

The Host and Microbial Determinants of Activity by Commensal *Clostridium immunis*

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy in the  
Department of Molecular Genetics and Microbiology in the  
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ABSTRACT

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## Abstract

The microbiota—the diverse collection of commensal microorganisms that normally colonizes mucosal and skin surfaces—plays a fundamental role in controlling various biological processes, including metabolism, immune responses and behavior. Although there have been increasing successes in identifying commensal bacteria that causally impact different host functions, the specific mechanisms by which these bacteria work remain poorly understood. Using *Clostridium immunis*—an immunomodulatory human gut commensal bacterium— we dissect the host and bacterial mechanisms of its activity. Through this, we unravel an intriguing and potentially generalizable strategy by which certain commensal bacteria uniquely regulate certain immune cells. In Chapters 3 and 4, we define the host cellular and molecular requirements for immunomodulation by *C. immunis*. In Chapter 3, we use transcriptomic analysis of *C. immunis* treated mice to reveal that *C. immunis* negatively regulates a lipid metabolism pathway driven by group 3 innate lymphoid cell (ILC3) activity. Using mice genetically deficient in ILC3s, we demonstrate using two distinct animal models—lipid metabolism and adiposity, as well as colonic inflammation, that group 3 innate lymphoid cell (ILC3) function is regulated by *C. immunis*. In Chapter 4, we further show that *C. immunis* regulates adiposity and colitis in a context- and ILC3 effector-dependent manner. In Chapters 5 and 6, we delineate the bacterial mechanisms

of activity. In Chapter 5, using comparative genomics and biochemical fractionation, we identify a *C. immunis*-derived exopolysaccharide (EPS) that is sufficient to recapitulate its ILC3-regulating activity. In Chapter 6, we demonstrate that a phosphocholine moiety on the EPS is associated with bioactivity. To attribute a causative role for phosphocholine on EPS, we performed targeted reverse genetics in *C. immunis* to disrupt its phosphocholine processing locus. Through this, we show that phosphocholine-modification of the EPS is essential for activity. Considered together, we identify a commensal bacterial species, its biochemical product, and a molecular determinant thereof that regulate ILC3 function in vivo. Broadly, our findings suggest that the specific ability of certain commensal bacteria to modulate host immunity is dependent on small-molecule modifications of classical microbial products.

## **Dedication**

To the pursuit of knowledge, and the friendships built during.

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# 1. Introduction

Exactly sixty years ago, in an impassioned speech about the future of mankind (1), Hilary Koprowski (who developed the first oral Polio vaccine) commented, “There is no greater nightmare to dream about the future than the creation of a germ-free man”. This is a prescient warning about the perils of humans existing without their microbial symbionts. We now know that the microbiota—the vast collection of commensal microbes that inhabit the human skin, lung, genitourinary and gastrointestinal tract—is, as Dr. Koprowski asserted, critical for many facets of health. Commensal microbes are responsible for a seemingly innumerable list of physiological functions, such as colonization resistance, metabolism, behavior, carcinogenesis, and regulating various functions of the immune system (2-7). Furthermore, the microbiota is dynamic; it varies with environmental stimuli like the circadian rhythm, diet and drugs (8-10). Understanding how the microbiota functions is therefore inexorably linked to understanding the active processes that underpin health and disease states.

## ***1.1 The microbiota and host immune system***

Perhaps the most well-studied microbiota-dependent host function is that of the intestinal immune system. It has been observed since the mid-1960s that germ-free animals have stigmata of an immature intestinal immune system, characterized by hypoplastic mesenteric lymph nodes and Peyer’s patches, and deficient antibody

production (11-15). As a result of their aberrant immunological state, germ-free animals have vastly different responses in models of various autoimmune and allergic diseases (16-18). It is now clear that the ontogeny, education and regulation of several major immune cell types are dependent on the microbiota (19, 20). Correspondingly, an appropriately educated immune system can respond adequately to infectious and neoplastic threats while tolerating autoimmune and allergic insults (21-24).

### ***1.1.1 Microbiota dependent regulation of immune cells***

The genesis of the immune system from bone marrow precursors is largely controlled by genetically determined differentiation programs and bone marrow niche factors (25), but certain immune cell subsets also require the microbiota for their normal development. A well-studied example is the ontogeny and regulation of CD4<sup>+</sup> T subsets cells by commensal microbes. Together with CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells make up the adaptive immune system. CD4<sup>+</sup> T cell subsets include T helper (Th)1, Th2, Th17 cells that require different lineage-defining transcription factors (Th1–Tbet; Th2–GATA3; Th17–ROR $\gamma$ t) for their development. Correspondingly, these different Th cell subtypes produce different effector cytokines and provide protection against a variety of intracellular and extracellular pathogens (26). To maintain immune homeostasis, their activities are negatively regulated by CD4<sup>+</sup> regulatory T cells (Tregs) that require the lineage-defining transcription factor FOXP3. In the colon, the activity of Th cells is

counterbalanced by the microbiota-dependent induction of a unique subset of FOXP3<sup>+</sup> RORγt<sup>+</sup> Helios<sup>-</sup> Tregs. This, in turn, is crucial for ensuring effective clearance of infections, while simultaneously restraining autoimmunity (27-30). Interestingly, although this Treg deficit in germ-free mice can be corrected by reconstituting mice with large consortia of select commensal taxa (31, 32), low-diversity microbial mixtures and even some individual species are also sufficiently effective, a finding that further highlights the profound immunological deficit in germ-free mice (27, 33-35). However, there exists some measure of specificity between commensal taxa and specific immune cell subsets. For example, segmented filamentous bacteria (SFB) is a poor inducer of Tregs but robustly induces the differentiation of Th17 cells in the intestine (30, 36). As a result of this unregulated Th17 response, SFB-colonized germ-free mice can rapidly clear colonic *Citrobacter rodentium* infection (36), but also suffer worse Th17-dependent autoimmune arthritis (37). Several other commensal bacteria that also increase Th17 cell frequency in the intestine have been identified, with some observed to worsen inflammatory disease (38-40). Clearly, significant progress has been made in identifying defined taxa that regulate certain immune cell subsets, particularly those of the adaptive immune system.

Cells of the innate immune system also require the microbiota for their normal function. For example, germ-free mice have significantly decreased bone marrow myeloid cell numbers (41-43), and as a result are unable to clear and survive systemic

Listeria infection (44). Additionally, the development and function of intraepithelial lymphocytes (IELs) are microbiota dependent. IELs are abundant cells that intercalate within the intestinal epithelial layer to sense microbial and dietary antigens. IELs expressing the  $\alpha\beta$  T cell receptor (TCR) originate in the thymus and migrate to the small intestine early in life, where their expansion relies on signals from the microbiota (45, 46). By comparison,  $\gamma\delta$ TCR-expressing IELs are comparable in abundance between germ-free and conventionally raised mice. Instead, the microbiota regulates their spatial distribution in the intestine and is critical for their ability to restrict mucosal injury (47-49). However, not much is known about specific microbial taxa that regulate IELs, though more recent work has identified *Bacteroidetes* and *Lactobacillus* species capable of inducing CD8 $\alpha\alpha^+$  and CD4 $^+$  IEL differentiation, respectively (50, 51).

Innate lymphoid cells (ILCs) are critical innate immune cells that are enriched in mucosal tissues whose development and/or function are also microbiota dependent (52-55). Being the innate counterparts to Th cells, group 1 ILCs (ILC1s), ILC2s and ILC3s respectively require the transcription factors Tbet, GATA3 and ROR $\gamma$ t for their development, and similarly mediate protective defenses against a variety of pathogens (56). ILCs and their Th counterparts cross-regulate and complement each other, and therefore in some sense perform redundant biological functions (57-60). However, because of their predominantly tissue-resident disposition within mucosal tissues, ILCs can respond rapidly to stimuli at these sites, which include signals from the microbiota.

For example, stomach ILC2 numbers are deficient in germ-free mice, restored when these mice are reconstituted with stomach microbiota, and produce immunoglobulin A that coats and ultimately clears pathogenic *Helicobacter pylori* (61). Like IELs, there are extremely few examples of specific commensal microbes that regulate the function of ILCs. Certain murine Clostridia species can induce IL-22 production by intestinal ILC3s and T cells that ultimately lead to protection against food allergen sensitization (21). Additionally, two murine non-gastric *Helicobacter* species decrease ILC3 proliferation (62), although it is less clear if this leads to changes in biological function and/or disease. Given the importance of ILCs in orchestrating homeostasis in mucosal tissue, it is imperative to understand how/if specific microbes regulate ILCs.

Ultimately, with continued efforts to identify immunomodulatory commensal microbes, one can begin to synthesize a comprehensive picture of how the microbiota regulates various aspects of immunity. Moreover, the immune system is increasingly appreciated to play roles that extend beyond the canonical functions of clearing infections and tumors (63-65). There is therefore growing interest in understanding microbiota-immune interactions as they pertain to metabolic disorders, behavioral abnormalities, and neurodegenerative processes (66-69).

### **1.1.2 Microbial mechanisms of immunomodulation**

In response to Koprowski's comments on the indispensability of the microbiota (Chapter 1), molecular biologist and Nobel laureate Joshua Lederberg thoughtfully wondered if certain microbial products may suffice in supplanting the microbiota, saying "... you could just take a shot of a mixed load of antigens every few weeks to keep yourself pepped up properly..." (1). We are still far from Dr. Lederberg's ideal of having a comprehensive "cocktail" that recapitulates the myriad functions of the microbiota. This is perhaps primarily due to the vast diversity of microbiota-derived molecules, and secondarily due the dearth of tools—partly mitigated by advances in metabolomics—to identify bioactive molecules at scale (70). A complete catalog of bioactive microbiota-derived products is therefore still in its infancy. Nonetheless, there are a few well characterized categories of microbe-derived products that regulate the immune system, such as bile acids, indole derivatives, short chain fatty acids and other microbial products/metabolites.

Host-synthesized primary bile acids are released into the small intestine during meals. The small amount (~5%) that is not reabsorbed is transformed by the colonic microbiota into secondary bile acids. This process first requires microbially-encoded bile salt hydrolase (BSH) activity to remove host-conjugated taurine or glycine, facilitating subsequent reactions such as oxidation by  $7\alpha$ -hydroxysteroid dehydrogenase ( $7\alpha$ -HSDH) (71). Virtually all commensal bacteria encode BSH, but only select taxa encode

specific enzymatic functions like 7 $\alpha$ -HSDH (72-76). While secondary bile acids have been implicated in numerous host processes (77, 78), their role in regulating immune function is only now beginning to unravel. For example, secondary bile acids produced through BSH and 7 $\alpha$ -HSDH activity by *Bacteroides* species induce FOXP3<sup>+</sup> ROR $\gamma$ t<sup>+</sup> Treg cells (79). This mixture of bile acids induce Treg cells via either the vitamin D receptor or farnesoid X receptor (FXR) and consequently ameliorates colitis (79). Consistent with these findings, a specific secondary bile acid, isoallothocholic acid, induces colonic Treg differentiation by enhancing *Foxp3* expression. However this effect is dependent neither on the vitamin D receptor nor FXR (80). In contrast, a closely related bile acid, 3-oxolithocholic acid, does not induce colonic Treg differentiation but potently suppresses Th17 cell differentiation by directly binding to its lineage-defining transcription factor, ROR $\gamma$ t<sup>+</sup> (80). Together, these findings provide early insights into the complexity underlying bile acid-mediated regulation of immune cells. However, it is probable that there exist hitherto unknown immunomodulatory bile acid compounds of non-*Bacteroides* provenance that warrant further exploration.

Compared to bile acids, indole and its derivatives represent a much better characterized group of microbial metabolites that regulate the immune system. Even before the molecular biology epoch, *Escherichia coli* and *Vibrio cholerae* were known to produce indole (81). Subsequently, indole was shown to ameliorate inflammation in cells, and that its formation from tryptophan is dependent on bacterial *TnaA* (82, 83).

More recently, a number of other bacterial genes required for indole derivative formation have been identified (84, 85). For example, the *Peptostreptococcus*-encoded *fldAIBC* gene cluster mediates the production of indoleacrylic acid, which suppresses inflammatory cytokine production in human cells (86). A different bacterial enzyme, aromatic aminotransferase (*ArAT*), is encoded by certain *Lactobacillus* species to produce indole-3-aldehyde, which possesses the ability to stimulate IL-22 production in the gut to facilitate immunological homeostasis (87). Additionally, indole-3-lactate produced by *L. reuteri* *ArAT* is sufficient to induce CD8 $\alpha$ <sup>+</sup> IELs in the intestine (51). Although diverse microbial enzymes mediate the production of multifarious indole derivatives, they are ultimately sensed by the host pregnane X receptor (PXR) or aryl hydrocarbon receptor (AhR) (88, 89). Specifically, AhR is expressed in a variety of immune cells and is required for the function of Th17 cells and group 3 ILCs (ILC3s) (59, 87, 90-93).

Therefore, indole derivative-mediated control of AhR activity is a critical strategy through which the microbiota regulates the immune system. More work is required to understand the unique and redundant contributions of the various microbe-derived indoles on AhR as they relate to immune function. In the same vein, given that diet-derived indoles and certain xenobiotic chemicals also act on AhR; it will be important to dissect their relative contributions towards AhR-mediated immune regulation (94, 95).

