Cooperative Microbe-Host Carbon Metabolism Drives *Drosophila* Regenerative Response

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Origins

This thesis project was completed in a turbulent time in history. I interviewed and joined the Goentoro Lab on Friday March 13 2020; two days later, COVID19 pandemic shutdowns began. My first year in the lab was reduced to catching up on literature , reviewing data from older colleagues, and planning experiments. In the beginning, my project was inspired by our postdoc, Aki Ohdera , who shared an unpublished finding that penicillin treatment induced jellyfish regeneration. In addition, a fellow graduate student in the lab, Yutian Li, demonstrated that penicillin decreased LGI efficacy in our *Drosophila* model. In my interpretation, these findings were our first evidence that bacteria can influence regeneration!

Turning to our *Drosophila* model, I had to consider what function could be removed when the microbiome is removed by an antibiotic. Due to my own preoccupation with culinary fermentation, I was already particularly familiar with some metabolic functions that the *Drosophila* microbiome, dominated by *Lactobacillus* and *Acetobacter* might provide *Lactobacillus* and *Acetobacter* are cornerstones of culinary fermentation because they siphon sweet and spew sour grinding sugars into acids. The first metabolites of interest therefore were lactic and acetic acids, the signature metabolites of *Lactobacillus* and *Acetobacter*.

ABSTRACT

Cooperative Microbe-Host Carbon Metabolism Drives *Drosophila* Regenerative Response

The gut microbiome is a collaborative intermediary between host diet and metabolism. Previous work from our lab in multiple species including in *Drosophila*, demonstrates that modulating nutrients can promote regeneration processes. Motivated by the roles of nutrients, in my thesis, I examined the role of the microbiome. I found that systemic administration of a single strain of *Lactobacillus brevis* promotes activation of regeneration processes in injured limbs. The regeneration-promoting effect of *Lactobacillus* can be recapitulated by lactate, TCA metabolites, and genetically overexpressing lactate dehydrogenase. Finally, in collaboration with Gloria Bates, we found that directly supplying lactate can promote partial limb regrowth. These experiments support growing evidence that bacteria can promote host regeneration processes, and propose a role for lactate in fuelling this host-microbe interaction.

PUBLISHED CONTENT AND CONTRIBUTIONS

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Z.J.C participated in data collection and analysis for single fly tracking and in editing the manuscript and the paper's response to comment.

TABLE OF CONTENTS

Acknowledgements ……………………………………………………….………......iii
Abstract…………………………………………………………...…………...iv
Published Content and Contributions…………………………..……….........v
Table of Contents………………………………………….…...…………vi
List of Illustrations and/or Tables……………………...……...……………vii
Nomenclature and Acronyms………………………………...……………viii
Chapter 1: Introduction to Regeneration Theory…………………………….1
Regeneration hinges on energetic and nutrient parameters………..……………1
Hosts are shaped by their microbes…………………………..…….………3
Drosophila gut microbiome is dominated by Lactobacillus and Acetobacter..….4
Can the gut microbiome enable or enhance distal host regeneration?.................4
Lactate as a key metabolite in animal metabolism……………………….…….. 6
Chapter II: To be Published Work and Discussion……………...……….10
Abstract……………………………………………………………………10
Microbiome influences how Drosophila responds to injury……………………10
Antibiotic treatment modulates microbiome composition in flies……………...12
Acetobacter also stimulated scabless healing, less so than Lactobacillus………13
LFM2 influences wound healing through something it secretes………………..14
Lactate, TCA metabolites, and expression of host lactate dehydrogenase can recapitulate the effects of LFM2……………………………………………………..…...…...16
Lactate supplementation induces partial limb regrowth……………….………20
Summary………………………………………………………….………22
Methods……………………………………………………………..………..22
Chapter III: Unpublished Work and Continued Discussion……...…..……27
Lactate and pyruvate are interconverted by LDH and transported with monocarboxylate transporters………………………………………………………………..27
Some SCFAs like acetic acid influences wound healing………………………28
Antioxidants glutathione and vitamin C modulate wound healing…………….29
Chapter IV: Conclusions and Future Directions…………………..……..31
Summary/Description of Findings…………………………………....…...31
Future Directions………………………………………………………..32
Bibliography…………………………………………………………….36

LIST OF ILLUSTRATIONS AND/OR TABLES

Number

1. **Figure 1. *Lactobacillus* modulates host injury response**
2. **Figure 2. *L. brevis* LFM2 does not directly act through the wound, must be metabolically alive to assert this effect, and does not require TOR activity in the fat body.**
3. **Figure 3. Lactate, TCA intermediates, and expression of lactate dehydrogenase can promote scabless wound healing.**
4. **Figure 4. Lactate promotes partial limb regrowth**
5. **Figure S1. Antibiotics selectively modify *Drosophila* microbiome.**

NOMENCLATURE AND ACRONYMS

Nomenclature

Commensal microbe –Bacteria commonly associated with healthy host microbiome

Fermentation - Microbial breakdown of carbohydrates into organic acids

Hemolymph – Body fluid in insects transporting nutrients and oxygen, comparable to blood in vertebrates.

Tarsus - The most distal segments of a fly limb

Tibia – The middle segment of a fly limb, target of amputation in the experiments described in this thesis

Femur - The most proximal large segment of a fly limb

Acronyms

LGI - Leucine, glutamine, and insulin

SCFAs – Short-chain fatty acids, carboxylic acids like acetate and butyrate produced by microbial carbohydrate fermentation

SFT - Single-fly tracking

LDH- Lactate dehydrogenase, an enzyme that reversibly interconverts pyruvate, the product of glycolysis, and lactate

PDH - Pyruvate dehydrogenase, an enzyme that irreversibly converts pyruvate to acetyl-CoA

TCA - Tricarboxylic acid cycle, a mechanism of oxidative phosphorylation, converts acetyl-CoA into CO_2, and generates roughly 15 times more ATP/carbon vs glycolysis alone

Chapter 1: Introduction to Regeneration Theory

Regeneration hinges on energetic and nutrient parameters

Simple wound healing is as critical to survival as it is ubiquitous to life. Even single-celled organisms like bacteria are able to survive and repair small holes in their cell membranes to prevent their cytoplasm from leaking out. Chemo- and electroporation of cells for plasmid insertion is reliant on this inherent capacity for repair. In multicellular organisms, small wounds are rapidly closed without cell/tissue replacement to seal leaking vascular systems and prevent infection.

Regeneration requires tissue/cell replacement. Plants and fungi are well known to regenerate whole body structures. However highly regenerative animals are phylogenetically few and far between. Only a select few animals are capable of regenerating complex body parts, such as salamanders, sea stars, and hydra. In many animals regeneration is much more limited. This spread of regeneration across the tree of life has led some biologists to suggest that regeneration is a basal trait. This hypothesis, championed by some including Thomas Hunt Morgan, proposes that regeneration is not a trait that has evolved many times, but rather an ancestral trait that has been reduced, deactivated or lost in some species. But why would it be advantageous to deactivate regeneration, and can latent regenerative healing responses be reactivated?

An alternative hypothesis is that regeneration is an evolved trait of a specific clade or organ. Other unique traits have convergently evolved separately numerous times; convergence is common and can occur even for complex traits. For instance, C4 photosynthetic plants including important agricultural crops like corn millet and sorghum thrive in high light environments and persist in other usually poor conditions like high soil salinity or low atmospheric CO_2. C4 plants do this by temporally and spatially separating their photosynthetic metabolism across multiple cells, using malate as temporary storage for CO_2. Despite requiring these large changes to development and metabolism, C4 photosynthesis has evolved separately at least sixty times (Schlüter and Weber, 2020).

Previous work from our laboratory studied how *Aurelia aurita*, moon jellyfish, symmetrize in response to injury. When amputated, *Aurelia* larvae reliably rearrange their existing body parts to become symmetrical again. This allows them to survive the injury, regain

motility and persist into their adult medusa stage normally (Abrams *et al*., 2015). Interestingly, it was subsequently observed that under high nutrient conditions, *Aurelia* begin regenerating appendages (Abrams et al., 2021). Importantly, the effect of nutrients can be recapitulated by the hormone insulin and the amino acid leucine. These observations emboldened our group to study regeneration induction in other poorly regenerating organisms, including in the fruit fly *Drosophila* (Abrams et al., 2021).

Drosophila as an experimental model offers a number of benefits, such as short lifespan/generation time, small body size, lower ethical constraints than mice, and a vast library of mutants already available from other labs and through public repositories. *Drosophila* imaginal discs, the primordial limb structure, are known to be regeneration capable (Hadorn and Buck, 1962). However, this regenerative ability is lost as the imaginal discs differentiate, and adult *Drosophila* are incapable of limb regeneration. Imaginal disc regeneration is regulated tumor suppressor proteins like TSC1 and TSC2 (key regulators of mTOR), PTEN (which works antagonistically to PI3K upstream of mTOR signalling) and the HIPPO Pathway (which controls organismal size; Hariharan and Bilder, 2006).

The insulin signaling pathway has a broad range of effects on *Drosophila* biology. *Drosophila* insulin-like peptides (DILPs) function similarly to insulin but expressed differentially. DILP expression increases cell size and number, glucose oxidation, lactate production, and reproductive rate and decreases longevity (Wu and Brown 2006).

We have used our *Drosophila* limb injury model as a high throughput testing platform. Our 2021 work (Abrams *et al*., 2021) highlighted the key importance of energetic parameters in regeneration. Specifically previous work from our lab found that regeneration processes in the adult fly limb can be promoted to begin using insulin, leucine, and glutamine, the same signals that worked in *Aurelia* In the same study, it was also demonstrated that similar nutrient signals, sucrose, leucine and glutamine, can promote digit regeneration in adult mice (Abrams et al., 2021). In Li et al. (in review) we characterized regeneration responses in *Drosophila* as modulation in wound healing and scab formation, enhanced tissue survival, and eventually regrowth of the limb segment.

These results suggest that perhaps high nutrient requirements prohibit organisms from regenerating under normal conditions. The high nutrient requirement may be above what is common in natural environments, so the organisms that retain it either have been living in a

relatively nutrient rich environment or require it for survival or reproduction in their niche. Organisms may in some capacity sense/detect whether their environment is nutrient rich enough to afford regeneration, while also maintaining life fecundity and healthspan.

It is still not well understood how regeneration is directly initiated or maintained. Work from other labs has also suggested the role of global, energetic parameters for development, growth and regeneration. For instance, insulin receptors are upregulated during limb regeneration in the invasive mitten crab and inhibiting those receptors downregulates myogenesis genes and reduces regeneration frequency (Li *et al*., 2022). In Staghorn coral, environmental nutrient and energetic metrics drive a tradeoff between regeneration and hard body growth (Denis *et al*., 2013). Hedgehog (Hh) is a segmentation protein expressed in imaginal discs that also functions as a starvation signal. Embryonic Hh functions as a morphogen, a diffusing tissue patterning developmental protein, helping form segments across its concentration gradient. Hh is expressed in larval imaginal discs and remains high through pupation, but Hh expression is usually low in adult limbs. Under starvation Hh expression increases in the gut and enables fat body fatty acid mobilization increasing survival (Rodenfels *et al*., 2014).

