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SYMBIOSIS

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To The Reader,

In this third issue of the Midwestern Journal of Undergraduate Sciences, we are excited to present a collection of review articles that explore the profound influences of the microbiome on the development of a healthy body. As research continues to uncover the intricate relationships between the trillions of microbes that inhabit our bodies and various aspects of human health, from immune function to mental well-being, it becomes increasingly clear that the microbiome plays a critical role in shaping our overall physiology. The reading list and reviews featured in this issue provide a strong introduction to current areas of research. We welcome you to make of this work to introduce yourself to this topic or to use in your own classes.

And, to young scientists and their mentors, we invite you to contribute your own research and insights to the Midwestern Journal of Undergraduate Sciences. By sharing your work with us, you become a part of a growing community of scholars dedicated to advancing scientific knowledge and fostering a culture of discovery. Whether you are conducting original research, developing new methodologies, or crafting thought-provoking reviews, we welcome submissions from all disciplines within the sciences. Join us in showcasing the innovation and excellence of undergraduate research—submit your work today!

Finally, KU Edwards needs to say goodbye to the Director of the Biotechnology Program, Randy Logan. It's sad to see you go after these many years. The Biotechnology program and KUEC have been made better by your influence and leadership. We wish you all the best out there and hope that your connections here remain strong!

-J.F. Treml

Contributors

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Cover Art
by Kaitlyn Sy

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Call for Papers

The Midwestern Journal of Undergraduate Sciences features research designed and performed by undergraduate scientists. The journal addresses a variety of disciplines and focuses on work done as senior capstone projects. We especially encourage works authored or co-authored by community college students and also by students pursuing master's degrees to submit. All submissions are due by the last Friday in May of each year.

For information for authors, as well as our submission site, visit: https://journals.ku.edu/MJUSc/about/submissions.

Living with It:

A Review of the Host / Microbiome Interactions

By J.F. Treml

Our understanding of gut / microbiome interactions has developed rapidly in the new century. This in no small part due to the increased availability of next generation sequencing and gene chip techniques that allow for a greater understanding of the makeup of our microbiota and the molecular mechanisms that mediate the impact these organisms have on their hosts. One unexpected finding is the magnitude of influence our diet and environment have on the diversity and richness of the gut microbiome. The benefits and harms of these diets extend well beyond the intestinal environment via the production of microbial products which enter the host's circulation or other direct interactions influencing allergy, obesity, type 2 diabetes, hypertension, and pulmonary inflammation. The following reviews highlight recent advances in our understanding of the microbiome and its effects in health and disease. They are further informed by a required undergraduate journal club course at the University of Kansas' Edwards Campus for which, the Spring 2024 reading list can be found following the reviews.

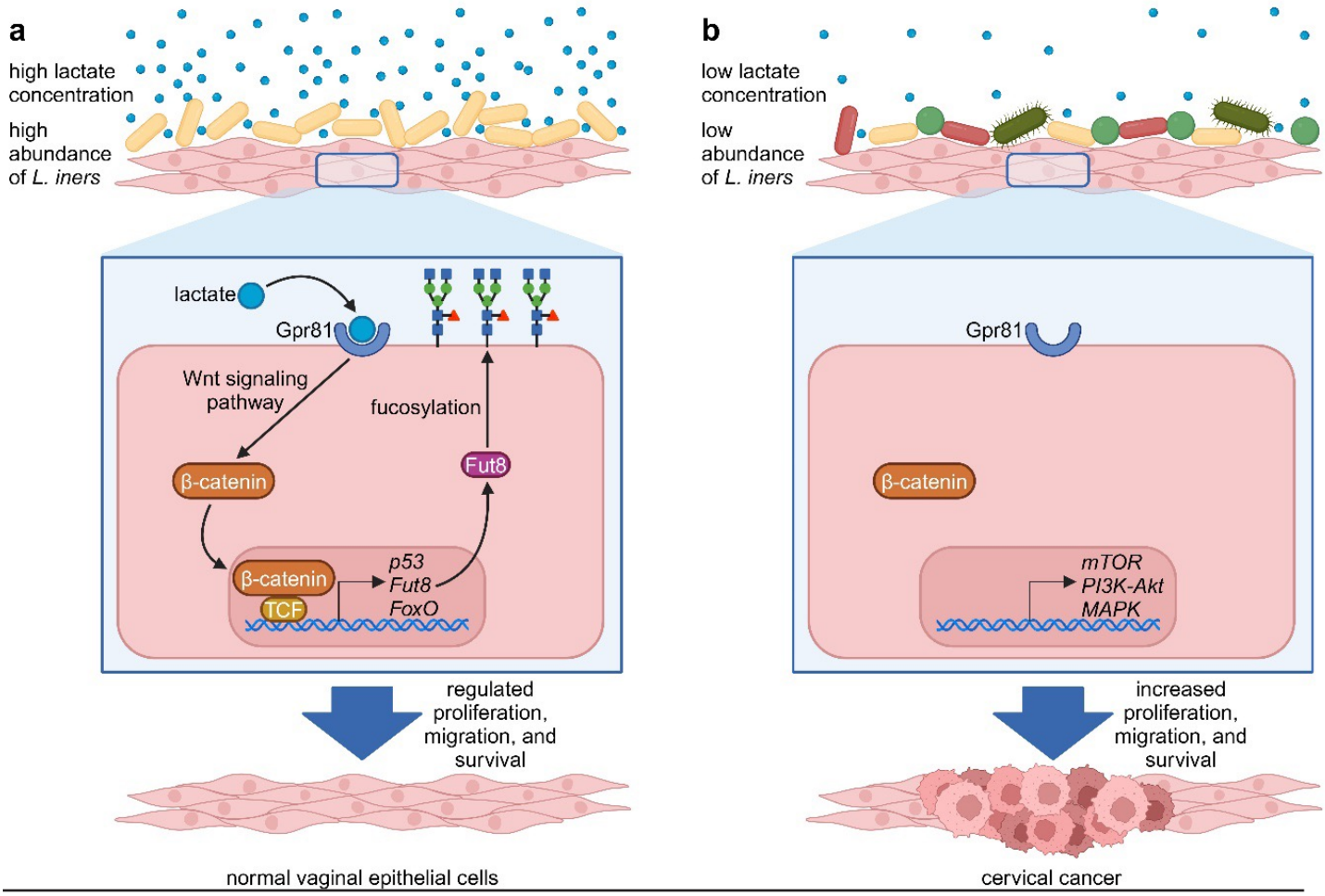
Giving Cancer Cells a Taste of Their Own Medicine: Lactic Acid from Vaginal Microbiota Protects Against Cancer

By Kaitlyn Sy

Cancer is a leading cause of morbidity and mortality worldwide, with cervical cancer ranking among the top four most common malignant tumors in females. Tumor microenvironments surrounding solid tumors, including cervical cancer, have often been found to contain high concentrations of lactic acid due to a preference for aerobic glycolysis, termed the Warburg effect. Several studies have implicated lactic acid in solid tumors' ability to evade immune defenses, but lactic acid's role in the human body is far from being one-sided. Lactobacillus species, the most common bacteria in the vaginal tract, have long been known to play a protective role in the vaginal microbiota by secreting lactic acid that inhibits the growth of pathogens. Recent research by Fan et al. uncovered that the beneficial effects of lactic acid produced by Lactobacillus species not only prevent vaginal infections but also protect against cervical cancer by regulating the fucosylation of vaginal epithelial cells. Hence, vaginal Lactobacillus gives cervical cancer cells a taste of their own medicine: using high doses of the very molecule that cancer cells may use to evade the immune system to suppress the cancer cells' growth.

Among females, cervical cancer ranks among the top four malignant tumors in both morbidity and mortality.[1] Consequently, understanding the processes that lead to cervical cancer and developing preventive measures and treatments is vital.

Cancer is characterized by uncontrolled proliferation of abnormal cells. It is a complex disease, affecting not only the malignant cells but also modulating the function of the surrounding cells. Solid tumors, including cervical cancer, have long been known to secrete lactic acid into the tumor microenvironment (TME) as a byproduct of their shift from aerobic respiration to fermentation, a phenomenon known as the Warburg effect.[2,3] Lactic acid quickly dissociates into lactate and H+. Lactate concentrations as high as 40 mM and pH levels as low as [5,6] have been reported in the TME, compared to the <3 mM lactate and pH 7.4 generally found under normal physiological conditions.[2] Lactic acid in the TME has been implicated in solid tumors' ability to evade immune defenses. For example, recent studies have reported that acidic conditions and high concentrations of lactate could independently impact the immune responsiveness of T cells,[4,5] and others have reported that tumor-derived lactate affects the phenotypes of natural killer cells6 and dendritic cells.[7] Despite the harmful effects of lactic acid on immune responsiveness in the TME, the role of lactic acid in the body is far from being one-sided, as lactic acid plays beneficial roles as well. Lactobacillus species, the predominant bacteria in the vaginal tract, have long been known to play a protective role in the vaginal microbiota by secreting lactic acid that inhibits the growth of pathogens.[8] In individuals where lactobacilli dominate the vaginal microbiota, pH levels around 3.5 and lactate concentrations above 110 mM are common.[9] Interestingly, the beneficial effects of lactobacilli seem to extend beyond protection against vaginal infections but also encompass anti-cancer defenses. Cervical cancer patients have been reported to be colonized by significantly fewer lactobacilli compared to healthy females, resulting in vaginal dysbiosis,[10] and changes in the vaginal mi-



crobiota have been proposed as a diagnostic marker for cervical cancer,[11] although neither a causal connection nor mechanism of action for the protective effects of lactobacilli against cervical cancer have been determined. However, groundbreaking research published by Fan et al. recently uncovered that lactic acid produced by Lactobacillus iners, a predominant member of the vaginal microbiota, protects against cervical cancer by regulating the fucosylation of epithelial cells in the vaginal mucosa.[12] Consistent with prior reports, Fan et al. observed that, compared to healthy fe-

Figure 1 | L. iners regulates fucosylation of vaginal epithelial cells and protects against cervical cancer.
(a) Lactobacillus species, especially L. iners, dominate the vaginal microbiota and secrete lactic acid that inhibits the growth of other bacteria. The lactate activates the Wnt signaling pathway via the lactate-Gpr81 complex, leading to the activation of TCF/β-catenin, which enhances the transcription of Fut8, p53, and genes involved in the FoxO signaling pathway. Fut8 mediates the fucosylation of vaginal epithelial cells, suppressing their transformation to a malignant phenotype and thereby inhibiting the growth of cervical cancer. **(b)** When the abundance of Lactobacillus species is decreased in a dysbiotic vagina, the deficiency of lactate prevents the normal fucosylation of vaginal epithelial cells. This leads to the upregulation of genes involved in the mTOR, PI3K-Akt, and MAPK signaling pathways, resulting in increased proliferation, migration, and survival, which could ultimately lead to cervical cancer if left unchecked. in HCMV-infected cells.

The University of Kansas Edwards Campus Biotechnology Program is made possible by the generous support from the

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males, cervical cancer patients had lower levels of protein core fucosylation in both cervical tissue and serum. Fucosylation is a post-translational modification comprising the attachment of a fucose residue to N-glycans and O-glycans most commonly mediated by α-1,6 fucosyltransferase (Fut8)[13] and has been reported to be decreased in cervical cancer patients, compared to healthy females.[14] To assess the role of fucosylation in cervical cancer, Fan et al. knocked out Fut8 in SiHa cells, a cell line derived from uterine tissue collected from a 55-year-old female patient with squamous cell carcinoma. Fut8-/- SiHa cells displayed increased migration as determined by scratch assay compared to Fut8+/+ SiHa cells, a phenotype reversible via the reintroduction of Fut8. Transcriptomic analyses revealed that knockout of Fut8 led to changes in gene expression in SiHa cells. Genes involved in the TNF signaling pathway were downregulated, whereas genes involved in the mTOR, PI3K-Akt, and MAPK signaling pathways and migration were upregulated. These changes in gene expression were predicted to lead to a more malignant phenotype, characterized by decreased apoptosis and increased growth, proliferation, migration, and survival. Fan et al. verified these effects in vivo by injecting SiHa cells into athymic female mice. Fut8-/- SiHa cells produced larger and heavier tumors than Fut8+/+ SiHa cells. Together, these results indicate that Fut8 plays a regulatory role in inhibiting the growth and progression of cervical cancer.

Considering that fucosylation of mucosal epithelial cells had previously been linked to the microbiota,[15] Fan et al. sought to elucidate the connections between the vaginal microbiota, fucosylation of epithelial cells in the vaginal mucosa, and cervical cancer. 16S rRNA sequencing showed that Lactobacillus was the only dominant bacterial genus in the vaginal microbiota of healthy females, whereas Clostridium, Staphylococcus, and Bacteroides dominated the vaginal microbiota of cervical cancer patients. Consistent with prior reports, the abundance of Lactobacillus—and especially L. iners—was significantly decreased in cervical cancer patients, compared to healthy females. This vaginal dysbiosis resulted in significantly higher vaginal pH and lower lactate levels in cervical cancer patients, due to decreased abundance of Lactobacillus metabolites. To assess the effects of L. iners metabolites on the growth of cervical cancer, Fan et al. treated SiHa cells with the supernatant and lysate of L. iners, resulting in decreased proliferation, central carbon metabolism, and survival. Transcriptomic analyses of SiHa cells indicated that L. iners metabolites upregulated IL-17, p53, Fut8, and genes involved in the FoxO signaling pathway. In vivo, abundance of L. iners in the vagina was positively correlated with serum levels of core fucosylation, suggesting that L. iners metabolites regulate the activity of Fut8.

In a previous study, this same research group reported that microbiota can upregulate core fucosylation of epithelial cells by activating the Wnt pathway,[16] so they sought to trace the mechanism by which L. iners metabolites regulate Fut8. While treatment of SiHa cells with L. iners metabolites increased the activity of TCF/β-catenin (transcription factors that significantly enhance the activity of Fut8), treatment with L. iners metabolites and DKK-1 (an inhibitor of the Wnt pathway) negated these effects, leading Fan et al. to suspect that lactic acid produced by L. iners activates the Wnt pathway via the lactate-Gpr81 complex. To test this hypothesis, Fan et. al compared levels of core fucosylation, wnt3, and β-catenin in SiHa cells treated with L. iners metabolites with and without 3-OBA, an antagonist of Gpr81. While SiHa cells treated with L. iners metabolites alone displayed increased levels of wnt3, β-catenin, and core fucosylation, these effects were negated in SiHa cells treated with L. iners and 3-OBA, supporting the authors' hypothesis.

The results reported by Fan et al. piece together a clear picture of the connections between the vaginal microbiota, fucosylation of epithelial cells in the vaginal mucosa, and cervical cancer (Figure 1). Lactobacillus species, especially L. iners, normally dominate the vaginal microbiota and secrete lactic acid that inhibits the growth of other bacteria. The lactate activates the Wnt signaling pathway via the lactate-Gpr81 complex, leading to the activation of TCF/β-catenin, which enhances the transcription of Fut8, p53, and genes involved in the FoxO signaling pathway. Fut8 mediates the fucosylation of vaginal epithelial cells, suppressing their transformation to a malignant phenotype and thereby inhibiting the growth of cervical cancer. When the abundance of Lactobacillus species is decreased in a dysbiotic vagina, the deficiency of lactate prevents the normal fucosylation of vaginal epithelial cells. This leads to the upregulation of genes involved in the mTOR, PI3K-Akt, and MAPK signaling pathways, resulting in increased proliferation, migration, and survival, which could ultimately lead to cervical cancer if left unchecked.

With these results in mind, several questions arise. For example, via what mechanisms does fucosylation of epithelial cells in the vaginal mucosa protect against cervical cancer? Or, considering the immunosuppressive effects of lactate, how do the high concentrations of lactate in a normobiotic vagina affect immune responsiveness in this area of the body? Although these questions are not addressed by Fan et al. in this paper, their discovery of the pivotal role of Lactobacillus species in protecting against cervical cancer has several implications. Considering the potentially harmful consequences of a dysbiotic vagina, clinical tests to assess the composition of the vaginal microbiota may be a useful measure for preventive screening and diagnosis of cervical cancer. Additionally, returning a dysbiotic vagina to normobiosis may have potential as a treatment for cervical cancer, alone or alongside other therapeutics. Conversely, providers must exercise caution when prescribing antibiotics for patients with cervical cancer or risk for cervical cancer, as microbial shifts in the vagina could not only lead to opportunistic infections but could also increase the chance of cancerous growth.

In conclusion, while questions remain to be answered, this study by Fan et al. significantly expanded the current understanding of the connections between the vaginal microbiota and cervical cancer, paving the way for future research.