Short chain fatty acids (SCFAs) are metabolic end products from the breakdown of dietary fiber by the microbiota. The most abundant SCFAs in humans and mice

comprise acetate, propionate and butyrate that individually have distinct roles in regulating physiology (96). SCFA derived from the microbiota is formed from a variety of biochemical pathways, such as the Wood-Ljungdahl, propanediol, and succinate pathways (97); the mode of SCFA formation from any of these pathways varies according to bacterial taxonomy (98). Crucially, the role of SCFAs in immune-mediated disease is well-studied. SCFAs are known inhibitors of host histone deacetylase (HDAC) as well as ligands of certain G protein coupled receptors, such as GPR41, GPR43 (FFAR2) and GPR109 (HCAR). Through activity at these host proteins, SCFAs profoundly alter immune function. For example, acetate protects mice against inflammatory and autoimmune disease in a GPR43-dependent manner (99), and also, via increasing FOXP3 expression in Tregs, suppresses allergic airway disease (100). The latter effect is likely due to the ability of acetate to increase FOXP3 promoter acetylation via HDAC9 inhibition. Furthermore, SCFAs have been reported to regulate the number and function of colonic Tregs, although there is some disagreement about whether SCFAs are sufficient and if so, which SCFA matters (30, 101, 102). Beyond Tregs, SCFAs—specifically acetate and propionate—also regulate dendritic cell development, an effect that requires their uptake by SLC5A8 and their inhibition of HDACs (103). Butyrate is an important energy source for colonic epithelial cells but is also sensed by GPR109 (HCAR) on dendritic cells and macrophages, that in turn restrain inflammatory immune responses (104, 105). GPR109 (HCAR) is also a receptor for niacin (Vitamin B3)

and the ketone body  $\beta$ -hydroxybutyrate (106, 107), and intriguingly, these three compounds are also HDAC inhibitors (108-110). Although this leads to potential redundancy in immune regulation, an interesting possibility is that immune cells can integrate microbial (butyrate), dietary (niacin) and metabolic ( $\beta$ -hydroxybutyrate) via GPR109 and HDAC, as the bioavailability of these compounds likely varies across anatomical regions and time. Considered together, acetate, propionate and butyrate are responsible for multiple effects on the immune system. Given that many commensal bacteria make these 3 SCFAs, it remains largely unclear if the provenance of an SCFA matters (for the microbe, and to the immune cell).

Lastly, there exist relatively few examples of immunomodulatory commensal-derived products that do not belong to the above three categories (bile acids, indole derivatives and SCFAs). Perhaps one of the first and most well-known example is that of *Bacteroides fragilis* polysaccharide A, a zwitterionic polysaccharide that regulates colonic Treg cell development and function, corrects Th cell imbalances in germ-free mice, and suppresses proinflammatory IL-17 responses in the intestine (33, 111, 112). *B. fragilis* also produces sphingolipids that restrict invariant natural killer T cell (iNKT) development in early life which protects against iNKT-mediated colitis in adulthood (113, 114).

Immunomodulatory lipids have also been identified in other commensal bacteria, such as a phospholipid from *Akkermansia muciniphila* that regulates TLR2 responses (115).

These examples are just the tip of the iceberg; “classical” microbe-associated molecular

patterns (116), comprising cell wall components (lipids, polysaccharides, proteins), flagellin and nucleic acids from commensal bacteria likely possess yet-to-be-discovered immunomodulatory activity.

## **1.2 Microbes as therapy for immune-mediated disease**

Fecal microbiota transplant (FMT) has been the historical archetype for microbial therapeutics. However, the lack of a real understanding of how FMT works, coupled with safety concerns (117), has led to a surge in interest towards defined microbial taxa as therapy. Yet, even with a biologically active probiotic, there exist several hurdles between preclinical efficacy and translation to treat human disease.

In the words of statistician George Box, “All models are wrong, but some are useful”, an adage that compels thoughtful selection of animal and disease models that best capture human biology. This process is even more pertinent in microbiota research, where the variations in microbial community composition across humans is undoubtedly larger than that in laboratory animals. A possible solution is to use more “representative” microbiotas, such as those of wild mice (118). Alternatively, mechanistic studies can be first pursued in animal models for which useful tools exist, revealing biology that helps guide clinical trial design. For instance, early mechanistic studies of T cell exhaustion and immune checkpoints were made in mice (119, 120), generating insights that led to the creation of not just checkpoint blockade

immunotherapy, but also a biomarker to stratify patient populations on the basis of PD-1 expression (121). Along similar lines, by dissecting the host and microbial mechanisms at play, one can identify human diseases and patient populations in which a probiotic has a higher pre-test probability of achieving clinical trial endpoints.

Moreover, it is important to understand the microbial mechanisms of action. Identification of the bioactive microbial product offers several advantages over treatment with the whole organism. Firstly, this obviates concerns commonly associated with the safety of using live probiotics, especially in immunocompromised populations (122, 123). Secondly, having a biochemical product in hand is crucial for structure-activity relationship studies, an important step to improve the efficacy and safety profile of the therapeutic. Lastly, a purified bioactive product is essentially a single ingredient compared to the thousands present on the whole bacterium. This mitigates unforeseen effects from other bacterial components if one were to give a probiotic, and can reduce concerns about dose escalation during early clinical trial phases. Collectively, understanding the host and microbial mechanisms that drive the activity of a probiotic is not just interesting from a basic science perspective, it is also extremely helpful, if not critical, for making the probiotic safe and efficacious.

### **1.3 Unresolved questions in microbiota-immune interactions**

Some of the seminal discoveries in host-microbiota interactions were made in the early 2000s using mono-colonized animals. These include the first description of Th17-inducing bacteria, the discovery of microbicidal angiogenins, and the survey of the bacterial determinants required for colonization (5, 36, 124). These reductionistic studies led to the revelation of new biology, and there is still a clear role for performing mechanistic studies of individual microbe-host interactions. However, over time, a role of the “terrain”, which comprises the host and other microbes (125), has been increasingly considered in conceptual and experimental design, but is still in its nascency. This progression somewhat mirrors the evolution of microbial pathogenesis through the 1990s and is perhaps best described by concepts within the damage response framework in 2003 (126). Ultimately, for biologically meaningful insights to be gained, immunomodulatory commensal bacteria identified through reductionistic approaches must be studied under experimental conditions that more closely model human lifestyles, which include variations in diet, microbiota, immune status and disease states. Therefore, to date, how commensal microbes regulate the host immune system under different biological contexts is mostly unknown, with some exception. For example, in recently described work, *Helicobacter* species differentially induce Tregs and follicular helper T cells, or inflammatory Th17 cells depending on the immune context (127, 128).

An enduring problem for most biological systems to solve is to generate order from complexity. This challenge similarly applies to microbiota-immune interactions. In a complex and diverse microbiota, there exist functionally redundant compounds within the “soup” of microbial products and metabolites in the body (129, 130). This presents a fundamental challenge as to how the host immune system senses and responds to specific bacteria, for which there exist a few possible solutions. A poor solution would be to encode one host receptor for each microbial product, a highly inefficient approach that would almost definitely impose a fitness penalty on the host. An improved solution is to use receptor combinations that, in a combination-dependent manner, can distinguish between different molecular patterns. This has been demonstrated where different TLR2 heterodimers vary in their affinity for different microbial products (131). Alternatively, specificity could be achieved efficiently by encoding receptors with different affinities for a common microbial product, and expressing them in a cell- or subcellular location-specific manner. Indeed, an example of this is seen with SCFAs, which alter HDAC activity at higher concentrations than those required for GPCR-dependent responses (132, 133). Given the fast clearance kinetics of SCFAs and evidence for their SLC5A8-dependent uptake in effectively inhibiting HDACs, only bacteria that produce large amounts of SCFAs could, in theory, regulate HDAC-dependent immunological functions. This provides a source of specificity encoded by subcellular localization-specific expression of different host receptors. Another interesting example

of specificity encoded by localization is that of mucus secretion in the colon; varying concentrations of microbial TLR ligands that differ relative to their penetration in the mucus layer leads to altered MUC2 secretion by sentinel goblet cells (134). Specificity can also be achieved by qualitative differences in microbial products that act via the same host receptor. Although LPS acts on TLR4, LPS from commensal *E. coli* is potentially immunostimulatory whereas LPS from *Bacteroides dorei* inhibits innate immune signaling at the same molar concentration (135). This finding suggests that the microbial community composition at a given juncture may contribute to the overall immunological tone. Yet, since *B. dorei* and *E. coli* LPS structures are vastly different (135), it remains unclear what actually determines their opposing activities on host TLR4. Smaller modifications to microbial products that drive their immunostimulatory/regulatory effects have also been documented. *B. fragilis* polysaccharide A requires a small lipid anchor for its immunomodulatory activity, though the bacterial genetic determinant of this specificity is less clear (136). A more recent report provides a molecular basis for how commensal-derived flagellins weakly stimulate TLR5 (relative to pathogens that typically induce a binary “all-or-none” response) (137). Flagellins from some pathogens contain a domain capable of allosteric activation of TLR5, leading to strong stimulation; other pathogens have mutated flagellins that are incapable of being bound by TLR5. Commensal-derived flagellin can still bind to TLR5 but lacked this allosteric activation domain, a finding that provides a

molecular explanation for how the host distinguishes between symbiont and pathogen. While these are the few examples of how commensal-immune interactions can achieve “order” from complexity, there likely exist other strategies which have yet to be uncovered.

#### **1.4 *Clostridium immunis* as a tool to study microbiota-immune interactions**

In previous work, Neil Surana pioneered microbe-phenotype triangulation to identify the family Lachnospiraceae from the human microbiota that is associated with protection against colitis (138). Bacteria belonging to Lachnospiraceae have been positively associated with health states and are depleted in inflammatory bowel disease but with unknown causal relationships (139, 140). Directed culture of Lachnospiraceae led to the identification of *Clostridium immunis*, a new bacterial species that was sufficient to protect not just germ-free, but also normal microbiota-harboring mice, against colitis (138). This provided us with an uncommon opportunity to gain mechanistic insights into how *Clostridium* species, and more broadly Lachnospiraceae, modulate the immune system. This was accomplished by reductionistic investigation of the host and bacterial mechanisms of activity by *C. immunis*. Work presented in the subsequent Chapters therefore reveal insights that not just help advance *C. immunis* as a therapeutic for certain diseases, but also provide some hints to how order can arise amidst complex microbiota-immune interactions.

## 2. Materials and Methods

### 2.1 Mice

*Ahr<sup>fl/fl</sup>* (stock #006203) (141), *Rorc-cre* (stock #022791) (142), and *Rag1<sup>-/-</sup>* mice (stock #002216) (143) were obtained from The Jackson Laboratory. *Csf2rb<sup>-/-</sup>* mice were obtained from Mari Shinohara (Duke University) (144). Gnotobiotic Swiss Webster MMB mice were obtained from Dennis Kasper (Harvard University) (145); these mice were co-housed with germ-free C57BL/6 mice to generate C57BL/6 MMB mice. All animals were bred and maintained in the animal facility at Duke University, and the gnotobiotic mice were bred and maintained in vinyl isolators in the Duke Gnotobiotic Core. Experimental manipulation of gnotobiotic mice was performed in autoclaved, individually ventilated cages in which animals received autoclaved food (LabDiet 5K67) and water. Mice used in experiments were sex- and age-matched and drawn randomly from the same litter, when feasible. All procedures were approved by Duke's Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines.

### 2.2 Bacteria

*C. immunis* was obtained from Dennis Kasper (Harvard University) (138). *C. immunis*, *C. symbiosum* (DSM 29356), and *C. clostridioforme* (DSM 933) were grown in peptone yeast glucose (PYG) broth (Anaerobe Systems) or on brain heart infusion-

supplemented (BHI-S) agar plates (ATCC Medium 1293). All clostridial strains were grown in an anaerobic chamber (Coy laboratories) with 2.5% H<sub>2</sub> and 0 ppm O<sub>2</sub> at 37 °C. *Escherichia coli* S17λPir was grown aerobically in LB at 37°C.

### **2.3 RNA-sequencing and analysis**

One week after orally administering *C. immunis* (200 µl containing ~10<sup>8</sup> colony-forming units [CFU]) or, as a control, sterile PYG media (200 µl) to male C57BL/6 MMb mice (12 weeks of age), the proximal 2 cm of colon was collected, frozen immediately in liquid nitrogen, and stored at –80 °C until needed. Tissues were homogenized in Trizol (Invitrogen) using a bead-beating approach, and RNA was purified according to the manufacturer’s instructions. RNA Integrity Number (RIN) scores for all samples used for RNA-seq were >7.0 as assessed on a TapeStation 2200 (Agilent). cDNA libraries were generated using a mRNA HyperPrep kit (KAPA) and sequenced on a NovaSeq6000 (Illumina; S4 flow cell with 150bp paired-end reads).

FastQC (version 0.11.9) was used to assess read quality and to perform trimming, STAR (release 2.7.9a) was used to align reads to the mouse genome mm10 (GRCm39) (146), and FeatureCounts was used to generate a count table (147). Differential gene analysis was performed with DESeq2 (version 1.28.1) (148), and a volcano plot was generated with EnhancedVolcano. A preranked gene set enrichment analysis was

performed using GSEA 4.3.2 using a previously described NFIL3-dependent gene set as input (149, 150). R (version 4.0.0) was used for all analyses.

## **2.4 Real-time qPCR analysis**

RNA was isolated from tissue as described above. For cells, Trizol was added to samples, and RNA was purified according to the manufacturer's instructions. cDNA was prepared using random hexamers and the High-Capacity cDNA synthesis kit (Applied Biosystems), and qPCR was performed on a Step One Real Time PCR system (Applied Biosystems) with iTaq Universal SYBR Green Supermix (Bio-Rad). The following primers were used: *Gadph* fwd: ACCACAGTCCATGCCATCAC; *Gadph* rev: TCCACCACCCTGTTGCTGTA; *Nfil3* fwd: CTTTCAGGACTACCAGACATCCAA; *Nfil3* rev: GATGCAACTTCCGGCTACCA; *Cd36* fwd: TCATATTGTGCTTGCAAATCCAA; *Cd36* rev: TGTAGATCGGCTTTACCAAAGATG; *Scd1* fwd: CTTCTTCTCTCACGTGGGTTG; *Scd1* rev: CGGGCTTGTAGTACCTCCTC; *Il23* fwd: CATGCTAGCCTGGAACGCACAT; *Il23* rev: ACTGGCTGTTGTCCTTGAGTCC; *Il1b* fwd: TGGACCTTCCAGGATGAGGACA; *Il1b* rev: GTTCATCTCGGAGCCTGTAGTG; *Il22* fwd: GCTTGAGGTGTCCAACCTTCCAG; *Il22* rev: ACTCCTCGGAACAGTTTCTCCC; *Csf2* fwd: AACCTCCTGGATGACATGCCTG; *Csf2* rev: AAATTGCCCCGTAGACCCTGCT; *Il17a* fwd: ATCCCTCAAAGCTCAGCGTGTC;

*Il17a* rev: GGGTCTTCATTGCGGTGGAGAG. The comparative Ct method was used to quantify transcripts that were normalized with respect to *Gapdh*.

