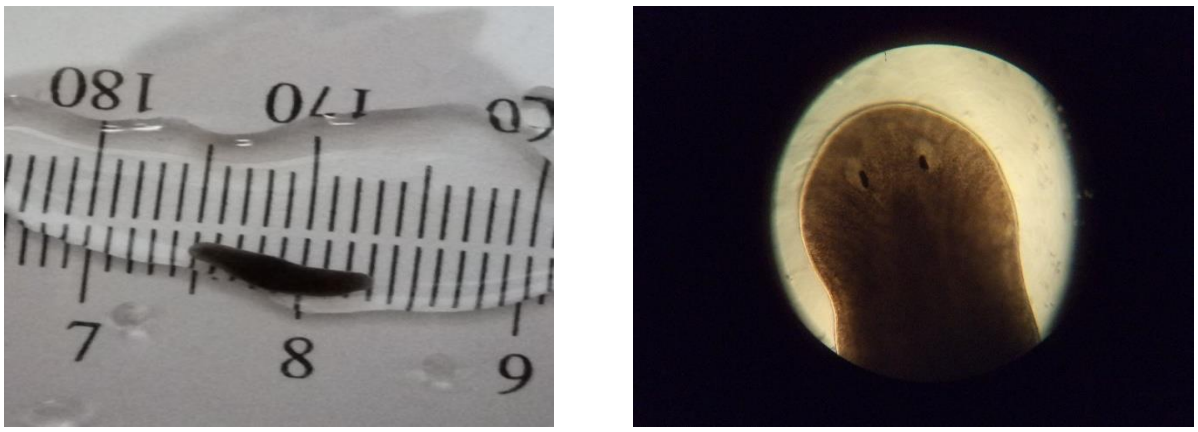


Fig 1.1 a) image of Schmidtea mediterranea along with a scale to look at the length b) image showing the eyespots of planaria taken under 4x in a compound microscope.

Like Humans and other organisms, planaria also have microbiota in them. To date, only one study has examined the microbiota and its effects on planaria. A study published in 2016 by Christopher Arnold showed that an increase in the number of proteobacteria, which includes gram negative pathogenic bacteria, hindered regeneration and increased tissue degeneration in *S. mediterranea*(Arnold et al. 2016). This experiment shows that vital processes such as regeneration can be hindered by shift in microbiota.

S.meditteranea are very easy to grow in a laboratory and can be cultured on liver or egg yolk. More individuals can be made by simply cutting an individual into two and allowing them to regenerate. It also contains all the organ systems that are present in humans and a diverse microbiota. Its ability to regenerate made them an ideal model system to study development but the presence of microbiota now makes an ideal system to study interactions between host and microbiota.

Regeneration in Planarian

In 1898, Thomas Hunt Morgan, the pioneer of genetics in Drosophila showed that 1/279th part of a planarian can regenerate into a complete organism(Morgan 1898). But this was not the first-time regeneration was mentioned in literature. Ideas of regeneration were present in Greek mythology dating back to 7th century BCE. In one such legend a titan named Prometheus would regrow his liver every day. Regeneration in the animal kingdom was first noted by Aristotle around 350 BCE. He noted that a lizard regrew its tail after amputation(Elliott and Sánchez Alvarado 2013). It wasn't until the 18th century CE that systematic studies to understand regeneration started. In an effort to determine whether hydra was a plant or an animal, Abraham Trembley discovered the remarkable regenerative capabilities of hydra in 1740s("Abraham Trembley (1710-1784) | The Embryo Project Encyclopedia" n.d.). This discovery not only put forth systematic study of organisms but also questioned the idea of Preformation, that a tiny human is already present in the head of a sperm and merely grow in size, that existed from Aristotle's time(Elliott and Sánchez Alvarado 2013).

The first mention of regeneration in planarian was made by Peter S Pallas (Elliott and Sánchez Alvarado 2013). The first systematic studies in these organisms were conducted by Harriet Randolph in 1870s a century after its 'discovery' by Pallas. She experimented on the different

ways in which the organism can be cut in order to regenerate and the smallest piece that can regrow. She concluded from her experiments that a piece that can be seen with the naked eye can regenerate into a full worm and she also showed that planarian have the capability to regenerate wounds(Randolph 1897). These studies formed a basis for TH Morgan to conduct experiments on planarian regeneration.

The regenerative capabilities in planaria raised many questions. What is different in planaria from other organisms for it to have these incredible regenerative abilities? How is the symmetry or polarity determined? While still much is left for us to understand regarding the molecular players involved in polarit establishment, many studies have examined the cells that give planaria their regenerative capabilities. These cells are called neoblasts, a term coined by Harriet Randolph (Baguñà 2012). Neoblasts are the resident stem cells in planaria that can produce all the other cell types including germ cells. The evidence that neoblasts are totipotent or at the least pluripotent comes from the experiments of performed in Peter Ridden's lab. In this experiment, they introduced a single neoblast cell into an irradiated worm (irradiation kills the dividing cells and neoblasts being the only dividing cells in planaria are removed) and found that the irradiated worm acquired the ability to regenerate (Baguñà 2012).

define polarity

During regeneration, proliferation of neoblast is not enough to regenerate the missing body parts(Reddien and Sánchez Alvarado 2004). Remodelling of pre-existing tissue or morphollaxis (a term coined by TH Morgan) plays as significant role as proliferation in regeneration(Reddien and Sánchez Alvarado 2004). Pellettieri et al conducted an experiment in Scmidtea mediterranea to understand the processes behind morphollaxis. After amputation, the amount of apoptosis was measured. Pellettieri and his team found that there is an increase of apoptosis near the wound region 1 to 4hrs after amputation but there was a systemic cell death in the body 3 days after amputation (Pellettieri et al. 2010).

Degrowth in planaria

Morphollaxis with the help of apoptosis is not just unique to regeneration but can also be found in another process in planaria, degrowth. Degrowth refers to the reduction in size of the organism in the absence of food. Planaria can survive up to or above 3 months by this process when starved(González-Estévez et al. 2012). It was found that the size of the organism can maintain a normal functioning even with 25 fold reduction in size (González-Estévez et al. 2012). It was also seen that during degrowth the proportion of different cells were maintained

The process of degrowth is not restricted to planarians. This process can also be seen in different phyla and taxa such as Cnidaria, nemertea, Annelida and gastropoda (Calow 2012). When a snail species called *Helisoma trivolvis* was starved, the researchers noticed that there was a reduction in 20-50% of the dry body mass (Russell-Hunter and Eversole 1976). In few species of nemertea, it was noticed that starvation can make the organism into ball of cells resembling the early embryonic stages. Hence there is not just a reduction in size but also reversal in aging. This was also observed in few species of planarian (Calow, 2012).

Reduction in body size can occur by two processes, one is by reducing the size of the cells and the other is by reduction in the number of cells. In planarian, degrowth occurs through reduction in number of cells(González-Estévez et al. 2012). This already points towards apoptosis playing a major role in degrowth. Cristina González showed that the amount of apoptosis does increase from 7 days to 20 days after starvation and again increase from the base-level 90 days after stavation(González-Estévez et al. 2012). It is speculated that this apoptosis of cells acts as energy source for the planaria to survive. autophagy not introduced?