Hosts are shaped by their microbes

Motivated by the increasing appreciation of the role of nutrients in regeneration, in my thesis, I investigated the role of microbiome.

The turn of the millenia brought with it a boom of microbiome science and a major shift in our understanding of our relationship with it. Increasingly available high-throughput sequencing came with an interest in the mechanistic effects of the microbiome, and advances in germ free experimentation enabled studies to interrogate these effects (Hiol *et al*., 2025). This new crop of studies revealed our microbiome to be a key switchbox for metabolism and the immune system (O'Hara *et al*., 2006). Our view of the microbiome was changing from a jungle to be tamed to farmland to be maintained.

Many microbiome studies have been primarily conducted in mice because of its short translational distance to humans and other similarities between models. Mammalian microbiomes are complex by nature and simplifying or removing it with antibiotics is deleterious to host health (Patangia *et al*., 2022).

The more we discover about the microbiome the more we find that it has its hands all over host physiology. Eons of host-microbe coevolution begot hosts shaped by their microbes. Microbes powerfully influence their host in an innumerable number of ways. Microbiota even influence mating preference in *Drosophila* (Sharon *et al*., 2010).

Drosophila* gut microbiome is dominated by *Lactobacillus* and *Acetobacter

Drosophila fruit flies are a model system used to dissect the roles of commensal bacteria (Wong *et al*., 2011; Ludington *et al*., 2020). On top of the same benefits of the *Drosophila* model described for our previous work, microbiome studies in *Drosophila* also benefit from a simpler microbiome composition. Laboratory *Drosophila* supports just three major bacterial genera, *Lactobacillus*, *Acetobacter*, and *Wolbachia*. The wild *Drosophila* microbiome however is more variable and slightly more diverse. Increased diversity is predominantly constrained to the order Lactobacillaceae, and the families *Acetobacter*aceae and Enterobacteriaceae, with new members primarily belonging to plant and soil microbiome members. In total at least 87 genera have been observed in collected wild *Drosophila* (Adair *et al*., 2018). The laboratory mouse microbiome is at least four times larger, likely containing greater than 367 genera (Kim *et al*., 2024). Yeasts and *Wolbachia* also make their home on or in *Drosophila*, but this work will focus on prokaryotes in the gut.

In lab grown larvae and young flies, *Lactobacillus* represent >75%, while elderly flies possess >75% *Acetobacter* (Wong *et al*., 2011). Wild flies accumulate their gut microbiome through feeding and shedding on food sources. Lab flies are unable to travel from food source to food source which limits their bacterial diversity as well as hard sets them into whatever was left behind from the microbiome of their parent generation. For the purpose of experiments, bacterial feeding and antibiotic supplementation can simulate flies moving to a new food source with a different microbial composition (Luddington *et al*., 2020).

Can the gut microbiome enable or enhance distal host regeneration?

Studies have revealed that microbiota influences host regeneration. *Xenopus laevis* tadpoles can fully regenerate their tails when amputated. Gentamicin treatment reduces tail regeneration frequency and quality. This treatment is complemented with commensal but not pathogenic lipopolysaccharide treatment, implicating Toll-like innate immune signalling in this model

(Chapman *et al*., 2022). Sea cucumbers fully regenerate their intestinal cavity after ejecting it as a defense mechanism or while under temporary stress. Even at low concentrations various antibiotics and antibiotics cocktails reduce intestine regeneration quality (Díaz-Díaz *et al*., 2021). Axolotl form blastema after limb amputation and are capable of complete limb regeneration. During blastema formation, *Flavobacterium* blooms in blastema tissue. Microbial nucleic acid synthesis peaks during regeneration initiation followed by rising lipid synthesis, which they compare to previously observed rises in polyamine and sphingomyelin concentrations in axolotl blastema (Altin *et al*., 2024). Hormone-induced axolotl metamorphosis decreases limb regeneration capacity, and their associated microbiomes shift enrichment away from Firmicutes to Proteobacteria (Demircan *et al*., 2018).

Drosophila microbiota increase intestinal stem cell proliferation rate driving epithelium renewal, authors implicated JNK JAK-STAT signalling (Buchon *et al*., 2009). Microbial lactate production induces gut epithelial dysplasia and increases epithelial ROS production in the *Drosophila* gut (Iatsenko *et al*., 2018). ROS-stimulated JNK signaling regulates intestinal stem cell activity and promotes epithelial regeneration via Ets21c (Mundorf *et al*., 2019). Ets21c is itself implicated in the transcriptional programming of blastema and regeneration in *Drosophila* imaginal discs (Worley *et al*., 2022).

One of the many ways microbiome influences host physiology is through energy homeostasis modulation. The gastrointestinal tract is both the entryway for environmental nutrients, and home to the metabolically active gut microbiome, giving the microbiome access to our nutrients before we do. Microbiome modifies available nutrients like excess sugars from the diet and produces intermediate metabolites. Vitamin A is increased by murine microbiome members like *Lactobacillus* (Bonakdar *et al*., 2022). Other energy related micronutrients may also be important. Evidence exists that NAD+, an important cellular electron shuttle and product of vitamin B3, is limiting in liver regeneration (Mukherjee *et al*., 2025). Influence over host available macro and micronutrients puts the microbiome in the position to modulate host nutrient availability and signalling.

Host energetic/signalling interactions with microbiota have been extensively studied. Germ-free mice require more chow and store less fat than conventionally raised controls. Additionally conventional mice had increased TCA intermediates (α-Ketoglutarate, citrate, malate, and NADH) in conventionally raised mouse calf muscle and liver suggesting increased

host TCA cycling (Backhead *et al*., 2004). Having conventional microbiota increases nutrient sensitivity and enhances mitochondrial function in zebrafish enteroendocrine cells (Alsudayri *et al*.,2023). Long-term antibiotic use worsens disease progression in non-obese diabetic mice (Brown *et al*., 2016).

Co-metabolism between *Lactobacillus*, *Acetobacter*, and host *Drosophila* enables growth in low protein conditions (Consuegra *et al*., 2020). Otherwise complete diets lacking an individual essential amino acid upregulate *Drosophila* odorant receptors that increase commensal microbe uptake (Ezra-Nevo *et al*., 2025). *Lactobacillus* produced lactic acid increases starvation resistance by promoting oxidative phosphorylation (Millington *et al*., 2026). The *Drosophila* microbiome itself may be able to modulate insulin/TOR signaling (Shin *et al*., 2011; Storelli *et al*., 2011). Honeybees and *Drosophila* have similar microbiota, *Lactobacillus* and other lactic acid bacteria *Bifidobacteria*, another sugar fermenter *Gilliamella*, and *Snodgrassella* which associate with the epithelium to form a negative oxygen gradient in the lumen to enhance sugar fermentation by fellow microbiome members in exchange for acetate. Germ-free honeybees have lower weight, expression of insulin receptor, and insulin production (Zheng *et al*., 2017).

These connections between microbiome and tissue regeneration motivated the work in my thesis to investigate how and the extent to which bacteria potentiate regeneration in *Drosophila*. In my thesis, I investigated how microbiome modulation influences organ regeneration in the host.

Lactate as a key metabolite in animal metabolism

One of the key metabolites that gut bacteria makes is lactate. And as I will describe in the next chapter, I find evidence that lactate mediates host-microbe interactions that are influencing regeneration processes. Lactate is indeed a key metabolite in animal metabolism. Our understanding of lactate's roles in energy metabolism has changed over time. Historically lactate has been seen as a metabolic waste product and stress signal. As highlighted by (Rabinowitz and Enerbäck 2021) positive opinion of lactate is growing, calling lactate, "the ugly duckling of energy metabolism". I really like the analogy used by (Brooks *et al*., 2020) lactate and by extension LDH are a "fulcrum of metabolism". Lactate usage and LDH expression allow for cell specialization between glycolytic and

Ox/Phos metabolism. Lactate acts as additional carbon storage when pyruvate is not limiting, and is used as a fuel when pyruvate is limiting (Rabinowitz and Enerbäck 2021). Pyruvate metabolism during periods of high ATP use, such as during regeneration, or low nutrients LDH can draw pyruvate from this lactate reservoir. Host glycolysis and commensal LAB produced lactate contribute to this lactate reservoir. This shifts the LDH kinetic balance promoting pyruvate production from lactate, which prevents glucose origin glycolytic carbon from leaking out of the TCA cycle. It is in this state of enforced TCA Oxidative metabolism with lactate concentration is high; increased cell respiration rate favors growth of oxidative muscle fibers over glycolytic (Zhang *et al*., 2024).

TCA rate is dependent on oxaloacetate and acetyl-CoA concentrations (Krebs, 1970), both of which can be made from lactate through pyruvate. In mice, blood serum lactate is the primary input to tricarboxylic acid (TCA) cycle (Hui *et al*., 2017). Germ-free mice have lower levels of TCA cycle intermediates (Bäckhed *et al*., 2004). *Drosophila* pack their blood fully with carbohydrates (Reyes-DelaTorre *et al*., 2012). Their fat body converts excess glucose into trehalose, a less reactive disaccharide that can accumulate to higher concentrations than and diffuse alongside glucose (Reyes-DelaTorre *et al*., 2012). High-energy metabolites atop the TCA cycle, lactate, acetate, and pyruvate, as well as metabolites in the TCA, oxaloacetate and citrate, stimulate a scabless response, while glycolysis precursors glucose and trehalose do not. Fermentation produced lactate acts like a carbon/energy reservoir that props up TCA by feeding into oxaloacetate and acetyl-CoA, kinetically stopping up a leak away from TCA. Along with being fed into TCA, acetyl-CoA can participate in an innumerable number of amino acid synthesis and fatty acid synthesis reactions, including steroid synthesis for hormone production.

Lactate, pyruvate, and ketone bodies are transported by monocarboxylate transporters (MCTs) transport. Oxidative heart mouse muscle cells express and localize MCT1 to plasma and mitochondrial membranes. MCT4, a lactate exporter, is expressed in and removes lactate from glycolytic mouse skeletal muscle cells, suggesting oxidative heart muscle cell metabolism is reliant on lactate produced elsewhere (Zhang *et al*., 2024).Monocarboxylate transporter-1 knockout (MCT1KO) prevents lactate transport in or out of skeletal muscle cells causing lactate to build up internally. MCT1KO muscle cells cannot export lactate doubling down on TCA for energy production. They do this by inducing mitochondrial biogenesis, reducing LDHA expression and increasing LDHB expression. This shifts the LDH kinetic balance promoting

pyruvate production from lactate, which prevents glucose origin glycolytic carbon from leaking out of the TCA cycle. It is in this state of enforced TCA Oxidative metabolism with lactate concentration is high; increased cell respiration rate favors growth of oxidative muscle fibers over glycolytic (Zhang *et al*., 2024).