## **2.5 Assessment of adiposity and serum triglycerides**

Mice (13–18 weeks of age) were orally administered either  $\sim 10^8$  CFU of bacteria, 4 mg of exopolysaccharide, or sterile PYG media. One week later, the gonadal fat pads were collected. The fat pads surrounding the ovaries and fallopian tubes were collected in female mice, while the epididymal fat pads were collected in male mice. The weight of these gonadal fat pads was normalized to the animal's body weight at time of sacrifice. For serum triglyceride and non-esterified fatty acid measurements, whole blood was collected in SST Microtainer tubes (BD Biosciences) from mice one week after oral administration of *C. immunis* or sterile PYG media. Serum was collected following manufacturer's instructions. Triglyceride and non-esterified fatty acids were quantified with the Duke Metabolomics/Proteomics Core Facility at the Duke Molecular Physiology Institute.

## **2.6 Dextran sodium sulfate (DSS) colitis**

DSS experiments were performed as previously described (151). Mice were orally administered *C. immunis* (200  $\mu$ l;  $\sim 10^8$  CFU), clarified *C. immunis* supernatant (200  $\mu$ l), or EPS as described below. For initiation of colitis, the mice were given DSS *ad libitum* in their drinking water for 7 days, with the DSS solution changed every 2–3 days. From

day 7 through day 10, the mice were given water without DSS. C57BL/6 mice were orally administered *C. immunis* or EPS (4 mg) on days -7 and 0, with day 0 representing initiation of 2.5% DSS (molecular mass 36–50 kDa; Affymetrix). Swiss Webster MMb mice were orally administered *C. immunis*, clarified *C. immunis* supernatant, or EPS (2 mg) three times per week starting on day -7 and continuing throughout the entire experiment. Consistent with our previous work (138), Swiss Webster MMb mice received 4% DSS (molecular weight 36-50 kDa; MP Biomedical). Animals were weighed every 1–2 days, and any mouse that appeared moribund was euthanized. Ten days after initiation of DSS, colons were harvested for qPCR and histological analyses. Two independent investigators conducted a histological assessment of colons, with scores representing the combination of epithelial damage, mucosal inflammation, and extent of disease; all elements were scored 0–3, with 0 being normal.

## **2.7 Cell culture**

All tissues and cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. MNK-3 cells were obtained from Maria Ciofani (Duke University) and maintained as previously described (152), adding recombinant mouse IL-7 (10 ng/ml; BioLegend) and IL-15 (10 ng/ml; BioLegend) every 3 days. After confirming no mycoplasma contamination, MNK-3 cells were authenticated by their ability to elicit the appropriate cytokine responses following stimulation as originally described (152). Cells ( $5 \times 10^5$ )

were plated in 24-well plates. The following day, cells were treated with bacterial supernatants (10% v/v) or EPS (0.5 mg/ml) for 4 hours and lysed for RNA isolation. For experiments with MNK-3 cells, bacterial supernatants were prepared by growing bacteria anaerobically in PYG overnight at 37 °C, resuspending the bacteria in an equal volume of peptone water (10 g/L peptone, 5 g/L sodium chloride), incubating anaerobically overnight at 37 °C, and collecting the supernatants after centrifugation.

## **2.8 IL-22 depletion**

Mice were injected intraperitoneally with either a neutralizing antibody against IL-22 (150 µg/dose; clone 8E11; Genentech) or an IgG1 isotype control (150 µg/dose; Genentech) every other day. For experiments assessing gonadal fat, the antibody dosing regimen began 2 days prior to treatment with *C. immunis*. For DSS colitis experiments, mice were treated with the antibodies beginning 2 days before the initiation of DSS.

## **2.9 Genomic comparisons of Clostridium species**

Genomic DNA was isolated from overnight cultures of *C. immunis*, *C. symbiosum*, and *C. clostridioforme* using a MagAttract HMW DNA kit (Qiagen). A multiplexed sequencing library was generated using a SMRTbell prep kit (PacBio) and sequenced on a PacBio RS. Flye (release 2.8.3) was used to perform de novo assembly on quality-filtered reads (153), which resulted in a complete and circularized genome for *C. immunis* (genome size of 5.34 Mbp), 108 contigs (genome size of 5.54 Mbp) for *C.*

*symbiosum*, and 18 contigs (genome size of 5.84 Mbp) for *C. clostridioforme*. Gene annotation was performed with RAST (154), and a genome-wide, sequence-based comparison of the three strains was performed using The SEED Viewer web server (version 2.0) (153). Genes that were uniquely present in *C. immunis* were further manually curated to identify genes that are likely to generate extracellular products.

## **2.10 Generation of *C. immunis*ΔLicABC**

We established that pMTL82151, one of the ClosTron plasmids (155), fails to replicate in *C. immunis* and therefore could be used as a suicide vector. We used the Q5 High-Fidelity DNA polymerase (NEB) to PCR amplify the *ErmB* gene that provides resistance to erythromycin (using pMTL83251 as the template) and two 800 bp regions (separated by 80 bp) that flanked the *LicA* target site. PCR primers used are as follows: LicA Left F: gctcgggtaccgggatcctAATACCGTCAGCTACACTG; LicA Left R: ctcggccggTAGGCCGGACATATTCTATATC; LicA Right F: gaatgtgtttTTTCTATATGGTGATCTTAGTAAAC; LicA Right R: agcttgcattgtctgcaggccTTTAATTGGACAAAGCGTC; ErmB F: gtccggcctaCCGGCCGAAGCAAACCTTAAG; ErmB R: catatagaaaAAACACATTCCTTTAGTAACGTG. After digesting these three PCR products with XbaI and XhoI, they were cloned into an XbaI- and XhoI-digested pMTL82151 plasmid using HiFi assembly (NEB). Electrocompetent *E. coli* S17λPir was

transformed with the ligation mixture, which generated S17 $\lambda$ Pir:pMTL82151 $\Delta$ LicABC. This strain was used to transfer pMTL82151 $\Delta$ LicABC into *C. immunis* using standard conjugation methods. Overnight cultures of *E. coli* donors and *C. immunis* recipients were mixed at a 10:1 donor-to-recipient ratio. Multiple aliquots (30  $\mu$ l) of the mixture were spotted onto a BHI-S agar plate and incubated anaerobically at 37 °C. After 24–48 h, the total growth on each plate was collected, resuspended in fresh media, and grown on BHI-S plates containing erythromycin (500  $\mu$ g/ml) and colistin (10  $\mu$ g/ml). To confirm that the plasmid was not present in *C. immunis*, the culture was also grown on BHI-S plates containing colistin (10  $\mu$ g/ml) and chloramphenicol (12.5  $\mu$ g/ml), against which the plasmid backbone confers resistance. Erythromycin-resistant colonies of *C. immunis* were confirmed to harbor the insertion of the *ErmB* gene and expected 80-bp deletion in *LicA* by PCR and Sanger sequencing.

### **2.11 EPS purification**

The supernatant from overnight bacterial cultures was concentrated ~50-fold using a 100 kDa molecular weight cutoff (MWCO) spin column (Amicon Ultra, Millipore). The retentate was incubated overnight with DNase I (50  $\mu$ g/ml) and Rnase A (50  $\mu$ g/ml) at 37 °C followed by an overnight incubation with proteinase K (500  $\mu$ g/ml) at 37 °C. The volume of the resultant mixture was increased to 15 ml with dH<sub>2</sub>O and concentrated back down to 1–2 ml using a 100 kDa MWCO column to remove

proteinase K. The retentate was precipitated with ice-cold ethanol (80% final volume) at  $-20\text{ }^{\circ}\text{C}$  overnight. Precipitates were air-dried and resuspended in distilled water.

### **2.12 Visualization of EPS by gel electrophoresis**

EPS (60  $\mu\text{g}$ ) was boiled for 5 mins in Laemmli buffer with  $\beta$ -mercaptoethanol and run on a 10% polyacrylamide gel (MiniPROTEAN TGX, BioRad) at 200 V for 1hr. Gels were visualized with a silver (Thermo Fisher Pierce), Sudan Black B (Thomas Scientific), or periodic acid–Schiff (PAS) stain. For PAS staining, the gel was washed in deionized water for 10 min, fixed in 12.5% trichloroacetic acid for 30 min, oxidized with 1% periodic acid for 1 h, washed in deionized water for 4–5 h, and stained with Schiff's reagent for 1 h. After staining, the gel was reduced with three 10 min washes in 0.5% sodium metabisulfite. For the Sudan black B-stained gel, *E. coli* 055:B5 lipopolysaccharide (75  $\mu\text{g}$ ; Sigma) was used as a positive control. To assess the presence of nucleic acids, EPS (60 $\mu\text{g}$ ) was run on a 1% agarose gel stained with a DNA Gel Stain (ApexBio) and imaged on an Odyssey XF system (LI-COR Biosciences).

### **2.13 Slot immunoblot**

EPS (5  $\mu\text{g}$ ) was added to a methanol-activated PVDF membrane housed in a slot blot manifold (Hoefer Inc.). The membrane was blocked in 3% bovine serum albumin in TBS containing 0.1% Tween 20 (TBS-T) for 1 h, followed by overnight incubation at  $4\text{ }^{\circ}\text{C}$  with an anti-phosphocholine antibody (1:500 dilution; clone BH8; Millipore). After

washing with TBS-T, the membrane was incubated with an HRP-conjugated secondary antibody (Thermo Fisher Scientific) for 1 h. Chemiluminescence generated by treatment with Clarity ECL (Bio-Rad) was imaged on an Odyssey XF system (LI-COR Biosciences). EPS-treated membranes were also stained with PAS as detailed above to visualize the amount of carbohydrate loaded.

### **2.14 Glycosyl composition analysis**

Glycosyl composition analysis was performed by combined gas chromatography-mass spectrometry (GC-MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis as described previously (156). Briefly, samples (150–300 µg) were heated with 1 M methanolic HCl in a sealed screw-top glass test tube for 18 h at 80 °C. After cooling and removal of the solvent under a stream of nitrogen, the samples were re-*N*-acetylated and dried again. The samples were then derivatized with Tri-Sil® (Pierce) at 80 °C for 30 min. GC-MS analysis of the TMS methyl glycosides was performed on an Agilent 7890A GC interfaced to a 5975C MSD, using a Supelco Equity-1 fused silica capillary column (30 m × 0.25 mm ID).

## **2.15 NMR Spectroscopy**

Each lyophilized EPS sample (~1–2 mg) was dissolved in 500  $\mu\text{l}$  of  $\text{D}_2\text{O}$  (99.9% D, Sigma) and placed in a 5-mm NMR tube. Sodium trimethylsilylpropanesulfonate (0.5  $\mu\text{l}$ ) was added as a reference. Liquid  $^1\text{H}$ -NMR data were obtained at 298 K on a Varian VNMRS spectrometer (1H, 599.66 MHz).  $^1\text{H}$ -NMR parameters: 2.0 s relaxation delay, 65536 Hz spectral width, 16384 data points and 64 transients with total recycle delay of 3.3 s between each transient. The experiment was performed with suppression of the HOD signal at 4.78 ppm by presaturation. Prior to the Fourier transformation, the data were apodized with an exponential decay function with line broadening of 0.5 Hz,  $90^\circ$  sine square, and zero-filled to 64k points. The baselines were corrected automatically by subtracting a 3rd-order Bernstein polynomial fit. The spectra were processed and analyzed with MestreNova (version 14.2.1-27684).

## **2.16 Phylogeny of *C. immunis* LicABC homologs**

Homologs of the *C. immunis* *LicABC* locus were identified using BLASTn (2.13.0). The gene sequences for these *LicABC* loci as well as the sequences from select organisms primarily found in the respiratory tract were aligned using ClustalW. A maximum likelihood phylogenetic tree was generated using RAxML as implemented with the default settings in MegAlign Pro (DNA Star).

## **2.17 Statistics**

Sample-size estimates for each experiment were based on previous laboratory experience. The investigators were not blinded to allocation during experiments and outcome assessment. Prism 9 (GraphPad Software) was used for all statistical analyses unless otherwise specified.