Apoptosis seems to play a very important role in the survival of planaria both during regeneration and degrowth. **If the planaria are able to disintegrate their own cells in order to prolong their survival, could it be possible that they are also disintegrating the**

designed (came up with is casual)

microbiota present in their bodies? To test this question, we came up with an experiment that involved determining the change in body size with normal and reduced amount of microbiota. The final size of the organism with reduced amount of microbiota would be lower if the microbiota is used as food.

Broadly, there are two aims for this experiment –

1. To determine the change in size of the organism in treatments with varying amount of microbiota during regeneration and degrowth.
2. To determine the change in microbiota during regeneration and degrowth.

Brief literature review of microbiota, regeneration and degrowth was given until now. We also established the question we are interested to study. In the next chapter, the methods and protocols used for the study will be described followed by results. The discussion of these results will be mentioned in the third chapter along with the results. The paper will end with conclusion and suggestions for future research.

new chapter, new page.
number subsections. see formatting guidelines

Chapter 2: Methods and materials

Organism and their maintenance-

The predecessor of the *Schmidtea mediterranea* that were used for the research were acquired from Dr. Dasaradhi Palakodeti's lab in National Centre for Biological Sciences, Bengaluru. Most of the organisms that were used for the experiment were sexual strains. The organisms were maintained at 18 – 20^{o} C in dark. They were fed the same batch of mashed up liver (beef), throughout the experiments, procured from a butcher shop in Sarjapura, Bengaluru. The planaria were fed the same batch of liver because different batches of liver that were procured from the butcher shop had different microbial growth when plated on a NB plate(Fig 2.1). The organisms were fed on alternative days to achieve maximum growth. The protocol for the preparation of the medium in which the organisms were kept is as follows.

To make 1 litre of 5X planaria medium, 1.6 ml of 5M sodium chloride (NaCl), 5ml of 1M calcium chloride ($Cacl_2$), 5ml of 1M magnesium sulphate ($MgSO_4$), 0.5ml of 1M magnesium chloride ($MgCl_2$), 0.5ml of 1M potassium chloride (KCl) are added to 900ml distilled water. To this mixture, 0.504g of sodium bicarbonate ($NaHCO_3$) is added and stirred. The whole volume of (1000ml) is made up after adjusting the pH of the solution to 7.00 using Hydrochloric acid (HCl). The 5X is diluted with distilled water to make 1X and final mixture is autoclaved to be used as media for planaria.

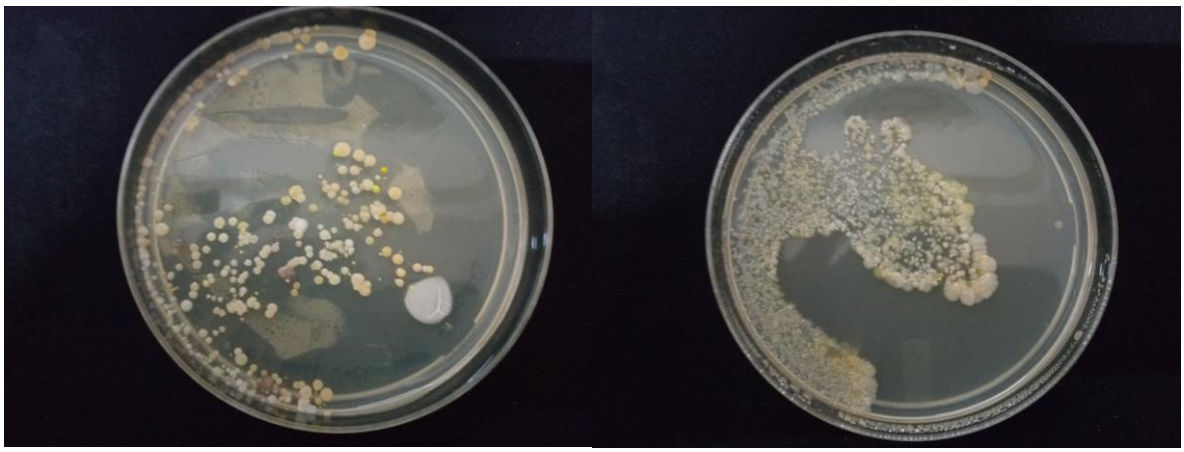


Fig 2.1 The images represent the microbial growth of two different batches of beef liver acquired from butcher shop in Sarjapura, Bengaluru.

Determining antibiotics to use

One way to test the effect of microbiota on regeneration and degrowth can be achieved by removing or reducing the amount of microbiota in an organism. This can be accomplished by treating the organisms with antibiotics. There are numerous antibiotics that are available in the market. In order to subset the antibiotics that can be used for the experiment, the following test was conducted.

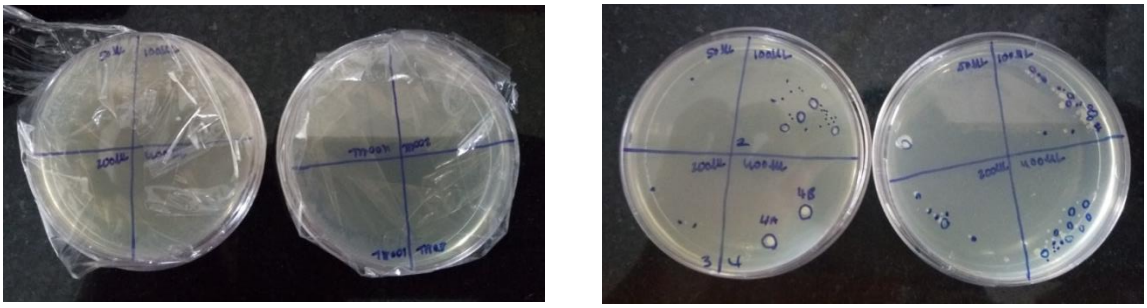


Fig 2.2 a) Agar plates (LB and NB) 12 hours after streaking and b) Agar plates (LB and NB) 72 hours after streaking

Two planaria specimens were smashed in 1ml effendorf tube, using a sterile steel rod. 50ul of Luria broth (LB) media was added to one of the effendorf tubes whereas 50ul of Nutrient broth (NB) media was added to the other one. The effendorf tubes were shook to distribute the crushed pieces and streaked onto respective agar plates. Different dilutions (100ul, 200ul and 400ul) were made and streaked on the same petri dish. Bacterial cultures usually are grown at 30^{o}C. As a preliminary test when the crushed planaria were streaked in various dilutions and left at 30^{o}C to grow, there was no growth observed after 5 days of plating. The same procedure was followed and the culture plates were left in 18^{o}C, there was growth in both LB and NB agar plates. And hence for this experiment the plates were left at 18^{o}C.

this is expected because the host thrives at 18C right?

did control plates have any growth?