Lactate is also an important oncometabolite. Cancerous uncontrolled unpatterned growth requires a lot of energy to maintain itself. A few major metabolic strategies emerge: glycolytic cancer cells that focus on grinding sugar into lactate, and oxidative cancer cells that take in lactate and perform OXYPHOS (Sharma *et al*., 2022). Oxidative cancer cell metabolism might therefore be similar to lactic acid treated or commensal associated fly metabolism compared to antibiotic treated flies which rely on host glycolysis. Some oxidative cancer cells induce glycolysis in surrounding tissue to feed on more lactate, and others team up with glycolytic cancer cells in a crossfeeding-like relationship (Sharma *et al*., 2022). Some oxidative cancers have even been shown to directly steal mitochondria from neurons and immune cells in order to more efficiently run their metabolism (Hoover *et al*., 2025; Terasaki *et al*., 2026).

Metabolites like lactate are key signaling molecules cancer cells use to both run their metabolism and the immune system (Mao *et al*., 2026). Lactate sensors like GPR81 are strongly expressed in breast cancers and its inhibition reduces cell proliferation, migration motility and invasion. Various studies refer to lactate as increasing cancer ‘stemness’ (Ishihara *et al*., 2022; Shegay *et al*., 2022). Cancer treatments targeting LDH are emerging. Local genetic inhibition of LDHA or LDHB reverses disease progression and reduces tumorigenesis in K-RAS and EGFR mouse models of non-small cell lung cancer. Small molecule LDHA inhibitor Quinoline 3-sulfonamide has been shown to reduce stem cell proliferation in the same model (Deng *et al*., 2022; Xie *et al*., 2014).

Finally, lactate is an important metabolite in the brain. Neurons require lots of energy which they obtain through high TCA flux efficiently generating ATP through oxidative phosphorylation. To do this neurons and surrounding astrocytes form an established metabolic unit (Bolaños *et al*., 2025; Jamadar *et al*., 2025). Oxidative phosphorylation in neurons is enhanced by glycolysis in astrocytes. Astrocytes function glycolytically, breaking sugar into pyruvate, use LDH to convert it into lactate, and MCTs like *Drosophila*’s CHASKI to export that lactate into neurons (Delgado *et al*., 2018). Those neurons use LDH to convert that astrocyte generated lactate back into pyruvate for TCA cycle entry. Neurons also operate glycolytic machinery further increasing the

availability of pyruvate and therefore flux through TCA. Similar to some of the cancers described in the previous section, glia have been shown to transfer mitochondria to surrounding neurons such that both of their metabolisms are enhanced (Xu *et al*., 2026).

Chapter 2: To be Published Work and Discussion

Abstract

The gut microbiome is a collaborative intermediary between host diet and metabolism. Gut bacteria absorb, use and/or modify nutrients from the diet, enhance host nutrient absorption and metabolism. Previous work from our lab demonstrated that modifying nutrient signaling promotes regeneration responses in poorly regenerating animals, including in *Drosophila*. In this chapter, motivated by the role of nutrients, I revealed a new key role *Lactobacillus* plays in influencing how the host *Drosophila* responds to injury. Antibiotics that eliminate *Lactobacillus* modulates host wound healing. Directly supplying *Lactobacillus brevis* (specifically strain LFM2), lactate and its downstream metabolic products indeed promotes pro-regenerative wound healing response and increases tissue survival following injury. Lactate dehydrogenase (LDH) overexpression in muscle cells recapitulates this effect. Finally, in collaboration with Gloria Bates, we found that directly supplying lactate promotes limb regrowth. .

Microbiome influences how *Drosophila* responds to injury

As an injury model, we amputated the limb (Fig. 1A). We chose the limb amputation because it can be quickly performed under gentle anesthesia, facilitating analysis with a large sample size. To rapidly assess regeneration activation processes, as described in previous work (Abrams *et al*., 2021; Li *et al*., in preparation), an early marker of regeneration activation can be detected within 1–3 days, from the way the wound heals. Flies typically heal wounds by forming a melanized scab (Fig. 1B; Bidla *et al*., 2005; Galkow and Krasnow, 2004). By contrast, in treated flies many wounds heal without forming a melanized scab (Fig. 1B).

For brevity, we hereon refer to this phenotype as “scabless”. Clotting to stop hemolymph flow still occurs within minutes, but it is not followed by any melanized hardening (Bidla *et al*., 2005). To facilitate quick screening, we began by assessing the wound healing phenotype, and subsequently used the strongest conditions we identified to assess tissue survival and growth phenotype.

To investigate how microbiome influences fly host response to injury, previous work from our lab showed that supplying leucine, glutamine, and insulin (LGI) promotes modulation

in wound healing that correlates with subsequent tissue survival and partial limb regrowth (Abrams et al., 2021; Li et al., in review). We therefore first tested antibiotics on LGI-treated flies (Fig. 1C). A broad-spectrum antibiotic (carbenicillin) markedly reduces the bacterial load (Fig. S1) and completely blocks the effects of treatment with LGI. Ampicillin that selectively inhibits *Lactobacillus* (Fig. S1) also markedly inhibits LGI treatment effects (Fig. 1C). By contrast, streptomycin that selectively inhibits *Acetobacter* (Campedelli et al., 2018; and Fig. S1) does not appreciably affect the scabless wound healing stimulation. These results suggest that the microbiome influences the host response to injury, and in particular *Lactobacillus*, is required for LGI stimulated scabless wound healing.

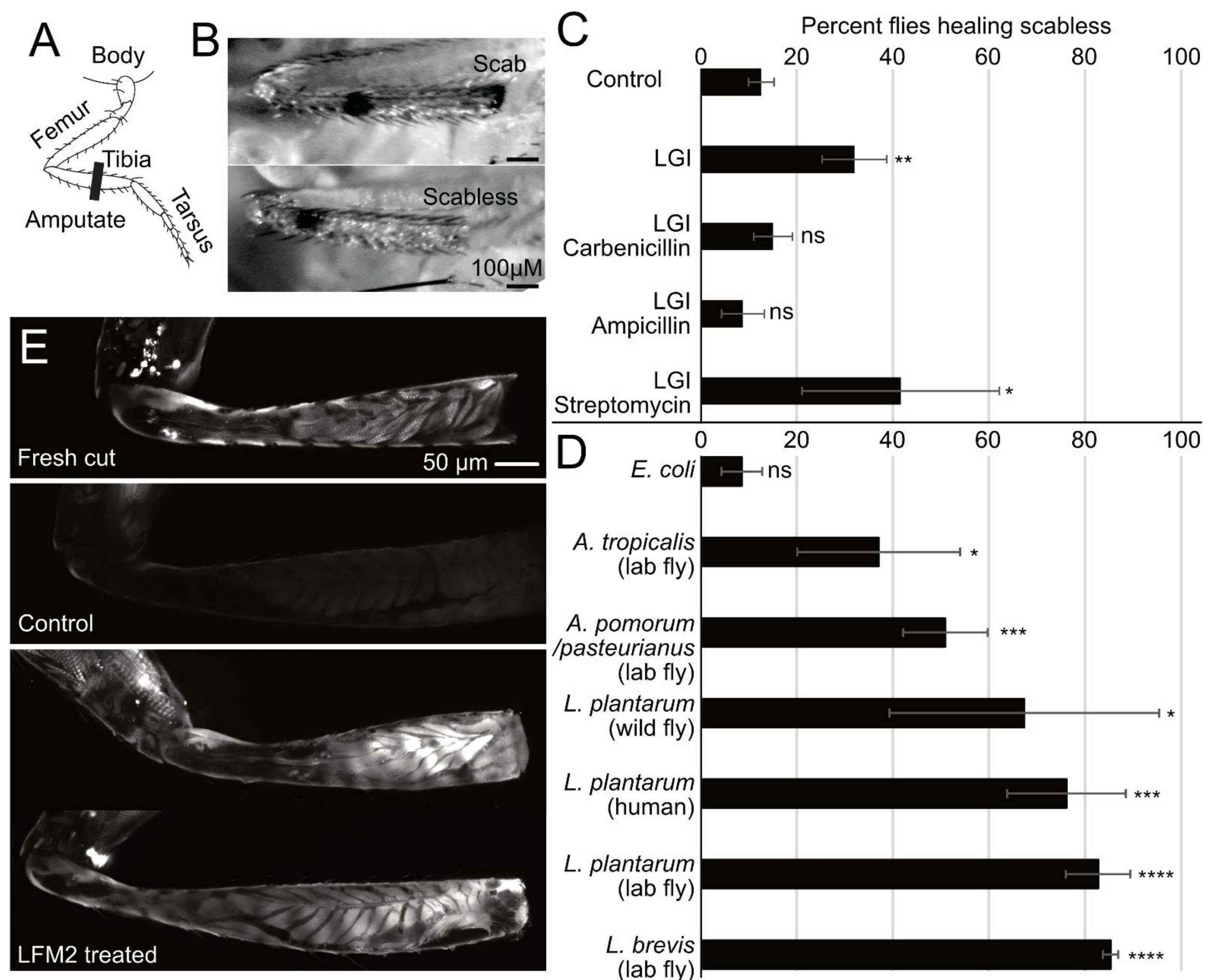


Figure 1. *Lactobacillus* modulates host injury response

(A) Fly limb was amputated medially across the tibia segment.

(B) The way the wounds heal within a week after amputation is an early marker of regeneration process activation. Shown here are residual tibia segments. Tibias normally heal amputation wounds by forming a

dark melanized scab at the wound site (top). In treated flies, many tibias heal without forming a melanized scab (bottom). Scale bar: 100 µm.

(C) Amputated flies were placed in regular food (control), food supplemented with leucine, glutamine, insulin (LGI), or food supplemented with LGI and 100 µg/mL antibiotic. Wound healing was assessed 7 days after amputation. Statistical significance, relative to control, was assessed using Student's t-test.

(D) To supplement bacteria, ~10^6 bacteria (in 100 µL volume) were coated onto the surface of each vial. Each vial housed six flies. Newly eclosed flies were fed with the indicated bacteria for four days, amputated, and then returned to new bacteria-supplemented vials. Wound healing was assessed 7 days after amputation. Statistical significance, relative to *E. coli* control, was assessed using Student's t-test.

(E) These experiments were performed in Mef2>RFP flies, which express red fluorescent protein (RFP) driven by the promoter of *Mef2*. Amputated flies were treated with *L. brevis* LFM2 as described in Fig D. At 2–3 weeks after amputation, the limbs were dissected, mounted, and imaged with confocal microscopy. Control limbs: only 2/14 tibias analyzed still showed residual and diffuse muscle signals by three weeks after amputation. Treated limbs: 10/11 limbs showed strong muscle signals. Scale bar: 50µm.

Antibiotic treatment modulates microbiome composition in flies.