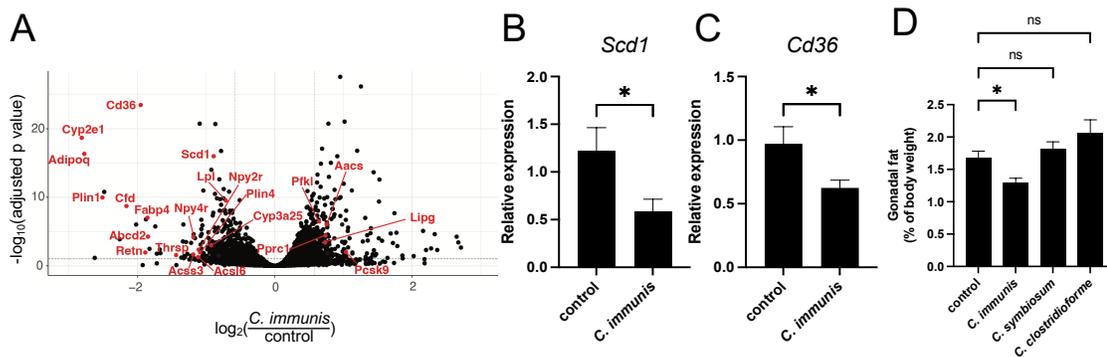
### **3. The Host Mechanisms Underlying *Clostridium immunis*–mediated Control of Fat Metabolism and Intestinal Inflammation**

#### **3.1 *C. immunis* negatively regulates adiposity**

To better understand how *C. immunis* protects against colitis, we treated gnotobiotic mice harboring a mouse microbiota (MMb) with or without *C. immunis* and compared their colonic transcriptional profile. Although we did not detect notable changes in immune-related genes, we observed that *C. immunis* treatment surprisingly led to suppression of genes related to lipid uptake and metabolism (Fig. 1A). We confirmed these findings extended to the small intestine, the primary site of lipid metabolism, by demonstrating *C. immunis* inhibits small-intestinal expression of *Cd36*, which encodes a transporter that imports fatty acids into cells (157), and *Scd1*, which encodes a stearoyl-coenzyme A desaturase (158) (Fig. 1B, C). Given that deletion of either of these genes results in decreased body fat (158, 159), we reasoned that treatment with *C. immunis* may similarly reduce visceral adiposity. Indeed, MMb mice orally treated with *C. immunis* had less gonadal fat than untreated mice, which demonstrates that *C. immunis* decreases visceral adiposity (Fig. 1D). Intriguingly, neither *Clostridium symbiosum* nor *Clostridium clostridioforme*, the two bacterial species most closely related to *C. immunis* (138) (genomic average nucleotide identity shared with *C. immunis* is 99%

and 70%, respectively), had any impact on gonadal fat (Fig. 1D), a finding that indicates *C. immunis* has unique functionality compared to these related bacterial species.

Although the microbiota generally promotes adiposity (149, 160), treatment with *C. immunis* decreased visceral fat, even in the presence of a complex microbiota.

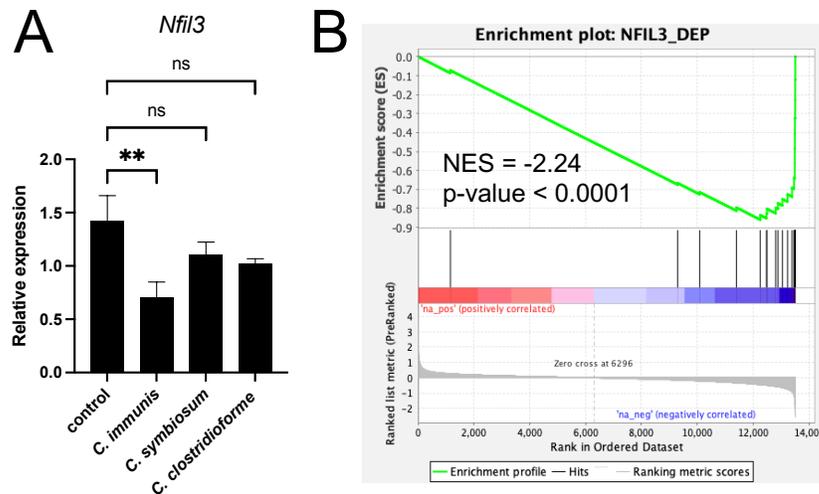


**Figure 1. *C. immunis* negatively regulates small intestinal lipid metabolism genes and visceral adiposity.**

(A) Volcano plot depicting differentially expressed genes in colons from MMB mice treated orally with or without *C. immunis* (n = 3 mice per group). Genes highlighted in red are related to lipid uptake and metabolism. (B, C) qPCR analysis of small-intestinal expression of *Scd1* (B) and *Cd36* (C) in MMB mice orally treated with or without *C. immunis* (n = 4–7 mice per group). (D) Gonadal fat mass normalized to body weight from MMB mice treated orally with or without the indicated bacteria (n = 4–6 mice per group). Data are pooled from two experiments and represent mean  $\pm$  s.e.m. . \*P < 0.05; ns, not significant by unpaired t test (B, C) or Brown-Forsyth and Welch ANOVA with Dunnett's T3 multiple comparisons test (D).

Recent work established that the microbiota positively regulates lipid metabolism genes and adiposity by increasing expression of the transcription factor NFIL3 through a pathway dependent on IL-22 secretion by group 3 innate lymphoid cells (ILC3s) (149, 161-163). ILC3s, which are critically important for orchestrating intestinal homeostasis (164, 165), are known to be regulated by the microbiota (39, 52, 166), with microbially-produced metabolites (e.g., indolic compounds, short-chain fatty acids) being one underlying mechanism (167). Although many commensal bacteria produce these metabolites, there are extremely limited examples of specific bacteria that regulate ILC3 function (21, 62). However, the mechanism of action of these known ILC3-modulating bacteria remains unclear. Given the ability of *C. immunis* to negatively regulate visceral adiposity, we speculated that it may do so via modulation of ILC3 effector function. Consistent with this notion, we observed that *C. immunis* decreases

expression of *Nfil3* and its target genes (Fig. 2A, B), which suggests that *C. immunis* may be working through this ILC3–IL-22–NFIL3 pathway.



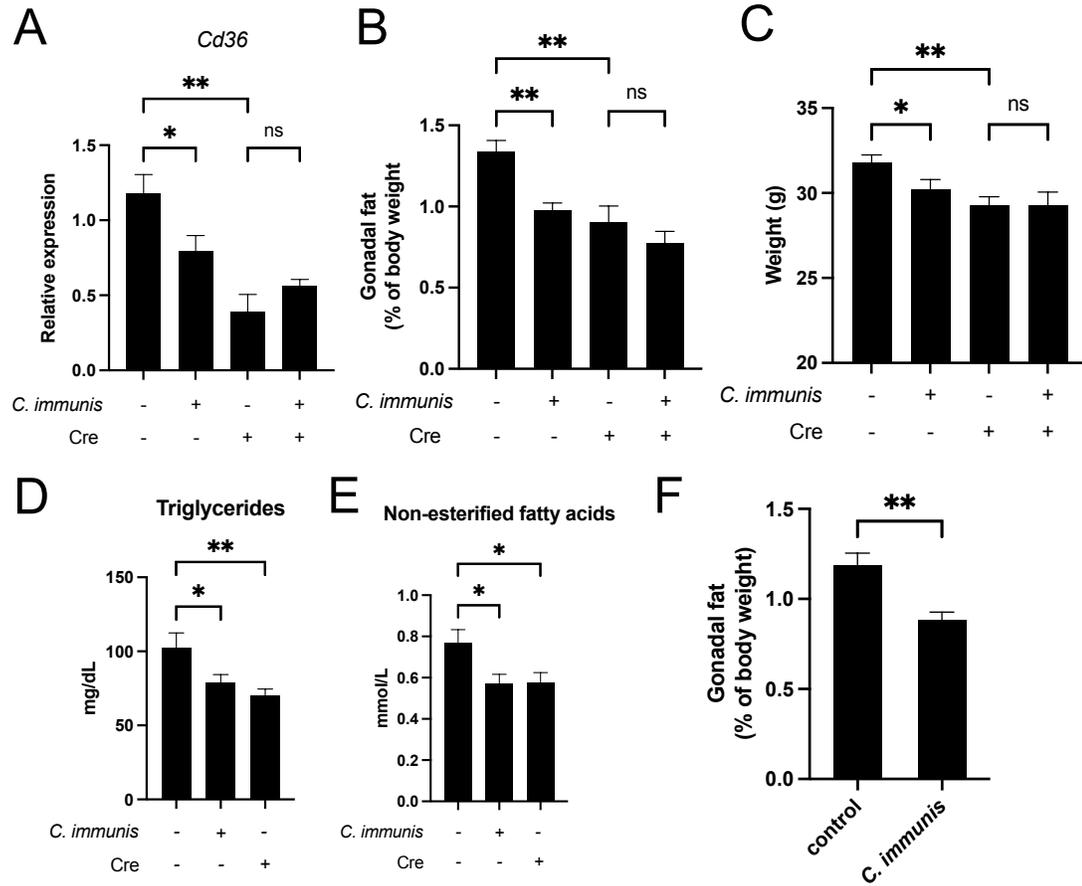
**Figure 2. *C. immunis* negatively regulates the expression of NFIL3 and NFIL3-target genes.**

(A) qPCR analysis of small-intestinal *Nfil3* expression in MMb mice orally treated with the indicated bacteria (n = 4–6 mice per group). Data are pooled from two experiments and represent mean±s.e.m. (B) Gene set enrichment analysis for NFIL3-dependent genes. NES, normalized enrichment score. \*\*P < 0.01; ns, not significant by ANOVA with Holm-Šidák correction for multiple comparisons.

### **3.2 C. immunis regulates lipid metabolism and adiposity via ILC3s**

To investigate this further, we generated ILC3-deficient mice by breeding *Ahr<sup>fl/fl</sup>* mice with *Rorc-cre* mice, as previously described (59). These ILC3-deficient mice had decreased *Cd36* expression, reduced gonadal fat, and lower body weights as compared to *Ahr<sup>fl/fl</sup>* littermate controls (Fig. 3A–C), findings that are consistent with the known role for ILC3s in obesity (149, 160). While *C. immunis* decreased these measures in *Ahr<sup>fl/fl</sup>* mice, it had no effect in ILC3-deficient mice (Fig. 3A–C). Moreover, *C. immunis* treatment, as well as ILC3 deficiency, led to decreased serum triglyceride and non-esterified fatty acid levels (Fig. 3D, E), findings consistent with the known fatty acid transporter role of small-intestinal CD36 and its decreased expression resulting from either *C. immunis* treatment or ILC3 deficiency (Fig. 3A). Of note, the ILC3-deficient *Ahr<sup>fl/fl</sup> Rorc-cre* mice also lack *Ahr* expression in T cells (59, 142), which have similarly been implicated in the microbial regulation of body composition (69, 163). We confirmed that these results are not related to T cells by showing *C. immunis* decreases visceral

adiposity in *Rag1*<sup>-/-</sup> mice (Fig. 3F). Taken together, these data establish that *C. immunis* decreases visceral fat in an ILC3-dependent manner.



**Figure 3. *C. immunis* modulates lipid metabolism and adiposity via ILC3s.**

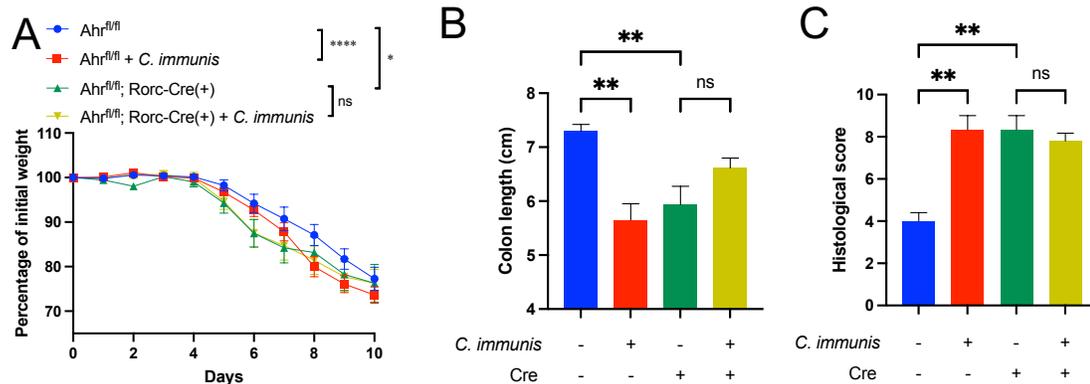
(A–C) Quantitative PCR (qPCR) analysis of small-intestinal *Cd36* expression (A; n = 3–8 mice per group), gonadal fat mass normalized to body weight (B; n = 5–10 mice per group), and body weight (C; n = 10–14 mice per group) from ILC3-sufficient (Cre-) or ILC3-deficient (Cre+) mice treated orally with or without *C. immunis*. (D, E) Serum triglyceride (D) or non-esterified fatty acid (E) concentrations from ILC3-sufficient (Cre-) or ILC3-deficient (Cre+) mice treated orally with or without *C. immunis*. (F) Gonadal fat mass normalized to body weight of *Rag1<sup>-/-</sup>* mice orally treated with or without *C. immunis* (n = 4–5 mice per group). Data are pooled from two (A, B) or three (C) experiments or are representative of two experiments (D, E, F) and represent mean ± s.e.m. . \*P < 0.05; \*\*P < 0.01; ns, not significant by one-way ANOVA with Holm-Šidák correction (A, C), Brown-Forsyth and Welch ANOVA with Dunnett's T3 multiple comparisons test (B), one-way ANOVA with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (D, E), or an unpaired t test (F).

### **3.3 C. immunis modulates colitis severity via ILC3s**

Since ILC3s also play a critical role in colitis (168), we speculated that *C. immunis* may similarly modulate colitis severity with ILC3s as the primary effector immune cell. To investigate this possibility, we orally treated ILC3-deficient mice and their *Ahr<sup>fl/fl</sup>* littermates with or without *C. immunis* and subjected them to dextran sodium sulfate (DSS)-induced colitis. *Ahr<sup>fl/fl</sup>* mice treated with *C. immunis* phenocopied ILC3-deficient mice, with increased weight loss, shorter colons, and greater histological evidence of disease compared to untreated *Ahr<sup>fl/fl</sup>* mice (Fig. 4A–C). Importantly, *C. immunis* treatment did not impact disease in ILC3-deficient mice, thereby demonstrating *C. immunis* modulates colitis severity through an ILC3-dependent pathway. Together, we have demonstrated that *C. immunis* impacts two distinct phenotypes, with ILC3s serving as a common immunological node. It is interesting that *C. immunis* worsens DSS-induced colitis in C57BL/6 mice but protects against disease in Swiss Webster mice (138). Although it remains unclear why, differences in mouse strain is well-known to variably impact the outcome of immune-mediated disease (169-172). Furthermore, *C. immunis* effectively protected Swiss Webster mice from colitis-induced mortality at 4% of DSS, a dose that was carefully titrated and optimized for the specific mouse strain and DSS severity. Interestingly, in C57BL/6 mice treated with 4% DSS, ILC3 deficiency led to less severe colitis (data not shown), a finding that supports a pathogenic role of ILC3s at this DSS dose. This finding is in contrast to a protective role of ILC3s at 2.5% DSS (Fig. 4A–

C). However, at 4% of DSS, colitis was too severe to be able to dissect the effect of *C. immunitis* to disease. Hence, a lower dose (2.5%) was used, in which we see that ILC3s were protective. Therefore, DSS dosage likely accounts in part for the differences observed between Swiss Webster and C57BL/6 mice, a finding that has been supported by others (173). Lastly, genetic differences across different mouse strains likely impacts

the endogenous microbiota, a variable that also may contribute to the discrepant effects in our studies of DSS on Swiss Webster and C57BL/6 mice.