Three days after plating (at 18^{o}C), the colonies were observed under naked eye and microscope (Fig 2.2). Six colonies from LB and six more from NB plates that looked phenotypically different were picked using a sterile toothpick and inoculated into test tube containing 5ml of LB media. The test tubes were left in a shaker (at 18^{o}C) for four days for the cultures to grow. Inoculates were then inoculated into 48 well plate containing solutions of LB media and various combinations of antibiotics (Fig.2.3). The antibiotics that were used for the test were Gentamycin (50ug/ml), Kanamycin (100ug/ml), Ampicillin (100ug/ml) and streptomycin (50ug/ml).

individual test tubes

what was this choice of antibiotics based on? you can be honest about this..

Fig 2.3. The image shows two well plates with LB medium and different antibiotics. Each row has different antibiotic whereas each column has different colonies.

Dissociation techniques

As mentioned in the introduction, change in body size occurs due to change in cell number and not cell growth in *Schmidtea mediterranea*. So, one way to quantify growth or "degrowth" is to look at the change in the number of cells. This can be done by dissociating the organism and counting the cells (both planarial and microbial) on a haemocytometer under a microscope. reference?

Two techniques were used to convert multicellular *Schmidtea mediterranea* into cell suspensions that can be counted. One was maceration protocol proposed by Baguna et al in his paper "Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians *Dugesia mediterranea* and *Dugesia tigrina*"(Baguñá and Romero 1981). This is a modified version of the David's technique of hydra disassociation medium. In this process, *Schmidtea mediterranea* were placed in 1ml solution made of methanol, glacial acetic acid, glycerol and distilled water in the ratio 3:1:2:14 at 8 – 10^{o}C. A schematic of this process can be found in Fig 2.4. After 24 to 48 hours, the test tubes were gently shook to separate and

spread the cells evenly throughout the solution. To this, 100ul of 20% formaldehyde was added to fix the cells. This solution is then put on a hemocytometer to obtain the cell count in the sample and the cell count of the whole organism can be estimated from this. As only one organism was used for the experiment and concentration of cells in 1ml was not high, the mixture was centrifuged and 800ul supernatant was removed. The tube was shaken to evenly distribute the cells.

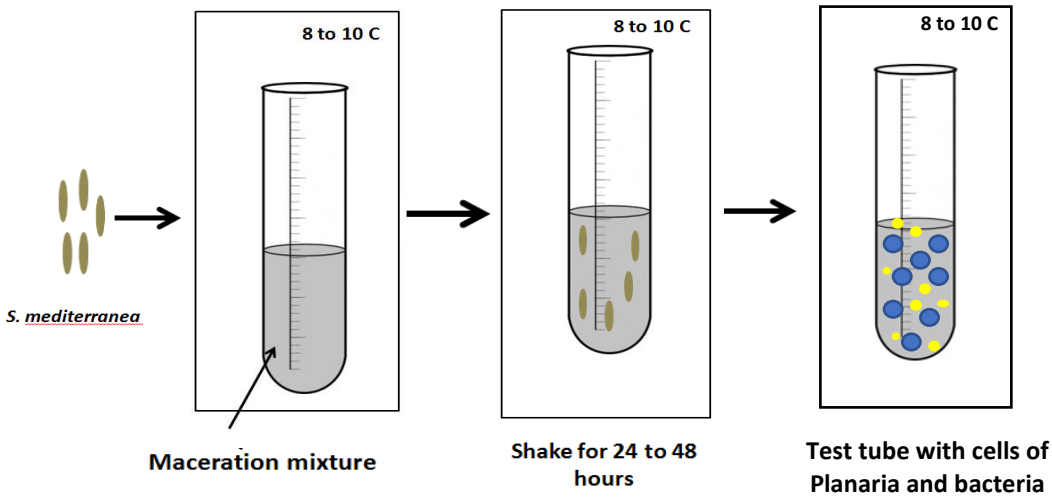


Fig.2.4. A schematic representing maceration protocol by Baguna et al

The maceration protocol mentioned in the above paragraph was very time consuming as it took around 1-2 days for disassociation to occur. A different protocol mentioned by Hayanshi et al in his paper, "A Unique FACS Method to Isolate Stem Cells in Planaria" was used. In this technique, *Schmidtea mediterranea* were placed on an ice stage, made by putting tissues and parafilm on top of an ice block. These organisms were then cut using a sterile knife. The cut pieces were then transferred to a 1ml effendorf tube containing 980ul 5/8 Holtfreter solution. 20ul 50x trypsin solution was added to the test tube. The mixture was incubated at 20^{o}C for 5-10 mins. The mixture was gently pipetted for 60 – 70 times to completely disassociate

organism. The tube was centrifuged for 5- 10 mins at 1,500g, washed twice and resuspended in 200ul of Holtfreter solution.

One of the biggest advantage of maceration techniques is that it allows us to quantify the number of microbial cells along with the planarial cells. This allow us to look at the change of planarial and bacterial cells throughout regeneration and degrowth in different treatments. The size of the planarial cells (eukaryotic) are much larger than microbes (which are mostly prokaryotes) and hence looking at them under a compound microscope should allow us to distinguish between the two. But, when the experiment was performed, no microbial cells were seen or it was hard to distinguish between the both.

There could be three reasons for the absence of microbiota –

1. The maceration mixture is disintegrating the microbes.
2. Planaria are kept in laboratory between 18 and 20^{o} C. The microbiota present in the planaria could have evolved to survive at these temperatures and leaving them at 10^{o}C could have resulted in their death. death and lysis? but you fix them - that would prevent their lysis?
3. The microbiota is unable to survive in the absence of the host.

To check whether the maceration mixture was degrading the bacteria, few cells of OP 50 strain were left for maceration using the technique by Baguna et al. No OP50 cells were visible after the maceration under the microscope. When the technique by Hayanshi et al was tried and the resulting cell suspension was looked under the Olympus inverted microscope, there were presence of bacteria like structures. no images of this?
so which was better? which was used, why or why not?

The inverted microscope could not be used regularly to count the cells and as it was difficult to distinguish in a compound microscope, a different way to quantify size (area measurement) was used.

Staining techniques

For better visibility of macerated cells, two staining techniques were tried. About 5ul of the solution containing dissociation media and cells were placed on a clean glass slide. The solution was left to air dry and fixed by gently heating it. Then it was either stained using crystal violet or gram staining. It was seen that gram staining allowed better visualization compared to crystal violet (Fig. 2.5). For crystal violet staining, 7-10ul of crystal violet was flooded on to the sample and left for about a minute. The stain was then washed off using distilled water.