Flies were placed in food supplemented with the indicated antibiotics (100 µg/mL) for 1 week. Sixty flies (30 males and 30 females) were processed for each condition, homogenized with mortar and pestle and diluted and plated onto multiple MRS agar plates which can permit *Lactobacillus* and *Acetobacter* growth. The plates were placed in a 37ºC incubator for two days, and then assessed for colony formation. *Lactobacillus* colonies appear first and are frequently larger because of their higher growth rate on MRS media. and puffy and white to cream in color. *Acetobacter* colonies appear later, are smaller, flatter, wetter, and are a duller grey color than *Lactobacillus*. Comparing Fig. S1 to Fig. 1C, it can be seen that removing *Lactobacillus* reduces LGI stimulated scabless healing.

Next, we assessed the effects of directly administering bacteria (Fig. 1D). *Drosophila* microbiome is dominated by two taxa: *Lactobacillus* and *Acetobacter* (Luddington and Ja, 2020). We tested directly administering *Lactobacillus* and *Acetobacter* strains isolated from flies (Fig. 1D). *Lactobacillus* supplementation can indeed strongly promote scabless wound healing. Even a *Lactobacillus* isolated from human saliva can induce scabless wound healing.

The strongest effect was produced by a *Lactobacillus brevis* isolated from wild-type lab flies (strain name LFM2). More than 80% of the flies supplemented with *L. brevis* LFM2

showed scabless wound healing (Fig. 1D). To further assess the influence of LFM2 on the host response to injury, we assessed the muscle tissues in the residual limb segment. Very few control flies spontaneously heal wounds scabless (~10%; Fig. 1C) and muscle tissues in their residual tibias degenerate within a week after amputation. By contrast, many LFM2-treated flies ~60%; heal with scabless wounds (Fig. 1D). Consistent with a concurrent study in the lab (Li *et al*., in preparation), LFM2-treated scabless flies preserve muscle tissues more than scab. This suggested that LFM2 may also initiate regeneration processes.

Despite multiple *Drosophila* commensal strains stimulating a scabless response, I focused on *Lactobacillus brevis* LFM2 for my next experiments because LFM2 produces a strong scabless response and grows consistently in culture. *Lactobacillus plantarum* strains formed gooey clumps/chains that made homogenization for vial prep difficult.

Acetobacter* also stimulated scabless healing, less so than *Lactobacillus

By contrast, the *Acetobacter* strains tested appear to be less effective. As a control, *E. coli* does not affect changes in wound healing. I suspect *Acetobacter* feeding is less effective for the following reasons. *Lactobacillus* growth rate is dramatically higher than *Acetobacter*. *Lactobacilli* double in a few hours even in the gut, *Acetobacter* doubles on the order of days. I observed this myself as *Acetobacter* growth took 2-3 days in a shaking incubator and reached far lower densities than the *Lactobacilli* were after an overnight in the incubator. Fewer bacteria around means less metabolic activity. Heterofermentative *Lactobacilli*, like the ones in my study, produce both lactate and acetate. I will discuss acetate in further detail in the next section. *Lactobacillus* feeding opens more *Acetobacter* niche space than vice versa. Under poor nutrient conditions *Lactobacillus* produced lactate enhances *Acetobacter* and subsequent host growth in *Drosophila*. Lactate increases larval developmental rate in conventionally raised and *Acetobacter* monocolonized flies, but slows growth in germ free larvae who heavily rely on glycolysis (Consuegra *et al*., 2020). *Lactobacillus* provided lactate also enhances *Acetobacter* growth in fermentation contexts as demonstrated by Adler *et al*., 2014 in cacao production.

LFM2 influences wound healing through something it secretes

Next we investigated how LFM2 acts. Since we supplemented LFM2 through the food, and flies walk on the food, we tested whether LFM2 might act directly on the wound. To test this, we directly delivered LFM2 to the amputation wound using a needle tip (Fig. 2A). Limbs directly given LFM2 scabbed at a rate similar to controls (Fig. 2A). Further, using a GFP-tagged LFM2, we confirmed that despite the large amount of bacteria placed in the food (~10^6 bacteria), in none of the limbs we analyzed (N=17), was LFM2-GFP ever found inside the injured limb (Fig. 2B). We therefore conclude that LFM2-GFP did not act by entering the injured limb.

Next, we assessed whether LFM2 could be acting as a passive food source for the fly, or whether its effect may require that it is metabolically active. To distinguish between these two possibilities, we administered heat-killed LFM2 to the food. Heat-killed LFM2 does not promote scabless wound healing (Fig. 2B). This demonstrates that LFM2 must be alive to influence host response to injury. Since LFM2 has to be metabolically active, we next asked whether LFM2 acts through the metabolites it secretes. To test this, we first placed LFM2 in the fly food, let it sit for 4 days, and then heat-treated the food. The LFM2-treated food can indeed stimulate scabless wound healing (Fig. 2A), although the effect is not as robust as supplying live LFM2, presumably because live LFM2 can continue multiplying, cycling in and out of the fly, and secreting the metabolites.

The metabolic activity of living LFM2 applying to cornmeal/molasses fly food may also be seen as ‘predigestion’. This gives microbiota more time and surface area with which to consume sugars in the fly food and convert them into lactate and acetate. Not only is bacteria being delivered to the fly, but the food itself is transformed by the bacteria.

Altogether, these results suggest that metabolite(s) secreted by LFM2 mediate its influence on host response to injury.

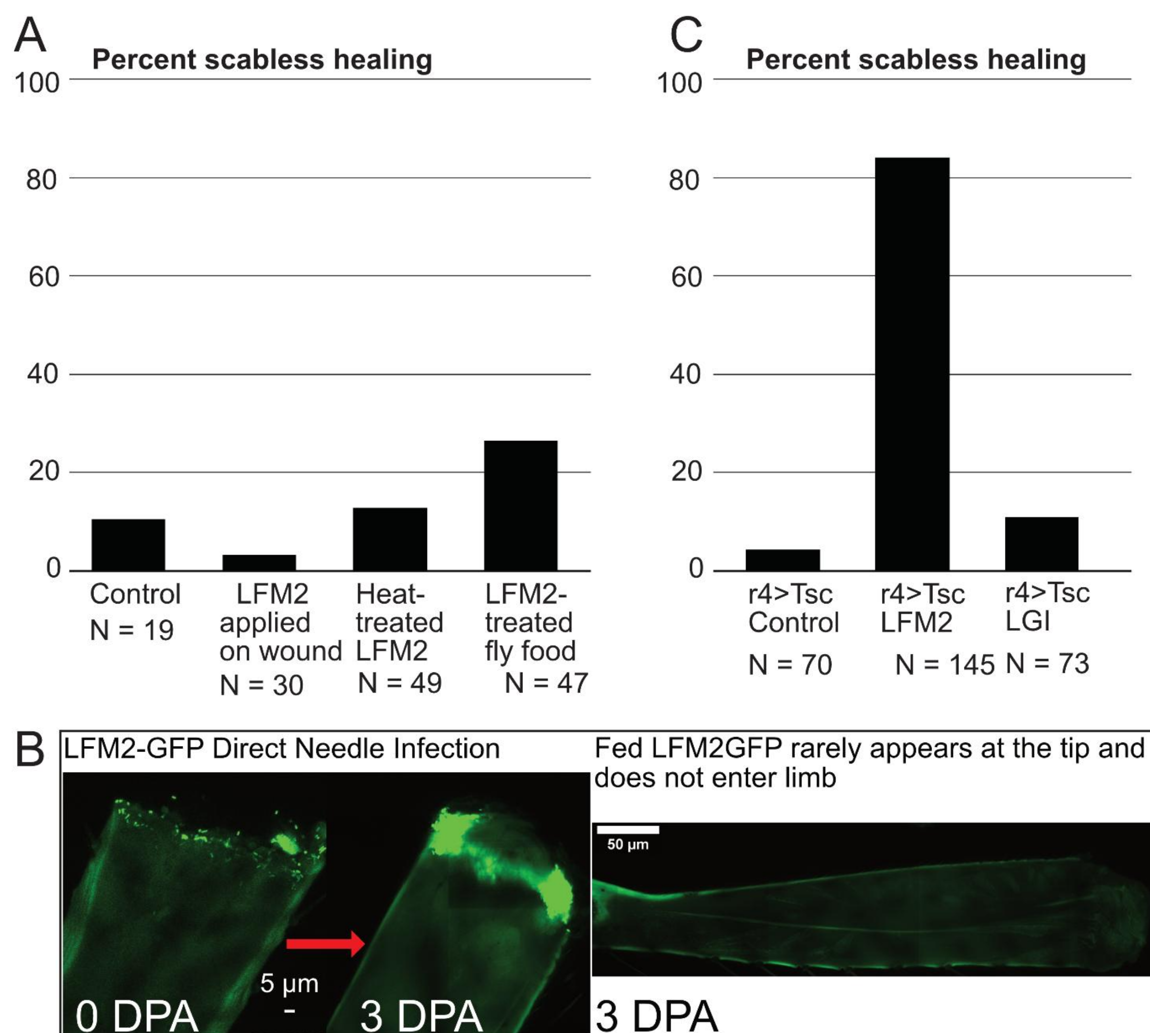


Figure 2. ***L. brevis*** **LFM2 does not directly act through the wound, must be metabolically alive to assert this effect, and does not require TOR activity in the fat body.**

(A) Flies were amputated as described in Fig. 1D, and then subjected to indicated treatment. Control: amputated flies were placed on regular food. Plotted on y-axis is the percentage of flies analyzed that heal wounds scabless, assessed at 1 week after amputation. To apply LFM2 directly to the wound, we dipped a needle in a pelleted LFM2, and then applied the needle to the freshly cut limb stumps. To heat kill LFM2, we placed the culture in 50ºF for 60 minutes. Death of bacteria was verified by plate streaking the heat-treated culture on agar plates and lack of colony formation. To make LFM2-treated fly food, LFM2 was placed in the food, 4 days later the food was heat-treated (50ºC for 12 h). Amputated flies were placed in this LFM2-treated food.

(B) Tibia was amputated and then directly given LFM2-GFP at the wound site. GFP signals were assessed at 1–3 days post infection. GFP signals were observed in 6 out of 6 limbs infected. Bottom right is a representative tibia from flies fed with LFM2-GFP, before and after amputation. The limbs were

imaged at 5-6 days post amputation. No GFP signals were observed inside the injured limbs (N=17). In 1 out of 17 limbs, we observed few bacterial cells (as indicated by GFP signals) on the wound.
(C) Gal4-r4>UAS-Tsc1 flies, with no treatment, treated with LFM2, or treated with leucine, insulin, and glutamine. For treatment, 3-5 day old flies were amputated and then placed in treated food for 1 week. Wound healing was scored at 1 week after amputation. To make treated food, bacteria (~a million cells) or molecules were mixed into the top layer of the food.