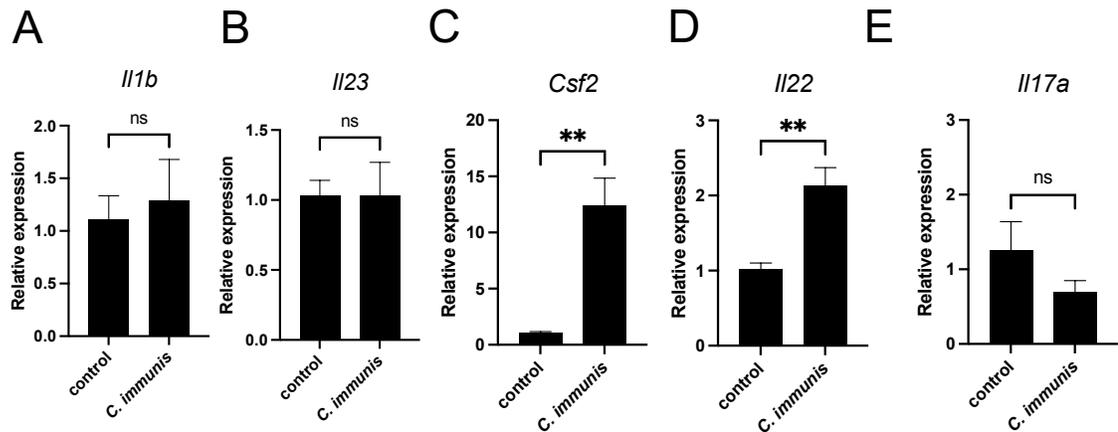
**Figure 4. *C. immunis* modulates colitis via ILC3s.**

(A–C) Weight change (A; n = 8–9 mice per group), colon length (B; n = 4–7 mice per group), and histological assessment of disease severity in the colon at day 7 (C; n = 3–5 mice per group) from ILC3-sufficient or ILC3-deficient mice treated orally with or without *C. immunis* and subjected to DSS-induced colitis. Data are pooled from two (A) experiments or are representative of 2 independent experiments (B, C). Data are represented as mean ± s.e.m. \*P < 0.05; \*\*P < 0.01; \*\*\*\*P < 0.0001; ns, not significant by comparison of fit by non-linear regression (A), or a Kruskal-Wallis test with correction for multiple comparisons (B, C).

### **3.4 C. immunis contextually regulates ILC3 effector function**

Given that numerous commensal bacteria affect myeloid cell secretion of cytokines (e.g., IL-23, IL-1 $\beta$ ) that alter ILC3 function (174-176), we explored whether *C. immunis* modulates ILC3s directly or influences their immunological milieu. We found that expression of *Il23* and *Il1b* was unchanged with *C. immunis* treatment (Fig. 5A, B), a finding that suggests *C. immunis* is not altering immune signals upstream of ILC3s. Furthermore, treating MNK-3 cells, an ILC3-like cell line (37), with *C. immunis* led to robust induction of *Il22* and *Csf2*, which encodes GM-CSF, with no impact on *Il17a* (Fig.

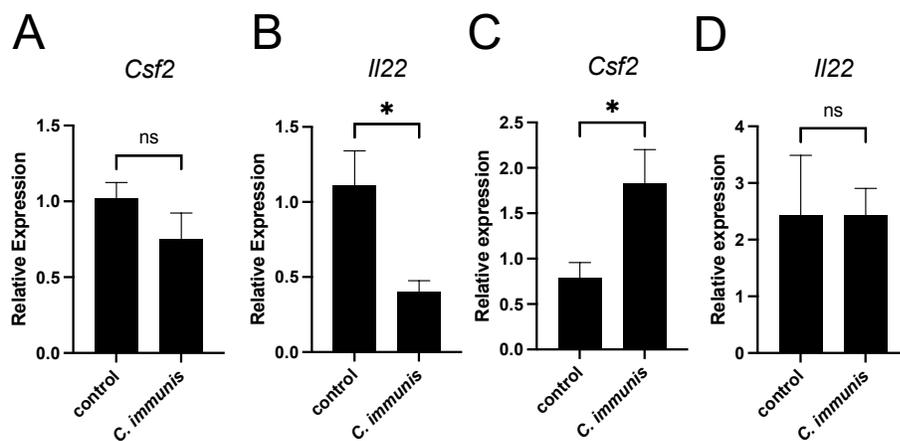
5C–E). Collectively, these findings support the notion that *C. immunis* can directly modulate ILC3 function, with expression of specific cytokines impacted.



**Figure 5. *C. immunis* directly regulates ILC3 effector function.**

qPCR analysis of *Il1b* (A) and *Il23* (B) expression in intestinal tissue obtained from MMB mice orally treated with or without *C. immunis* ( $n = 6$  mice per group). Data are pooled from two independent experiments and represent mean  $\pm$  s.e.m. ns, not significant by Mann Whitney U test.

As ILC3s are major sources of intestinal IL-22 and GM-CSF (166, 176, 177), we reasoned that treatment of mice with *C. immunis* leads to altered expression of these cytokines. In healthy SPF mice, *C. immunis* led to no change in *Csf2* expression with decreased levels of *Il22* (Fig. 6A, B); however, in mice subjected to colitis, *C. immunis* increased expression of *Csf2* with no effect on *Il22* expression (Fig. 6C, D).



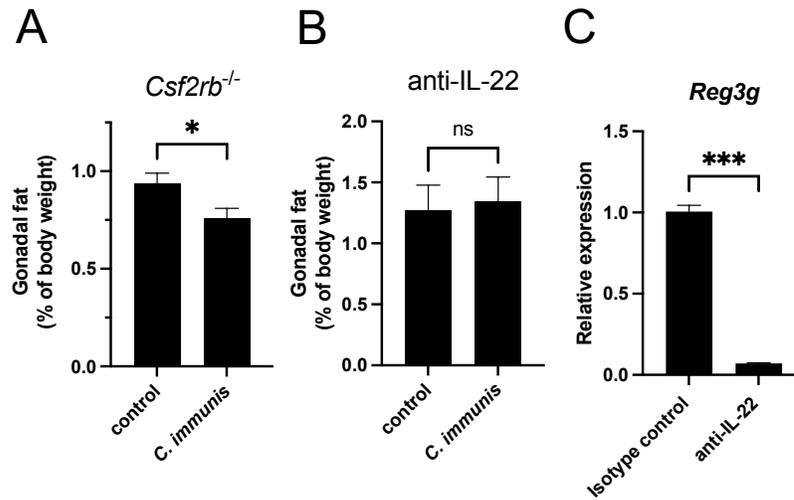
**Figure 6. *C. immunis* contextually regulates ILC3 cytokines in vivo.**

(A, B) qPCR analysis of small-intestinal expression of *Csf2* (A) and *Il22* (B) in mice orally treated with or without *C. immunis* ( $n = 6-7$  mice per group). (C, D) qPCR analysis of colonic expression of *Csf2* (C) and *Il22* (D) in mice orally treated with or without *C. immunis* and challenged with DSS-induced colitis for 7 days ( $n = 5-8$  mice per group). Data are representative of  $\geq 2$  experiments. Data are represented as mean  $\pm$  s.e.m.

\* $P < 0.05$ ; ns, not significant by Mann Whitney U test.

### **3.5 C. *immunis* modulates adiposity and colitis via contextual regulation of different ILC3 effector cytokines**

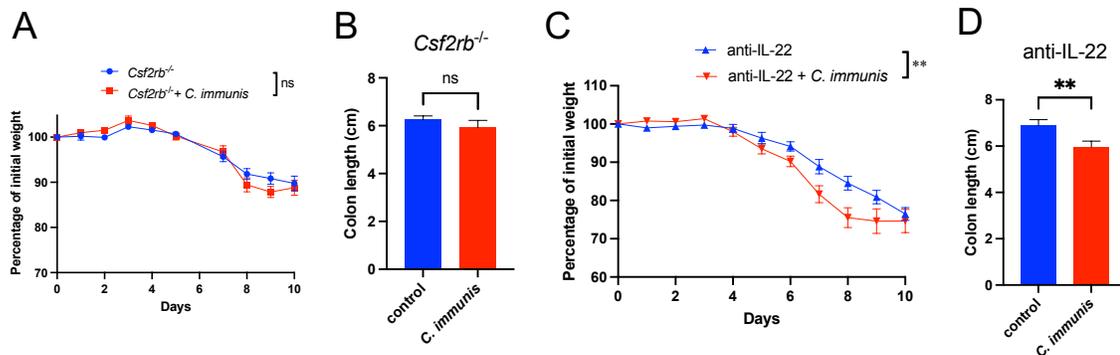
Given this distinctive regulation of ILC3-related cytokines that varies with inflammatory states, we hypothesized IL-22 and GM-CSF are differentially required for *C. immunis*-mediated modulation of adiposity and colitis. Indeed, the ability of *C. immunis* to reduce visceral fat was maintained in mice lacking CSF2RB, the receptor for GM-CSF, and lost when IL-22 was depleted (Fig. 7A–B). Additionally, we confirmed the efficacy of antibody-mediated depletion of IL-22 by measuring the expression of *Reg3g*, a known IL-22 dependent gene in the small intestine (Fig. 7C).



**Figure 7. Regulation of adiposity by *C. immunis* is dependent on IL-22 but not GM-CSF.**

(A, B) Gonadal fat mass normalized to body weight in *Csf2rb*<sup>-/-</sup> mice (A;  $n = 11$  mice per group) or *Rag1*<sup>-/-</sup> mice treated with an IL-22 depleting antibody (B;  $n = 8$  mice per group). Mice were orally treated with or without *C. immunis*. (C) qPCR analysis of *Reg3g* expression in small-intestinal tissue from mice in (B). Data are pooled from two (A) experiments or are representative of  $\geq 2$  experiments (B, C). Data are represented as mean  $\pm$  s.e.m. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; ns, not significant by unpaired t-test (A, B) or Mann Whitney U test (C).

In contrast, *C. immunis* was able to modulate colitis severity in IL-22-depleted animals but not in *Csf2rb*<sup>-/-</sup> mice (Fig. 8A–D). These findings demonstrate that *C. immunis* differentially requires—and contextually regulates—IL-22 and GM-CSF for modulation of adiposity and colitis, respectively. Considered together, we have established that *C. immunis* directly modulates ILC3 effector function, thereby controlling ILC3-mediated phenotypes.



**Figure 8. Modulation of colitis by *C. immunis*, in contrast, is GM-CSF dependent but IL-22 independent.**

Weight change (A, C) and colon length (B, D) of *Csf2rb*<sup>-/-</sup> mice (A, B; *n* = 10 mice per group) or mice treated with an IL-22 depleting antibody (C, D); *n* = 6 mice per group).

Mice were treated with or without *C. immunis* and subjected to DSS-induced colitis. Data are pooled from two (A, B) experiments or are representative of ≥2 experiments (C, D).

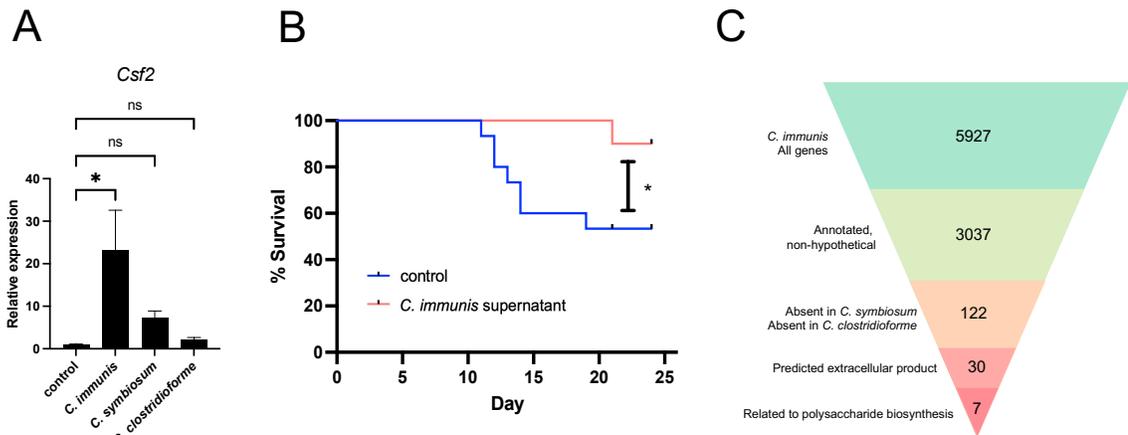
Data are represented as mean ± s.e.m. \*\**P* < 0.01; ns, not significant by Mann Whitney U test (B, D) or comparison of fit by non-linear regression (A, C).

## 4. A Bioactive Exopolysaccharide Revealed by Comparative Genomics

Having elucidated ILC3s as the key immunological determinant of *C. immunis* activity, we sought to identify the bacterial factors required for modulating ILC3-mediated diseases.