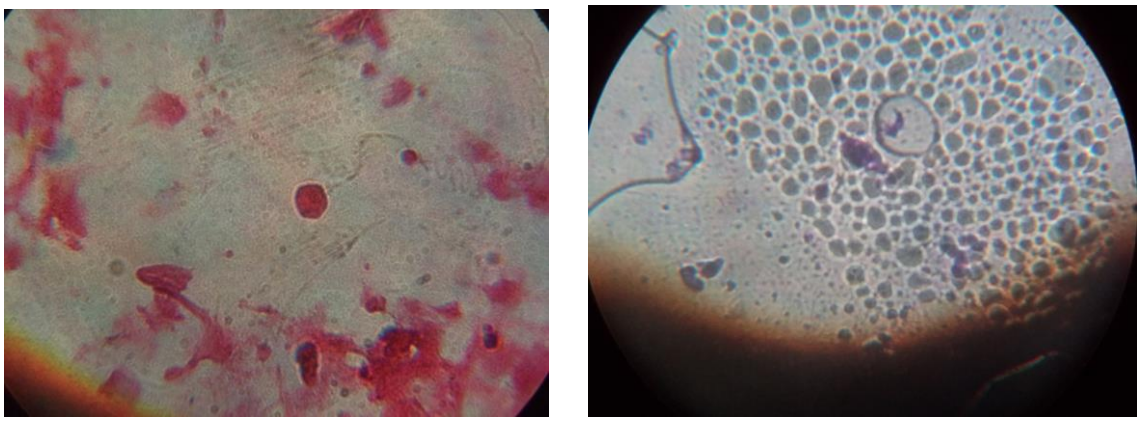


Fig 2.5. a) Planaria cell with gram staining under 100x b) Planaria cell with crystal violet under 40x

Gram staining is a technique used to differentiate gram positive from gram negative bacteria. In this technique, the dried slide is added with crystal violet. After a minute, it is washed off using distilled water. Then gram's iodine is left on the sample for a minute and washed off with distilled water. The slide is then flooded with decolouriser, in this case ethanol. After washing the slide with distilled water, safranin is added to the sample and left for about a minute and rinsed. So, now the gram-negative bacteria acquire the pink colour of the safranin whereas gram positive bacteria acquire the violet colour of the crystal violet.

In this experiment, gram staining technique was seen as way to differentiate between eukaryotic and bacterial cells as gram staining reagents were supposed to colour only bacterial

cell walls. After performing the experiment, it was seen that eukaryotic cells were also stained. Even though this staining allowed us to visualize the cells better, it was not helpful in differentiating the bacterial from planarial cells.

Imaging

Another way to quantify the change in size is to take snapshots of the organism (growth and degrowth) through time and use the image to make measurements of length, width or area. Planaria are very plastic organisms and are constantly changing their length and width while moving. This makes the measurement of area more reliable than length and width.

To take the images, the organisms were placed in a petri dish with planaria medium. A dropper was used to move the planaria under the objective and images were taken under 8x in Leica microscope. Five images of each planaria are taken in different stages of its movement. All these images were taken under the same light conditions with the same magnification. The mean area of all the five images is taken as the area of the organism.

The images were taken throughout regeneration and degrowth. For regeneration experiment, images were taken every day for 3 weeks. Images were taken once every 3 days for organisms undergoing degrowth for the first 1 month and it was later shifted to once a week for the next 2 months.

Image analysis

R programming was used to analyse the images taken during degrowth and regeneration. An algorithm made by Pooja Pravinbabu (personal communication) was used to measure the area of each planaria. The logic behind the algorithm is that a plain background image without the planaria is subtracted from an image with planaria whose area we need to determine. The resultant images are then converted into a black and white image from which the area is calculated in pixels and

changed into mm^2. The code was elegantly designed such that output of the function is not just the area of a single image of planaria but rather mean of areas of a collection of images of a planaria. As mentioned above, planaria are very plastic in their movements and using mean of multiple images is a much better indicator of the true area than area acquired through a single image.

Microbial colonies

One way to visualize the microbes in planaria was to macerate the planaria and see the cells under a microscope. But another way to look at them is by observing their colonies. With the restriction placed by instruments in the labs it was hard to look at prokaryotic cells in a mass of eukaryotic cells let alone identify the different morphologies of these cells. Unlike cells, colonies are visible to the naked eye and help us differentiate between different communities.

Inside the LAF, Planaria specimens used for the experiment were cut into as tiny pieces as possible using a surgical knife on a sterile glass slide. The cut pieces were then transferred to a effendorf tube containing 200ul of sterile NB media. The solution in the effendorf tube was pipetted a few times to spread the cut pieces. This solution was then plated on to a petri dish with NB agar. The plates were kept in an incubator at 18 – 20^{o}C. It took around 1-2 days for the colonies to appear.

didn't you do a pilot study? mention this?

we had to identify another metric to ...

The colonies rapidly than expected and hence we had to come up with a metric to quantify the colonies. So, we chose four bins in which the colonies can be sorted to. One is 0-1 in which almost no microbial growth was observed. The second bin is between 1-100 to represent lower number of colonies. The third bin consists the values from 100-200 to show higher but countable number of colonies. The fourth bin consists of all the values that are above 200, this

plates, not colonies

bin contains all the plates in which the colonies were extremely crowded to count or had films in which the colony number cannot be found.

this paragraph is a very awkward way to describe a histogram. consider rewriting it.

margins?

new chapter/?

Results and discussion:

To achieve the aims of the study twenty-four planaria (eight in each treatment) were cut into two pieces and left to regenerate in three different treatments (Planaria medium without any antibiotic, with ampicillin and with kanamycin) for three weeks. To study the effect of microbiota on degrowth, thirty (ten in each treatment) planaria were left in above mentioned three treatments and were not fed until the end of the experiment (i.e., 3 months). After regeneration and degrowth, the organisms were chopped and plated to determine the quantity and diversity of the microbiota present in them. The microbiota of ten individual that did not undergo regeneration or degrowth was measured to determine microbiota present in 'normal' worms. A schematic of this experiment can be seen below (in Fig. 3.1)