A previous study demonstrates that *Lactobacillus* (a *L. plantarum*) supports growth in *Drosophila* through influencing the TOR signaling in the fat body (Storelli *et al*., 2011). Moreover, TOR activity in the fat body is necessary for the effects of insulin/leucine treatment (del Rio Salgado and Goentoro, in review; and reproduced here in Fig. 2C). To assess whether the effect of LFM2 also depends on TOR in the fat body, we used a fat body driver to express Tsc1, a negative regulator of TOR. Expression of Tsc1 in the fat body does not affect the effect of LFM2 on wound healing (Fig. 2C). Therefore, this data suggest that TOR signaling in the fat body is not required for how LFM2 influences the host response to injury.

To test whether LFM2 influences host response to injury through TOR signaling first we used Sapanisertib, a potent inhibitor of TOR, to globally inhibit TOR signaling. Globally inhibiting TOR reduces but does not eliminate the effect of LFM2 like it does to LGI treatment. The results from the inhibitor treatment is informative, but has the caveat that we are affecting the whole animal, which may have non-specific effects. This suggests that while global TOR signaling is still important to LFM2 scabless induction, TOR signaling in the fat body is not required for the effect of LFM2 effect. This indicates lactate may be functioning as a signaling molecule and a metabolite.

Lactate, TCA metabolites, and expression of host lactate dehydrogenase can recapitulate the effects of LFM2

We also observed that flies emerging from LFM2 treated vials have a high scabless response rate, suggesting a role for metabolites produced by *Lactobacilli*. We next looked into the major metabolites that LFM2 secrete. *Lactobacilli* typically cannot perform tricarboxylic acid (TCA; Morishita *et al*., 2014). *Lactobacilli* rely on glycolysis for their central metabolism and secrete lactate.

We therefore tested if directly administering lactate can recapitulate the effects of

supplementing LFM2. Lactic acid bacteria acidify their environment by producing lactate to concentrations less than 0.17M (Popova-Krumova et al., 2024). Concentrations at the upper end of biologically relevant give robust scabless responses. At 0.05M lactate supplementation strongly promotes scabless wound healing (in 58% of the flies, Fig. 3A).

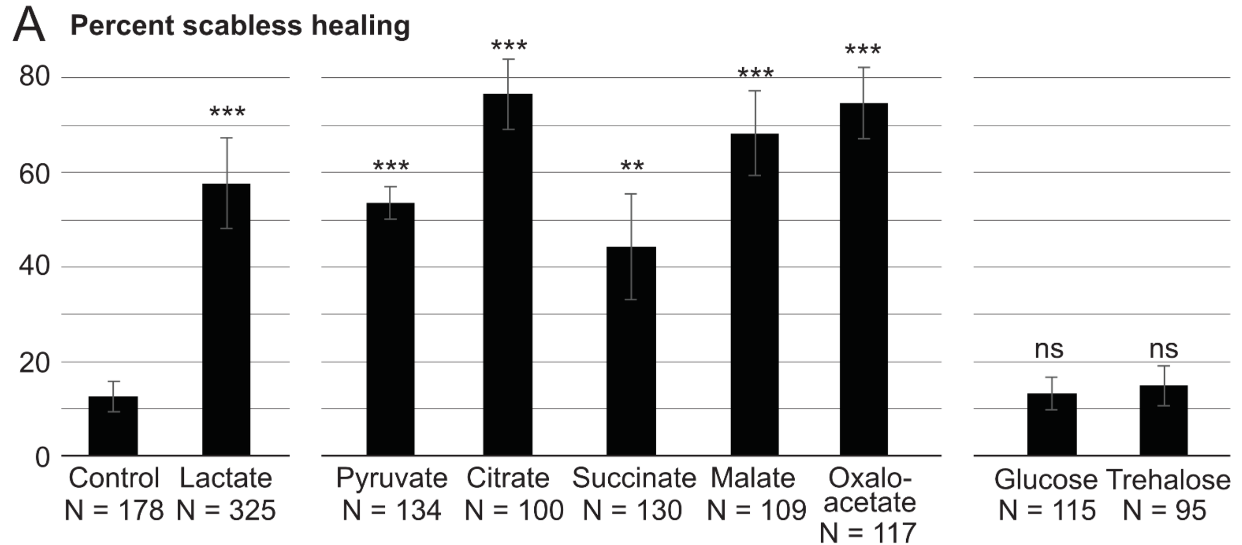
In mice, blood serum lactate is the primary input to the TCA cycle (Hui *et al*., 2017). To further assess the role of lactate, we examined the metabolic pathways downstream of lactate we tested directly administering TCA metabolites. Administration of pyruvic, oxaloacetic, citric, malic, and succinic acids readily increases the proportion of scabless wound healing in the flies. The effects are not general to all metabolites as, by contrast, administration of glucose or trehalose, at the same concentrations, did not promote scabless wound healing (Fig. 3A). This suggests that modulation in TCA metabolites strongly influences response to injury.

To verify the role of lactate, lactate fuels the TCA cycle through the action of the enzyme lactate dehydrogenase (LDH), which converts lactate into pyruvate. LDH is broadly expressed in heart and skeletal muscle as well as organs like the liver and kidneys (Farhana and Lappin, 2023). We therefore tested overexpressing the human LDHA using a muscle-specific Gal4 driver, Mef2-Gal4. Mef2-Gal4>UAS-LDHA flies spontaneously showed, without any supplementation, a marked increase in the frequency of scabless wound healing (Fig. 3B). I also tested supplementing Mef2-Gal4>UAS-LDHA flies with 0.1 M lactate, which further increased scabless response rate. This shows that lactate does not become toxic and suggests that microbial lactate is being metabolized by the flies.

Another adult organ system in which dLDH is known to be expressed is the gut (Hongjie Li *et al.*, 2022). To test if overexpressing LDH in another organ suffices to produce this effect, or if there is a specific role for LDH in muscle, we tested overexpressing LDH in the gut using the Mex1 midgut driver. We found that overexpressing LDHA using the midgut driver Mex2 does not influence scabless response (Fig. 3B).

We wanted to reconnect changes in host genetics with their microbiome. If commensal produced lactate enables the effect of Mef2>LDHA on wound healing, antibiotics that remove lactate producers should reduce that response. As we showed in Fig. S1 ampicillin selectively reduces *Lactobacillus*, and streptomycin selectively reduces *Acetobacter*. Ampicillin but not streptomycin treatment reduced Mef2>LDHA scabless frequency, reinforcing that *Lactobacillus* produced lactate is key to Mef2>LDHA scabless response. These results suggest that lactate, the

major fermentation product of *Lactobacillus*, can recapitulate the effect of LFM2 on wound healing.



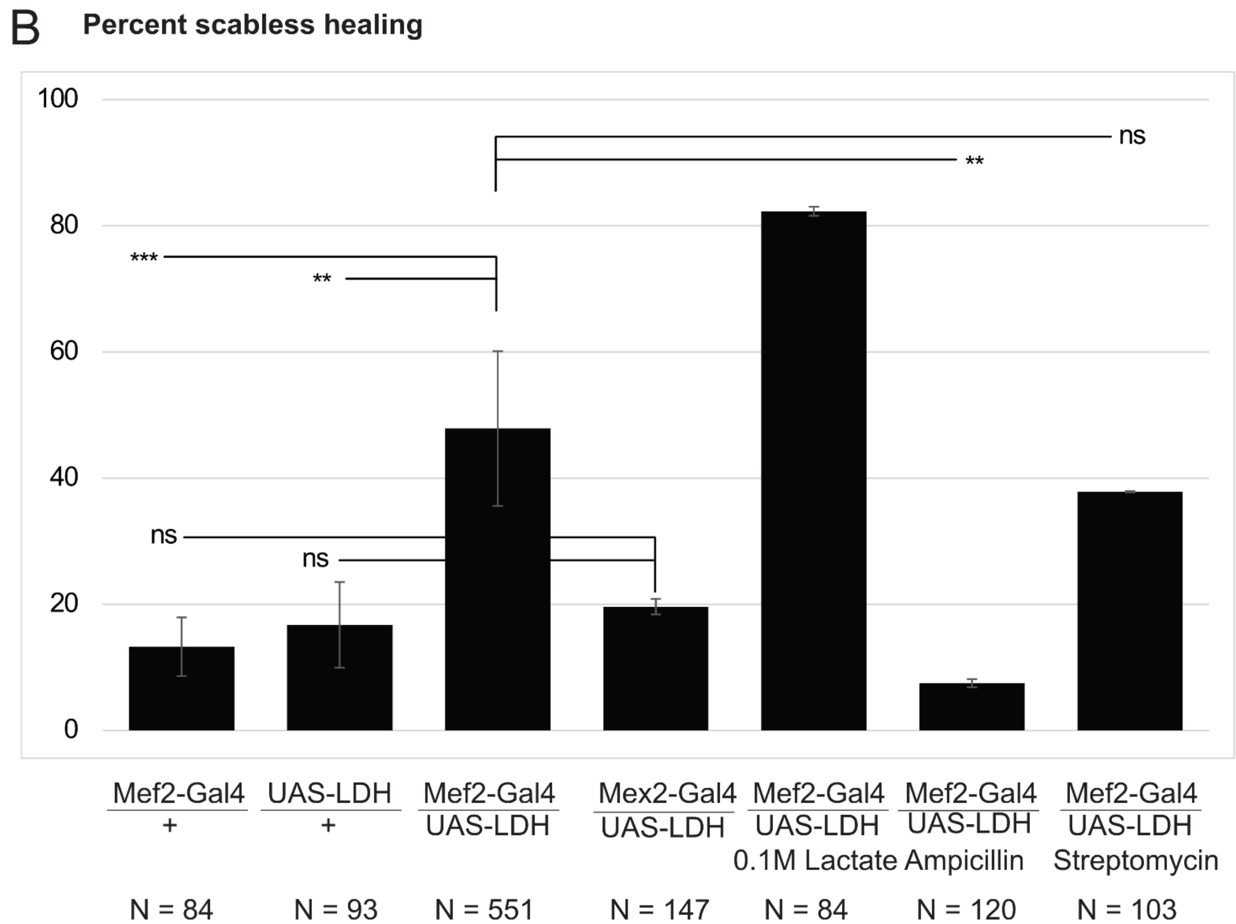


Figure 3. Lactate, TCA intermediates, and expression of lactate dehydrogenase can promote scabless wound healing.

(A) Flies were amputated and then placed on food supplemented with the metabolites at 50 mM mixed into regular food. Wound healing was assayed at 1 week after amputation. Each dataset was obtained from 2–5 independent experiments, with the total flies analyzed shown (N). Statistical analysis: t-test against the control dataset.

(B) Lactate dehydrogenase (human LDHA) was expressed using the muscle-specific Gal4 driver (Mef2-Gal4>UAS-LDH), and the midgut specfic Mex1 driver (Mex1-Gal4>UAS-LDH). Mef2-Gal4>UAS-LDH flies are also tested with 0.1M lactate, streptomycin 100uM, or ampicillin 100uM. As a control, we assessed wound healing in the heterozygous parental lines. Each dataset was obtained from 2–6 independent experiments, with the total flies analyzed shown (N). Statistical analysis: t-test.