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A Tale of Three Cytokines: How Gut Bacteria Regulate Immunity

By Alexander Rees

If you have been scrolling social media recently, you may have noticed an increase in articles or people talking about the gut microbiome. Many claims suggest that the gut microbiome may influence your energy levels, acne, mental health, and even hair loss. While some of these claims may come from social media influencers jumping on the latest trend without scientific backing, studies illustrate the significant impact the gut microbiome has on overall health. Examples include microbiome-mediated prevention of allergic inflammation in the lungs and its influence on BMI, body fat percentage, and insulin resistance.[1,2]

Growing evidence also suggests that the gut microbiota plays a large role in immune responses, including the maintenance of homeostasis and the shaping of mammalian immune responses.[3] Part of this immune response in mammals involves interferons, which are part of the frontline response in the immune system, helping to restrict viral propagation in locally infected areas of the body. Type 1 interferons (IFNs), including IFNα and IFNβ, create proinflammatory, antiviral, and antimicrobial responses through the activation of interferon-stimulated genes (ISGs).[4] Perhaps surprisingly, a healthy microbiota also triggers the production of IFNs in a manner required to maintain competently tuned immunity.[5] Ayala et al. explored whether and by what means commensal bacteria-produced IFNs may elicit a tolerogenic response.[6]

Using germ-free (GF) and specific pathogen-free (SPF) mice, ISG expression was examined in colon tissue under steady-state conditions. In the absence of commensal bacteria, IFNα—but not IFNβ—was found. However, the introduction of Bacteroides fragilis (Bf) restored IFNβ expression in the GF mice. This response was repeated in vitro, where Bf, B. thetaiotaomicron, or B. vulgatus all elicited IFNβ production, demonstrating a specific role for these microbiota in priming IFN responsiveness.

These results were further observed using colon explant tissue stimulated with the Toll-like receptor 3 (TLR-3) agonist, polyinosinic–polycytidylic acid (poly I:C). Because CD11c+ dendritic cells (DCs) are responsible for the constitutive expression of type 1 IFNs in the intestines,[7] the interaction between these cells' IFNβ production was investigated in the presence or absence of commensal bacteria in vitro, establishing a direct role for commensal priming of DC responsiveness. Of particular interest, Bf-monocolonized mouse tissue responded similarly to SPF-derived tissue, rather than GF-derived tissue. Further, the ISG response to stimulation was not reliant on differential IFN-receptor 1 (IFNAR1) expression, but only on the presence of commensals.

Bf has been shown in previous studies to help dendritic cells achieve immune tolerance.[8] What is less understood are the signaling pathways that are used to accomplish this. To identify how a deficiency of type 1 IFN would change the production of cytokines downstream, IFNAR1-deficient bone marrow-derived dendritic cells (BMDCs) and wild-type (wt) BMDCs were treated with Bf in vitro. The results showed a decrease in the expression of the anti-inflammatory cytokines IL-10 and IL-27 in mice lacking this receptor.

Without IFNAR1, BMDCs create a pro-inflammatory environment. To examine if regulatory T cells (Tregs) played a role in this, wt and IFNAR1-deficient BMDCs were co-cultured with wt CD4+ T cells and Bf. Where wt BMDCs showed production of IL-10 and the induction of Foxp3 (a transcription factor for Tregs) in Tregs, IFNAR1-deficient cells did not show these results, clarifying the involvement of type 1 IFNs for Treg-mediated tolerance.

Lastly, Zhang et al. have previously shown that IL-27 facilitates IL-10 expression in certain CD4+ T cell groups.[9] Knowing this, it was theorized that IL-27 could mediate commensal-initiated tolerance by Tregs. Using a CRE-Lox system, the tolerance-promoting IL-10 production by Tregs in response to co-culture with Bf was found to be IFNAR1-dependent in vitro and in vivo.

The diminished Treg environment has been associated with an expansion of other T cell subgroups, such as Th17 or Th1. Since the IFNAR1-deficient mice had a greatly reduced Treg population, it would make sense that there would be an increase in one of these groups. As expected, there was a significant increase in Th17-produced IL-17 in the IFNAR1-deficient animals compared to their wt counterparts, even in the presence of Bf.

The data generated by Ayala et al. shows just how important commensal bacteria are in IFN signaling and bringing about appropriate T cell responses. It remains to be seen by what mechanisms commensal-induced IFN signaling differs from pathogenic bacteria, which uniformly stimulate much higher levels of IFN compared to that elicited by commensal microbes.

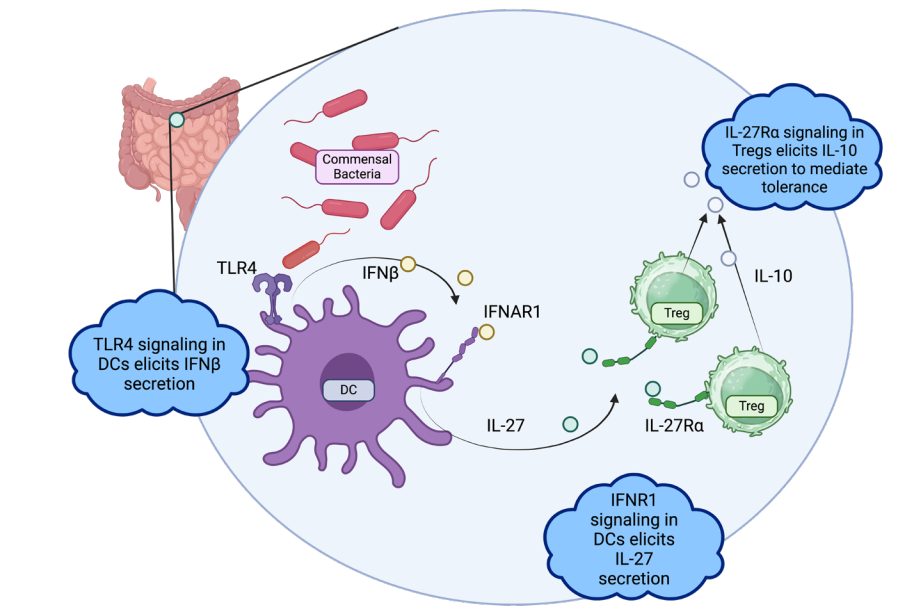

Figure 1 | Commensal Organisms initiate tolerance via Dendritic Cell signaling to Regulatory T Cells.

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Short-Chain Fatty Acids

A Potential Therapy for Colorectal Cancer

By Samia Chergui

Short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, have garnered significant attention due to their anti-inflammatory properties within the human body.[1] Expanding on these studies, recent research has revealed that SCFAs exhibit selective antitumor effects on colorectal cancer cells (CRC cells). The findings from these investigations have illuminated the influence SCFAs can have on the development and progression of colorectal cancer. These discoveries underscore the therapeutic potential SCFAs might offer in the management and treatment of colorectal cancer.

Short-chain fatty acids (SCFAs) are produced in the colon during the fermentation of indigestible dietary fibers. The average molar ratio of acetate: propionate: butyrate found in the colon and feces at physiological conditions is 60:25:10 mmol/L.[2] These SCFAs can exert regulatory effects on various processes, including phagocytosis, the production of chemokines, and signaling pathways that control cell growth and apoptosis. Although a significant portion of research has been dedicated to exploring the specificity of the individual SCFAs toward CRC cells, the recent study by Gomes et al. instead focuses on evaluating the additive anti-cancer and possible synergism of SCFA mixtures. The results of this study provide a promising foundation for exploring the modulation of the gut microbiota-SCFAs axis as a potential avenue for CRC treatment or prevention.[3]

Previous studies have primarily investigated the impact of SCFA treatments on immune cells, cell cycle control and apoptosis, metabolic processes, and other aspects of cellular physiology. SCFAs, particularly butyrate, have demonstrated a role in stabilizing hypoxia-inducing factor (HIF), which is disease-protective against colitis.1 Individual SCFA exposure has also been shown to reduce cell proliferation rates on cultured cell lines derived from normal human colon mucosal epithelium (NCM460), colon carcinoma (RKO), and colorectal adenocarcinoma (HCT-15) with more selectivity toward CRCs.[3] RKO colon cancer cells exposed to butyrate were observed to have suppressed MAPK/ERK signaling, leading to upregulation of the endocan gene, which is thought to contribute to anti-cancer effects such as inhibition of cellular proliferation and migration.[4] The SCFA propionate was shown to promote proteasomal degradation of euchromatic histone-Lysine N-methyltransferase 2 (EHMT2) in cultured adenocarcinoma cell lines HCT116 and LS174T cell line. This epigenetic control influences the downstream pathways involved in cell proliferation. One direct target that EHMT2 acts on can induce apoptosis in colorectal cancer cells. By promoting EHMT2 degradation, propionate inhibits the growth of colorectal cancer cells.[5] Acetate treatment was observed to inhibit cell proliferation and viability of the colorectal cancer cell lines HT29 and HCT116. Additionally, under normoxic conditions, acetate modulates the mitochondrial function. It diminishes glycolysis activity, a metabolic pathway cancer cells rely heavily on for energy production to support their rapid cell growth and division.[6]

To evaluate the additive effect of SCFAs, Gomes et al. first predicted the joint toxic effects using a concentration addition model. The SCFAs were combined at the molar ratio typically found in the human colon (60 acetate:15 butyrate:25 propionate). In the RKO cell line, the experimentally observed effects of the SCFA mixtures closely matched the effects predicted by the CA model, indicating that the SCFAs exhibited an additive effect when combined. The observed effects were more pronounced for the HCT-15 cell line than the predicted additive effects, suggesting a potential synergistic interaction at these concentrations. The SCFAs, individually and in the mixture, induced cell-cycle arrest, lysosomal membrane permeabilization (LMP), and decreased cytosolic pH in CRC cells (**Fig. 1**). The lysosomal dysfunction and acidification of the cytosol are proposed to be part of the mechanism leading to apoptosis.[7] The sensitivity of CRC cells to the effect of SCFAs could be explained by the higher activity of multiple lysosomal enzymes in many cancer tissues compared to normal cells. The compromised lysosomal membrane releases lysosomal enzymes, such as cathepsins, into the cytosol. The released cathepsins can then trigger apoptosis by activating various apoptotic pathways.[3]

The data from the study by Gomes et al. implies that butyrate, even at a relatively lower concentration than acetate and propionate, exerted a dominant influence on the mixture effects.[3] From the individual dose-response curves and IC50 calculations (concentration required for 50% inhibition of cell growth), butyrate was shown to have a lower IC50 among the three SCFAs. This indicates that butyrate has a higher potency in inhibiting the growth of colorectal cancer cells. Although the potentially dominant mechanisms of butyrate-induced cell growth inhibition are not explicitly stated, butyrate's specific mechanisms of action, such as histone deacetylase inhibition or other epigenetic modulations, could explain why it is the more potent component of the SCFA mixture.[8]

In conclusion, the study by Gomes et al. highlights the important effects SCFAs have on CRC cells. However, relying on in-vitro experiments alone fails to recapitulate the complex tumor microenvironment and systemic effects observed in vivo. Furthermore, the study may have overlooked the potential synergistic or antagonistic effects of other untested SCFAs and other metabolic byproducts of the gut microbiota. Overall, to translate these findings into effective therapeutic approaches for treating colorectal cancers, it is crucial to conduct further investigations into the possible synergistic or antagonistic effects of a much larger array of SCFAs and to understand better how diet can impact the variety of metabolic byproducts produced by the gut microbiota.

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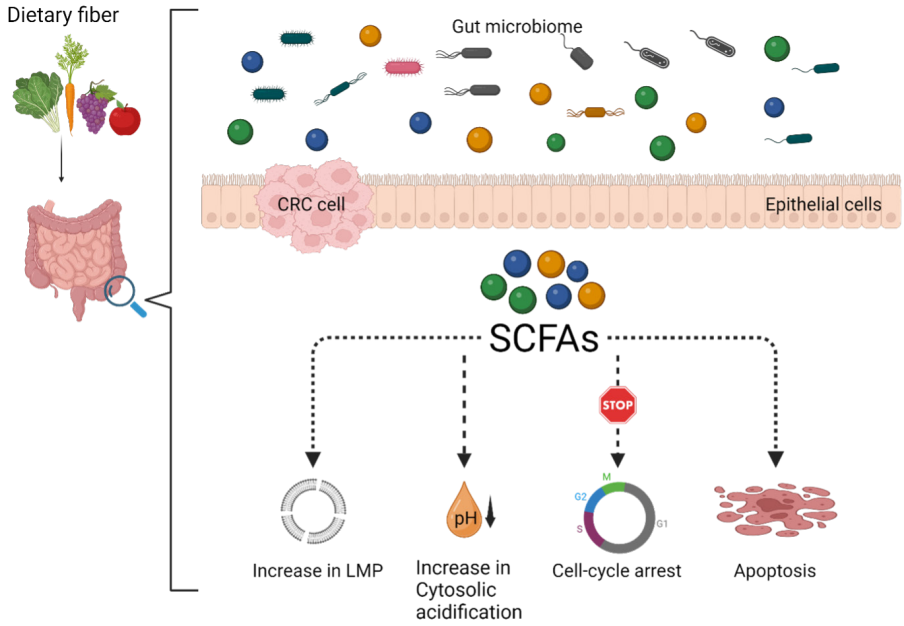

Figure 1 | The effect of short-chain fatty acids (SCFAs) on CRC cells. Gut microbiota ferments dietary fiber and produces the SCFAs acetate, butyrate, and propionate. In CRC cells, the combination of SCFAs shows an additive effect in inducing cell apoptosis and cell cycle arrest. They also lower the pH of the cytosol and promote an increase in lysosomal membrane permeability (LMP).

The crosstalk between microbial sensors ELMO1 and NOD2 shape intestinal immune responses

By Bridget Ruhme

Microbial sensors are integral to maintaining cellular homeostasis and orchestrating immune responses within the intestinal environment. The mechanisms contributing to differential immune responses and their link to inflammatory bowel diseases (IBD) are not yet fully understood. IBD is an umbrella term used to describe disorders that cause chronic inflammation of the gastrointestinal tract. This review explores The crosstalk between microbial sensors ELMO1 and NOD2 shape intestinal immune responses during enteric infections of AIEC-LF82 and Salmonella published in the February 2023 Virulence Journal by Aditi Sharma *et al.*

The two most common forms are ulcerative colitis and Crohn's disease (CD).[1] Recently, ELMO1 (Engulfment and Cell Motility Protein-1) has emerged as a critical player in this process, working in direct association with the bacterial sensor protein, Nucleotide-binding oligomerization domain-containing protein 2 (NOD2). ELMO1 has been implicated in the inflammatory cascade of IBD by sensing microbes associated with NOD2 and triggering pro-inflammatory cytokines secretion. Mutations in NOD2, an intracellular receptor for the bacterial cell wall component muramyl dipeptide (MDP),[2] are among the strongest risk factors for disease.[3] The most common mutation, a frameshift of NOD2 (L1007fs) resulting in premature termination, was previously found to be defective in its recognition of MDP.[4] Until now the direct interaction between ELMO1 and NOD2 regulating bacterial sensing was unknown. This knowledge has the potential to modernize our approach to preventing and treating NOD2-mediated inflammatory bowel diseases, and other immune-related disorders. Effective bacterial sensing is a critical first-line defense against infection and impairments in this process have been linked to the development of several autoimmune and inflammatory conditions, including CD.[5,6] pattern recognition receptors like ELMO1 and NOD2 are essential for microbial sensing between commensals and pathogens by identifying pathogen-associated molecular patterns associated with microbes.[7] In this study Sharma *et al*., report the direct link between these two important microbial sensors, and the role they have in determining host response to pathogens. By using a stem-cell approach to stimulate normal gut physiology, and intestinal bacteria (*Salmonella*), Adherent-invasive *E. coli* strain LF82 (AIEC-LF82) to assess the guts' function to stress, due to its association with CD. They found that the C-terminal region of ELMO1 was sufficient for interaction with the Leucine-Rich Repeat (LRR) region of NOD2 and that the absence of either or both proteins results in dysregulated antibacterial response in the case of AIEC-LF82 and *Salmonella* infection.

They started by assessing the physical interaction of the C-terminal domain of ELMO1 with NOD2. The C-terminal region of ELMO1 is known to be essential for bacterial phagocytosis due to the interaction with DOCK 180 by its PH domain regulating the GTP-ase, Rac, which is involved in cytoskeletal remodeling.[8] The C-terminal of ELMO1 binds with bacterial effectors through the signature WxxxE motif to induce various immune responses between pathogens and commensals by interacting with several bacterial effectors.[9] Similarly, the LRR domain of NOD2 recognizes bacterial components and was identified in the binding of the bacterial cell wall component MDP.[10,11] The interaction between ELMO1 and the LRR region of NOD2 suggests a coordination between the two sensing systems that may have significant effects on the immune system's ability to detect and respond to bacteria by influencing bacterial recognition, bacterial engulfment and clearance, and regulation of the immune response.

They next examined the functional effects of the CD-associated L1007fsinsC NOD2 mutant in epithelial and immune cells through ELMO1-NOD2 interactions. This mutant has previously been shown to reduce NFκB activity compared to wild-type NOD2.4 In, this study Sharma *et al*. found that the Leucine frame-shift mutation did not interrupt ELMO/NOD2 binding, yet did impede NFκB activity and reduce the phagocytosis of bacteria while.

Although initial phagocytosis of bacteria was reduced in knockout cells, these cells were later found to contain more bacteria than their wt counterparts indicating that while cells could still phagocytize some bacteria, they were unable to eliminate these organisms.