### 4.1 *C. immunis* contains unique genes relative to *non-ILC3* regulating *Clostridium* species

Given *C. symbiosum* and *C. clostridioforme* were unable to modulate adiposity (Fig. 1D), we speculated that these organisms do not impact ILC3 function. Indeed, neither bacterium induced *Csf2* expression in MNK-3 cells (Fig. 9A), which strengthens the notion that *C. immunis* has some unique function lacking in these other closely related bacteria. With this in mind, we sequenced the genomes for *C. symbiosum* and *C. clostridioforme* and performed genomic comparisons with *C. immunis*. Ultimately, we identified 122 genes that were uniquely present in *C. immunis* (Fig. 9C). Based on the findings with MNK-3 cells, we realized the bioactive molecule was present in the bacterial supernatant, which we additionally confirmed was sufficient to modulate colitis outcomes (Fig. 9B). As such, we manually curated the list of 122 genes to those likely to produce extracellular products, which reduced the list to 30 genes (Fig. 9C, Table 1).



**Figure 9. Comparative genomics reveals unique *C. immunis* genes associated with activity.**

(A) qPCR analysis of *Csf2* expression in MNK-3 cells incubated with a control (sterile culture media) or supernatants from the indicated bacteria. (B) Survival of Swiss Webster MMB mice orally treated with a control (sterile culture media) or *C. immunis* supernatant ( $n = 10-15$  per group) and subjected to DSS-induced colitis. (C) Schematic of comparative genomics analysis for *C. immunis* genes associated with activity. Data are representative of 2 experiments (A) or pooled from three independent experiments (B). Data are represented as mean  $\pm$  s.e.m.  $*P < 0.05$ ; ns, not significant by Kruskal-Wallis test with Dunn's multiple comparisons test (A) or log-rank test (B).

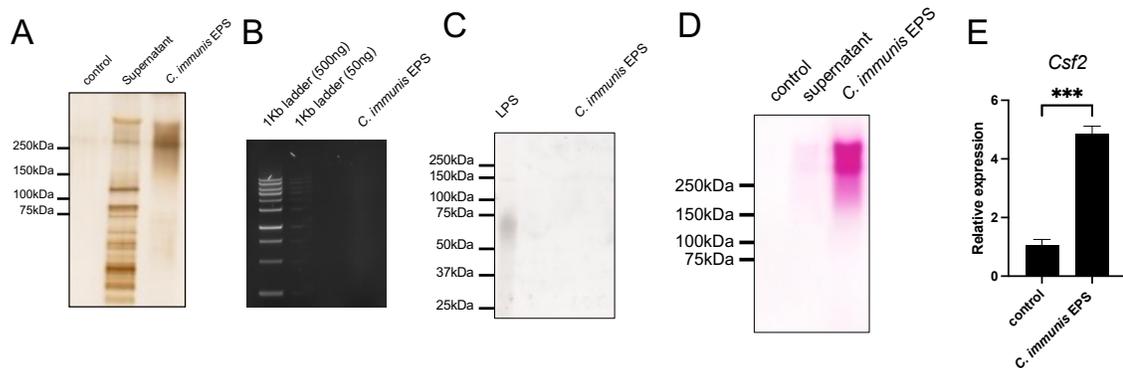
**Table 1. Genes present in *C. immunis* but absent in closely related *Clostridium* strains that likely produce extracellular products**

Genes involved in polysaccharide synthesis are listed in bold.

<b>Gene ID</b>	<b>Function</b>
Ci_66	Tripartite tricarboxylate transporter TctA family
Ci_131	NAD-dependent oxidoreductase
Ci_271	Protein-export membrane protein SecD/SecF
Ci_554	Hydrolase of alpha/beta superfamily
<b>Ci_818</b>	<b>capsular polysaccharide biosynthesis protein Cps4F</b>
<b>Ci_820</b>	<b>Capsular polysaccharide synthesis enzyme Cap5F</b>
Ci_821	UDP-N-acetyl-L-fucosamine synthase
Ci_891	Pantothenate:Na <sup>+</sup> symporter
Ci_1692	Branched-chain amino acid transport system permease protein LivM
<b>Ci_2065</b>	<b>capsular polysaccharide biosynthesis protein</b>
<b>Ci_2145</b>	<b>Pyrophosphorylase involved in lipopolysaccharide biosynthesis (LicC)</b>
<b>Ci_2146</b>	<b>Capsular polysaccharide biosynthesis protein (LicB)</b>
Ci_2162	Acyltransferase 3
Ci_2655	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA
Ci_2783	Multi antimicrobial extrusion protein (Na <sup>+</sup> )/drug antiporter
Ci_2978	PTS system, mannitol-specific component
Ci_3330	Glycosyltransferase, group 2 family
Ci_3460	Cell surface protein
Ci_3691	Glycoside-Pentoside-Hexuronide transporter
Ci_3700	UDP-N-acetyl-L-fucosamine synthase
<b>Ci_3701</b>	<b>Capsular polysaccharide synthesis enzyme Cap5F</b>
Ci_3710	Galacturonosyl transferase
Ci_3711	Alpha-1,3-N-acetylgalactosamine transferase PglA
Ci_3992	Type IV fimbrial assembly protein PilC
Ci_4441	Sodium/glutamate symporter
Ci_4572	Bacteriocin-like protein
Ci_5479	Polygalacturonase
<b>Ci_5817</b>	<b>Putative enzyme of poly-gamma-glutamate biosynthesis (capsule formation)</b>
Ci_5925	Choline binding protein A
Ci_5927	Choline binding protein A

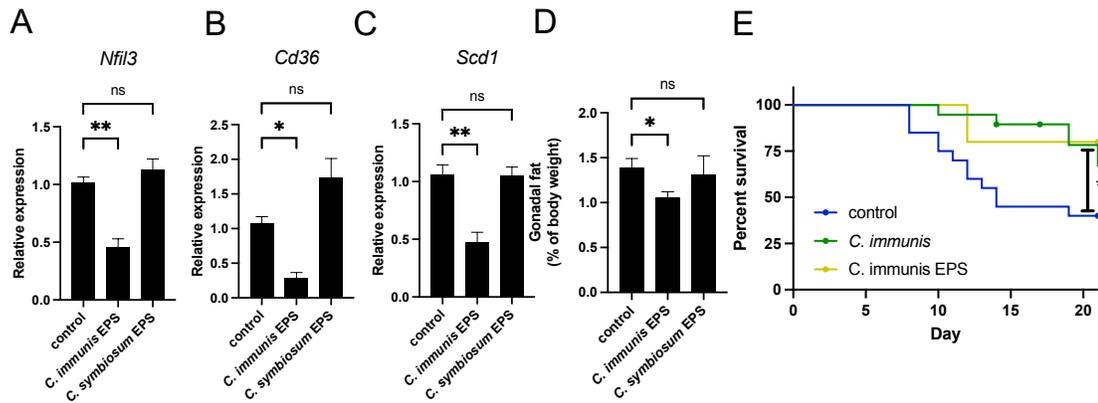
## **4.2 Identification of a bioactive exopolysaccharide from *C. immunis* guided by comparative genomics**

Intriguingly, nearly one-quarter of these genes were related to biosynthesis of an extracellular polysaccharide, a well-described mechanism by which commensal bacteria modulate the immune system (111, 178, 179). As such, we purified from the *C. immunis* supernatant high-molecular weight exopolysaccharides (EPS; Fig. 10D), which contained no appreciable protein, nucleic acid, or lipid contamination (Fig. 10A–C). This purified EPS was sufficient to induce *Csf2* expression in MNK-3 cells (Fig. 10E). Moreover, mice treated with EPS from *C. immunis*—but not EPS purified from *C. symbiosum*—had decreased small-intestinal expression of *Nfil3*, *Cd36*, and *Scd1* as well as reduced levels of gonadal fat compared to control animals (Fig. 11A–D). Finally, *C. immunis* EPS phenocopied *C. immunis* in its ability to protect against colitis-associated death in Swiss Webster MMb mice (Fig. 11E).



**Figure 10. A high molecular weight exopolysaccharide from *C. immunis* is sufficient to regulate ILC3 function in vitro.**

(A) Silver-stained SDS-PAGE gel of a control, *C. immunis* supernatant, and *C. immunis* EPS. (B) Safe DNA-stained agarose gel of *C. immunis* EPS. Two different amounts of the ladder were included to demonstrate sensitivity. (C) Sudan Black B-stained PAGE gel of *E. coli* lipopolysaccharide and *C. immunis* EPS. (D) Periodic acid–Schiff-stained SDS-PAGE gel of a control, *C. immunis* supernatant, and purified *C. immunis* exopolysaccharide (EPS). For (A) and (D), the control included sterile bacterial media that went through the same purification process as *C. immunis* EPS, and all samples represent an equal amount of culture volume. (E) qPCR analysis of *Csf2* expression in MNK-3 cells incubated with or without *C. immunis* EPS. Data are pooled from two (E) experiments or are representative of 3 independent experiments (A–D) . Data are represented as mean±s.e.m. \*\*\* $P < 0.001$  by unpaired t-test (D).



**Figure 11. *C. immunis* exopolysaccharide recapitulates the adiposity- and colitis-regulating functions of the whole bacterium.**

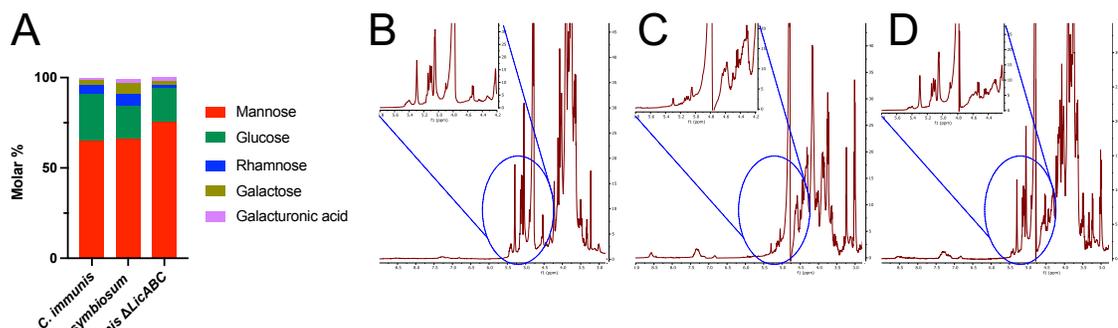
(A–C), qPCR analysis of small-intestinal expression of *Nfil3* (A), *Cd36* (B), and *Scd1* (C) in *Rag1*<sup>-/-</sup> mice treated with control or EPS isolated from *C. immunis* or *C. symbiosum* ( $n = 5–9$  per group). (D) Gonadal fat mass normalized to body weight in *Rag1*<sup>-/-</sup> mice treated with control or EPS isolated from *C. immunis* or *C. symbiosum* ( $n = 10–17$  per group). (E) Survival of Swiss Webster MMB mice treated with a control (sterile culture media), *C. immunis*, or *C. immunis* EPS following DSS-induced colitis ( $n = 5–19$  per group). Data are pooled from two (A–D) or three experiments (E). Data are represented as mean $\pm$ s.e.m. \* $P < 0.05$ ; \*\* $P < 0.01$ ; ns, not significant by Kruskal-Wallis test with Dunn’s multiple comparisons test (A–D) or log-rank test (E).

Taken together, we have conclusively established that this EPS is the relevant bioactive molecule from *C. immunis* that regulates ILC3s function in vitro and in vivo. These data, which are consistent with the notion that ILC3s may directly sense bacterial ligands (180), extend the molecular mechanisms by which the microbiota regulates ILC3 function beyond a limited number of common bacterial metabolites (167).

## **5. The Genetic and Structural Determinant of the *C. immunis* EPS Activity**

### **5.1 A phosphocholine moiety on *C. immunis* EPS is associated with activity**

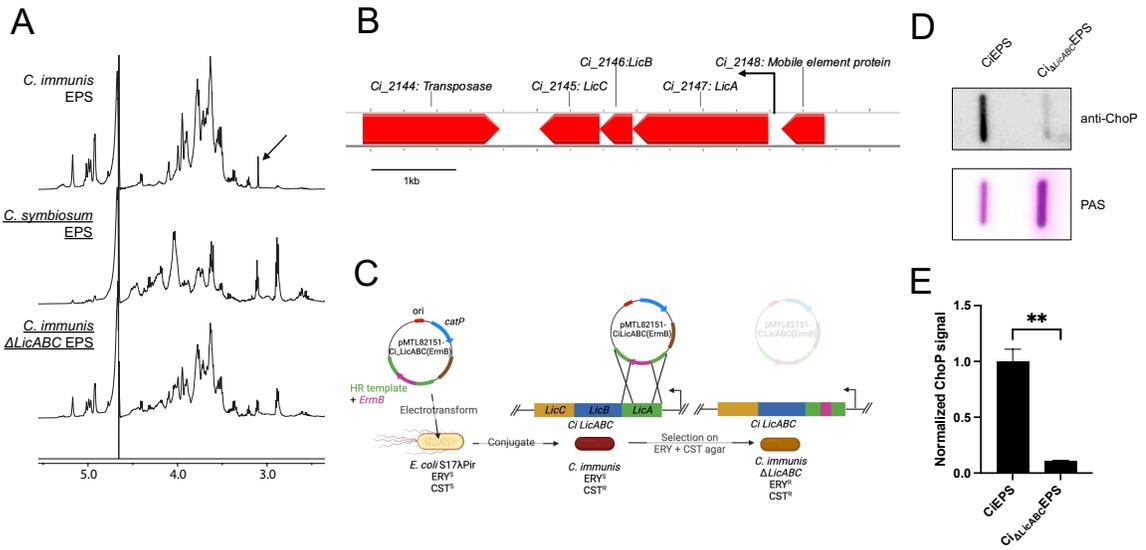
In collaboration with the Complex Carbohydrate Research Center at University of Georgia, we performed glycosyl compositional analysis on the EPS from *C. immunis* and *C. symbiosum* to gain insight into the structural determinants required for activity. Interestingly, the overall monosaccharide composition did not differ markedly between the two species, with both EPS structures composed primarily of mannose and glucose (Fig. 12A). Although this mannan-like composition is common in plants and fungi (181, 182), it is rare in bacterial polysaccharides. The <sup>1</sup>H-NMR spectra for both EPS purifications were very similar with respect to the anomeric signals that form the carbohydrate portion (Fig. 12B–D).