Lactate supplementation induces partial limb regrowth

Having identified the role of lactate in modulating wound healing response, we further verified the regeneration processes induced by lactate. As shown in previous work, scabless wound healing correlates with better tissue survival in the residual limb at week-scale and eventually regrowth at month-scale (Abrams *et al*., 2021; Li *et al*., in review).

In collaboration with Judah Bates, we tracked individual flies for a month after amputation to assess for regrowth (Fig. 4). The tracking enables us to compare side-by-side the residual tibia at different time points. First, to establish the validity of the assay, we performed the tracking on untreated flies As expected, no growth was indeed observed in the injured tibias from the control flies In the flies supplemented with lactate, we observed growth.
We observed that, similarly, lactate supplementation leads to better tissue survival in the residual limb at week-scale (Fig. 4A).

To assess regrowth, we tracked the flies for one month (Fig. 4B). As expected, no growth was observed in the injured tibias from the control flies (Fig. 4A, and as quantified in Fig. 4B). Long- term exposure to higher lactate concentrations seems to impair motor function to some extent giving the flies a ‘drunken’ appearance. In spite of which, flies supplemented with lactate some showed limb regrowth (Fig 4B). In the flies analyzed, 20% showed change in length that was beyond the 95% confidence interval for untreated flies

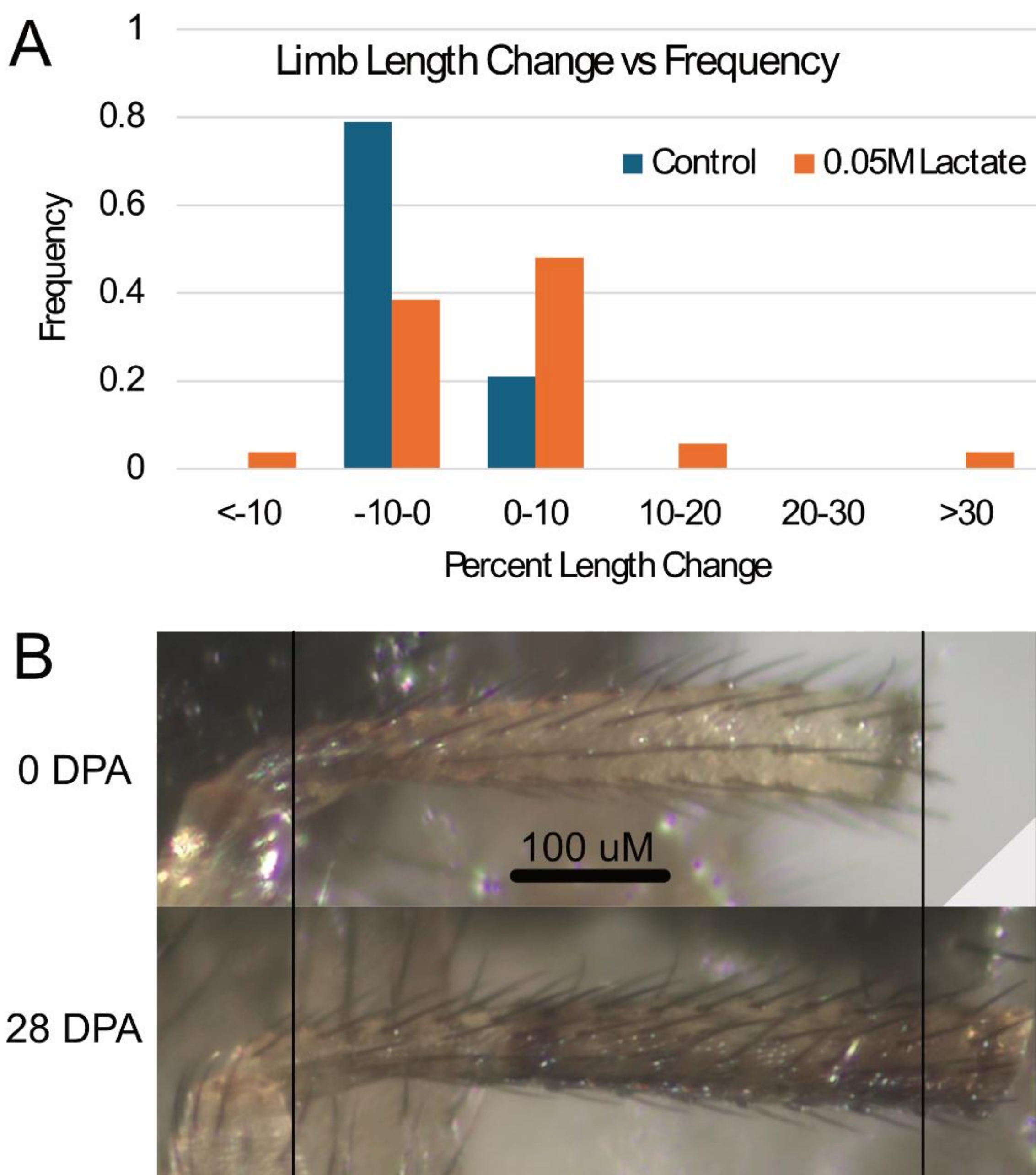


Figure 4. Lactate promotes partial limb regrowth

(A) Single Fly Tracking (SFT) length change frequency data. Residual fly limbs were imaged immediately following amputation and placed on 0.05M lactate treated or control fly food vials. Vials were refreshed weekly with 0.05M lactate treated or control fly food vials respectively. 28 days post amputation the fly limbs were imaged again. Pixel lengths of each limb were measured and compared for change in length. 20% of treated flies exhibited limb regrowth greater than control flies. To ensure limbs were aligned perpendicular to the camera for imaging, flies were restrained under a glass microscope slide

with adequate coverslips attached on each side as spacers. This perpendicular camera angle is confirmed by the sharpness of superficial hairs at either end of the residual limb.
(B) Confocal microscopy of freshly amputated limb and 28 days post amputation, with notable new limb length. The leftmost line aligns the inside of the femoral-tibial joint, the middle line indicates the amputation site, and the rightmost line is the furthest extent of regrowth.

Summary

In this chapter, I characterized how supplementing *Lactobacillus* LFM2 promotes activation of regeneration processes in the host. The effects of *Lactobacillus* can be recapitulated by its fermentation product, lactate. Our data further suggest that lactate promotes activation of regeneration processes by fueling the TCA cycle, and that expression of lactate dehydrogenase influences how flies respond to injury. Finally, supplementing lactate directly modulates wound healing, promotes tissue survival in the injured organ, and eventually promotes activation of partial limb regrowth.

Methods

Antibiotic/LGI/metabolite supplementation. The following antibiotics were used: Ampicillin (RPI, A40040-1.0), carbenicillin (RPI, C46000-1.0), and streptomycin (TCI, S0585). To mix antibiotics into the fly food, the food was melted by microwaving, and the antibiotic added to achieve a final concentration of 100 μg/mL).

Bacterial Fly Food Modification Treatment Method.
To make bacterial MRS broth media, 55 grams powdered medium was mixed in 1 L distilled water, autoclaved and stored at room temperature to use within a month. Bacterial cultures were prepared by inoculating 50 mL media in a Falcon tube from frozen glycerol stock. Larger batches (~250-500 mL), bacteria were grown in an Erlenmeyer flask. Bacterial cultures were incubated at 37°C overnight with no shaking (typically 16-18 hours), reaching maximum growth density at stationary phase.

Cultures were washed with 50 mL of cold PBS and centrifuged at 3000 rcf for 10 minutes at 4°C and the supernatant poured off. The bacterial pellet is resuspended in 25 mL of PBS by alternating vortexing and vigorous shaking. The pellet was washed 2 more times with 50 mL of

PBS until the supernatant is clear. After the final wash, the resulting bacteria paste was thick, not watery but still flowing. Each 50ml vial of MRS typically yielded ~400 ul of LFM2 bacterial paste.

The surface of each treated fly food vial was covered with 50 uL of bacterial paste, such that the flies have a thin layer of bacteria between them and their food. This equates to approximately 10^6 CFU per vial. To pretreat the flies, newly eclosed flies (0–1 day olds) were transferred to bacterial-supplemented vials and left for 4 days. Flies were then amputated, and then returned to fresh bacterial-supplemented vials for 1 week then put on fresh food vials. Freshly loaded bacteria treatment vials must be held sideways after adding flies to prevent anesthetized flies drowning in sticky/wet bacterial paste.

Fruit fly lines and culture.

Flies were kept in a 12hr/12hr light/dark cycle, at room temperature (21-23ºC). The following lines were used in this study: CantonS (gift from Kai Zinn lab at Caltech), Mef2-Gal4>UAS-RFP (BDSC #26882), r4-Gal4 (BDSC #33832), Mef2-Gal4 (BDSC #27390), UAS-Tsc1 (BDSC #80963), UAS-LDHA (BDSC #79190).

Fly amputation protocol

Only adult flies aged 3-5 days are used. Flies are anesthetized using carbon dioxide under low flow rate (below 4 liters/minute)for no more than 2 minutes at a time. Only six anesthetized flies are loaded into each vial to prevent overcrowding and allow time for anesthesia procedures to be completed in under 2 minutes.

To amputate, we used surgical spring scissors (Fine Science Tools, 91500-09) to cut across the tibia (see Figure 1A). For the experiments shown, we amputated mid and hind-limbs randomly, roughly 50:50.

Freshly loaded bacteria treatment vials are held sideways after adding flies to prevent anesthetized flies drowning in sticky/wet bacterial paste. Mortality rate should be <10% if handling procedure is done carefully around the sticky bacteria paste. After 1 week, flies were placed back on regular lab food. Wound healing and tissue survival were analyzed at 1-3 weeks after amputation.