To investigate why the bacteria were able to persist in immune cells, the pro-inflammatory cytokines, MCP-1, IL-6, and IL-8, secreted by these cells and Reactive Oxygen Species (ROS) levels induced within these cells were examined. Indeed, depleting ELMO1 and NOD2 resulted in reduced pro-inflammatory cytokine levels in response to enteric pathogens and decreased ROS levels. This suggests that ELMO1 and NOD2 play a role in regulating the inflammatory response and ROS production, which are important for combating bacterial infections. The depletion of these proteins impairs the ability of cells to mount effective immune responses, making them more susceptible to bacterial infection and persistence.

While this research provides new insights, it was limited to specific bacterial challenges and a small number of NOD2 and ELMO1 mutations, including complete knockouts and specific CD-associated mutations.

Sharma et al. are the first to report a direct interaction between NOD2 and ELMO1, important microbial sensors, which play a significant role in determining host response to pathogens. These direct interactions of NOD2 and ELMO1 influence the course of bacterial infection by regulating bacterial survival/clearance, ROS generation, and immune responses during AIEC-LF82 and Salmonella infections. Future research should focus on uncovering the structural and molecular details of this interaction and the subsequent pathway involved. This knowledge can potentially reveal alternative therapeutic targeting CD where defective sensing of luminal bacteria contributes to the disease's pathogenesis.

Taken together, a role for ELMO1 might be to stabilize NOD2 in an active conformation where it can interact with MDP to elicit an acute, transient upregulation of pro-inflammatory signals including IL-6 and ROS. Unlike the knockout NOD2s, mutant proteins associated with CD, such as L1007fs, may, with the assistance of ELMO1, be unable to refold into an inactive form (**Figure 1**). While trapped in an active conformation, NOD2 continues to promote pro-inflammatory signals, even in the absence of infection, resulting in chronic inflammatory conditions.

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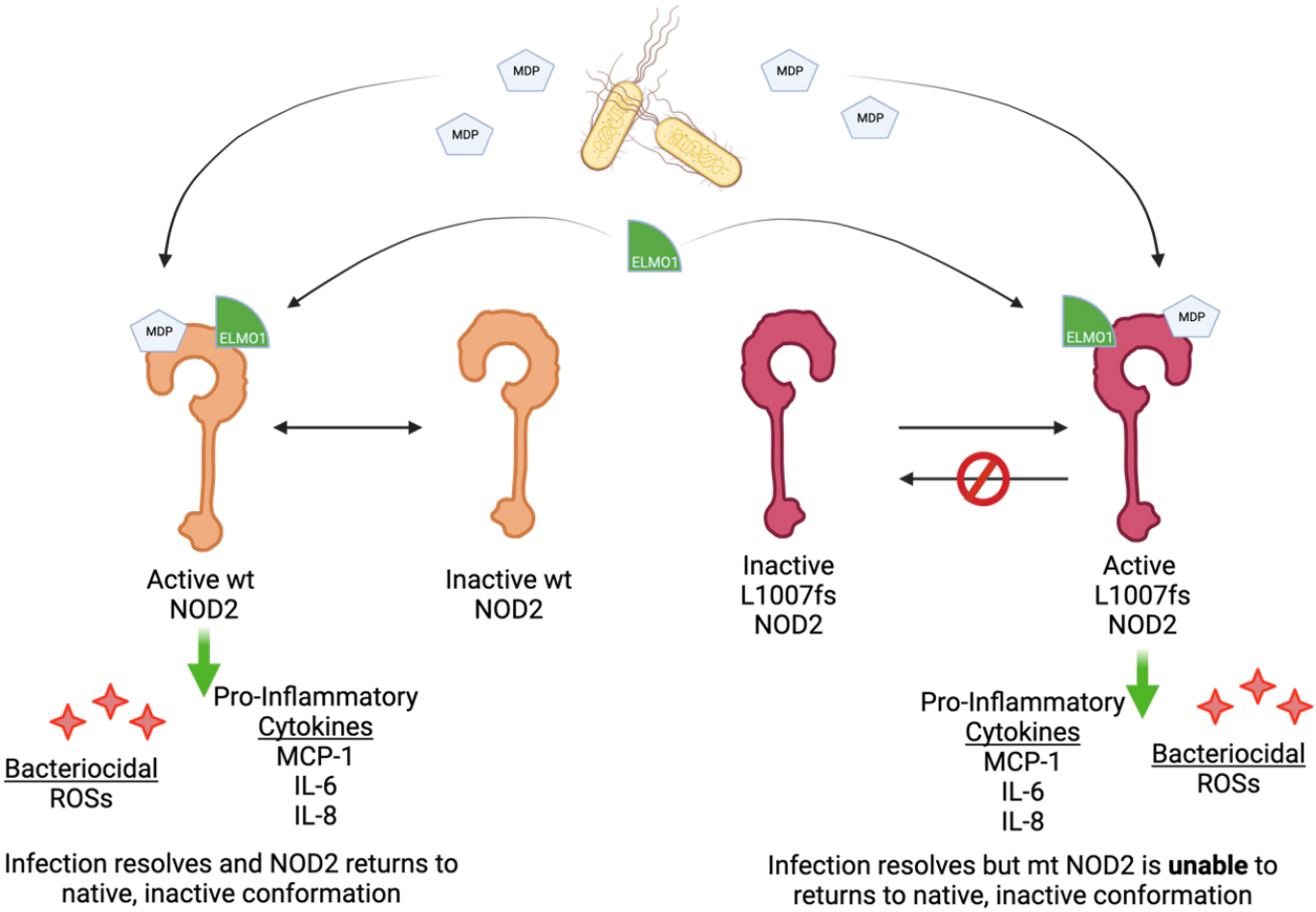

Figure 1 ELMO1 binding stabilizes NOD2 in an active conformation | ELMO1 binds wt NOD2 at the LRR domain to transiently upregulate inflammatory and bacteriocidal reactions. ELMO1 binds L1007fs NOD at the LRR domain to chronically upregulate inflammatory and bacteriocidal reactions resulting in CD and other chronic inflammatory conditions.

Microbes helping Microbes

Evidence for gut microbiota establishing conditions permissive to pulmonary infection with *M. Tuberculosis*

by Olive Njoroge

MicroRNAs, small non-coding RNAs that regulate gene expression post-transcriptionally, have emerged as key players in immune regulation and disease pathogenesis.1 Among these, miR-21 is one of the most highly expressed miRNAs in various tissues and has been implicated in modulating numerous inflammatory responses and immune functions. While the impact of miRNAs on bacterial pathogen-host interactions has been extensively studied, our understanding of their role in gastrointestinal (GI) tract immunity, particularly in the context of Tuberculosis (TB), remains limited.

In the article "MiR-21 is Remotely Governed by the Commensal Bacteria and Impairs Anti-TB Immunity by Down-Regulating IFN-γ," Yang et al. examine the interaction between gut microbiota, miR-21, and immunity in TB pathogenesis. MicroRNAs are known to regulate gene expression, and previous work specifically indicates roles in regulating immune function.[1] A growing body of research has explored the connection between the gut microbiome and lung diseases, a concept termed the "gut-lung axis."[2] This area of study suggests that gut-derived factors, including surviving bacteria, metabolites produced by gut bacteria, or other molecules, may travel into the circulatory system by way of the lymphatic system, potentially triggering immune responses in the lungs.[3] Through a "loss of function" model involving antibiotic-induced dysbiosis of the gut microbiota, the authors investigate the impact of this dysbiosis on immune responses to pulmonary *M. tuberculosis* infection, identifying miR-21 as a potential mediator of host-microbiota interactions in TB.[4]

Understanding the mechanisms by which commensal bacteria regulate interferon gamma (IFN-γ) production via miR-21 offers valuable insight into TB pathogenesis and potential treatment targets and vaccines.[5,6] Traditionally, TB infection occurs through aerosol absorption of *M. tuberculosis* by lung tissues. However, intraperitoneal (i.p.) infection in mouse models has been shown to mimic the chronic TB infection seen in low-dose aerosol exposure, providing several advantages for research. The model used in this study established a steady state level of bacterial burden in organs, resembling clinical latency characterized by low bacillary loads in humans. Furthermore, i.p. infections enable reliable, dose-dependent challenges, with higher doses triggering systemic immune responses relevant to TB pathogenesis.[4]

A key finding of this research is the modulation of miR-21-3p expression by gut dysbiosis induced by antibiotics. MiRNAs, which play critical roles in both innate and adaptive immunity, have been linked to commensal bacteria and disease development. Specifically, upregulated miR-21 expression has been associated with immune evasion by *Mycobacterium leprae*. In the context of TB, dysbiosis-driven changes in miR-21-3p expression affect immune responses, notably by inhibiting IFN-γ production, which is crucial for anti-TB immunity. Mechanistically, the presence of gut microbiota is required for miR-21-3p expression, which directly targets IFN-γ expression by T Cells resident in the lungs, highlighting its role in modulating immune protection against TB (See **Figure 1**). Additionally, fecal microbiota transplantation (FMT) from healthy mice restored gut microbiota and increased miR-21-3p expression in the lungs, providing further evidence linking gut microbiota, miRNAs, and immune responses in TB pathogenesis.

This research underscores the importance of understanding host-microbiota interactions in TB pathogenesis. By elucidating the regulatory pathways involving gut dysbiosis, miR-21-3p, and IFN-γ, the study provides a foundation for developing targeted therapies to enhance immune protection against TB. Given the global prevalence of TB, Tuberculosis, a disease claiming nearly 2 million lives each year, further investigations into the gut-lung axis and miRNA-mediated immune regulation hold promise for advancing TB control strategies and improving global health outcomes.[6]

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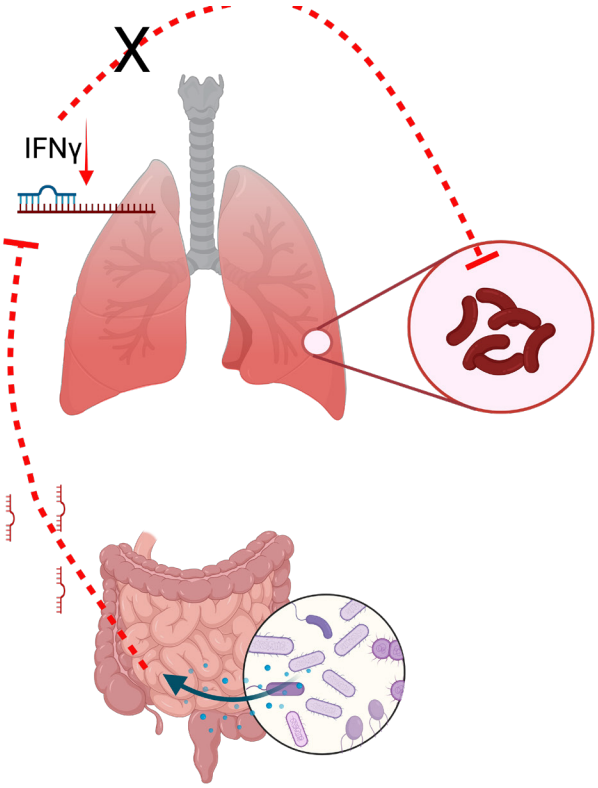


Figure 1: MiR-21 produced in the gut in response to microbiota modulates the expression of IFN-γ by lung-resident T Cells, resulting in an inability to clear pulmonary *M. tuberculosis infection.*

Selected Topics
A Gutsy Reading List

J.F. Treml

The following articles were assigned for group presentations in the Spring 2024 Selected Topic Class at the University of Kansas, Edwards Campus. Articles assigned for presentation are in bold. Background articles are left unbolded.

1. Commensal Influence on Gene Expression

a. **Hooper, L. V. et al. Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. Science 291, 881–884 . 2001.**

b. Thaiss, C. A., Zmora, N., Levy, M. & Elinav, E. The microbiome and innate immunity. Nature 535, 65–74. 2016. [Review]

2. Toll Like Receptor (TLR) Signaling

a. **Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. & Medzhitov, R. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell 118, 229–241 . 2004.**

b. **Venkatesh, M. et al. Symbiotic Bacterial Metabolites Regulate Gastrointestinal Barrier Function via the Xenobiotic Sensor PXR and Toll-like Receptor 4. Immunity 41, 296–310. 2014.**

c. Adrian Hall, Hugues Chanteux, Karelle Ménochet, Marie Ledecq, and Monika-Sarah E. D. Schulze. Designing Out PXR Activity on Drug Discovery Projects: A Review of Structure-Based Methods, Empirical and Computational Approaches. J. Med. Chem. 64, 10, 6413–6522. 2021.

3. Transcriptional Regulation

a. **Kelly, C. J. et al. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe 17, 662–671. 2015**

4. TLR-Independent TRAF Signaling

a. **Vlantis, K. et al. TLR-independent anti-inflammatory function of intestinal epithelial TRAF6 signalling prevents DSS-induced colitis in mice. Gut 65, 935–943 . 2016.**

5. Innate Immunity and Inflammation

a. **Nenci, A. et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 446, 557–561. 2007.**

6. Reactive Oxygen Species

a. **Kumar, A. et al. Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. EMBO J. 26, 4457–4466. 2007.**

7. Ubiquitin

a. Patrick, S. et al. A unique homologue of the eukaryotic protein-modifier ubiquitin present in the bacterium Bacteroides fragilis, a predominant resident of the human gastrointestinal tract.

Microbiology 157, 3071–3078. 2011

8. Host Immune System Development

a. **Chung, H. et al. Gut Immune Maturation Depends on Colonization with a Host-Specific Microbiota. Cell 149, 1578–1593. 2015.**

b. **Zhang, D. et al. Neutrophil ageing is regulated by the microbiome. Nature 525, 528–532. 2015.**

9. Regulation of Vesicular Trafficking

a. **Zhang, Q. et al. Commensal bacteria direct selective cargo sorting to promote symbiosis. Nat. Immunol. 16, 918–926. 2015**

10. Host Regulation of Microbiota

a. **Ramanan, D., Tang, M. S., Bowcutt, R., Loke, P. & Cadwell, K. Bacterial Sensor Nod2 Prevents Inflammation of the Small Intestine by Restricting the Expansion of the Commensal Bacteroides vulgatus. Immunity 41, 311–324. 2014.**

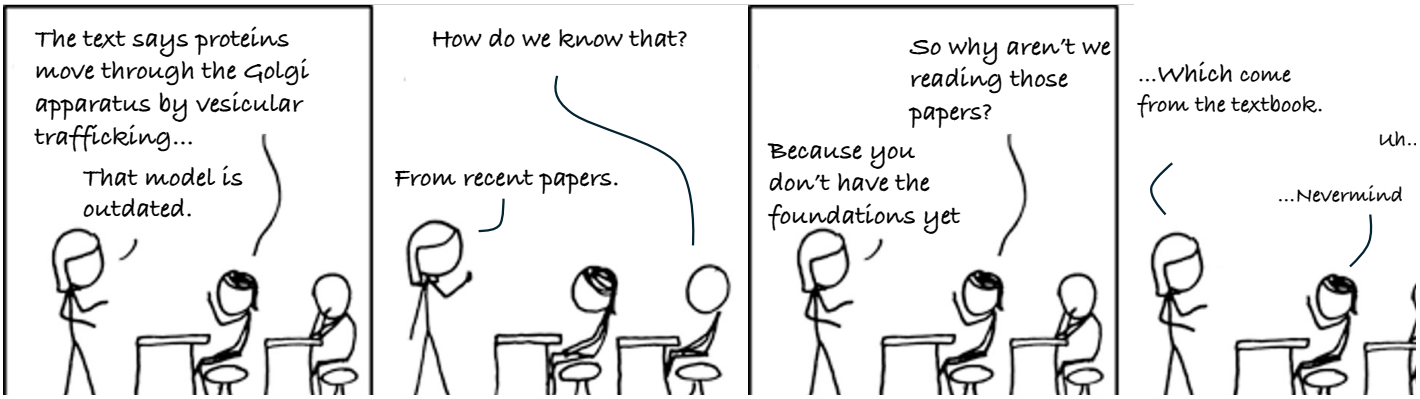
11. Dietary and Adiposy Regulation of Microbiota

a. **Trompette, A. et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat. Med. 20, 159–166. 2014.**

b. Le Chatelier, E. et al. Richness of human gut microbiome correlates with metabolic markers. Nature 500, 541–546. 2013.

c. Koeth, R. A. et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat. Med. 19, 576–585. 2013.

Keeping up on the Literature



Student Research

In recent years, there has been growing recognition of the need for students to demonstrate "real-world" or "authentic" research experience through a senior capstone or thesis project. Industry partners have consistently expressed their trust in the quality of our graduates, but they also emphasize the importance of ensuring that those entering the workforce possess skills beyond field-specific knowledge. They seek graduates who can plan, communicate effectively, and lead others.

At the KU Edwards Campus, we have expanded the senior capstone research model from the biotechnology program to include independent research across various disciplines. These courses encompass not only the design and execution of a research project but also essential skills such as budgeting, inventory management, project scheduling with Gantt charts, and troubleshooting.

This year, we are pleased to feature the first article contributed by a student from outside our (biotech) program, reflecting our commitment to broadening the scope of these “authentic” research experiences. As these student-led projects demonstrate, the ability to navigate unexpected challenges and refine methods fosters a deeper understanding of science and its application in the real world.

We look forward to including more articles from a variety of disciplines and hope to include work from other universities and community colleges whose students also perform exemplary work.