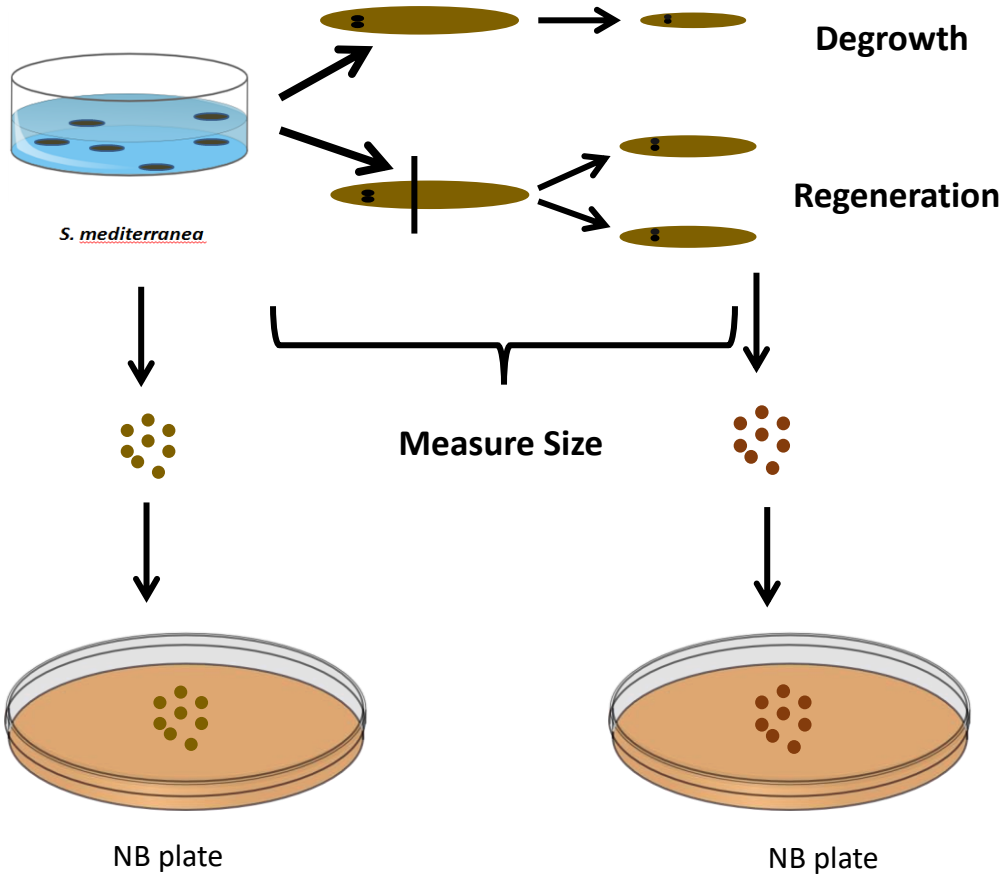


Fig 3.1 A schematic showing the experiment

This final setup was established along with and after trying out different experiments mentioned in methods section.

Choice of antibiotics

To subset the antibiotics that can be used for the experiment, planaria were crushed and plated. The microbes that grew were inoculated into well plates with LB media and antibiotics. The changing of clear growth media to a turbid one shows the presence of microbes. When the well-plates were observed after inoculation, it was seen that ampicillin did not allow growth of any microbial samples for the first two days, gentamycin and streptomycin allowed 9 out of 14 bacteria to grow and kanamycin allowed around 6 out of 14 (as can be seen in the table 3.1) by

the end of 2 days. In the wells where no antibiotic was present almost all the samples except one grew.

Box 1

16hrs after inoculation

	LB1A	LB2A	LB2B	LB3A	LB3B	LB4A	LB4B	No bacteria
Gentamycin								
Kanamycin								
Streptomycin								
Ampicillin								
No antibiotic								

40hrs after inoculation

	LB1A	LB2A	LB2B	LB3A	LB3B	LB4A	LB4B	No bacteria
Gentamycin								
Kanamycin								
Streptomycin								
Ampicillin								
No antibiotic								

Box 2

16hrs after inoculation

	NB1A	NB2A	NB2C	NB3A	NB3B	NB4A	NB4B	No bacteria

Gentamycin								
Kanamycin								
Streptomycin								
Ampicillin								
No antibiotic								

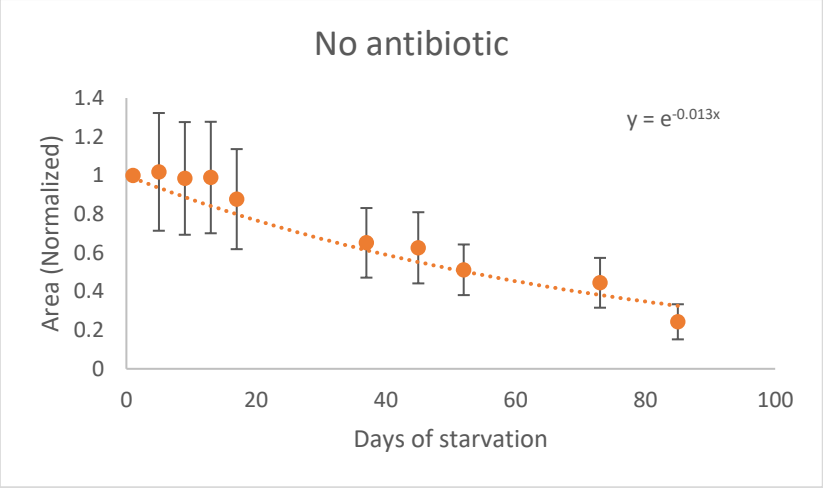
40 hrs after inoculation

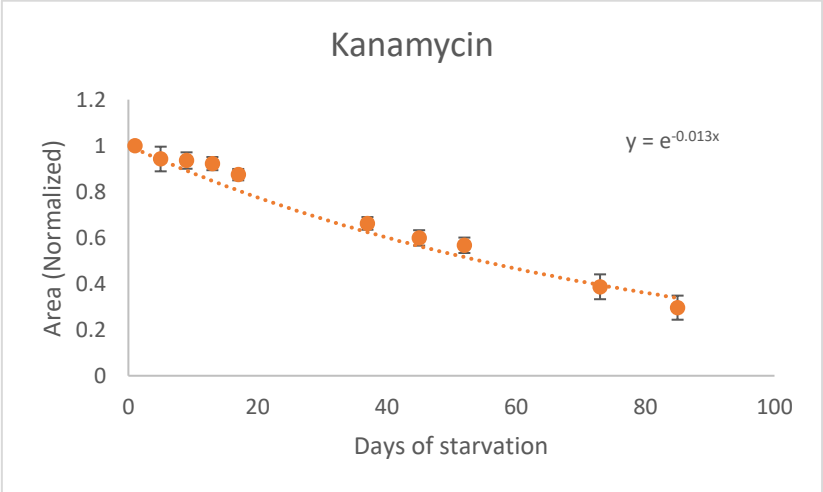
	NB1A	NB2A	NB2C	NB3A	NB3B	NB4A	NB4B	No bacteria
Gentamycin								
Kanamycin								
Streptomycin								
Ampicillin								
No antibiotic								

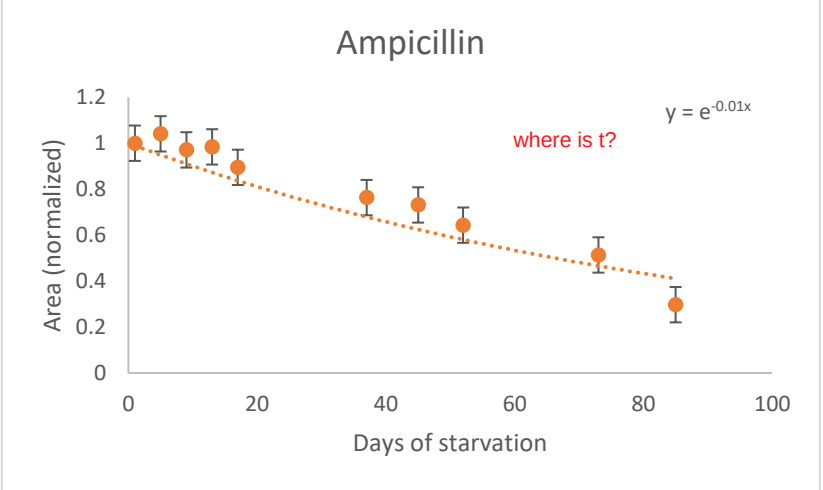
Table 3.1 The above tables show the growth of bacterial colonies in different antibiotic conditions 16hrs and 40hrs after inoculation. The green colour represents presence of bacteria whereas red represents absence. top row is colony name?