**Figure 12. EPS isolated from *C. immunis*, *C. symbiosum* and *C. immunis*  $\Delta$ *LicABC* have comparable monosaccharide composition and carbohydrate anomeric signals.**

(A) Glycosyl composition analysis of EPS isolated from *C. immunis*, *C. symbiosum* and *C. immunis*  $\Delta$ *LicABC*. (B–D)  $^1\text{H}$  NMR spectra of EPS samples from *C. immunis* (B), *C. symbiosum* (C) or *C. immunis*  $\Delta$ *LicABC* (D).

However, the *C. immunis* EPS contained a narrow signal at 3.22 ppm suggestive of phosphocholine (Fig. 13A); this sharp singlet is absent in the *C. symbiosum* EPS, which instead has a triplet slightly downfield (Fig. 13A). Of note, phosphocholine moieties on polysaccharides from mucosal pathogens enhance virulence by modulating the host immune system (183). For example, *Streptococcus pneumoniae* contains wall teichoic acids and lipoteichoic acids contain repeating units that are phosphocholine-conjugated (184), a modification that is critical for adherence and invasion of airway epithelial cells (185). Moreover, the rodent helminth parasite, *Acanthocheilonema vitae* secretes a protein that contains carbohydrate-linked phosphocholine that is critical for suppressing inflammatory responses (186). The role phosphocholine plays in diverse host-pathogen interactions therefore serves as an important precedent of its potential to regulate host-commensal interactions. Moreover, the structural presentation of phosphocholine (i.e., the macromolecular scaffold is conjugated to) is likely important for its specific effects on the host (187). We therefore hypothesized that phosphocholine modification of *C. immunis* EPS was relevant for its activity. Indeed, upon closer examination of the genes identified as distinguishing *C. immunis* from *C. symbiosum* and *C. clostridioforme*, we found two genes, Ci2145 and Ci2146, that were homologous to a phosphocholine transferase (*LicC*) and a choline transporter (*LicB*), respectively (188). These genes reside in a three-gene operon that also contains a choline kinase gene (*LicA*; Fig. 13B).



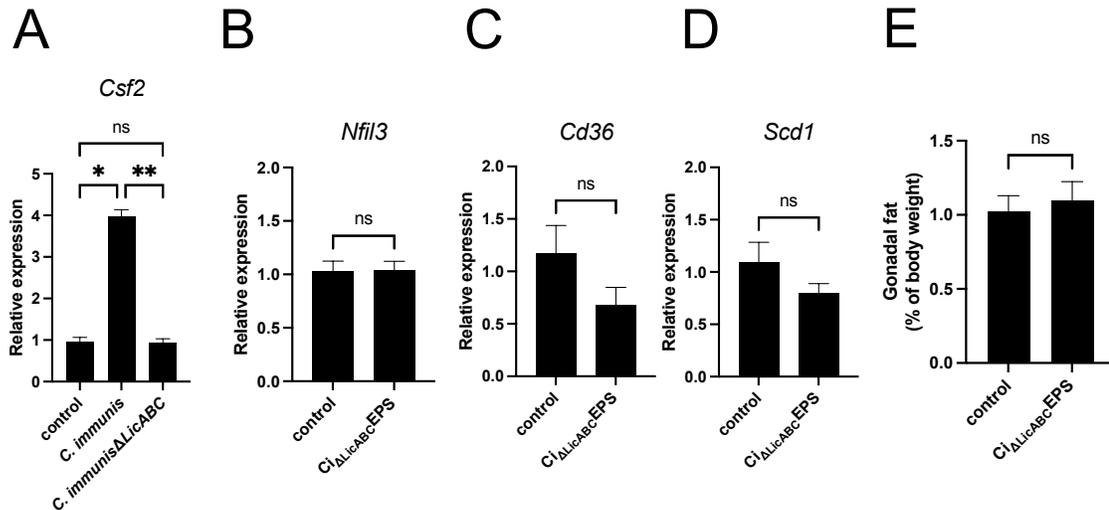
**Figure 13. The *C. immunis* EPS contains phosphocholine (ChoP), which is abrogated by deleting *LicABC*.**

(A)  $^1\text{H-NMR}$  spectra of EPS purified from *C. immunis*, *C. symbiosum*, or *C. immunis* $\Delta$ *LicABC*. The arrow highlights a peak corresponding to phosphocholine, which is only present in EPS from *C. immunis*. (B) A schematic of the *C. immunis* genetic locus containing *LicABC* and flanking genes. The predicted promoter is indicated by the arrow. (C) A schematic of the genetic strategy used to delete *LicABC* in *C. immunis*. *E. coli* was used to conjugally transfer into *C. immunis* a suicide plasmid containing an erythromycin resistance gene (*ErmB*) flanked by homology arms to *LicA*. Erythromycin-resistant ( $\text{Ery}^{\text{R}}$ ) *C. immunis* mutants contained the *ErmB* gene and an 80-bp deletion in *LicA*. (D) Immunoblot of EPS purified from *C. immunis* (CiEPS) or *C. immunis* $\Delta$ *LicABC* (Ci $\Delta$ *LicABC*EPS) that is probed with an antibody against phosphocholine (ChoP; top blot). Samples were also visualized with periodic acid–Schiff stain (PAS; bottom blot). (E) Quantification of the band intensities in panel (D), with values from the immunoblot normalized to that from the PAS-stained blot. Data are pooled from 3 experiments (D, E). Data are represented as mean $\pm$ s.e.m.  $**P < 0.01$  by unpaired t-test (E).

## **5.2 LicABC-encoded phosphocholine modification is essential for the bioactivity of *C. immunis***

To determine whether this *LicABC* operon is required for activity, we first needed to develop a system to genetically manipulate *C. immunis*, a feat that is still challenging for most commensal organisms (189). Although CRISPR-Cas9 has been a useful approach to make isogenic mutants in some clostridial species (190, 191), expression of Cas9 was toxic to *C. immunis*, similar to its effect in many other commensal bacteria (192). Instead, we created a suicide plasmid that contained an erythromycin resistance cassette flanked by DNA homologous to *C. immunis LicA* to facilitate homologous recombination that disrupted the entire *LicABC* locus (Fig. 13C). EPS isolated from the *C. immunis* $\Delta$ *LicABC* isogenic mutant ( $C_{i\Delta LicABC}EPS$ ) still had the same monosaccharide composition as that from wild-type *C. immunis* (Fig. 12A), but it lacked phosphocholine as assessed by  $^1H$ -NMR and immunoblot (Fig. 13A, D, E). Thus, using 3 orthogonal methods— $^1H$ -NMR, bacterial genetics, and immunoblotting—we have demonstrated *C. immunis* EPS contains phosphocholine. Consistent with phosphocholine being critical for the activity of *C. immunis* EPS, supernatant from *C. immunis* $\Delta$ *LicABC* was unable to induce *Csf2* expression in MNK-3 cells (Fig. 14A). Moreover, mice treated with  $C_{i\Delta LicABC}EPS$  had small-intestinal expression of *Nfil3*, *Cd36*, or *Scd1* and levels of adiposity that were indistinguishable from control animals (Fig.

14B–E). Collectively, these data clearly demonstrate that the phosphocholine present on *C. immunis* EPS is critical for its ability to regulate ILC3 function.



**Figure 14. Phosphocholine modification of *C. immunis* EPS is essential for its in vitro and in vivo activity.**

(A) qPCR analysis of *Csf2* expression in MNK-3 cells incubated with a control (sterile culture media) or supernatants from *C. immunis* or *C. immunis*Δ*LicABC*. (B–D) qPCR analysis of small-intestinal *Nfil3* (B), *Cd36* (C), and *Scd1* (D) in *Rag1*<sup>-/-</sup> mice treated with a control or *Ci*Δ*LicABC*EPS. (E) Gonadal fat mass normalized to body weight in *Rag1*<sup>-/-</sup> mice treated with control or *Ci*Δ*LicABC*EPS ( $n = 6–7$  mice per group). Data are pooled from 3 experiments (A) or are representative of  $\geq 2$  experiments (B–E). Data are represented as mean  $\pm$  s.e.m. \* $P < 0.05$ ; \*\* $P < 0.01$ ; ns, not significant by Kruskal-Wallis test with Dunn’s multiple comparisons test (D) or Mann Whitney U test (B–E).

## 6. Discussion and Future Work

The microbiota and the immune system are intimately connected, and there has been a concerted effort to leverage these microbiota-immune interactions to develop therapeutics for immune-mediated diseases. Implicitly, this task requires the answer to three separate issues, **1. Identify bacteria causally related to the phenotype of interest, 2. Understand how the bacterium and its bioactive product(s) modulates host physiology, and 3. Determine what is functionally unique about this bacterium that confers its activity.** Indeed, the past several years have seen rapid advances in each of these domains; however, there remain exceedingly few examples where all three issues have been addressed for a given host-microbiota relationship. In discovering *C. immunis* as a new microbial species that protects mice against colitis-mediated death, the first point has been addressed with earlier published work. My PhD is focused on identifying answers to the other two points.

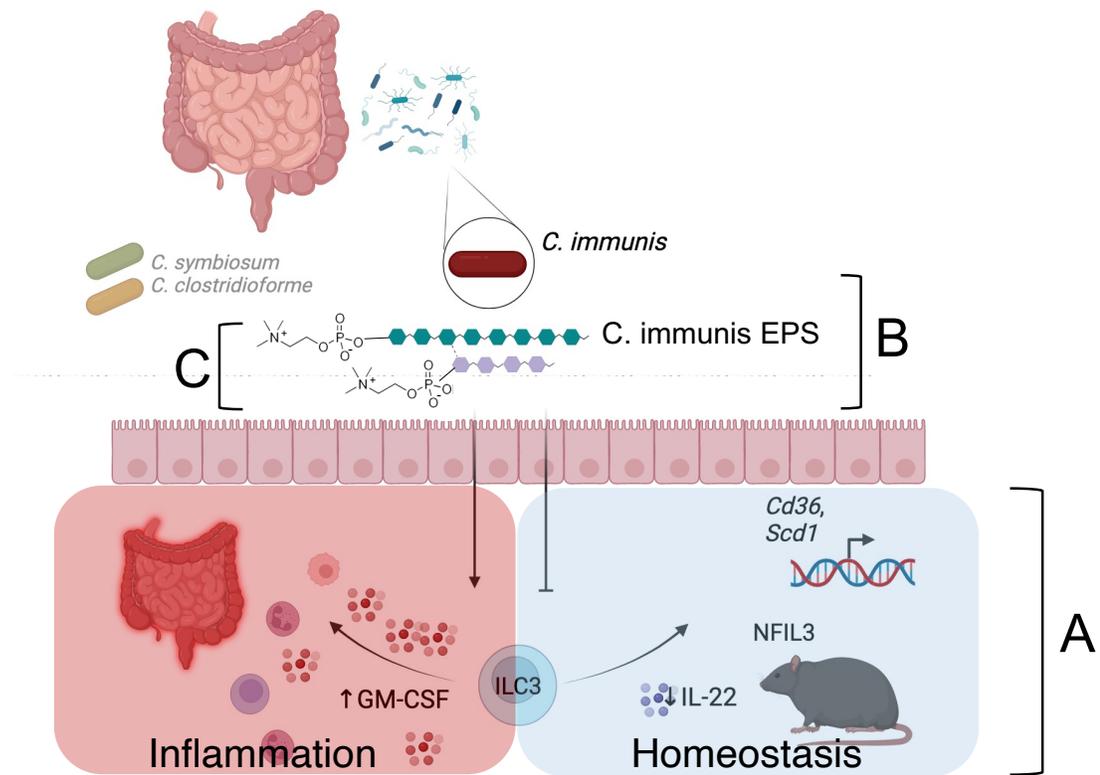
First, we find that *C. immunis* directly and contextually regulates the effector functions of ILC3s, and through this, it modulates the outcomes of two disparate disease models (adiposity and colitis) (Fig. 15A). These findings establish *C. immunis* as the first known human-derived commensal bacterium that modulates ILC3 function.

Additionally, we establish a mechanistic link between a specific commensal bacterium, cellular and molecular components of the immune system, and a metabolic phenotype.

Although it is known that the microbiota can regulate metabolism via immune-mediated

effects, we now show that a specific microbe can regulate systemic immunometabolism in mice harboring native microbiota. An earlier finding that *C. immunis* is unique (relative to other Clostridium strains) in its ability to regulate host adiposity led us to hypothesize that its ability to regulate ILC3s is also unique, which we validated in culture. Guided by comparative genomics between *C. immunis* and inactive Clostridium strains, we performed directed purification of the active compound and discovered an EPS that recapitulates the colitis- and adiposity-regulating function of the whole bacterium (Fig. 15B). Collectively, these findings address point 2.

We performed structural analysis of the *C. immunis* EPS, which led us to molecular insights (i.e., phosphocholine modification) that might account for its unique activity. However, to causally attribute phosphocholine modification to activity, we had to identify a method to remove phosphocholine from EPS. In the absence of enzymatic approaches, we decided to delete the relevant genetic locus in *C. immunis*. This necessitated the development of genetic tools for *C. immunis*, which belongs to a taxonomic clade notorious for its genetic intransigence. Ultimately, we succeeded in using reverse genetics to disrupt the *LicABC* locus in *C. immunis*, abrogate phosphocholine modification of its EPS and hence, its in vitro and in vivo activity (Fig. 15C). In doing so, we not just address point 3 but also uncover a new “rule of engagement” between a microbe and the immune system.



**Figure 15. A schematic summary of the mechanisms underlying *C. immunis* modulation of host physiology.**

(A) The host cellular and molecular mechanisms underlying contextual modulation of adiposity and colitis by *C. immunis*. (B) The *C. immunis*-derived bioactive molecule is an expolysaccharide (EPS). (C) Phosphocholine decoration of the *C. immunis* EPS is critical for its activity.