Assessing the effects of lactate on the immune responsiveness of T cells *in vitro*

Kaitlyn A. Sy*, R. Logan, and J. Treml*†

T cells play a key role in protecting the body from cancer. While T cells are normally effective in destroying abnormal cells, there are times when malignancies grow seemingly unchecked by the immune system. Chimeric antigen receptor (CAR)-T cell therapy implements T cells that have been genetically engineered to recognize and kill cancer cells. While CAR-T cell therapy has demonstrated efficacy in treating hematological malignancies, it has shown less promise in treating solid tumors. A possible explanation for the decreased efficacy of T cells and T cell-based immunotherapies in destroying solid tumors may lie in a phenomenon that scientists have long been aware of. The Warburg effect, first described in 1924, is the observation that cancer cells tend to consume significantly more glucose than other cells due to their shift from aerobic respiration to fermentation, resulting in the accumulation of the waste product lactate in the tumor microenvironment. This research investigated the effects of lactate on the immune responsiveness of T cells, using Jurkat E6.1 cells and human primary T cells as models. The effects of lactate on viability, CD3 surface expression, and proliferation were assessed via flow cytometry. While lactate concentration and exposure time had no significant effects on T cell viability, marked decreases in CD3 surface expression and proliferation were observed following exposure to lactate. These findings may shed light on how solid tumors evade immune detection and immunotherapies and have implications for future approaches to treating solid tumors.

Cancer is a devastating disease characterized by uncontrolled proliferation of abnormal cells. Approximately two in every five individuals will be diagnosed with cancer at some point in their lifetime,[1] and cancer accounts for nearly one in every six deaths,[2] making it a leading cause of morbidity and mortality worldwide. Consequently, understanding how cancer takes a foothold in its victims and how it can be prevented or treated is an important area of research.

Recently, the interaction between cancer and the immune system has been a hot area of study. T cells play a key role in protecting the body from cancer.[3] T cells are equipped with T cell receptors (TCRs) that interact with peptide antigens displayed by major histocompatibility complexes (MHCs) on most of the body's cells, including cancer cells. Recognition of the peptide antigen by the TCR triggers a multistep signaling cascade known as TCR signaling.[4] In cytotoxic ($CD8^+$) T cells, TCR signaling leads to the direct killing of cancer cells by inducing apoptosis.[3] In helper (CD4+) T cells, TCR signaling leads to a variety of effector functions, including directly killing cancer cells, activating and maintaining the anti-tumor responses of CD8+ T cells, and driving B cells to produce antibodies against tumor antigens.[5] While T cells are normally effective in destroying abnormal cells, there are times when malignancies grow seemingly unchecked by the immune system. The latest advances in cancer therapeutics have been targeted at enhancing or assisting the body's immune response against cancer. For example, chimeric antigen receptor (CAR)-T cell therapy implements a patient's own T cells that have been genetically engineered to recognize and kill cancer cells.[6] While CAR-T cell therapy has demonstrated efficacy in treating hematological malignancies,[3] it has shown less promise in treating solid tumors.[7] The current understanding of how solid tumors evade the immune system and immunotherapies is incomplete at best.

A possible explanation for the decreased efficacy of T cells and T cell-based immunotherapies in destroying solid tumors may lie in a phenomenon that scientists have long been aware of. The Warburg effect, named after the physiologist who first described it in 1924, is the observation that cancer cells tend to consume significantly more glucose than other cells due to their shift from aerobic respiration to fermentation.8 In some solid tumors, the shift to fermentation may be due to hypoxia when the tumor grows beyond the diffusion limit of the local blood supply,[9] but cancer cells still prefer fermentation over aerobic respiration even under normoxic conditions, a phenomenon known as aerobic glycolysis.[10] Considering that glycolysis alone is a less efficient means of energy production compared to complete glucose oxidation, this phenomenon has long puzzled scientists. A recent study discovered that increased flux through the glycolytic pathway drives the regeneration of NAD^+, a cofactor required for catabolic and anabolic reactions that enable rapid cell proliferation.[11] Shifting to fermentation may be advantageous for surviving in hypoxic

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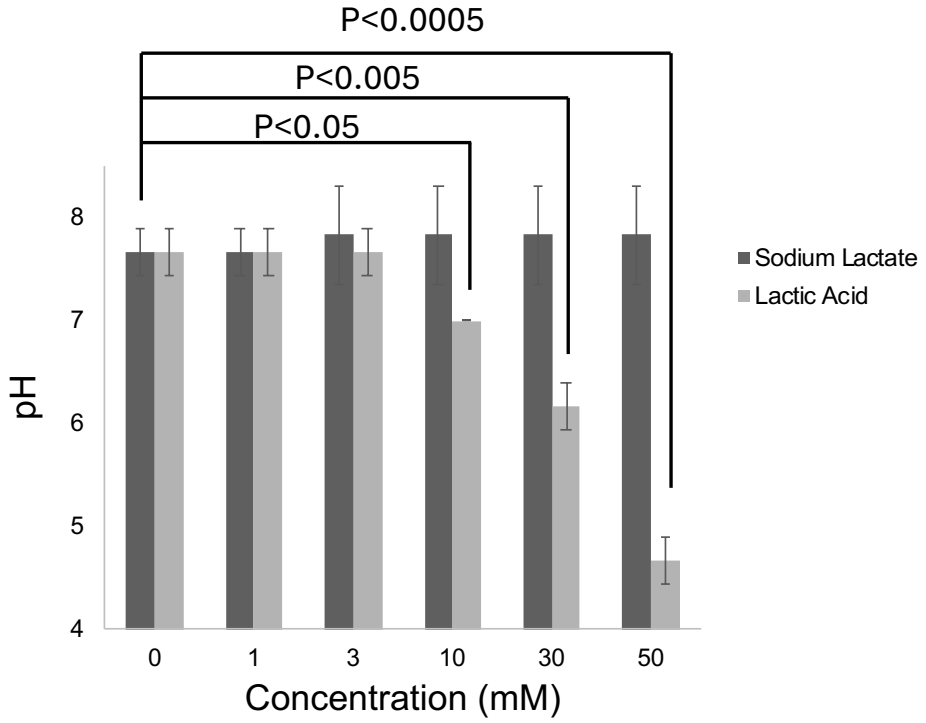

Figure 1 | Addition of sodium lactate to complete cell culture media does not change pH.
The pH of complete cell culture media containing different concentrations of lactic acid or sodium lactate was measured using universal pH indicator paper. Data from three independent experiments is displayed. Statistical significance was determined via two-tailed, homoscedastic t-tests.

environments and proliferating rapidly, but it could also have a role in immune evasion. Cancer cells performing fermentation produce large amounts of lactic acid, which quickly dissociates into lactate and H^+, acidifying the intracellular space. To combat the harmful effects of this acidification, cancer cells efflux lactate and H^+ into the tumor microenvironment (TME) through monocarboxylate transporters.[10,12] Lactate concentrations as high as 40 mM and pH levels as low as 5.6 have been reported in the TME.13 Our lab previously reported that exposure of Jurkat E6.1 cells, an immortalized $CD4^+$ T cell line, to acidic conditions potentially led to decreased TCR signaling upon stimulation of CD3.[14] However, the immunosuppressive effects of cancer cells' shift to fermentation may extend beyond acidifying the TME, as tumor-derived lactate has been demonstrated to impact the phenotypes of dendritic cells[15] and NK cells,[16] and the effects of lactate on other immune cells have yet to be investigated. Interestingly, aerobic glycolysis is not unique to cancer cells, but has also been observed in other rapidly proliferating cells, such as T cells and fibroblasts.[17,18] While it is plausible that high concentrations of lactate may inhibit the continued proliferation of cells performing aerobic glycolysis via negative feedback mechanisms, the opposite has been observed in some cases. For example, lactate has been demonstrated to stimulate the proliferation of fibroblasts in vitro[19] and promote protein synthesis in wound healing.[20]

In this study, we sought to investigate the effects of lactate on the immune responsiveness of T cells in the context of the TME—without changes in pH—to determine whether lactate itself has immunomodulatory properties. This was done by assessing viability, CD3 surface expression, and T cell proliferation in primary and immortalized T cells following exposure to lactate.

Materials & Methods

Preparation of 100x Lactic Acid and Lactate Stock Solutions

A 5 M lactic acid stock solution was prepared by dissolving the appropriate mass of sodium lactate (Thermo Scientific Chemicals, Cat. No. L14500.06) in 5 M HCl (Fisher Scientific, Cat. No. A144-212). A 5 M sodium lactate stock solution was prepared in water. The 5 M stock solutions were passed through a 0.2 micron filter (PALL Gelman Laboratory, Cat. No. 4192) and diluted with sterile-filtered diH_2O to concentrations of 100 mM, 300 mM, 1 M, and 3 M to make 100x stock solutions for the following working concentrations: 1 mM, 3 mM, 10 mM, 30 mM, and 50 mM. DiH_2O was used as a vehicle control. All stock solutions were stored at 4°C.

Jurkat E6.1 Cell Culture

The Jurkat E6.1 cells were kindly donated by Dr. Jon Houtman and Dr. Gary Weisman (previously purchased from ATCC: TIB-152TM). Jurkat E6.1 cells were grown at 37°C in 5% CO_2 using complete RPMI-1640 Medium (Sigma-Aldrich, Cat. No. R8758) supplemented with 10% FBS (Gibco, Cat. No. A52567-01). Cultures were maintained between 1x105 and 1x106 cells/mL at passage numbers below 20.

Lactic Acid, Lactate, and Media pH Assay

1 mL of complete RPMI-1640 Medium supplemented with 10% FBS was aliquoted into microcentrifuge tubes and spiked with 10 µL of the appropriate 100x lactic acid or sodium lactate stock solution. The pH of the media was measured using universal pH indicator paper (Cytiva, Cat. No. 2613-991). Data from three independent experiments were averaged. Two-tailed, homoscedastic t-tests were used to determine statistical significance with a threshold of $p \leq 0.05$.

Timepoint Viability and CD3 Surface Expression Assay

Jurkat E6.1 cells were plated (2.5x105 cells/well) 30 hours prior to analysis and incubated at 37°C in 5% CO_2. Samples were treated with sodium lactate (at working concentrations of 0, 1, 3, 10, 30, and 50 mM) 30 hours, 1 hour, 30 minutes, and 15 minutes prior to analysis. Samples were then harvested, washed, and resuspended in 1 mL DPBS (2.7 mM KCl [Fisher Scientific, Cat. No. BP366-500], 1.5 mM KH_2PO_4 [Fisher Scientific, Cat. No. BP362-1], 136.9 mM NaCl [Fisher Science Education, Cat. No. S25541A], 8.9 mM $Na_2HPO_4 \cdot 7H2O$ [EMD Millipore Corp Cat. No. 56747]). Unstained samples were analyzed using the Attune NxT Flow Cytometer.

Samples were then stained with one drop of SYTOX AADvanced Ready Flow Reagent (Invitrogen, Cat. No. R37173) and 5 μL of FITC-labelled anti-CD3 (Invitrogen, Cat. No. 11-0037-42), incubated on ice in the dark for 40 min, then analyzed using the Attune NxT Flow Cytometer. A SSC-A vs. FSC-A density plot of all events was used to visualize populations and gate the Jurkat E6.1 cells. A histogram plot of the Jurkat E6.1 cells was used to visualize fluorescence intensity detected through the BL3 channel (used to detect SYTOX) to gate live and dead cells. Counts of live and dead cells were used to calculate percent viability. A histogram plot of the live Jurkat E6.1 cells was used to visualize fluorescence intensity detected through the BL1 channel (used to detect FITC) to assess relative surface expression of CD3. X mean values of stained cells were normalized to X mean values of unstained controls and then normalized to the appropriate vehicle control for each time exposure. Data from three independent experiments were averaged. Two-tailed, homoscedastic t-tests were used to determine statistical significance with a threshold of $p \leq 0.05$.

Primary Leukocyte Isolation

Whole blood was collected from a healthy adult male human volunteer via venipuncture into BD Vacutainer EDTA tubes (Becton Dickson and Company, Cat. No. 366643). The research protocol of this study was approved by the Institutional Review Board of The University of Kansas (STUDY00150912). The volunteer who donated blood for this study gave informed consent for participation. The whole blood was diluted two-fold with DPBS, layered on top of Histopaque-1077 (Sigma-Aldrich, Cat. No. 10771-100ML), and centrifuged at 400 x g for 30 min. The buffy coat (containing leukocytes) was transferred to a clean tube, washed twice, and resuspended in DPBS. Leukocytes were used immediately after isolation.

T Cell Proliferation Assay

Freshly isolated primary leukocytes were resuspended in CFSE staining solution (5 uM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester [Sigma-Aldrich, Cat. No. 21888-25MG-F], 0.1% DMSO [Fisher Chemical, Cat. No. D128-4] in DPBS) and incubated for 10 min in a water bath set to 37°C. An equal volume of complete RPMI-1640 media with 10% FBS was added to the staining solution and incubated for 5 min to quench the staining. The stained cells were resuspended in complete RPMI-1640 media supplemented with 10% FBS and plated (500,000 cells/well). Samples were treated with sodium lactate (at working concentrations of 0, 3, 10, and 30 mM) and 60 ng/mL human IL-2 (Miltenyi Biotec, Cat. No. 130-097-743). Samples were split into stimulated and unstimulated groups. Stimulated groups were treated with 25 μL/mL of ImmunoCultTM Human CD3/CD28 T Cell Activator (STEMCELL Technologies, Cat. No. 10971). Samples were incubated at 37°C in 5% CO2 for 4 days. Samples were then harvested, washed, and resuspended in 1 mL DPBS. Samples were stained with 1 μL reconstituted LIVE/DEAD Fixable Red Dead Cell Stain (Invitrogen, Cat. No. S10274) and 5 μL PE-Cy5.5-labelled anti-CD8 (Invitrogen, Cat. No. 12-0088-42), incubated on ice for 30 min, and analyzed using the Attune NxT Flow Cytometer. A histogram plot of all events was used to visualize fluorescence intensity detected through the BL2 channel (used to detect LIVE/DEAD Fixable Red Dead Cell Stain) to gate live and dead cells. A density plot of BL3 (used to detect PE-Cy5.5) vs. BL1 (used to detect CFSE) was used to visualize proliferated and non-proliferated $CD8^+$ T cells. A histogram plot of $CD8^+$ T cells was used to visualize fluorescence intensity of CFSE to gate proliferated and non-proliferated $CD8^+$ T cells. Counts of proliferated and non-proliferated $CD8^+$ T cells were used to calculate the percentage of proliferated cells. Data from three replicates were averaged. Two-tailed, homoscedastic t-tests were used to determine statistical significance with a threshold of $p \leq 0.05$.

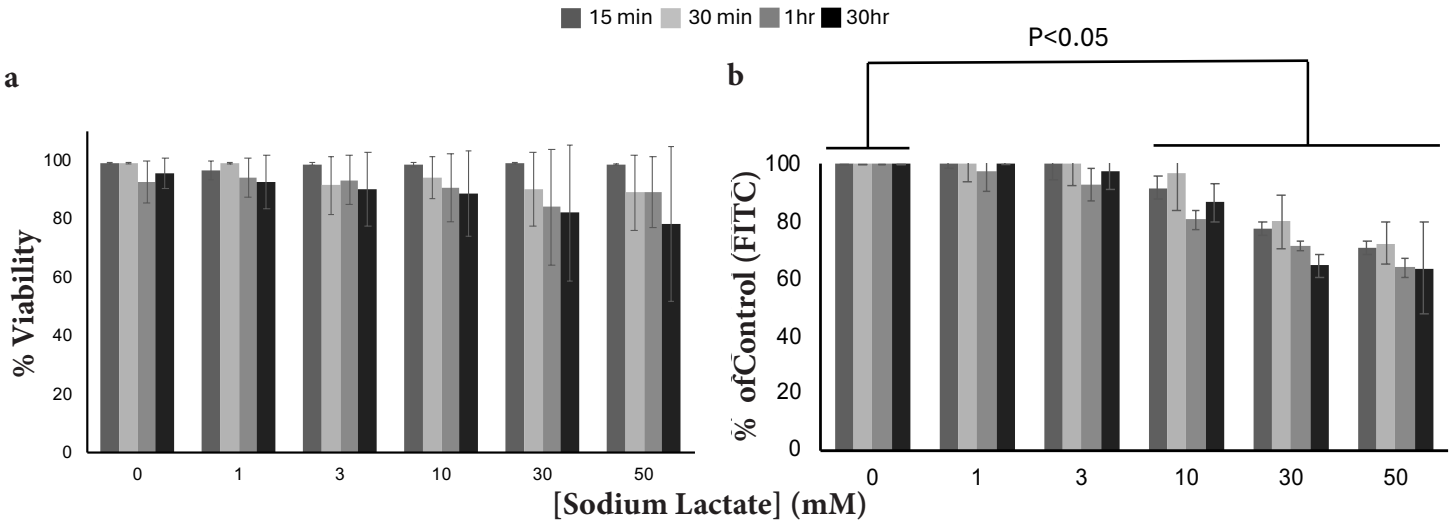

Figure 2 | Exposure to lactate decreases CD3 surface expression, but not viability, of Jurkat E6.1 cells. Jurkat E6.1 cells incubated with different concentrations of lactate for variable exposure times were analyzed via flow cytometry. **(a)** Cell viability was determined via staining with SYTOX AADvanced Ready Flow Reagent. **(b)** CD3 surface expression of live cells was determined via staining with FITC-labelled anti-CD3. Data from three independent experiments is displayed. Statistical significance was determined via two-tailed, homoscedastic t-tests. (Note that the CD3 surface expression in Jurkat E6.1 cells exposed to 10 mM lactate for 30 minutes did not differ significantly from the vehicle control.)