Supplemental Figure

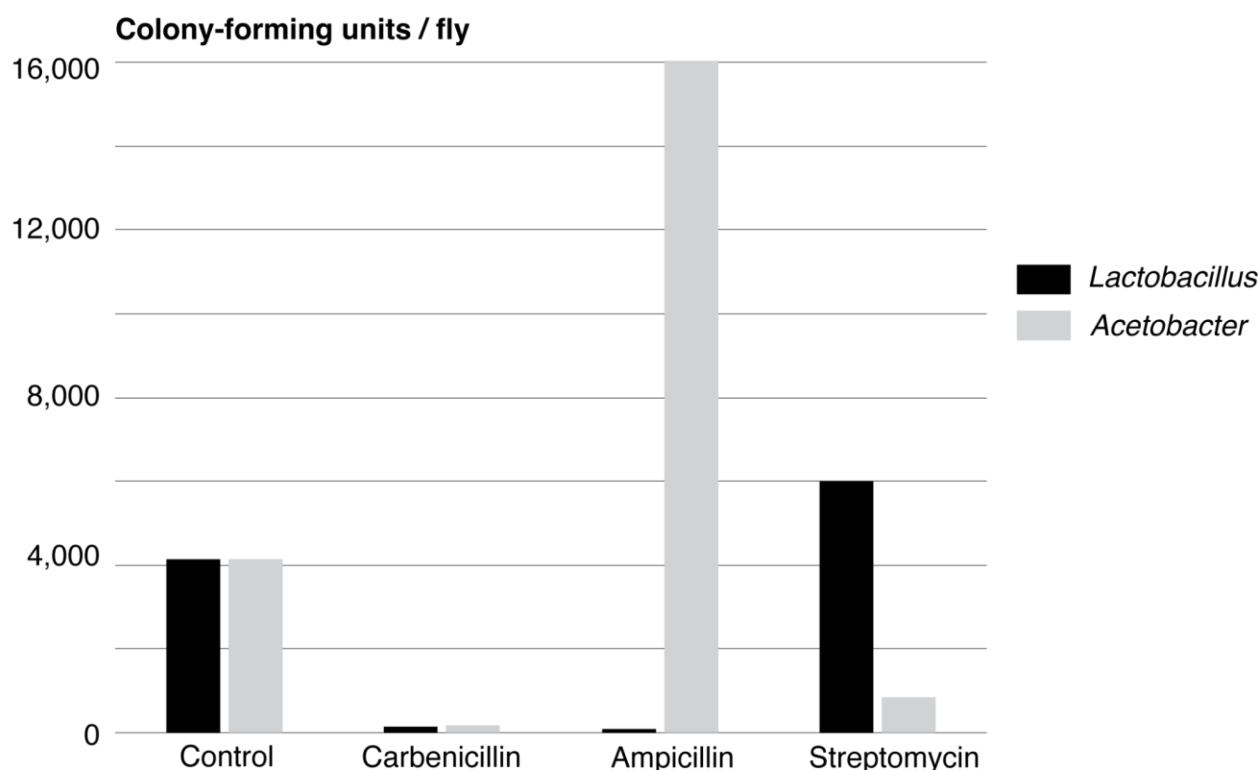


Figure S1. Antibiotics selectively modify *Drosophila* microbiome. Each antibiotic is diluted into food at 100 µg/mL from 1000X 100mg/ml refrigerated stock. 30 flies of each sex were homogenized by microcentrifuge pestle in 100 uL of PBS and dilution plated onto MRS agar for growth analysis. *Lactobacillus* and *Acetobacter* were differentiated and counted separately by order of appearance, size, shape and color. 3 dilution plates were averaged together for each sample and back calculated to CFU per fly.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strain		
Lactobacillus brevis LFM2	Luddington 2022	LFM2, Lb
Lactobacillus brevis LFM2-GFP	Luddington 2022	LFM2-GFP
Lactobacillus plantarum	Luddington 2022	Lp

Acetobacter indonensis	Luddington 2022	Ai
Acetobacter orientalis	Luddington 2022	Ao
Escherichia coli Dh5α	Thermofischer	*Dh5α*
Reagents		
D/L Lactate 90% Leucine Glutamine Insulin Ampicillin Tetracycline		
Streptomycin		
Experimental models: organisms/strains		
Fruit Fly *Drosophila melanogaster* WT, Canton S	Kai-Zinn Lab Caltech	WT, CS
Fruit Fly *Drosophila melanogaster* Mex1 Gal4 Driver	Bloomington Drosophila Stock Center	Mex1-Gal4, 91368
Fruit Fly *Drosophila melanogaster* Mef2 Gal4 Driver	Bloomington Drosophila Stock Center	Mef2-Gal4, 27390
Fruit Fly *Drosophila melanogaster* UAS Lactate Dehydrogenase A +HA tag	Bloomington Drosophila Stock Center	LDHA-, 79190
Fruit Fly *Drosophila melanogaster* UAS Lactate Dehydrogenase B +HA tag	Bloomington Drosophila Stock Center	LDHB-, 79193
Fruit Fly *Drosophila melanogaster* UAS Lactate Dehydrogenase B	Bloomington Drosophila Stock Center	LDHB, 97487

Other

Kimwipe	Kimberly Clark	
Spring scissors	Fine Science Tools	Item# 91500-09
Caltech fly Food	Caltech Fly Food Kitchen	
de Man, Rogosa and Sharpe Broth (MRS medium for *Lactobacillus* growth)	Fisher Scientific	RPI cat # 50488730
Luria broth (LB medium for *E.coli* growth)		
Acetobacter broth (*Acetobacter* Medium for *Acetobacter* growth)	HiMediaLabs	Himedia cat# M1717 and Himedia cat# M1718

Chapter III: Unpublished Work and Continued Discussion

Abstract

Over the duration of my degree I performed various experiments with interesting results that did not quite make the cut or would stem toward the beginning of a new project. In this section I will focus on detailing how I understand my unpublished experiments fit with my own published work as well as the literature, rather than what further study I would like to see which will be included in Chapter 4: Future Directions.

Lactate and pyruvate are interconverted by LDH and transported with monocarboxylate transporters

Lactate is a 'dead end' metabolite of a reversible reaction, microbial and host produced lactate needs to be excreted or it will build up to concentrations that suppress its production with respect to the enzyme coefficient K of available lactate dehydrogenase tetramers.

Humans have two LDH subunit variants, LDHA and LDHB, which tetramerize to perform their catalytic function. All tetramer combinations are capable of catalysing the reversible interconversion of lactate and pyruvate, but have differing kinetics favorabilities. Pyruvate is another pivotal monocarboxylate involved in energy production, amino acid synthesis, cell size and growth regulation (Montero and Finley, 2025). LDHA subunits favor rapid conversion of pyruvate to lactate and LDHB subunits favor rapid conversion of lactate to pyruvate. However even the homotetramers of LDHA and LDHB are capable of catalyzing both the forward and reverse directions, kinetically governed by substrate concentration through mass action. LDHA expression in skeletal muscle allows for quick removal of glycolytic lactate under temporary anaerobic exercise stress. LDHB expression in heart muscle ensures that heart tissue can rapidly utilize lactate for TCA, because heart tissue is always oxygen and lactate rich. This is also related to heart muscle's increased mitochondrial load compared to skeletal muscle.

Drosophila lactate dehydrogenase (dLDH) was first discovered as a gene stimulated in the imaginal disc during morphogenesis (Natzle *et al*., 1985). *Drosophila* dLdh KO mutants develop normally, they maintain redox balance by overproducing G3P and running the glycerol

phosphate shuttle to restore redox balance. (Li *et al*., 2019). Perhaps when metabolizing microbial lactate *Drosophila* could be producing G3P for the same purpose. dLdh KO mutants cannot export excess pyruvate as lactate and accumulate excess pyruvate without increasing the rate at which pyruvate enters the TCA cycle (Li *et al*., 2019).

Given how key dLDH is to this project, I tested flies carrying a fluorescently tagged dLDH driven by the native promoter for dLDH (P_{dLDH}-dLDH-GFP flies). I observed that P_{dLDH}-dLDH-GFP regenerating tips would glow brighter than muscle in the residual limb or femur. However at the time we disregarded this result because we had not yet quantified the autofluorescence associated with regenerating tissues in our model. Interestingly, in the new single-cell transcriptomic data that the lab has now produced (Li *et al*., in preparation), dLDH is found to be highly upregulated in the new muscle cell subtype found in the regenerating limb, suggesting a hypothesis that this new cell type is differentially allocating glycolytic metabolites.

In my to-be published work, I focus on Mef2>LDHA overexpression mutants. However, I also extensively tested Mef2>LDHB overexpression mutants, as LDHB has different enzymatic kinetics to dLDH. (explain the difference between LDHA and LDHB here). These Mef2>LDHB mutants healed scabless even more frequently than the Mef2>LDHA mutants. Unfortunately the LDHB double and half parentals also spontaneously heal scablessly more frequently than CantonS controls. I expect this is ‘leaky’ UAS promoter activation, which impedes and complicates data interpretation.

Some SCFAs like acetic acid influences wound healing

Short chain fatty acids (SCFAs) SCFAs are a popular topic in mammalian gastro-microbiology. They are produced by members of the mammalian microbiome, enhance gut barrier function, and interact with bile acids to enhance absorption of dietary fat (Mukhopadhya *et al*., 2025).

The one-carbon SCFA, formic acid, is a well known insecticide. At low concentrations it causes neuroexcitation and at high concentrations it lethally interferes with mitochondrial metabolism (Song and Scharf, 2008). It is used to treat beehives for mite infestations. The mites are more sensitive and die and the bees are exposed to sublethal but still neuroexcitatory doses. Bees treated with that dose are better at learning to correlate and remember a sucrose solution by smell without increasing general sucrose responsiveness (Bachert *et al*., 2023). I did not test

formic acid because I suspect that if it were to influence regeneration frequency, there would be great difficulty up-titrating a signaling molecule that is lethal at non-trivial doses.

The two-carbon SCFA, acetic acid, vinegar. Acetic acid is *Acetobacter's* primary metabolic output, but is also produced by our heterofermentative *Lactobacilli*. Like lactic acid, acetic acid was also observed to stimulate scabless wound healing and muscle survival. Perhaps bacterial provided acetate also resupplies the Ac-CoA pool in regeneration stress or increases insulin/DILP sensitivity (Mitrou *et al*., 2015). Removal of acetate by pathogenic *V. cholerae* or elimination of microbiota deactivates host insulin signaling (Hang *et al*., 2014).

The three and four-carbon SCFAs propionate and butyrate are less common in laboratory *Drosophila* but can be used as a fuel source by mammalian cells and enable bile acid fat solubilization. This may become increasingly important in the mouse model because there is also interest in testing the 'western style' high fat and/or sugar mouse chow. High fat and/or sugar diets are commonly used to study metabolic syndromes like obesity and diabetes in mice. Some but not all butyrate producing bacteria are associated with higher insulin sensitivity in diabetics (Cui *et al*., 2022). Neither 0.05M propionic acid nor 0.05M butyric acid stimulated an appreciable scabless response.

Larger fatty acids are also produced and metabolized by microbiota, but are more biologically relevant to future mammalian experiments. My major takeaway from all of these SCFA experiments is that nonmetabolizable acids do not seem to stimulate scabless wound healing or regeneration, separate from food pH change alone.

Antioxidants glutathione and vitamin C modulate wound healing

The only other experiments I performed that dramatically increased scabless response were antioxidants. 25mM glutathione stimulated an 82% scabless response in 44 flies, but is relatively expensive and unstable. The flies are highly tolerant to ascorbic acid (the highest concentration of vitamin C that I tested was rather high maybe 500mg/~5mLvial and the flies survived more than a week), and the scabless response also very high (50mg, >70%) (100mg, >90%).

Ramping up metabolism increases redox stress. Maybe antioxidants are *oiling the gears to allow the machine to run faster without damaging itself* so to speak, and raising a cap on metabolism. On the other hand, the melanization reaction (the scab forming reaction) requires oxidative polymerization of phenols into melanin by Prophenooxidases (PPO1 and PPO2;

Ryckebusch *et al*., 2025). Perhaps the large quantities of vitamin C I used just swamped the oxidases either systemically through ingestion or locally because the food they step in coats the wound with vitamin C. This could imply that vitamin C is relevant to scab prevention. Whether that translates to growth has not been tested.