For the experiment, we did not want to have more than three treatments as the population of planaria that can be used were insufficient to run a lot more treatments. So, we chose kanamycin and ampicillin as the two experimental treatments. The former allowed a small subset to grow, while latter did not allow any of the bacterial species to grow.

Degrowth-


No antibiotic
y = e-0.013x
Area (Normalized)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Days of starvation


Kanamycin
y = e-0.013x
Area (Normalized)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Days of starvation


Ampicillin
y = e-0.01x
where is t?
Area (normalized)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Days of starvation

Graph 3.1. The above graphs show a scatter plot change in area of planaria with respect to time in three different treatments (No antibiotic, kanamycin and ampicillin). The scatter plot was fitted with exponential decay curve.

To look at the change in area during degrowth, images of planaria were taken and analysed to measure the area. These areas were then normalized by dividing the area of all the days with the initial area (area at beginning of experiment) to account for difference in sizes for different treatments, yielding an area of one as the initial condition. These normalized areas were then plotted on y axis with days of starvation on x axis (Graph 3.1). In a paper published by González et al called "Decreased neoblast progeny and increased cell death during starvation induced planarian degrowth", an exponential line was fitted to degrowth data (González-Estévez et al. 2012). Replicating this, we also fitted the degrowth data with an exponential fit and found that the R^2 value, which represents how well the trendline fits the data, was high ranging between the values 0.85 to 0.96. The equation for the trendline was acquired using R to compared the rates of degrowth among different treatments. We found that the values of t, which gives the rate of decay are very similar across conditions.

with y=...e^tx????

you mention a paper, but explain why exponential.
remember sriram's suggestion/criticisim

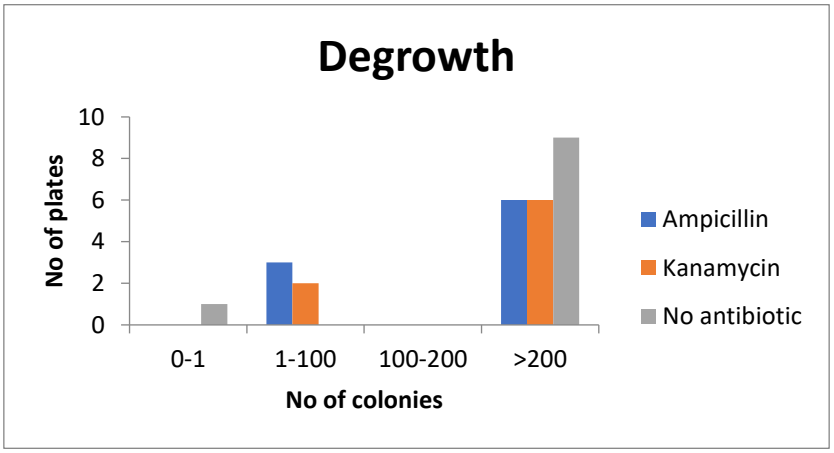
	Ampicillin	**Kanamycin**	**No antibiotic**
t	-0.01	-0.013	-0.013
R^2	0.8589	0.9675	0.9617

Table 3.2. shows the t values from the equation of exponential decay and R^2 values which show the fit of the trendline to the data.

what is the equation?

There seems to be no drastic difference in the rates of degrowth in planaria among different conditions. To verify whether the antibiotic treatments actually caused reduction in the number of microbes we extracted the microbiota from the worm by macerating the worm (as describe in the methods section) and plating them on NB plates. The resulting colonies were counted the colonies from all the treatments and plotted as a histogram. It was seen that the number of colonies did vary in different treatments, with no antibiotic condition having more than 200 colonies in 9 out of 10 plates compared to kanamycin and ampicillin which only has 6 plates with more than 200 colonies (Fig 3.2).

did you have the same number of plates in all conditions?
otherwise these results are meaningless right?
or, you must normalize to the total number plates in each condition

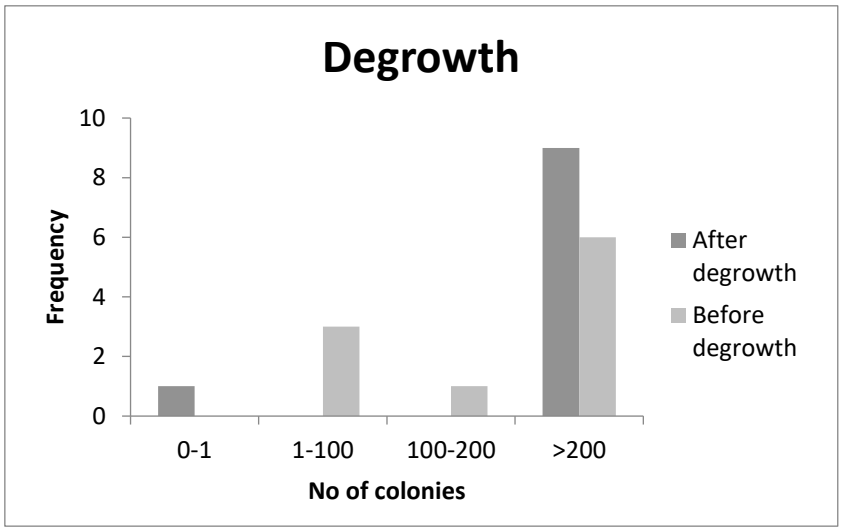


Graph 3.2 is a histogram with no of colonies on x axis and no of plates on y axis. The amount of colonies found in different treatments were plotted

number

While this method is not very quantitative in measuring the relative quantity of microbiota in the worm, it does indicate qualitatively that antibiotic treatment results in a reduction of microbes within the planarian host. However, this difference in quantity of microbiota and the similarity in the rates of degrowth seem indicate that microbiota may not have any effect on the size of planaria in the process of degrowth. If microbes did have an effect during degrowth, the rates of degrowth should be higher in ampicillin and kanamycin treatments compared to control (No antibiotics).

The second goal of this work was to achieve was to measure the change in the quality and quantity of the microbiota before and after degrowth. To achieve this, we plated smashed planaria before and after degrowth and found that higher number of plates in 'after degrowth' condition had more colonies than in 'before degrowth' condition.



Graph 3.3. shows the change in number of colonies before and after degrowth.

These results obtained are quite contrary to the expected outcome. We predicted that the quantity of microbiota will be higher before degrowth and would reduce by the end of degrowth experiment. This hypothesis was made with the assumption that microbiota becomes food for the planaria as there is no external food being provided for the organism. If microbes might not don't play a role in degrowth, then why is the quantity of microbes higher after degrowth? What could have caused the increase in microbes during degrowth? it is a bit awkward to write two questions like this

It is possible that degrowth increases the susceptibility of the planaria to bacterial colonization. However, until further studies are conducted, we will not know the reason for this result. In humans, it was seen that a receptor called nucleotide-binding oligomerization domain-like

receptor (NLR) regulated the microbiota by detecting the microbes and their products (Biswas and Kobayashi 2013). There is constant regulation of microbiota in humans. Are the planaria also constantly regulating their microbiota and does the failure to regulate during degrowth result in the increase in microbiota?