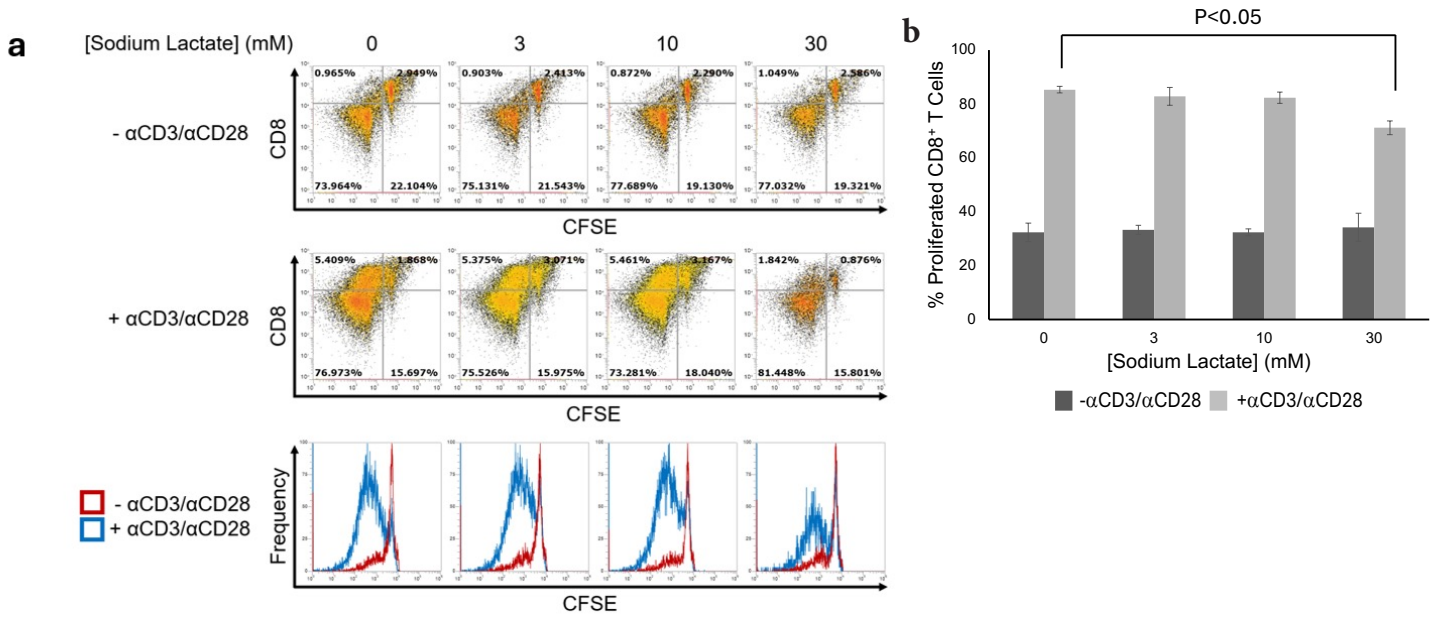

Figure 3 | Exposure to lactate impairs the proliferation of primary CD8+ T cells.
Proliferation of primary leukocytes activated via CD3/CD28 costimulation and incubated with different concentrations of lactate was analyzed via flow cytometry. (a) Top panel displays density plots of CD8 vs. CFSE for live leukocytes that were left unstimulated. Middle panel displays density plots of CD8 vs. CFSE for live leukocytes that were activated via CD3/CD28 costimulation. Bottom plot displays overlays of histogram plots of CFSE for live $CD8^+$ T cells activated via CD3/CD28 costimulation (blue) and unstimulated controls (red). (b) Data from the bottom panel of Figure 3a was replotted as a bar graph to display numerical differences in proliferation among the four treatment groups. Data from three replicates is displayed. Statistical significance was determined via two-tailed, homoscedastic t-tests.

Results

Addition of sodium lactate to complete cell culture media does not change pH

The goal of the present study was to assess the effects of lactate on the immune responsiveness of T cells independent of changes in the pH of the tumor microenvironment. To verify that the addition of sodium lactate to complete cell culture media does not change the pH of the media, we measured the pH of complete cell culture media containing the following concentrations of lactate: 0, 1, 3, 10, 30, and 50 mM. For sake of comparison, we also measured the pH of complete cell culture media containing 0, 1, 3, 10, 30, and 50 mM lactic acid. While lactic acid acidified the cell culture media at concentrations of 10 mM and above, sodium lactate did not significantly affect the pH of the cell culture media at all concentrations tested (**Figure 1**).

Exposure to lactate decreases CD3 surface expression, but not viability, of Jurkat E6.1 cells

Having established that lactate does not affect the pH of cell culture media, we next wanted to assess whether exposure to lactate decreases T cell viability and CD3 surface expression, using Jurkat E6.1 cells (an immortalized CD4+ T cell line) as a model. CD3 is an important part of the TCR complex and is vital for TCR signaling.4 CD3 surface expression in Jurkat E6.1 cells was previously verified by our lab.14 However, Jurkat E6.1 cells have been known to down-regulate CD3 surface expression over time in culture (Jon Houtman, personal communication), so we assessed CD3 surface expression of our Jurkat E6.1 cell cultures periodically via flow cytometry to ensure strong expression (data not shown). To model exposure of T cells to lactate in a TME, we exposed Jurkat E6.1 cells to complete cell culture media containing different concentrations of lactate. 1 and 3 mM lactate simulated normal physiological concentrations of lactate; 10, 30, and 50 mM lactate simulated lactate concentrations that might be found in a TME.13 0 mM lactate was used as a vehicle control. We incubated the Jurkat E6.1 cells with the lactate for four different lengths of exposure—15 minutes, 30 minutes, 1 hour, and 30 hours—to simulate infiltration of circulating and tissue-resident T cells in the TME.21 Following incubation, the cells were collected and analyzed via flow cytometry. Jurkat E6.1 cell viability remained high (>80% viable) following exposure to all lactate concentrations and all exposure times tested (**Figure 2a**). However, there was a significant decrease in CD3 surface expression in Jurkat E6.1 cells exposed to lactate concentrations of 10 mM and above (Figure 2b). This decrease was observable following 15-minute exposure times but was more pronounced following longer exposures. However, CD3 surface expression in Jurkat E6.1 cells exposed to 10 mM lactate for 30 minutes did not differ significantly from the vehicle control, possibly due to variability among repeats.

Exposure to lactate impairs the proliferation of primary CD8+ T cells

Considering the vital role of CD3 in TCR signaling, we wanted to investigate the implications that the lactate-induced decrease in CD3 surface expression had for T cell proliferation, a downstream effect of TCR signaling.3 Jurkat E6.1 cells already proliferate rapidly without stimulation, so primary T cells were used for this experiment. Leukocytes were isolated from whole blood collected from a healthy adult male human volunteer. Normal counts of peripheral blood mononuclear cells (PBMCs) in the volunteer's blood were veri-

fied via flow cytometry (data not shown). T cells were determined to strongly express CD3 and either CD4 or CD8 (data not shown). To model the stimulation of T cells in a TME, we activated primary T cells via CD3/CD28 costimulation in media containing different concentrations of lactate. CFSE was used to track cell proliferation. Following a four-day incubation, the cells were collected, and viability and proliferation of CD8+ T cells were analyzed via flow cytometry. Similar to the Jurkat E6.1 cells, the primary cells displayed no significant changes in viability across all lactate concentrations tested (>90% viable, data not shown). CD8+ T cells stimulated in media with lactate concentrations of 10 mM and below proliferated normally, but CD8+ T cells stimulated in media with 30 mM lactate demonstrated impaired proliferation (**Figures 3a and 3b**).

Discussion

T cells play a vital role in the body's defense against abnormal cells, including cancer cells.[3] T-cell-based immunotherapies—such as CAR-T cell therapy—have been explored as treatments for cancer, when the body's natural immune response is ineffective in eradicating malignant cells. While immunotherapies like CAR-T cell therapy have demonstrated success in treating hematological malignancies,[3] they have shown less promise in treating solid tumors,[7] necessitating research into how solid tumors evade the immune system and immunotherapies. The tumor microenvironments found in and around solid tumors have commonly been reported to be acidic and to contain high concentrations of the metabolic byproduct lactate, due to the Warburg effect.[13] Considering previous findings that exposure to acidic environments potentially impacted the immune responsiveness of T cells,[14] and that tumor-derived lactate impacted the phenotypes of immune cells like natural killer cells and dendritic cells,[15,16] recent evidence points to the Warburg effect as a possible explanation for the ability of solid tumors to evade the immune system and immunotherapies. In the present study, we assessed whether lactate could impact the immune responsiveness of T cells independent of a change in pH by exposing primary and immortalized T cells to different concentrations of lactate simulating both normal physiological conditions and tumor microenvironments. We first verified that the addition of lactate to complete cell culture media does not change the pH of the media. We then assessed the effects of lactate on the viability and CD3 surface expression of T cells, using Jurkat E6.1 cells as a model. While lactate had no significant effect on Jurkat E6.1 cell viability, high concentrations of lactate significantly decreased CD3 surface expression following both short (15 minutes) and long (30 hours) exposures. We investigated the implications of this finding on T cell proliferation, using primary $CD8^+$ T cells as a model. We found that, following CD3/CD28 costimulation and a four-day incubation, primary $CD8^+$ T cells treated with 10 mM lactate or less proliferated normally, whereas cells treated with 30 mM lactate proliferated significantly less

Future Directions

This study suggests that lactate—independent of a change in pH—could impact CD3 surface expression and proliferation of T cells. This corroborates the findings of other studies regarding the immunosuppressive effects of tumor-derived lactate. However, it would be interesting to further characterize the effects of lactate on the immune responsiveness of T cells—namely, to assess the effects of lactate on TCR signaling itself and downstream effects of TCR signaling, such as cytotoxicity and cytokine production. Additionally, while the mechanism by which cancer cells produce lactic acid and efflux it into the tumor microenvironment is well understood, the mechanisms by which lactate impacts immune responsiveness have not been characterized. Tracing these mechanisms may uncover potential targets for therapeutics that could be used to improve the body's natural immune response or increase the efficacy of immunotherapies against solid tumors.

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Kaitlyn Sy is a senior in the Biotechnology program at the University of Kansas Edwards Campus. After completing her undergraduate degree, she will pursue her M.D. at the University of Kansas School of Medicine. She is devoted to improving outcomes for cancer patients through research, clinical practice, and community service.

Author's Contributions

K.A.S. contributed to the experimental work, design, and writing of this work; R.L.L. contributed to the design and editing of this work; J.F.T. contributed to the design and editing of this work.

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Integrative Review of Riparian Buffers Benefits in Urbanized Watersheds

Paige Denning* and Scott Schulte*†

Riparian buffers or riparian corridors are areas of vegetation in the floodplains and areas surrounding a stream. By the early 2000s, numerous national reports and studies of riparian buffer benefits established that vegetation near streams is helpful in protecting the stream from increasing urban runoff, minimizing bank erosion, reducing flooding, and improving overall water quality. However, influential studies concluded that buffer benefits dwindle as urbanization increases, eventually becoming ineffective. This study evaluates recent research that suggests riparian buffers are more effective at countering urbanization impacts than previously understood and considers the extent to which we can quantify these benefits and identify the factors that maximize their effectiveness (i.e. greater efficiency based on buffer distance from the stream, extent of stream setbacks, and percentage of impervious cover in the area). Much of the research has been conducted in the Kansas City area in the Blue River Watershed, which begins in Kansas and flows into the Missouri River east of downtown Kansas City, Missouri. About 800,000 residents live in the watershed, which includes some of the region's fastest-growing areas. It is critical to protect this major resource, and other regional rivers and streams, for residents of the Kansas City Metropolitan area and the ecosystems that depend on them. It is also critical to provide the latest and best information to the community-of-practice currently updating regional stormwater management planning and design guidance.

Introduction

The Mid-America Regional Council (MARC), the Kansas City Metropolitan Chapter of the American Public Works Association (APWA), and its member communities and consultants have worked to define and quantify the benefits of riparian corridors for 30 years to develop more effective regional policies and design criteria. The work was undertaken with assistance from the Center for Watershed Protection, state and Federal agencies, regional and national not-for-profit environmental organizations, and academic researchers. The efforts are built upon fundamental research from pioneers in the field and early stream protection and restoration practitioners.

The purpose of this review is to integrate available resources including multiple peer-reviewed and other published studies, watershed plans, and modeling over the last twenty years to define and update our understanding of riparian buffer benefits. The emphasis of this paper will be how riparian corridors help reduce bank erosion, minimize flooding, protect the area from urban runoff, and overall improve stream health and quality. Furthermore, this analysis will evaluate distinct types of riparian corridors and where they can be most effective (i.e., woody vegetation vs. partially vegetated corridors). The conclusion of the paper will also provide a few recommendations for how riparian buffers can be utilized along the Blue River.

To understand riparian buffer benefits and potential protection strategies for urbanizing watersheds, it is also important to understand the Impervious Cover Model, stream setback ordinances, and how the negative effects of urbanization can be countered by riparian zones. Based on this analysis, the paper will present updated conclusions about how stream buffers protect urban streams, what factors improve their benefits, and how communities might incorporate this knowledge into policies.

Urbanization's Impact on Streams

Urbanization converts rural land and increases impervious cover that degrades stream quality in many ways. "Contaminants, habitat destruction, and increasing streamflow flashiness resulting from urban development have been associated with the disruption of biological communities."[1] Reduced stormwater infiltration as vegetation is replaced by buildings and pavements increases flooding, bank erosion, and loss of biodiversity in surrounding streams. Increasing pollution impacts all urban streams. From 2001-2003, Jeffery Deacon studied water quality in New Hampshire to correlate concentrations of different chemicals and organisms in streams as well as the overall water quality/habitat scores in areas with various levels of urbanization and buffer coverage. Deacon found that areas with higher impervious cover and urbanization had higher concentrations of E. Coli, nitrite and nitrate yields, pesticide detection, etc.[2]

Impervious Cover and the Impervious Cover Model

Impervious cover includes human-caused structures that do not allow for water infiltration in the surrounding area. Impervious cover has been widely known to affect stream quality in many ways: the urbanization of land causes urban runoff, loss of biodiversity, bank erosion, and increased flooding. However, it has been found that stream buffers may be able to combat the negative impacts that impervious cover has on water/stream quality.