Chapter IV: Conclusions and Future Directions

Summary/description of findings

Partially metabolized carbon substrates downstream of glycolysis like lactate, but not upstream sugars like glucose and trehalose, enhance muscle survival after injury opening the path to patterned tissue regeneration. The *Drosophila* gut microbiome enhances host wound healing as described by fermenting excess dietary carbohydrates into partially metabolized substrates like lactate and acetate.

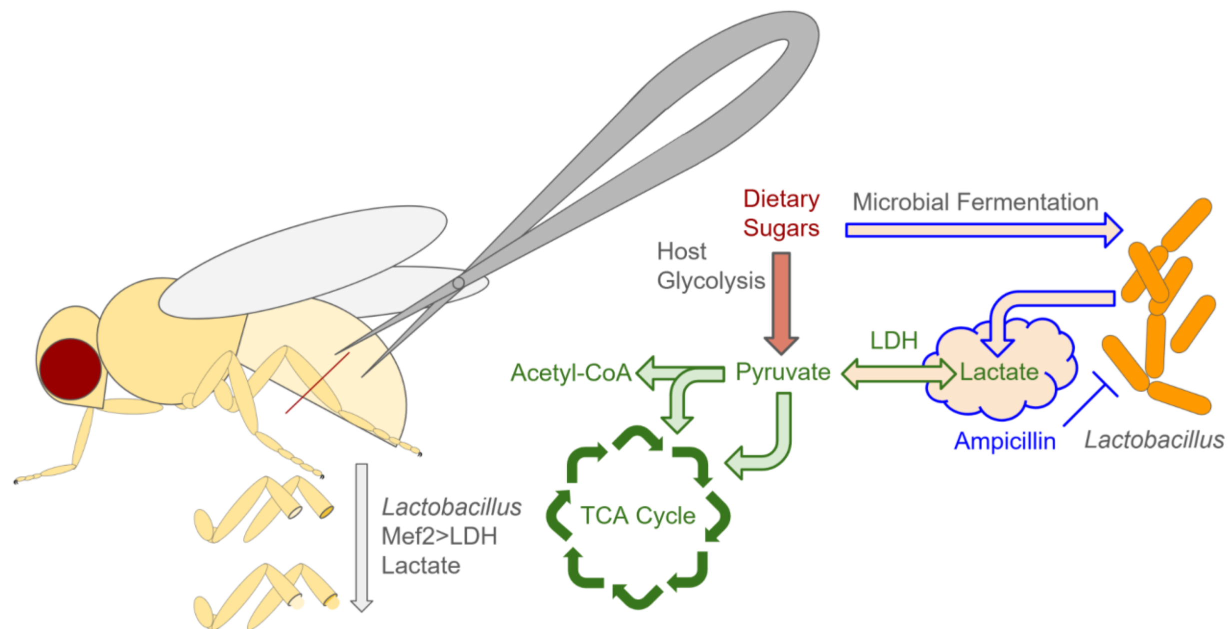


Figure 5. Resident *Lactobacillus* produced lactate enables regeneration in *Drosophila*

Under wound healing stress, anabolism and catabolism must work in concert to provide both cellular building blocks and the energy to assemble them. Dietary sugars are broken down by both host glycolysis and microbial fermentation, increasing the pool of host available lactate. Ampicillin treatment (blue) kills off resident *Lactobacillus* and shuts off access to an external source of lactate. Here we have shown that doing so inhibits regeneration induction with LGI. We further showed that *Lactobacillus*, lactate treatment, or muscular Lactate Dehydrogenase overexpression can induce regenerative response.

Future directions

Why do TCA metabolites keep damaged/regenerating tissue alive/stable? Does TCA generate new/more valuable intermediates compared to glycolysis, or is glycolysis energy generation already maxed out and the only way to push further energy generation is through TCA? What roles does oxygen supply have? How could we apply what we learned from our *Drosophila* model that we could apply to more complex models like mice and humans? In this final section I ask a lot of questions and speculate about possible future experiments, and list a few more genes I would look out for.

One potential next direction is to track where the carbon from lactate entering the TCA cycle is going. Track radiolabeled carbon from sugars vs lactate and Acetate and in LGI treated vs untreated animals. Does carbon leave the TCA cycle more frequently at a different reaction step when it comes from sugars or lactate or acetate or in LGI treated animals vs controls? If so, which downstream products are favored and why might those products be important to regeneration? Carbon intermediates just upstream of lactate may be just as important as those downstream. When exogenous lactate is taken in, glycolytic intermediates past irreversible reactions at the bottom of glycolysis may build up. 3-phosphoglycerate can be used to generate serine for example.

FRET sensors bearing *Drosophila* have been created to track intracellular lactate pyruvate and other metabolites *in vivo*. These FRET mutants have been used to quantify changes in metabolic flux that indicate switching between or to Warburg and Ox/Phos metabolism in anoxic stress and tumor induction (Gándara *et al*., 2019). Given that dLDH is upregulated in the new muscle cell subtype from Yutian Li's scRNA-seq, we could use these mutants to visualize which cell types in the regenerating limb are importing or producing these important metabolites. This could also corroborate my observation that P_{dLDH}-dLDH-GFP regenerating tips would glow brighter than muscle in the residual limb or femur.

Further exploration of food consumption under treatment is needed to determine whether various treatments increase feeding immediately, through gustatory effects, or indirectly through changes in feeding behavioral genes over a longer period of time.

Our lab has uncovered multiple methods of regeneration induction treatments in the normally unregenerate *Drosophila*, what differences/similarities exist in host genetic response to various treatment methods? RNAseq comparison of different regeneration inducing treatments could

reveal which host genetic pathways that are nonuniquely activated/suppressed in regenerating animals. Mitochondrial enzyme expression levels may also indicate which reactions are favored under different treatment conditions, assisting with Aim 1. List of genes I would be interested in looking for: dLDH, DILPs, AMPK, Hedgehog (Hh), and G3P dehydrogenase (GPDH1).
Given TCA cycling's vastly higher efficiency to glycolytic metabolism, is ATP state or flux important? ATP can be used as a biomarker to differentiate OXPHOS to glycolysis dependence (Merin *et al*., 2025). Merin *et al*., 2025 developed a method using flow cytometry to measure fluorescent multicolored ATP sensors in a variety of immune cell types. An intracellular ATP sensor is available in *Drosophila* RRID:BDSC_94566, and has been used to show how glycolytic ATP reduces Hh Hedgehog signalling (Spannl *et al*., 2020). Mutants like these could allow you to see where ATP is compartmentally within regenerating tissue.

AMPK is a cellular energy sensor, activating when ATP concentration falls relative to AMP or ADP concentrations. Among other functions, active AMPK suppresses mTOR through TSC1 and TSC2. This is the mechanism by which Metformin inhibits mTOR signalling (Hasanvand *et al*., 2022). In this way AMPK functions as a sensor for low cellular energy state. Knocking out both AMPK β subunits to disable its function in mouse muscle reduces mitochondria count and muscle glucose uptake inducing a lethargic poorly exercising phenotype (O'Neill *et al*., 2011).

We could utilize our *Drosophila* regeneration model to investigate the roles of AMPK in poorly regenerating animals. AMPK's enzymatic core *AMPKα* knockout insertion is not lethal and available at Bloomington Drosophila Stock Center RRID:BDSC_63274. AMPK overexpression via UAS using fly lines such as RRID:BDSC_62100 or RRID:BDSC_32108. Differentially de/activating AMPK may be optimal, for example AMPK overexpression in the fat body is likely bad for TOR signaling. Active AMPK functions as a starvation stress signal that increases noncarbohydrate metabolite input into TCA. But it is also used to power energy-costly developmental transitions like dendrite pruning in neurons (Marzano *et al*., 2021). For this reason I expect muscle AMPK overexpression to be beneficial
We have observed that prolonged oxygen limitation, as happens during CO_2 anesthesia >2 minutes, can nonlethally reduce scabless wound healing. However in our moon jellyfish model anoxia induced by bubbled nitrogen gas can stimulate regeneration. While this may be due to jellyfish's vastly higher hypoxic tolerance, this may also be due to higher exchange with the

environment that jellyfish have by nature of being fully immersed in water at all times. Being able to easily export glycolytic 'waste' lactate more easily.

These animals exist in unique and changing contexts. Fly larvae live in environments with very high nutrients particularly carbohydrates and compete with each other and bacteria for inwardly diffusing oxygen as they burrow deeper into their media. After eclosing into a ~20% oxygen circulating atmosphere, they take in and distribute that oxygen by pushing air through small tracheae and simple diffusion through hemolymph to the rest of the body. *Drosophila* heme is believed to be mostly used for oxygen buffering rather than as a dedicated oxygen carrier (Burmester 2007). While Caltech fly food has more yeast protein content than other formulations, it is still just under 20% carbohydrates by weight with the majority remaining as water. Jellyfish live exclusively in a circulating <1% dissolved oxygen environment but are sessile as a polyp. Jellyfish do not have hemes and passively absorb oxygen across their whole body. Jellyfish feed on high protein brine shrimp, and 'superfeeding' to induce regeneration is only a temporary exposure as rotting food spoils too rapidly in the jellyfish model. I do not think that anoxia being pro-regenerative for the jellyfish model devalues the potential for oxygen to enhance regeneration, given different environmental and nutrition factors.
Satisfying and safe ways to test this are limited. An anaerobic chamber could be rigged into an 'aerobic' chamber. Or have a high oxygen gas mix piped into each vial at a slow low volume per minute through a needle like we do with CO_2. The glaring problem is that it is dangerous to maintain an oxygen enriched atmosphere in confined spaces like a vial with a cotton plug as this presents a fire hazard.

If I could arbitrarily set an oxygen percent for treatment I would test 25-35% Oxygen. Atmospheric oxygen peaked at ~35% ~300 million years ago. During this time insects which rely on inefficient thin trachea throughout their bodies to distribute oxygen flourished and were capable of growing to larger sizes. If oxygen is a limiting factor in insect size growth, could increased oxygen also enhance insect regrowth.

The vastly more diverse mouse microbiome paired with the vastly more complex vertebrate adaptive immune system leaves a lot more loose ends to tie up. However, facing these challenges would make this research more translatable because they are the same roadblocks that exist in the human system. Mammals are also able to metabolize more microbial fermentation products than flies specifically including SCFAs propionate and butyrate.Larger animal models

also allow for different treatment delivery methods. For example, does lactate injected directly into the bloodstream increase or decrease treatment effectiveness compared to dietary supplementation?

Diet is known to influence host phenotypes and microbiome composition. The mouse treatment from (Abrams *et al*., 2021) was additional sucrose in the water, essentially a high sugar diet. Do the ‘western style’ high fat mouse chows also induce spontaneous digital regeneration? This is where microbiome produced SCFAs help bile acids solubilize fats aiding absorption comes into play. The potential here is that these fatty acids are beta-oxidized into acetylCoA.

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