In *C.elegans* worms, the amount of OP50(the food on which the worms are grown in the lab) increase in the gut(Cabreiro and Gems 2013). This was correlated to reduction in the efficiency of the gut in disintegrating OP50 (McGee et al. 2011). Is the efficiency of the microbe inhibitory mechanism reduced? Is this reduced inhibiting being traded off for better survival?

these two para may be better in discussion section

All the data that was collected was done as meticulously as possible but the results we got should be read with a lot of caution. One of biggest assumptions we have made is that the petri plates on which the microbes are grown reflects the microbiota of the organisms. But this might not be the case. 16s rDNA sequencing of planaria's microbiota showed that there were at least 350 distinct species(Arnold et al. 2016). In the plates, there were not more than 14 phenotypically distinct colonies. It is possible that there is higher quantity of microbiota in planaria in ampicillin condition but it did not reflect on the culture plate because those species could not cultured on the petri dish. This problem can be combated by using much finer techniques such as 16s DNA sequencing to determine the difference in microbiota in different treatments. Even though the cost of sequencing has reduced drastically, it is still expensive to use it to analyse such large samples.

our study

did you use the same conditions? in either case you should compare teh conditions

what about differences in micrbiome itself? what if you really had such few species and they had more?

Regeneration-

Schmidtea mediterranea's ability to regenerate into a complete worm even from a tiny piece sets it apart from other organisms. We have established that based on the experiments we have

conducted, there seems to be no effect of microbiota on the process of degrowth. We then wanted to see if the microbial content has any effect on the process of regeneration. Similar to what was done in degrowth, the images of regenerating worms were taken and analysed and their normalized data was plotted with respect to time. As we do not know the complete mechanism by which regeneration occurs, it is hard to determine which fit makes sense biologically. Instead of using fit to determine the change in size, we calculated the reduction in size with the formula ((initial size-final size)/initial size)*100).

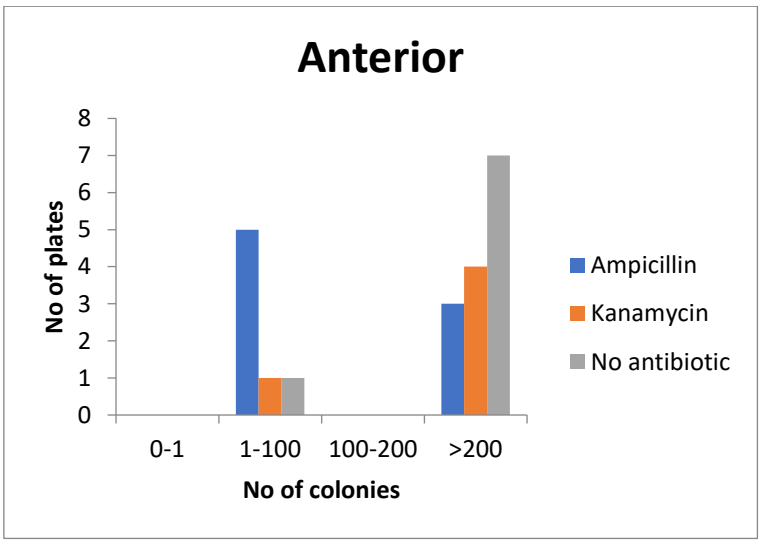
not sure so many decimal places are significant?!

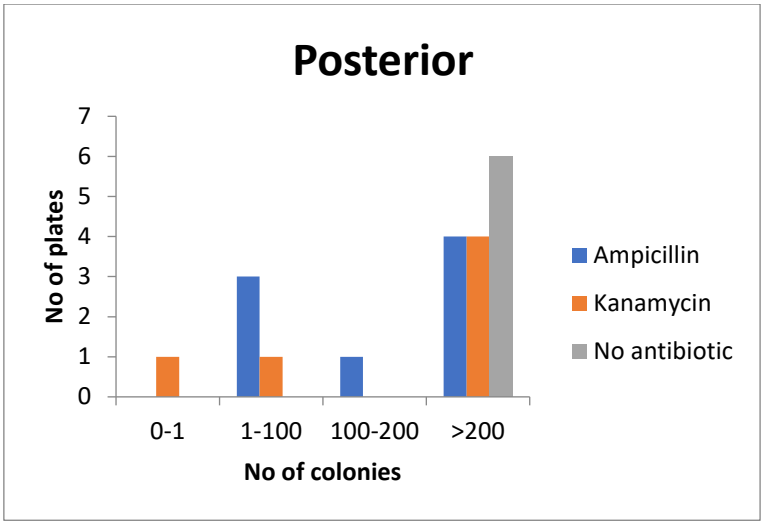
Treatment	Anterior or posterior	Initial size (mm^2)	Final size (mm^2)	Reduction in size (%)
No antibiotic	Anterior	9.28646 (SE = 1.969438)	6.29522 (SE = 1.447258)	32.21077
	Posterior	8.326332 (SE = 1.297055)	6.100384 (SE= 1.106487)	26.73384
Ampicillin	Anterior	9.833676 (SE = 1.252658)	7.041922 (SE= 1.473289)	28.38973
	Posterior	8.610548 (SE = 0.938923)	6.674132 (SE = 0.522691)	22.48888
	Anterior	13.36965 (SE = 1.619357)	8.773642 (SE = 1.120605)	34.37643

Kanamycin				
	Posterior	9.693828 (SE= 1.069825)	7.666302 (SE = 0.82806)	20.91564

Table 3.3. shows the reduction in size during regeneration ensure table is on on page

We have seen that even though the reduction in size is not very different among treatments, there is a difference in size reduction between anterior and posterior. We plotted the number of colonies in order to see that there was a difference in amount of microbiota in different conditions. As expected, we found that there are higher number of plates with more bacteria in control than the other two treatments for anterior and posterior pieces.