Thomas Schueler revisited the impervious cover model (ICM) in 2018. The ICM model was first introduced in 1994 and has been amended since then. The ICM documented a direct correlation between the percentage

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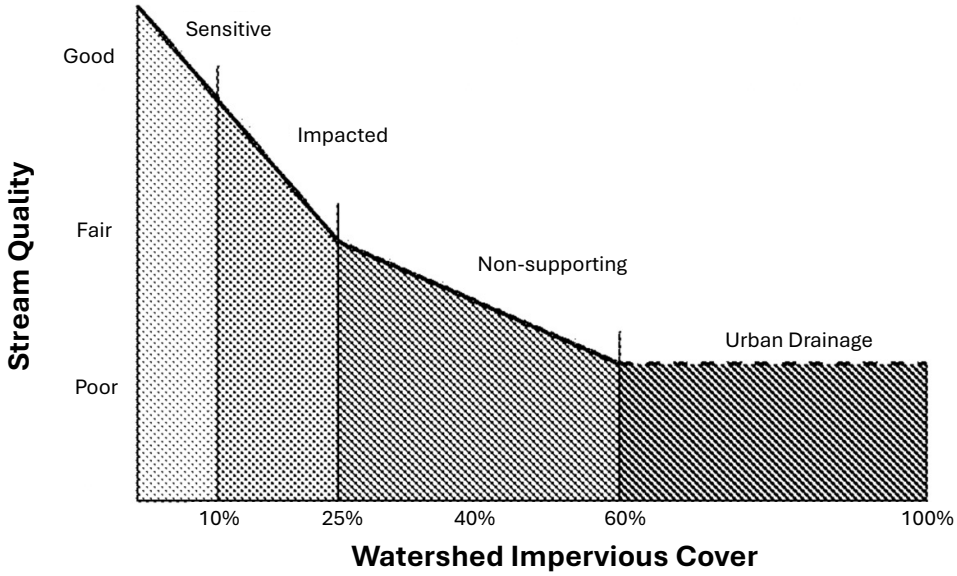

Figure 1 | Stream quality v. impervious coverage.[3]

of impervious coverage and stream quality and is still used today to provide insight into how much it can affect urban watersheds.[3]

Riparian Buffer Benefits for Urbanization

As it is clear that impervious cover impacts receiving streams, research has shifted to how to mitigate these negative effects – and riparian buffers may be the answer. Schueler (2018, p. 97) noted significant stream degradation "... reported in watersheds that had less than 10% IC, with eight notable outliers. These outliers had greater IC (25 to 35%) but similar B-IBI scores. These outliers are unique in that they had a large upstream wetland and/ or a large, intact riparian corridor upstream (i.e., >70% of stream corridor had buffer width >100 feet)". The Index of Biotic Integrity scores (B-IBI scores) assess the effects of human disturbance (i.e., impervious cover) on stream health and quality in wetlands.[4]

Riparian buffers provide resisting forces against urbanization impacts that contribute to stream health and quality in several ways. The vegetated zones slow runoff as the stems create friction that reduces the velocity of urban runoff entering the stream, which helps prevent bank erosion and reduces flooding as the roots absorb and infiltrate water that is flowing more slowly.[5] Riparian vegetation further protects streambank structure as greater amounts of vegetation and native plant roots fortify the bank and lessen the likelihood of bank erosion.[1]

When riparian vegetation is present, soil loss decreases exponentially (see **Figure 2**). The soil loss in the figure is due to canopy coverage. When more vegetation is present, "Some precipitation is intercepted by plant foliage and evaporated back to the atmosphere, but most of it reaches the soil."[5] Vegetation also builds healthy soil that allows for better infiltration of this water into the ground, further curbing the flow of urban runoff into the stream. Riparian zones contribute organic material (such as leaf litter and debris) to the streams and nutrients and the aquatic organisms they support, and deposit organic material in the riparian corridor, increasing soil health. Soil health is important because healthy soil can absorb more water, pollutants, and sediment, which protects streams from these harmful pollutants. But in an area without vegetation, the soil is degraded and water cannot infiltrate, making it even more likely to erode into the nearby streams.[5]

Several national and regional sources have offered important insights on riparian buffers' effectiveness in combatting the negative effects of impervious cover on stream quality. A 2001 study by Horner and May illustrated a correlation between the Habitat Quality Index (HQI) score and the presence of riparian buffers, even in watersheds with total impervious areas (TIA) approaching 40%. Riparian integrity was defined as buffer widths wider than 30 meters (m) over at least 70% of the corridor, less than 10% of the corridor with buffers under 10 m in width, riparian continuity (fewer than two breaks in the corridor per kilometer of stream), and riparian quality (more than 80% of the corridor as forest or wetland cover)."[6] The results are shown in **Figure 3**. A more recent and extensive study conducted by the U.S. Geological Survey (USGS) collected water quality data in nine metropolitan areas: Portland, Oregon; Salt Lake City, Utah; Birmingham, Alabama; Atlanta, Georgia; Raleigh, North Carolina; Boston Massachusetts; Denver, Colorado; Dallas, Texas; and Milwaukee, Wisconsin. Multiple, extensive sampling events focused on the riparian buffer and impervious cover effects on water quality.[1] In **Figure 4**, the percentage of urban development correlates to the channel cross-sectional area per watershed unit area, as increased runoff volumes and velocities generally erode and widen channels. However, Milwaukee's data shows a much less channel widening, which was attributed to the resisting forces from much greater riparian vegetation, that increased the strength of the bank structure in the region's streams. This further supports that riparian vegetation can be useful against the negative effects of urbanization and impervious cover.

Kansas City Regional Studies

Much of the riparian buffer research conducted in the Kansas City region has been focused on the Blue River Watershed, which begins in Kansas and flows into the Missouri River east of downtown Kansas City, Missouri. About 800,000 residents live in its watershed, which includes some of the region's fastest-growing areas. It is critical to protect this major resource, and other regional rivers and streams, for residents of the Kansas City Metropolitan area and the ecosystems that depend on them. Numerous not-for-profit and governmental partners have developed watershed management and conservation plans for the Blue River, all of which recognize the need to protect riparian buffers.[7]

In 2005, Patti Banks Associates and Black

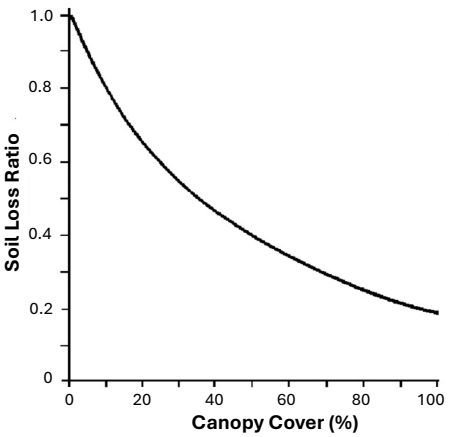

Figure 2 | Soil loss v. canopy cover ratio.[5]

& Veatch Corporation conducted a Stream Asset Inventory of 289 locations in Kansas City, Missouri, including many in the Blue River Watershed.[8] The SAI procedure was developed by practitioners in the Kansas City region to address a lack of urban stream assessment methods, using indicators of relative stream stability, terrestrial and aquatic habitat, and water quality. The results are presented in **Table 1**.

SAI stream types rank stream quality with Type I being the highest level of stream quality and Type V being the lowest, based on a statistical distribution of assessed streams; Type III streams represent the median streams. Type I and II streams are significantly higher quality, and Type IV and V streams are significantly lower. Type I and II streams are generally stable and correspond to the "sensitive" streams in the ICM, while the Type III streams correspond to the ICM's "impacted" streams. Type IV and V streams are "non-supporting" in ICM terms.[8]

However, the Blue River watershed stream types were not what the ICM predicted. "Type IV streams were found in the upper reaches of the Wolf Creek watershed, while Type II streams were identified in the Blue River main stem and Camp Branch sub-watersheds."[8] The Type II stream reaches along the Blue River main stem had drainage areas with about 8% imperviousness, while the Wolf Creek and Camp Branch sub-watersheds both exhibited impervious cover of 2 to 3%.[8] It was observed that more extensive and higher quality vegetation surrounded the Type II streams; and across the data set, the quality and quantity of riparian buffer vegetation correlated moderately to strongly with overall stream scores. Thus, it further supports that riparian corridors can negate the effects of impervious cover/urbanization.[8]

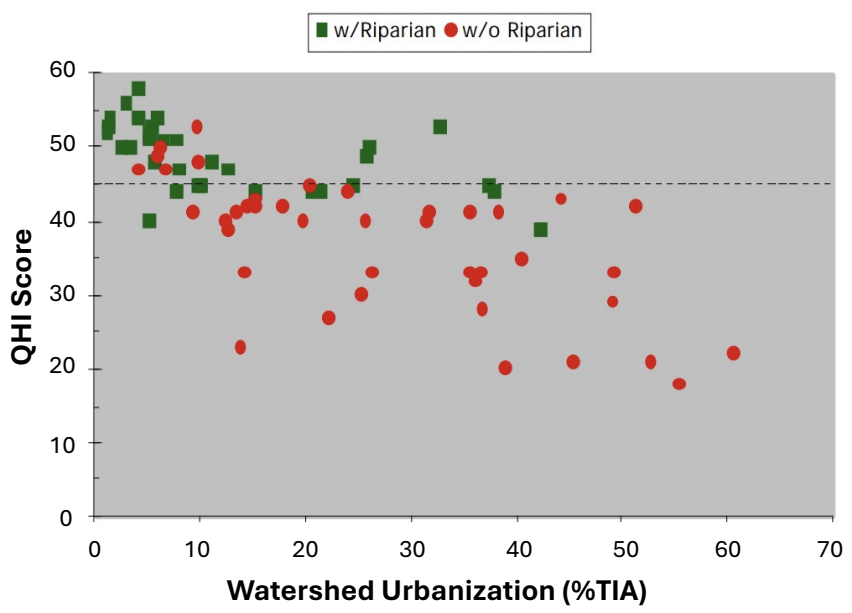

Figure 3 | QHI Score v. Watershed urbanization percentage[6]

Results and Discussion

The studies described above indicate that riparian buffers provide greater benefits for urban streams than the ICM predicts. Wherever present, buffer vegetation continues to protect streams from increased runoff volume, velocity, erosion, sedimentation, and contaminant loads, even as impervious cover increases. Given this result, riparian buffers should be protected and restored in the Kansas City region. The following sections suggest guidelines for prioritizing and designing effective riparian buffers.

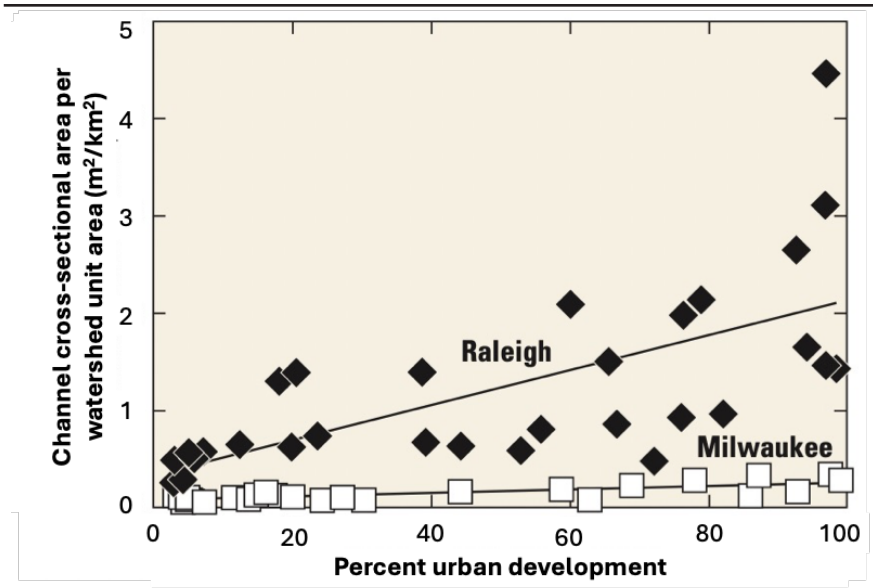

Figure 4 | Cross-sectional area v. percentage of urban development[1]

Prioritizing Areas of Concern

Areas, where urbanization is rapidly increasing, are in dire need of riparian corridor protection to prevent further stream degradation, and the data indicate that riparian buffer restoration may also help reverse negative impacts. Tools are needed to prioritize protection and restoration. Approaches like the two presented below may be complementary for assessing which riparian corridors need the most attention in places like the Blue River Watershed.

Watershed models can help quantify riparian buffer benefits for reducing flooding and erosion, and forecasting increases in impervious cover can help identify where riparian corridor protection is needed the most. Kansas State University developed a hydrologic and hydraulic model of the Blue River watershed and found that riparian buffers fully vegetated with native species reduced stream discharge by up to 20% for frequent storms (occurring on average every 1- to 2-years) that cause most stream erosion, and even some larger storms that cause flooding. Loss of native vegetation increased discharge while restoring native vegetation reduced it; however, turf grass provided no benefit. Kansas State then used future land use forecasts and Geographic Information System (GIS) mapping to estimate where impervious coverage would increase, and

Summary Distribution						
	KC-One Data (2005)		KC SAI Data (2002-03)		Overall	
Stream Type	**Count**	**% Total**	**Count**	**% Total**	**Count**	**% Total**
Type I	3	1.0%	0	0.0%	3	0.6%
Type II	39	13.5%	18	9.5%	57	11.9%
Type III	196	67.8%	141	74.2%	337	70.4%
Type IV	42	14.5%	28	14.7%	70	14.6%
Type V	9	3.1%	3	1.6%	12	2.5%
Total:	289	100.0%	190	100.0%	479	100.0%

Table 1 | Stream Types[8]

to identify which areas (**Figure 5a**) of the Blue River watershed would benefit the most from riparian corridor protection or would be negatively impacted by riparian vegetation loss.[9]

Another promising model from a study in northern central Texas helps predict water quality. A 2020 study of a public water supply reservoir's watershed in the Dallas-Fort Worth area utilized GIS mapping to assess 40-meter-wide corridors in 90 sub-watersheds.[10] The Water Quality Corridor Management for Restoration (WQCM-R) model is "a spatially explicit modeling and mapping technique"[10] used to "(1) utilize easily accessible data for the purpose of identifying and assessing potential water quality issues and (2) to classify stream segments in order of riparian quality in order to prioritize potential restoration activities as a component of an overall watershed management plan."[10] **Figure 5b** shows how the model sorted streams reached based on their relative protection and restoration opportunities.

Buffer Design Guidelines

Once priorities for protection and restoration are set, it's vital to optimize stream setback and corridor widths to improve stream health and achieve important goals, including minimizing bank erosion, reducing urban runoff volume and velocity, filtering pollutants, and minimizing flooding. Kansas State University's Blue River watershed modeling found that fully vegetated 100-year floodplains (and wide buffers on headwater streams) provided the greatest benefit with native vegetation.[9]

Gary Bentrup of the US Department of Agriculture (USDA) consulted a slew of research to develop more than 80 riparian corridor design models to help protect soil, alleviate bank erosion, improve water quality, reduce flooding, and increase biodiversity. These models help planners assess stream corridors and determine the minimum vegetation widths to provide the desired benefits; **Figure 8** illustrates how buffer width helps quantify the effectiveness of trapping pollutants.[11] One model specifically focuses on buffer widths to minimize urban runoff and can help planners assess stream corridors and development projects.[11]

After setting the buffer width, it is important to consider which types of vegetation can be most beneficial. A diverse mix of native plants is recommended for riparian corridors. Streamside plants include "herbaceous plants with fibrous root systems" and woody species.[11] In the Blue River, a variation of native streamside plants could include native grasses, willow trees, and other flood-tolerant woody species including Elm, Oak, and Dogwood.

Future Directions

As stated throughout this paper, riparian buffers can provide many benefits to the stream health of urban watersheds. It is critical to provide the latest and best information to planners, stormwater managers, consultants, developers, policymakers and elected officials. Preliminary results from this study have been provided to the community-of-practice currently updating regional stormwater management planning and design guidance, APWA 5600 (MARC, 2024), but more research is needed.

More work is needed to identify riparian buffer assessment and design models that optimize protection and restoration benefits based on stream and watershed characteristics. Understanding and using models like the Buffer Width Tool, proper vegetation selection, and watershed assessments like Kansas State's watershed model and the

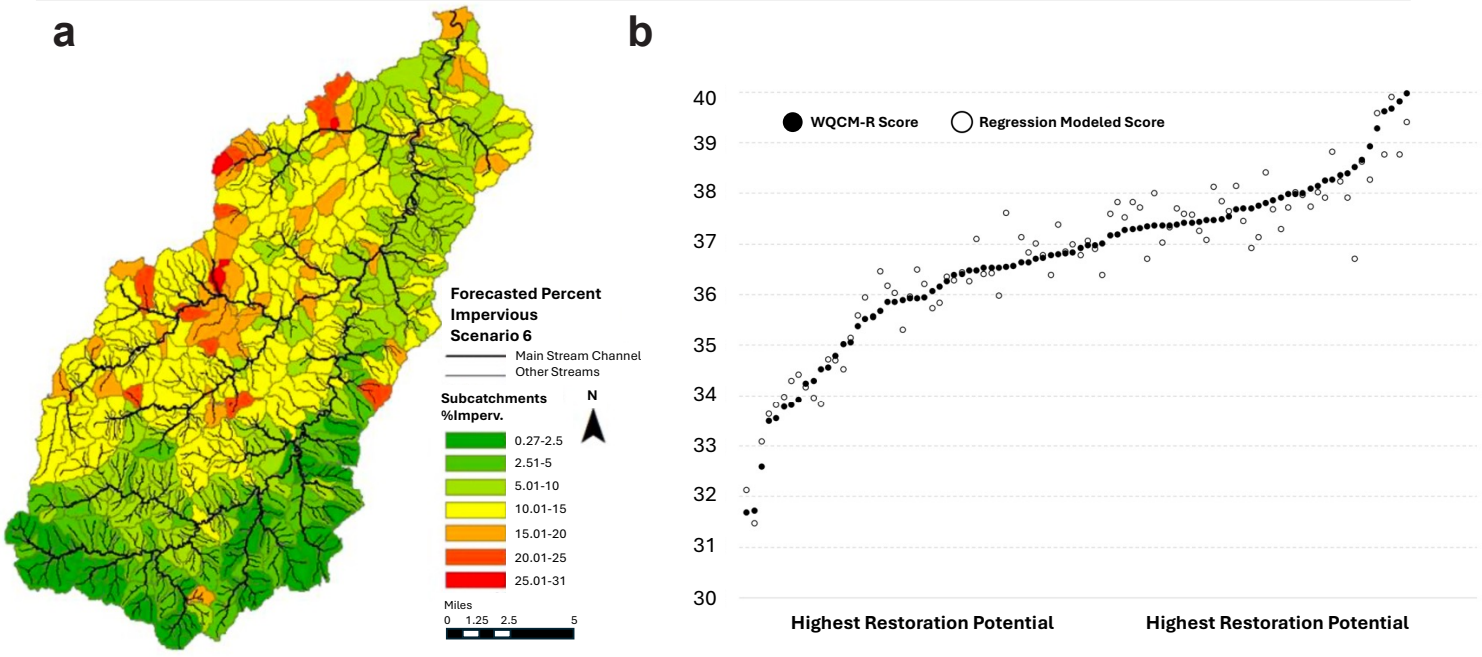

Figure 5 | (a) Forecasted impervious coverage percentages[9] & (b) Restoration potentials[10]

WQCM-R rating systems are crucial in improving riparian corridor spaces and their stream health. Ultimately, the region needs a quantitative model that can better predict and quantify the impacts of riparian corridor protection and restoration to help guide watershed managers, policymakers, elected officials, developers, and conservationists.

