

AWARD NUMBER: W81XWH-21-1-0481

TITLE: Drugs from Bugs: Developing New Inflammatory Bowel Disease Drugs from Gut Bacteria-Derived Bioactives

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14. ABSTRACT Gut bacteria produce bioactive metabolites that are absorbed by the intestine and distributed throughout the body, impacting both local and distant sites. In our previous successful Discovery Award project, we identified several human gut-derived bacteria capable of producing anti-inflammatory compounds. In this expansion award, we will identify the structure of the small molecules and optimize their structure to improve stability and solubility and test their efficacy in pre-clinical animal models (Aim 1). Optimized version of the small molecules will be assessed for pharmacodynamic, pharmacokinetic and toxicity in animal models (Aim 2). We will attempt to identify the host target molecule, cell and organ of action of the therapeutic agents (Aim 3). We will assess the impact of the small molecules on the microbiota of using the optimized therapeutic compound, but also the natural product to reveal bacteria-bacteria communication in the gut. At the conclusion of this project, we aim to have optimized three unique structures of anti-inflammatories ready for human trials.					
15. SUBJECT TERMS microbiota, anti-inflammatory, inflammatory bowel disease, ulcerative colitis, Crohn's disease, NF-κB, epithelial cells					
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1. INTRODUCTION:

This Expansion project is based on a successful Discovery project that identified several gut-derived bacterial strains with anti-inflammatory activity. In this Expansion project, we hypothesized that active compounds produced by the bacteria could be harnessed as human therapeutics for the treatment of inflammatory bowel disease. In this project, we will work towards developing one partially optimized and two as yet un-identified anti-inflammatories for therapeutic use. We aim to optimize the structure of the active molecules, identify the host target, assess pharmacokinetic and dynamics and identify any toxicities. Additionally, we will examine the impact of the compounds on the growth and composition of the gut microbiota and define the metabolism of the bacteria-derived compounds by other bacteria. At the conclusion of this project, we aim to develop three novel small molecule therapeutic candidates for human clinical trials.

2. KEYWORDS:

microbiota, anti-inflammatory, inflammatory bowel disease, ulcerative colitis, Crohn's disease, NF- κ B, epithelial cells

3. ACCOMPLISHMENTS:

- What were the major goals of the project?

	Timeline	Site 1
Major Task 1. Optimize and validate Hit #1 as a potent anti-inflammatory with a known target, and drug properties.	Months	
Subtask 1 – Obtain local and USAMRDC Human Research Protection Office (HRPO) approval for use of human cells lines and human subject material <i>Complete. Continuing approval of E20374.1 noted 17 May 2022</i>	-4 to -1	A/Prof Begun, Dr. Davies
Subtask 2 – Obtain local and USAMRDC Animal Care and Use Review Office (ACURO) approval for all animal experiments <i>Complete. Approval dated 28 June 2021</i>	-4 to -1	A/Prof Begun, Dr. Davies
Subtask 3 – Optimize structure-activity relationship - Bioassay is an NF-κB reporter in human CaCo2 cells. - Add tags, verify activity <i>Ongoing. Anticipate completion on schedule</i>	1-12	Prof Capon
Subtask 4 – Identify target Proteomic profiling, interaction validation, cellular localization <i>Ongoing. Anticipate completion on schedule</i>	6-24	A/Prof Begun, Dr. Davies
Subtask 5 – Measure microbiota interaction with bioactives	6-30	Prof Morrison, A/Prof Begun, Dr. Davies

<i>In vitro</i> measurement of bioactive breakdown and changes in microbial communities <i>In vivo</i> measurement of bioactive breakdown and changes in microbial communities Ongoing. Probes generated, cultures in the planning stage. Anticipate completion on schedule		
Milestone(s) Achieved: Complete optimization and characterization of Hit #1	30	
Major Task 2. Identify, optimize and validate Hit #2		
Subtask 1 – Determine efficacy in colitis animal models Winnie and Rag1KO models. Measure efficacy of bacterial culture supernatant to reduce inflammation Ongoing. First cohorts completed.	1-6	A/Prof Begun, Dr. Davies
Subtask 2 – Identify and optimize the chemical structure of the bioactive - Generate library of analogues, optimize structure activity relationship using NF-κB reporter assay - Add tags, verify activity Not yet begun. Anticipate completion on schedule	1-24	Prof Capon, A/Prof Begun, Dr. Davies
Subtask 3 – Determine <i>in vivo</i> properties - Time-course of distribution to different tissues with varying routes of administration (oral, ip, rectal) - Toxicity study - Visualize distribution in tissue Not yet begun. Anticipate completion on schedule	12-24	Prof Capon, A/Prof Begun, Dr. Davies
Subtask 4 – Identify target - Transcriptomic profiling - Proteomic profiling, interaction validation, cellular localization Not yet begun. Anticipate completion on schedule	12-36	A/Prof Begun, Dr. Davies
Subtask 5 - Measure microbiota interaction with bioactives <i>In vitro</i> measurement of bioactive breakdown and changes in microbial communities <i>In vivo</i> measurement of bioactive breakdown and changes in microbial communities	12-36	Prof Morrison, A/Prof Begun, Dr. Davies