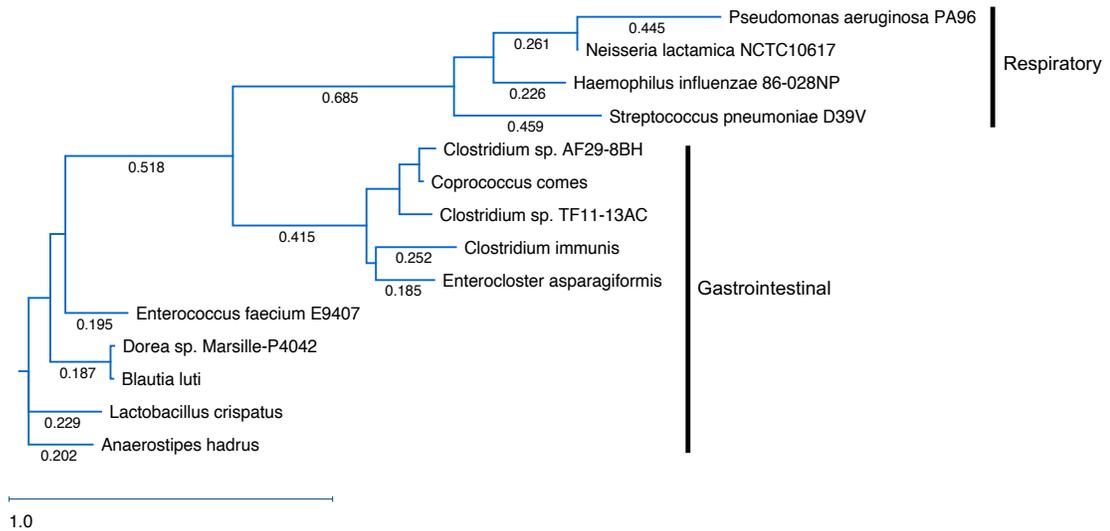
By elucidating the host and bacterial mechanisms of immunomodulation by *C. immunis*, we have made two observations that highlight the biological intricacy of commensal-host interactions. Firstly, although the regulation of adiposity and colitis by *C. immunis* is dependent on ILC3s, they are reliant on two different ILC3 effector cytokines, IL-22 or GM-CSF, respectively. This phenomenon is consistent with the fact that under non-inflamed conditions, IL-22—but not GM-CSF—expression is downregulated by *C. immunis*, and with a known role of IL-22 in regulating metabolic phenotypes (149, 193). Under inflammatory conditions in DSS colitis, we observe a converse pattern, where GM-CSF—but not IL-22—expression is differentially upregulated and specifically required for modulation of colitis severity. This observation is also consistent with a known proinflammatory role of GM-CSF in colitis (177). Collectively, our findings suggest that the immunological milieu in different disease states may in turn regulate how certain commensal bacteria alter the immune system, a phenomenon that has been recently described for other commensal bacteria (127, 128). However, given that ILC3s are a known source of GM-CSF, it is unclear why the abrogation of ILC3s would lead to worse DSS colitis outcomes. It is possible that a secondary cellular source of GM-CSF, coupled with the lack of IL-22 upregulation to mitigate epithelial barrier disruption, might account for the worse colitis outcomes seen in ILC3-deficiency (194). In support of this, we have preliminary data suggesting that ILC3-deficient mice with DSS colitis have elevated colonic GM-CSF levels but

virtually no IL-22 (not shown). Work by others also illustrates how colonic GM-CSF reinforces proinflammatory phenotypes in intestinal macrophages, which in turn further activate ILC3s (195), mechanisms that underscore a nuanced and complex interplay of ILC3s, GM-CSF and other immune cells in the inflamed colon. It will be interesting, in future work, to identify the other immunological factors that account for context-specific regulation of IL-22 and GM-CSF regulation by *C. immunis*. More broadly, since ILC3s also play important roles in defense against enteric pathogens (particularly via IL-22), it will also be useful to test if *C. immunis* can regulate infection outcomes via ILC3s.

Secondly, we discover an atypical (mannose- and glucose-containing) EPS from *C. immunis* that recapitulates its bioactivity. Although bacterial polysaccharides represent a classical microbe-associated immune ligand, there are at present only a handful of commensal-derived polysaccharides for which biological function have been ascribed (111, 178, 196). Furthermore, while there has been increasing recognition of the mechanisms by which the microbiota modulates various components of the adaptive immune system (33, 113, 197), there are strikingly fewer mechanistic examples for how specific commensal bacteria influence innate immune responses (135, 198, 199). Here we not only identified a specific commensal bacterium that regulates ILC3-mediated phenotypes, but we have also provided mechanistic insight into how it does so. Moreover, we identified phosphocholine as the molecular determinant required for full activity of the *C. immunis* EPS. We speculate that addition of phosphocholine as a

structural modification may be a common strategy employed by intestinal commensal bacteria in modulating host function, similar to its described role in respiratory pathogens. Indeed, we identified several taxonomically diverse commensal bacteria that contain a *LicABC* locus homologous to that of *C. immunis* (Fig. 16). Intriguingly, the gene sequences for bacteria primarily found in the respiratory tract cluster separately from intestinal organisms, which itself forms two distinct clades (Fig. 16). Given the phylogeny of the gene sequences is distinct from the bacterial taxonomic relationships, it is possible the *LicABC* locus is spread via horizontal gene transfer. Along these lines, it is interesting to note that the *C. immunis* *LicABC* operon is flanked by mobile genetic elements (Fig. 13B), which may help explain why closely related taxa lacked these genes.

More broadly, this mode of microbial ligand modification by *C. immunis* represents an additional possible solution for producing order from the complex microbiota-immune interplay (Chapter 1.3). It is tempting to speculate that modification of common microbe-associated molecular patterns with phosphocholine and other similar small moieties may act to fine-tune the host response, thus providing increased specificity in how commensal bacteria regulate host responses.



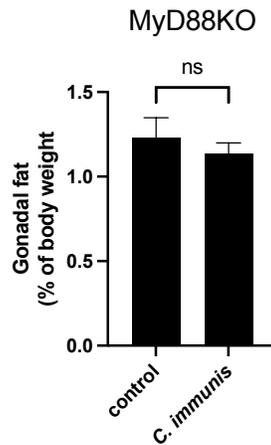
**Figure 16. *LicABC* genes are present in taxonomically diverse commensal organisms.**

*LicABC* genes homologous to the *C. immunis* locus were identified by BLAST and are depicted in a randomized accelerated maximum likelihood (RAxML) phylogenetic tree, with the *LicABC* genes from four respiratory organisms added in for reference.

It will be interesting to see if phosphocholine addition to *C. immunis* EPS is also differentially regulated under various in vivo conditions, thereby providing *C. immunis* contextual control over its ability to modulate ILC3s. This ability has been observed with certain pathogens that control phosphocholine modification of their glycans (e.g., by phase variation of *Lic* genes) as a strategy to regulate their virulence (200). At present,

the *C. immunis* EPS receptor and signaling pathway(s) on ILC3s are unknown. Our preliminary studies have shown that the in vivo adiposity modulating effects of *C. immunis* requires the TLR signaling adaptor MyD88 (Fig. 17), a finding that suggests that the EPS is sensed by TLRs. Indeed, we observe that *C. immunis* EPS potently agonizes TLR2 but not TLR4 in a reporter cell line (Fig. 18). However, in a preliminary study, this effect was not abrogated with EPS from *C. immunis* $\Delta$ *LicABC* (Fig. 19), findings that suggest TLR2 may be necessary but not sufficient in recognizing phosphocholine-decorated EPS by ILC3s. There could be a second receptor, such as CD36 or Dectin-1 (136, 201) that recognizes phosphocholine that together with TLR2 stimulation by the glycan chain, provides the necessary signals for GM-CSF upregulation in ILC3s. In work beyond this dissertation, we will identify the ILC3 receptor(s) and signaling pathways necessary for responding to phosphocholine-decorated EPS, work that will lend insight into how host factors distinguish specific chemical motifs from a plethora of general microbial products. Furthermore, we have only begun to get insight into the structure of

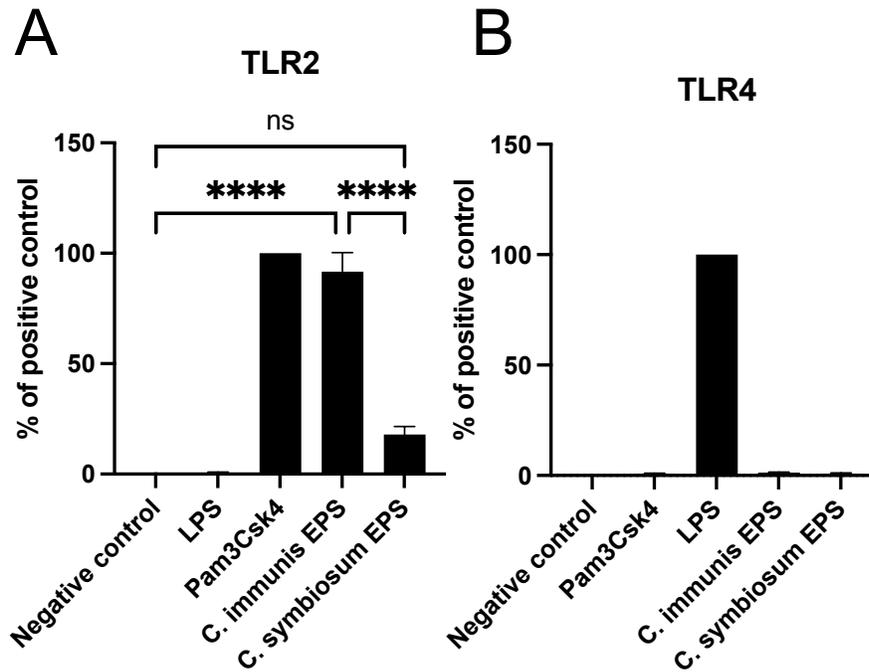
the EPS; further biochemical characterization with  $^{13}\text{C}$  and 2D-correlation spectroscopy NMR is needed to facilitate full structural characterization of the *C. immunis* EPS.



**Figure 17. *C. immunis* modulation of adiposity is MyD88-dependent.**

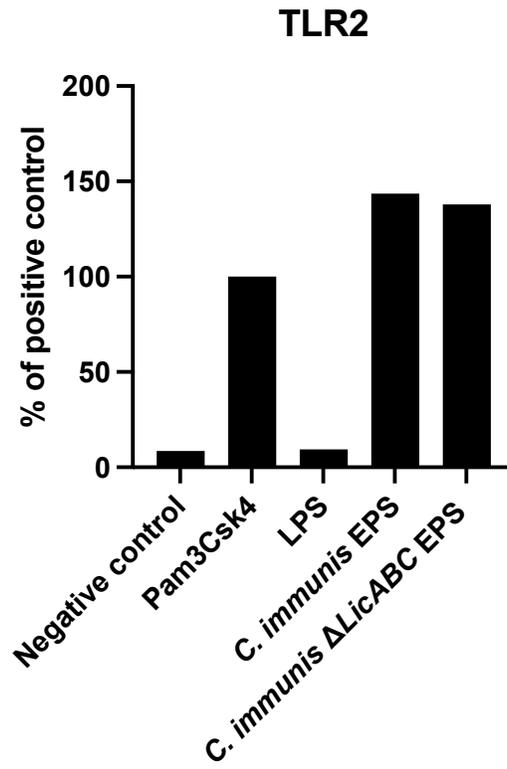
Gonadal fat mass normalized to body weight in *MyD88*<sup>-/-</sup> mice treated with control or *C. immunis* ( $n = 5-6$  mice per group). Data are pooled from 2 independent experiments.

Data are represented as mean  $\pm$  s.e.m. ns, not significant by unpaired t test.



**Figure 18. *C. immunis* EPS, but not *C. symbiosum* EPS potently stimulates TLR2 but not TLR4.**

(A) TLR2 or (B) TLR4 reporter activity when treated with controls (Negative control: culture media; LPS: positive control for TLR4; Pam3Csk4: positive control for TLR2) or 0.01mg/mL of EPS from *C. immunis* or *C. symbiosum*. TLR activity is normalized to the respective positive controls. Data are pooled from 3 independent experiments. Data are represented as mean $\pm$ s.e.m. \*\*\*\* $P < 0.0001$ ; ns, not significant by ANOVA with Šidák's multiple comparisons test.



**Figure 19. TLR2 agonism by *C. immunis* EPS is not dependent on phosphocholine-modification.**

TLR2 reporter activity when treated with controls (Negative control: culture media; LPS: negative control for TLR2; Pam3Csk4: positive control for TLR2) or EPS from wildtype or phosphocholine-deficient *C. immunis* ( $\Delta$ LicABC). TLR activity is normalized to the respective positive controls. This experiment is from one biological replicate.

## 7. Conclusion

Through a combination of computational, genetic, and biochemical approaches, we have conceptually fulfilled molecular Koch's postulates for the role of the *C. immunis* *LicABC* locus in modulating ILC3-regulated diseases (190). As the ability to culture and genetically manipulate commensal organisms continues to advance, demonstrating the genetic and structural basis for how the microbiota impacts host physiology will become more commonplace, just as it has become de rigueur in microbial pathogenesis.

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## **Biography**

Chin Yee Tan was born in Singapore. He obtained a Bachelor of Science at the National University of Singapore (NUS) in May 2015. Working in Professor Ong Siong's research group as an honors student in 2014, Chin Yee was inspired towards a career in medicine and science. He enrolled in Duke-NUS Medical School as an MD-PhD student in July 2015. In August 2017, Chin Yee began his PhD studies in the Department of Molecular Genetics and Microbiology at Duke University, where he joined the laboratory of Dr. Neeraj (Neil) Surana. He will return to complete his medical training in Singapore in March 2023.