Author's Contributions

P.D. contributed to the experimental analysis, design, and writing of this work; S.S. contributed to the design and editing of this work.

Author's Biography

Paige Denning is a recent graduate from the University of Kansas with a Bachelor of Science degree in Environmental Studies. Throughout her final semester, she worked on a capstone project to understand the extent of benefits riparian corridors for stream quality as watersheds urbanize. The research supported updates to regional stormwater management planning and design guidance. Paige is currently pursuing work in water quality testing in the Kansas City region.

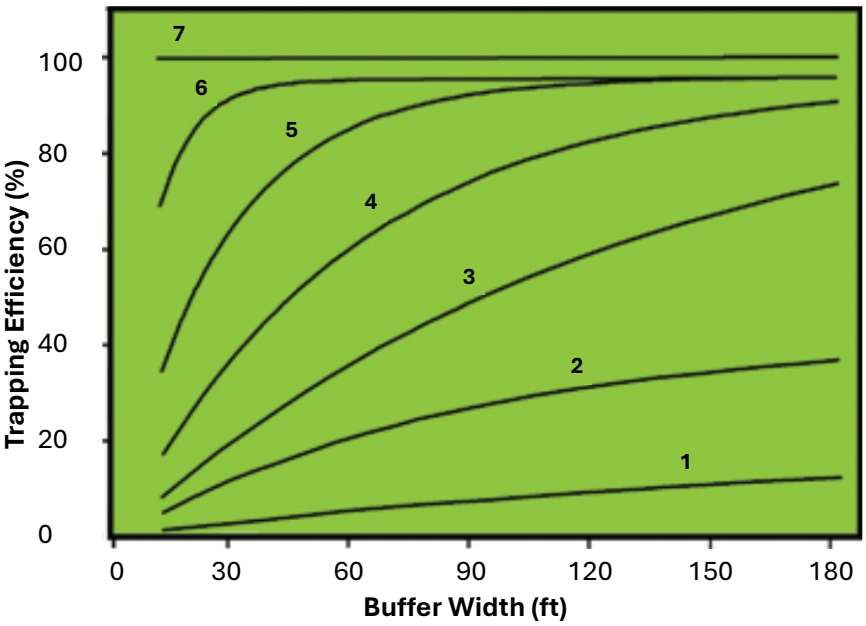

Figure 6 | : Trapping efficiency v. buffer width[11]

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Harnessing Natures Palette: Bioengineering Yeast for Sustainable Textile Dyeing with Betalains

Bridget Ruhme*, R. Logan*, and J.F. Treml*†

The fast-paced digital era has sparked an alarming surge of micro-trends in the fashion industry. Many companies prioritize mass production of textiles to fuel this culture of disposable fashion. This mass production of dyed textiles relies heavily on the use of synthetic azo dyes and, in some processes, the use of heavy metals such as lead, mercury, and chromium VI. Consequently, our planet's landfills are inundated with an overwhelming volume of clothes that can leach toxic or carcinogenic chemicals into the environment. In response, scientists are researching sustainable alternatives that can harness living organisms to produce biosynthetic dyes that have less environmental impact. To contribute to this research effort, I aimed to develop a bioengineered strain of yeast that expresses red pigments called Betalains, a gene derived from beetroots. To achieve this, I cloned a three-enzyme polyprotein (RUBY) comprised of the requisite enzymes to express Betalains from a construct made for plant-based production into a plasmid for yeast expression. The expressed biosynthetic dye was then purified and its fabric-standing properties were characterized. Ultimately, this bioengineered yeast strain can be used to mass produce the red biosynthetic dye, Betalain, which could replace environmentally harmful synthetic dyes. This research project serves as a proof-of-concept study that could lay the foundation for broader adoption of more sustainable and ethically conscious textile dyeing processes for decades to come.

With the increase of fast fashion, people are increasingly aware of the textile industry's environmental impact. This impact includes the discharge of large quantities of chemical pollutants into the environment, such as sulphur, naphthol, vat dyes, nitrates, acetic acid, soaps, enzymes chromium compounds and heavy metals like copper, arsenic, lead, cadmium, mercury, nickel, and cobalt.1It is estimated that textile dyeing and finishing treatments contribute to 20% of the industrial wastewater pollution. Among the toxic chemicals in wastewater from textile producers, 72 substances have been detected, and due to inadequate effluent treatment methods, 30 of these cannot be removed.[1,2] If we look back 168 years before William H. Perkins developed the first synthetic, mass-produced chemical dye, we can see the potential to change the future with eco-friendly dyes derived from microorganisms, fungi, and plants.[3]

Turning to nature, inspiration is found in the wide pallet of color pigments beet plants provide, from red to violet and yellow. This project sought to take advantage of a pigment pathway in beets and clone the required enzymes into yeast cells.

Beetroot stands out as a notable source of betalains, including vivid red betacyanins and yellow betaxanthins. Derived from betalamic acid, betalains are nitrogen-containing compounds exclusive to plants within the Caryophyllales order. These water-soluble chromoalkaloids are primarily located within plant vacuoles, defining the unique coloration of Caryophyllales species. [4–10]

Many genes responsible for the biosynthesis of pigments have already been cloned, and are available, including those found in the UBQ: RUBY plasmid created by Zhang et al. The biosynthesis of betalains can be broken down into three required enzymatic reactions to convert the amino acid tyrosine to betalain. The three betalain biosynthetic genes required for this, Cytochrome P450 76AD1, l-DOPA 4,5-dioxygenase (DODA), and Glucosyltransferase were fused into a single open reading frame called RUBY, which has been expressed using a single promoter and terminator.[11,12]

The goal of this work was to bioengineer a yeast strain that produces betalain as a sustainable pigment for use as a dye. We obtained a version of the pPICZc yeast expression vector, MtPT4, consists of an AXO1 promoter and terminator flanking a multiple cloning site bearing the MtPT4 gene from Addgene.[13] Given the availability of the amino acid tyrosine, it was hypothesized that the RUBY cassette could be cloned into the pPICZc plasmid in place of the MtPT4 gene for expression of these enzymes in yeast to direct the synthesis of the red pigment, betalain.

The use of yeast over plants offers many benefits. Plant have a number of drawbacks including limited availability yearround and sensitivity to environmental conditions. Furthermore, extensive plant use may jeopardize a valuable species .[14] Production of pigments in yeast boasts advantages like easy and rapid growth in cost-effective mediums, simple processing methods, and independence from weather conditions, making them a compelling option for sustainable pigment production.[14]

Natural dyes often offer a limited and dull color range, low colorfastness, and no guarantee of safety over their synthetic counterparts. Their safety depends on factors like the mordant used which allows garments to maintain color. For example, the mordant chromium, is highly toxic and can significantly impact wastewater quality. We found a plant-based mordant alternative using the symplocos plant from Indonesia.

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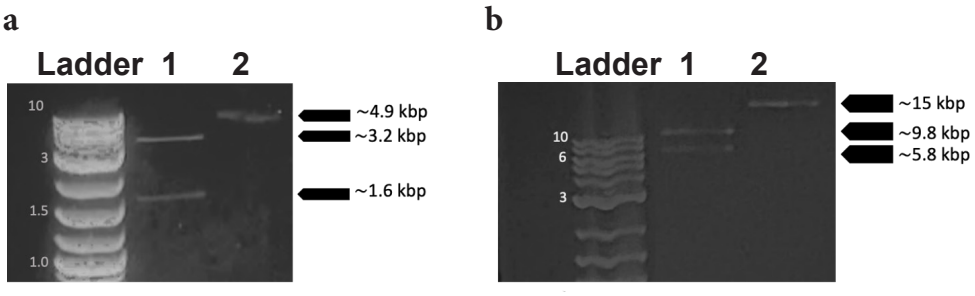

Figure 1 | Gel electrophoresis of restriction digests for plasmid verification of MtPT4 and UBQ:RUBY.
Agarose gel electrophoresis of **(a)** MtPT4 on a 1.5% gel. Lane 1 cut with restriction enzymes EcoRI and XhoI, lane 2 uncut and **(b)** UBQ:RUBY on a 1% gel. Lane 1 cut with XhoI, and HindIII, lane 2 uncut

Fallen leaves from symplocos are abundant in alum content, which is commonly used to treat cellulose fibers like cotton. Many beautiful, vibrant, and permanent colors can be achieved using only alum, a relatively safe mordant. Powdered leaves from symplocos trees offer a sustainable replacement for mined aluminum sulfate and alum acetate used in traditional dye recipes. This innovation not only expands the color palette but also reduces reliance on potentially harmful mordants like chromium, contributing to eco-friendlier dyeing practices. 1,[16–19]

The future of sustainable fashion is upon us, requiring exploration for eco-friendly replacements to synthetic dyes and mined mordants. As an alternative to plant-extracted betalains production of individual betalains by genetically engineered yeast could facilitate the continuous, controlled production of pigment with greatly reduced environmental dependencies and impacts. [11,20]

Materials and Methods

Plasmid growth and verification of MtPT4 and UBQ:RUBY

The UBQ:RUBY plasmid was a gift from Yunde Zhao via Addgene (plasmid # 160909).21 MtPT4 was a gift from Geoffrey Chang via Addgene (plasmid # 117096).13 Both plasmids were grown from separate bacterial stabs: MtPT4 was grown on Lennox-LB plates supplemented with 50 µg/mL Zeocin (ThermoFisher Scienctific, Cat. No. R25001), while UBQ:RUBY was grown on Miller-LB (Fisher Bioreagents Cat. No. BP1425-500) plates supplemented with 50 µg/mL Spectinomycin (ThermoFisher Scientific Cat. No. J66034-03).

Bacterial incubations were overnight at 37°C with shaking at 220 rpm. Single colonies, of each plasmid, were selected from their agar plate for bacterial cultures. The Monarch Miniprep Kit (NEB Cat. No. T1010S) was used to purify DNA from the liquid cultures and all plasmid isolations were verified by restriction digests.

The restriction enzymes XhoI (Cat. No. R0146S), EcoRI (Cat. No. R0101S), and HindIII (Cat. No. R0104S) were obtained from New England Biolabs. Digestion products were analyzed by agarose gel electrophoresis using a 2 and 1.2% agarose (Fisher Bioreagents Lot. No. 188373) stained with ethidium bromide. Gel electrophoresis was conducted at 100 V for 45 minutes in 1x TAE buffer. The resulting DNA fragments were visualized under UV light. The digestion pattern/bands were compared with an expected pattern based on the plasmid map and a 1 kb DNA Ladder (NEB Cat. No. N0550S).

DNA manipulation of RUBY construct from UBQ:RUBY plasmid

The RUBY construct (bases 2,140-6,096) was PCR amplified using a Mastercycler Nexus Gradient from Eppendorf (serial no. 6331ER710937) The PCR reaction mixture consisted of a forward cloning primer, RUBYf, (Integrated DNA Tech Cat. No 472031126), 5'-CC-ATGGATCTGCGAC-CCTCGCCATGATCC-3', reverse cloning primer, RUBYr (Integrated DNA Tech Cat. No. 472031127), 5'-GCGGCCGC-CTAT-CACTGGAGGCTTGGCTCAAG-3', for a 1 µM concentration of each, 1 µL Taq Polymerase (Promega Cat. No. M712B), and 1.5 µL (~10ng) of UBQ:RUBY DNA from the miniprep mentioned previously. The cycling conditions were as followed: initial denature at 95°C for 2 minutes, followed by 30 cycles of denaturing at 95°C for 15 seconds, annealing was at 53°C for 15 seconds, and extension at 72°C for 5 minutes. A final extension step was run at 72°C for 1 minute. The PCR products were analyzed by gel electrophoresis using a 1.5% agarose gel in 1x TAE. The product bands were excised and purified using the Monarch Gel Purification kit (NEB Cat. No. T1020S).

Generation of Betalains

Betalains were extracted from the beet plant by washing and peeling the top layers then mashed in a mortar pestle. 10g of beet peels were subjected to a nonpolar extraction in a beaker with 100 mL of MeOH: H2O (80/20, v/v) as the matrix. The beaker was wrapped in tinfoil and left to incubate in an orbital shaker for 120 minutes at 45°Cshaking at 100 rpm. The liquid was filtered using a Q8 filter paper (Fisher Scientific Cat No.09-790-B). The polar extraction was done by blending 100g of beets with Reverse Osmosis (RO) water to create a homogenous mixture and spilt into four 50 mL conical tubes. All tubes were centrifuged for 30 minutes at 3500 rpm to form a pellet then the supernatant was filtered with Q8 filter paper. Both extraction methods underwent a 2-fold dilution scheme a total of seven times in a 96-well plate before an absorbance spectrum from 350nm to 750nm was ran using a spectrophotometer (Molecular devices SpectraMax M3).

Biphasic Extraction and Crude Purification of Betaxanthins

10g of beet peels were weighed and submerged in a beaker with 50 mL of RO water and 50 mL of chloroform. The solution was covered in tinfoil, mixed, and left to form two layers while incubating in an orbital shaker for 90 minutes at 45°C shaking at 100 rpm. The top organic layer containing betaxanthins was filtered with Q8 filter paper into a 50 mL Erlenmeyer flask for further purification by evaporating the chloroform at 100°C to create a crude product. This was reconstituted in 10 mL of RO water. The betaxanthin's underwent the same dilution scheme and absorbance spectrum as above.

Polar Extraction of Betacycanins

10g of beet peels were weighed and submerged in a beaker with 100 mL of DI water and covered in tinfoil to incubate in an orbital shaker for 90 minutes at 45°C shaking at 100 rpm. After incubation the liquid

was filtered with Q8 filter paper to remove any insoluble materials. The betacycanins underwent the same dilution scheme and absorbance spectrum described above.

Symplocos mordant treatment and beet dye

This method established four groups; fabric 1: untreated fabric, fabric 2: symplocos treated fabric, fabric 3: untreated dyed fabric, fabric 4: dyed symplocos treated fabric. The mordant was made by boiling 22g of symplocos in water for an hour. Fabrics 2&4 were submerged in the pot with the symplocos to simmer for an hour, rung out, rinsed to remove any residual powder, and air dried. The beet dye was generated by boiling chopped, fresh beets for an hour. Beets were removed, leaving a solution of pigments. Fabrics 3&4 were submerged in the beet dye for an hour, then left to dry and rinsed with warm sink water the next day.

Results

Confirmation of Plasmid Identity via restriction digest and gel electrophoresis

To ensure the integrity of our experimental materials, a thorough verification of the identity of the two plasmids was crucial to our bioengineering of the yeast. Utilizing restriction digest and gel electrophoresis techniques, we confirmed the identity of the MtPT4 and UBQ: RUBY plasmids, by generating restriction fragments consistent with the pattern expected for these vectors. MtPT4 was digested with EcoRI and XhoI then electrophoresis of the digest was performed on an agarose gel against the 1kbp+ DNA ladder and imaged under UV light. As expected, two bands in lane 1 were observed at 3.2 kbp and 1.6 kbp on the gel **(Figure 1a)**. UBQ:RUBY was digested with HindIII and XhoI then followed the same visualization steps as MtPT4. As expected, two bands in lane 1 were observed at 9.8 kbp and 5.8 kbp on the gel **(Figure 1b)**. For each digest an uncut sample of the plasmid was run in lane 2 to serve as a control and reference point. These results help confirm that each plasmid of interest is present and suitable for further manipulation.

RUBY cassette amplification via PCR and gel electrophoresis

The UBQ:RUBY plasmid was engineered for betalanin synthesis in plants cells via the enzymes contained in the RUBY cassette, Cytochrome P450 76AD1, l-DOPA 4,5-dioxygenase (DODA), and Glucosyltransferase. We amplified this cassette for cloning into a yeast expression vector (MtPT4) for betalanin production in yeast cells. PCR amplification targeting bases 2140 to 6096 of the RUBY cassette yielded a distinct band of 3965 base pairs on gel electrophoresis, indicative of successful amplification **(Figure 2)**.