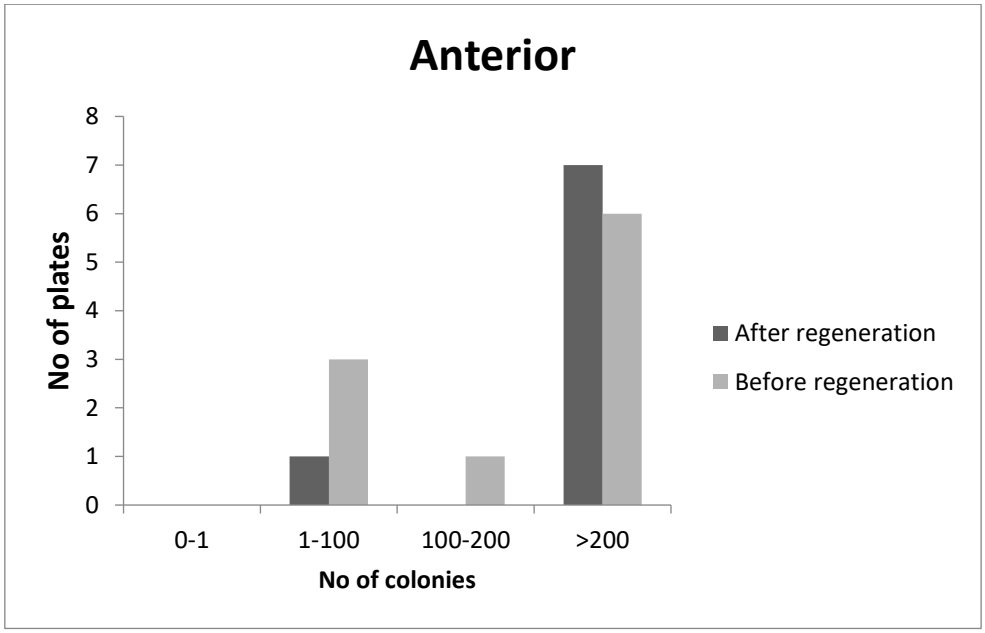


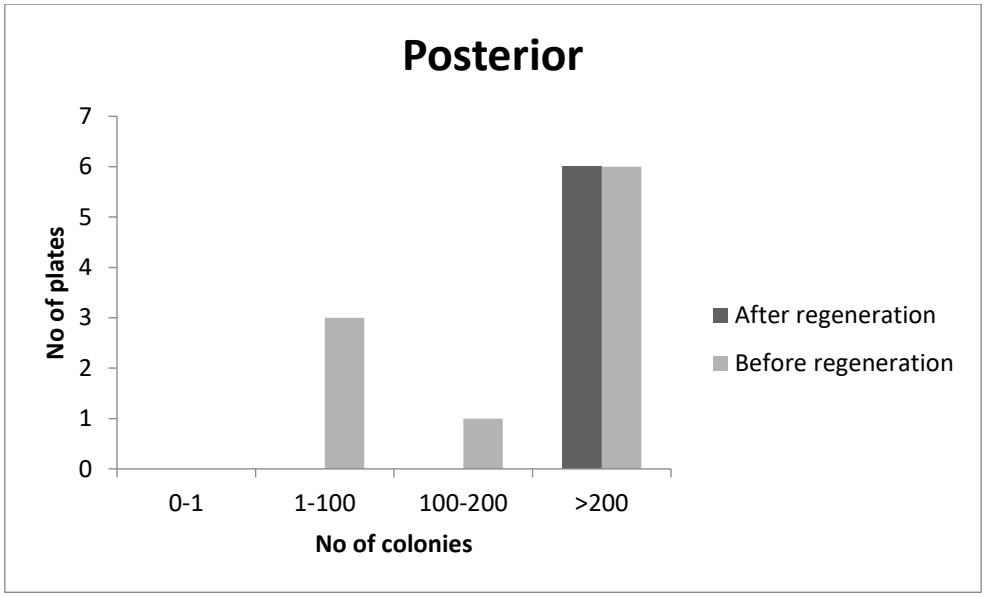


Graph 3.4. shows the difference in amount colonies. The data for the anterior and the posterior region were separated.

graph vs figure? not clear. please name them all Figure 3.5, Figure 3.4 etc.

We found that there was no difference in the amount of microbiota before cutting the planaria and after its regeneration both in the anterior and posterior parts of the organism.





Graph 3.5. shows the number of colonies before and after regeneration in anterior and posterior parts of planaria

We can see from the results above that there was difference in size reduction of the anterior and posterior parts but this difference does not seem to be caused by the quantitative difference in microbiota as there was no difference in microbiota between anterior and posterior. Then could it be because of qualitative difference in microbiota? When we looked at the plates of anterior and posterior parts, we found that there were phenotypically different colonies in anterior and posterior part of a same organism.

These difference in microbiota between anterior and posterior could have been a product of the box and medium they were kept in. To check if the planaria medium they were placed had any bacteria content we plated 200ul of planaria medium on both LB and NB agar and found that there was no bacterial growth seen even after four days. This show that different bacteria may be present in the anterior and posterior regions of the planaria.

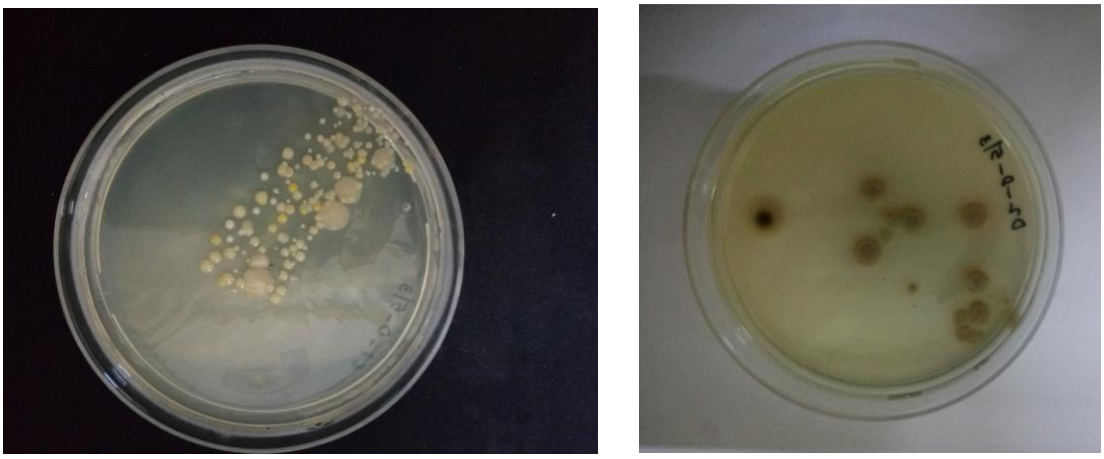


Fig 3.1. a) shows the colony morphology in NB plate of the anterior region whereas b) shows the colony morphology of posterior region of the same planaria colonies look significantly different!

The difference in the reduction in size between anterior and posterior could also be because of the different parts the anterior and posterior pieces have to regrow. The anterior piece has to regenerate the tail and pharynx whereas the posterior has to regenerate the head part which also contains the eye spots. It is known that neural ganglia and eye spots which might be expensive to produce are in the anterior region of the planaria. This means that there should be larger reduction in size in the posterior part compared to the anterior. This was not what was observed. What would have caused the anterior parts to reduce in size more than the posterior even though they have regenerate lesser energy expensive regions?

When we compared the images of anterior part to the posterior part, we found that regeneration image of what? refer to figure in the anterior was complete in many organisms whereas in posterior the eye spots have not fully developed and the colour of the anterior part was slightly less brown than posterior indicating that complete regeneration has not occurred. The rate of regeneration seems to be faster in anterior than posterior and hence a larger decrease in size in the same amount of time.

conclusion and future work is ...

Conclusion

Through these experiments of regeneration and degrowth, by changing the microbiota present in the organisms we discovered answers for few questions but lot more questions unearthed. Our experiment of degrowth supports the earlier observations of González et al. that planaria can survive more than 3 months in the absence of food. We also found that the microbiota present in planaria might not have any effect on the change in size during degrowth.

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