Qualitative Analysis of betalains presented in beets using different extraction techniques via spectrophotometer

The qualitative analysis of betalains and its subclasses was performed using extractions using both polar and nonpolar techniques, followed by the measurement of absorbance between 350 and 750 nm at 10 nm intervals on the spectrophotometer. Betalain was first extracted with a nonpolar technique by incubating beet peels in MeOH: H2O (80/20, v/v) at 45°C for 120 minutes while shaking at 100 rpm. Two distinct peaks are observed for the two subclasses of pigment. The higher absorbance peak at 480 nm corresponds to betaxanthin, while the lower peak at 540 nm corresponds with betacyanin **(Figure 3a).** The presence of these two peaks in the spectrum was consistent with expected data confirming a successful extraction of both betacyanin and betaxanthin. The second betalain extraction method used a polar technique by blending beets with water to create a homogenous mixture that was centrifuged at 3350 rpm for 30 minutes then filtered. We expected to visualize peaks on the absorbance spectrum to reflect the differential polarity of these substances compared to the previous non-polar extraction. As predicted the betaxanthin peak at 480 nm was slightly smaller than the betacyanin peak at 540 nm (**Figure 3b**). Overall, these results suggest that both betalain molecules were present in a raw extract from beets. To further investigate the subclasses of pigments in beets, betaxanthin and betacyanin, were differentially purified with two different extraction techniques developed based on chemical structures and their respective polarities. Of the two subclasses, betacyanin is more polar, due to the sugar moiety, making it favorable in a polar extraction method with an expected single peak at 540nm based on data from the second extraction method above. Betacyanin was extracted by incubating beet peels in water at °C for 90 minutes while shaking at 100 rpm. Another absorbance spectrum was run. As expected, one large peak at 540nm was observed, indicating a successful isolation via extraction **(Figure 3c)**. Conversely, a nonpolar organic solvent will favor the extraction of betaxanthin over betacyanin. Betaxanthin was extracted through a biphasic extraction, with equal parts chloroform and water, following the same incubation steps as betacyanin. The organic phase of chloroform, containing betaxanthin, was removed, filtered, and heated to 100°C to evaporate the chloroform, resulting in a crude purification of betaxanthin. This was reconstituted in water and reanalyzed by spectrophotometry. The absorbance spectrum resulted in a large peak at 480nm, as expected based on the data from the first extraction, the slight shoulder observed shows this extraction method didn't result in a pure product **(Figure 3d)**.

Dyeing cotton with Symplocos mordant and beet dye

The treatment of fabric with a mordant, typically a heavy metal, is a crucial step for a garment's colorfastness. Here, the typical heavy metal mordant was replaced with one derived by the symplocos plant from Indonesia. The symplocos plant represents a sustainable source of alum, used as a mordant by grinding the leaves into a fine powder. To treat fabric, a symplocos bath was made by boiling the symplocos powder at 60°C for an hour. Fabric 2 was treated by simmering in the symplocos bath, allowing the alum to etch away at the fabric's fibers exhibiting in a tinted pale-yellow hue. Upon comparison with the untreated fabric

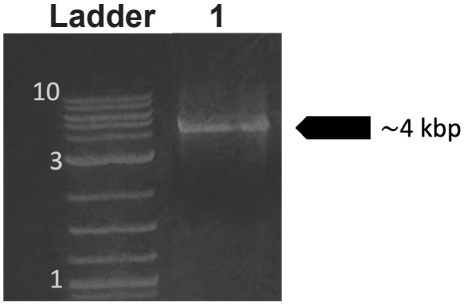

Figure 2 | The RUBY fragment was amplified properly through PCR. Agarose gel electrophoresis of RUBY PCR fragment amplified from UBQ:RUBY plasmid using Ruby forward and reverse primers. Electrophoresis. was performed on a 1.5% gel. A 1 kb plus DNA ladder was used. The single band at 4 kb confirms the RUBY cassette was amplified during PCR.

1 we can confirm the symplocos mordant treatment was successful **(Figure 4a)**. The beet dye was made by boiling chopped beets for an hour then removing it from the heat. Fabric 4 was treated with the mordant as described for Fabric 2 above. While fabric 4 dried, fabric 3 was submerged in the beet dye bath for an hour and rinsed with warm water until the water ran clear then air dried. After fabric 4 was dry it underwent the same dyeing steps as fabric 3. A comparison of the fabric samples revealed that fabric 4 showed superior retention of red pigment, observed by the brighter hue compared to the salmon color of fabric 3 **(Figure 4b)**. This was also evident by the shorter rinse time until the water ran clear. The enhanced colorfastness in fabric 4 can be attributed to the formation of a coordination complex between fabrics fibers and the beet dye facilitated by the alum treatment in the symplocos mordant. These results suggest the symplocos plant-based could serve as a good replacement for conventional heavy-metal dyes.

Discussion

Beets are known to produce betalain, which serves as basis for the RUBY cassette, utilized in this experiment. To establish a refence for my bioengineered yeast dye, the spectral properties of dye extracted from beets was characterized. Whole beets were obtained from a local grocery store to be washed, peeled, chopped, and blended. The two expected betalain pigment types were successfully extracted with absorbance maxima for betacyanin at 540 nm and betaxanthin at 480 nm.[4,5,8,9,20]

We examined manipulating betalain production yields to obtain different color pigments from a single source. Through analysis of the chemical structures of betaxanthin and betacyanin, two techniques were created to develop to standardize each specific class. The polar extraction for betacyanin was successful, while the biphasic extraction for betaxanthin needs to be further investigated to create a pure product, potentially with a second round of extraction.

Continuing the analysis, we investigated whether the pigment was

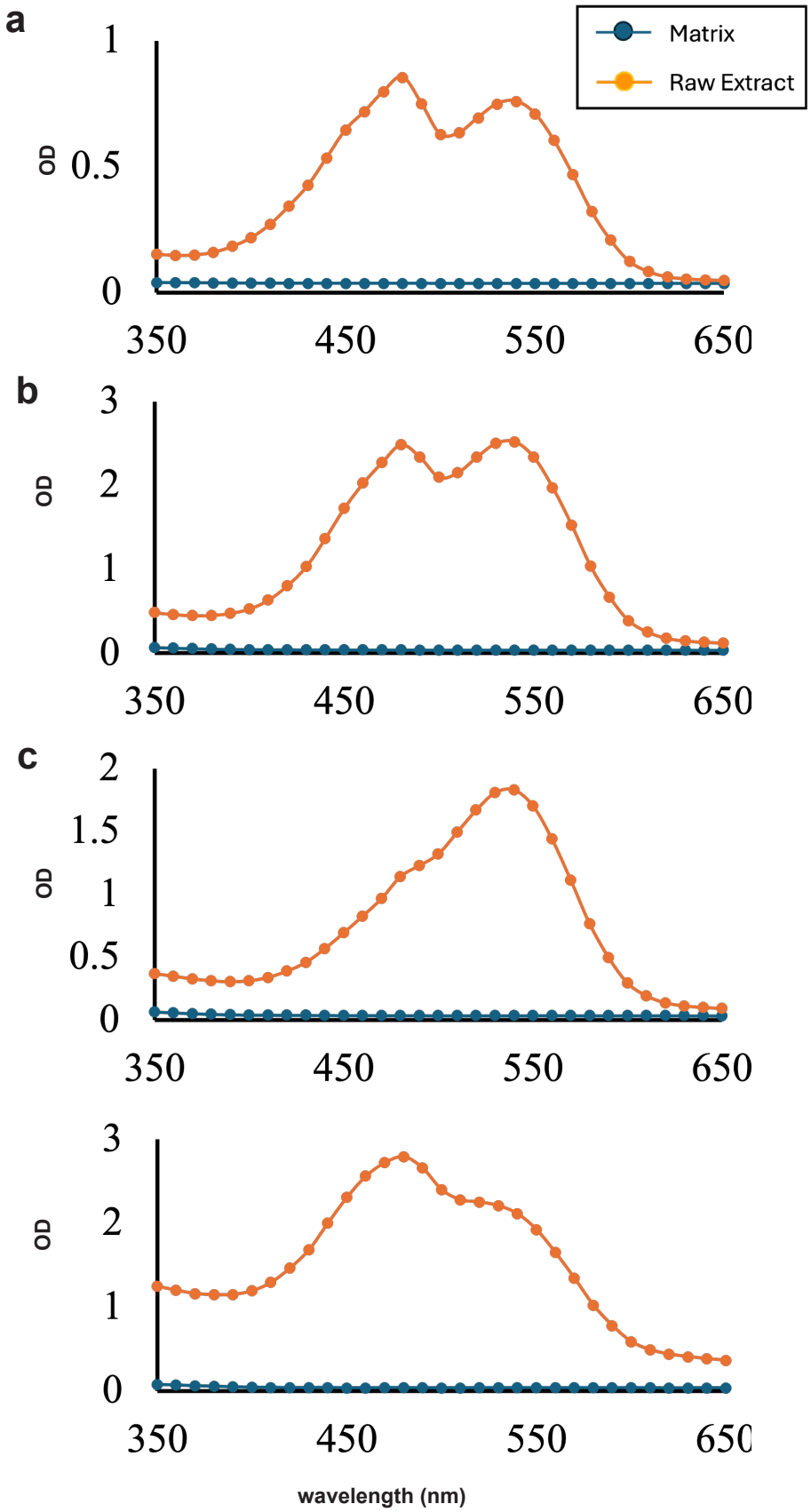

Figure 3 | Qualitative Analysis of betalains present in beets using different extraction techniques via spectrophotometer. 150 µL of each pigment classes, extracted from beet peels, underwent a 2-fold dilution scheme six times in a 96-well plate using the proper matrix. An absorbance spectrum from 350nm until 750nm was ran on a spectrophotometer, picking the best dilution factor (DF) to represent the data (a) Nonpolar extraction; 1:2 dil. of betalains extracted with a nonpolar matrix of MeOH: H2O (80/20, v/v) from peeled beets (b) Polar extraction; 1:8 dil. of betalains extracted with a polar matrix of di-water from blended beets (c) 1:8 dil. of betacyanins extracted with a polar matrix of di-water from peeled beets (d) 1:16 dil. of betaxanthins extracted with chlorofom during a bipahsic extraction then purfied and reconstiituted in water.

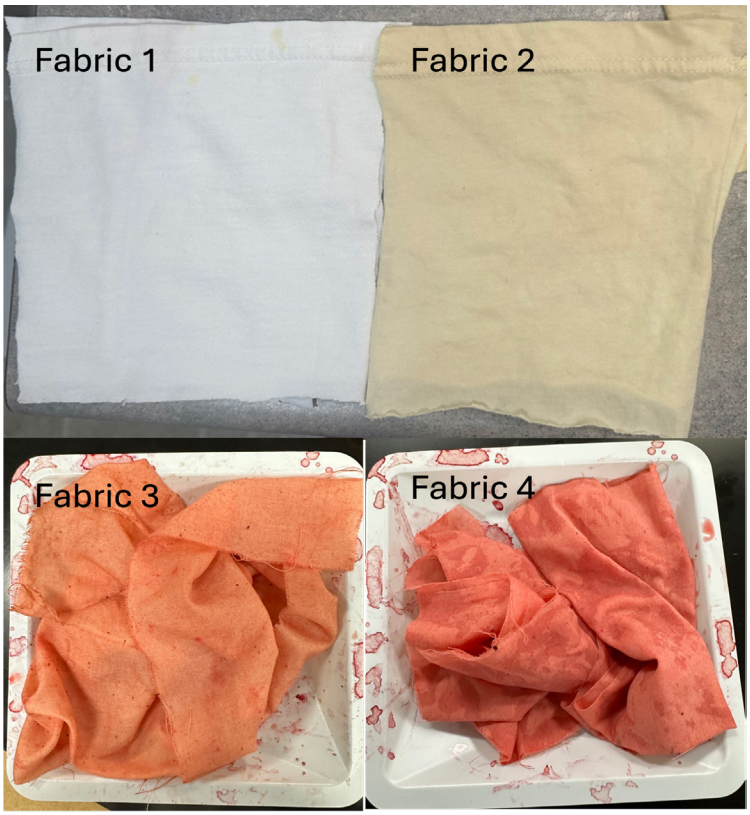

Figure 4 |Dyeing cotton with symplocos mordant and beet dye.
Comparison of 22 grams of fabric treated with or without symplocos then dyed. Untreated samples on the left, treated on the right (a) Undyed comparison to test symplocos mordant; fabric 1 untreated, fabric 2 was treated for an hour in the symplocos mordant bath (b) comparison of dyed fabric that had been treated against fabric that hasn't, both fabric samples were dyed in the beet bath for 1 hour; fabric 3 untreated and dyed appears more salmon and dull, fabric 4 treated with symplocos and dyed appears brighter red

pH-dependent by exposing the liquid from polar and nonpolar extractions to various pH buffers ranging from 1 to 12. Due to the stability of betalain within the pH range 3 to 7 [4,8,9,22], the only observed color change occurred at pH of 12. Suggesting the pigment is pH-dependent where pH 12 marked the transition from red and purple to orange to yellow (data not shown).

The plant-based mordant, symplocos, was obtained as dried leaf powder from the forests of Indonesia. The yellow-tinted fabric and enhanced colorfastness observed indicates symplocos offers an alternative for a commercially available alum mordant. By using the fallen leaves, we supported a sustainable crop rather than industrial mining.[23]

Overall, this work serves as progress toward a proof of concept study with numerous potential applications.

Future Directions

Future work will continue to develop the reagents and processes required for the production of pigment dyes in yeast. We expect that once the RUBY cassette is under a yeast promoter and terminator, yeast cells will exhibit a red phenotype, confirming a successful transformation. Liquid cultures of the bioengineered yeast cells will be assessed for pigment expression followed by the development of a purification scheme for one or both betalains. Betalain extraction from the supernatant or cell portion of harvested cultures using various extraction methods mentioned will confirm their presence, which will then be to be further divided into betacyanin and betaxanthin. To assess the purity of the red dye derived from betalains chemical fingerprinting must be conducted by HPLC analysis against a known standard.

The observed color shift at pH 12 presents promising options for further exploration and warrants further investigation which would extend the potential for these pigments. Once the purity of the dye is confirmed it will be tested in different applications with various textiles, evaluating the colorfastness of these dyes in combination with symplocos or other plant-derived other plant-based mordants.

Overall, these future steps aim to advance of the capacity for betalain-based dye production in a sustainable, scalable manner and explores its potential application in textile dyeing.

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Author Biography

In May 2024, Bridget Ruhme graduated with a Bachelor of Applied Science in Biotechnology from The University of Kansas. Bridget plans to work in the biotech industry during which time she will seek research residencies within the new sustainable design practices from bio-mediums to expand her capstone work.

Author Contributions

B.R.R. contributed to the design, experimental, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

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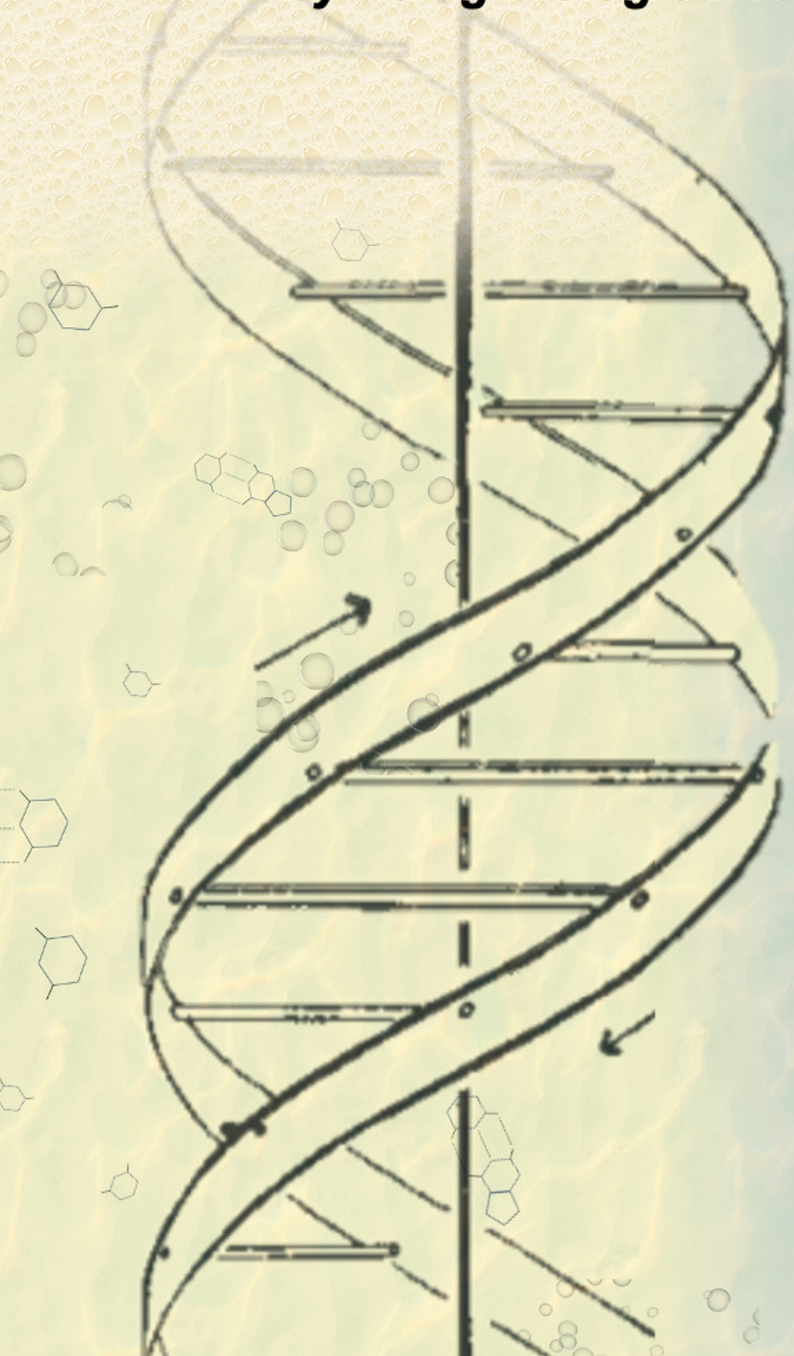

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