Not yet begun. Anticipate completion on schedule		
Milestone(s) Achieved: Complete identification, optimization and characterization of Hit #2	36	
Major Task 3: Identify, optimize and validate Hit #3		
Subtask 1 – Determine efficacy in colitis animal models Winnie and Rag1KO models. Measure efficacy of bacterial culture supernatant to reduce inflammation Ongoing. First cohorts completed.	1-6	A/Prof Begun, Dr. Davies
Subtask 2 – Identify and optimize the chemical structure of the bioactive • Generate library of analogues, optimize structure activity relationship using NF-κB reporter assay • Add tags, verify activity Not yet begun. Anticipate completion on schedule	1-24	Prof Capon, A/Prof Begun, Dr. Davies
Subtask 3 – Determine <i>in vivo</i> properties • Time-course of distribution to different tissues with varying routes of administration (oral, ip, rectal) • Toxicity study • Visualize distribution in tissue Not yet begun. Anticipate completion on schedule	12-24	Prof Capon, A/Prof Begun, Dr. Davies
Subtask 4 – Identify target • Transcriptomic profiling • Proteomic profiling, interaction validation, cellular localization Not yet begun. Anticipate completion on schedule	12-36	A/Prof Begun, Dr. Davies
Subtask 5 - Measure microbiota interaction with bioactives • <i>In vitro</i> measurement of bioactive breakdown and changes in microbial communities • <i>In vivo</i> measurement of bioactive breakdown and changes in microbial communities Not yet begun. Anticipate completion on schedule	12-36	Prof Morrison, A/Prof Begun, Dr. Davies
Milestone(s) Achieved: Complete identification, optimization and characterization of Hit #3	36	

- What was accomplished under these goals?

Initial work on this project began when contracts were agreed between the three institutions. This occurred on January 31, 2022, rather than the proposed start date of July 1, 2021. This means we had a seven-month delay in our SOW timeline and have only completed 5 months of work to date.

Major Task 1. Subtask 3: Optimizing structure-activity relationship of Hit #1.

Previously, we identified that the active fraction of Hit #1 supernatant contained 6 unique molecules which we hypothesized accounted for its suppressive activity. Initial MS/MS analysis allowed us to approximate a putative structure (HSL), which we synthesized and tested in our NF- κ B bioassay for activity. The putative structure displayed significant activity and an initial modification to produce a more stable compound was also active (HSC) (**Figure 1A**), which we described in our final report for the Discovery project (W81XWH-17-1-0279). In this Expansion project (W81XWH-21-1-0481), we are defining and optimizing both the natural product (HSL) and the therapeutically optimized compound (HSC).

Why optimize both structures? Identifying and optimizing both structures promotes two different goals. Firstly, identifying the chemical structure of the active molecule produced by the bacteria (natural product, HSL) will allow us to measure whether other bacteria make the active molecule and whether it is detectable in human specimens, including stool and blood. This will allow us to track the presence and metabolism of the product and measure bacteria-bacteria interactions as would occur naturally in the gut. Secondly, optimizing the structure of the more stable therapeutic candidate will produce a structurally tractable molecule with the best therapeutic characteristics including narrow specificity and low toxicity (HSC). To this end we have produced a series of tools to investigate the structure activity relationship of both the natural HSL and the potential therapeutic HSC.

HSL series – We have identified the active structure class which includes analogues that are mono and di hydroxylated on the lipid chain. We are currently developing and applying a synthetic strategy to prepare an array of HSL analogues, with varying levels of hydroxylation, oxidation and chain length, to both definitively identify the original bacterial bioactives and optimize the associated pharmacophore. We have already demonstrated that monohydroxylated lipid analogues show superior potency compared to analogues not bearing hydroxylation on the lipid chain (**data not shown**).

HSC series – In our preliminary studies we demonstrated that conversion of the chemically non-rigid lipid tail of the HSL series to a more rigid and chemically defined cholic acid tail in the HSC series imparted superior potency. We are currently assembling a library of HSC analogues to better explore and optimize the pharmacophore. We have already prepared and tested several HSC analogues (**Figure 1B** and **data not shown**)

Major Task 1. Subtask 4: Identify Target of Hit #1.

To determine pathways altered by the host-targeted therapeutic molecule HSC (HSC-1) we performed RNAseq on HT29 cells. This was performed prior to the start of the Expansion project and paid for with private funds. The results of this analysis were presented in the project narrative for the Expansion award. As expected, genes downstream of NF- κ B transcriptional activity were inhibited in the presence of HSC-1. Pathways specifically inhibited included “Granulocyte Adhesion and Diapedesis”, Inhibition of Matrix Metalloproteases”, “Oncostatin M signaling” and “Role of IL-17A in Arthritis”. This analysis confirmed our screening assay results that the target of HSC-1 lies in the NF- κ B pathway.

To identify potential interacting partners, we assayed the regulation of a suite of proteins in the NF- κ B pathway using a commercially available proteome profiler assay (Cell Signaling). This assay measures total protein levels, not phosphorylation states (**Figure 2A**). We found that pre-treatment with HSC-1 reduced the total level of several proteins, including SHARPIN, I κ B α and JNK2 following TNF stimulation. To more closely investigate the impact of HSC-1 on I κ B α levels we performed Western blot analysis. Pre-treatment with HSC-1 prevented the near complete loss of I κ B α induced by TNF, suggesting that HSC-1 activity occurred upstream of I κ B α (**Figure 2B**). Exploring canonical NF- κ B signaling upstream of I κ B α ubiquitination led us to investigate whether IKK α and IKK β were the interacting partners for HSC-1. Using a cell-free enzymatic assay we assessed the activity of IKK α

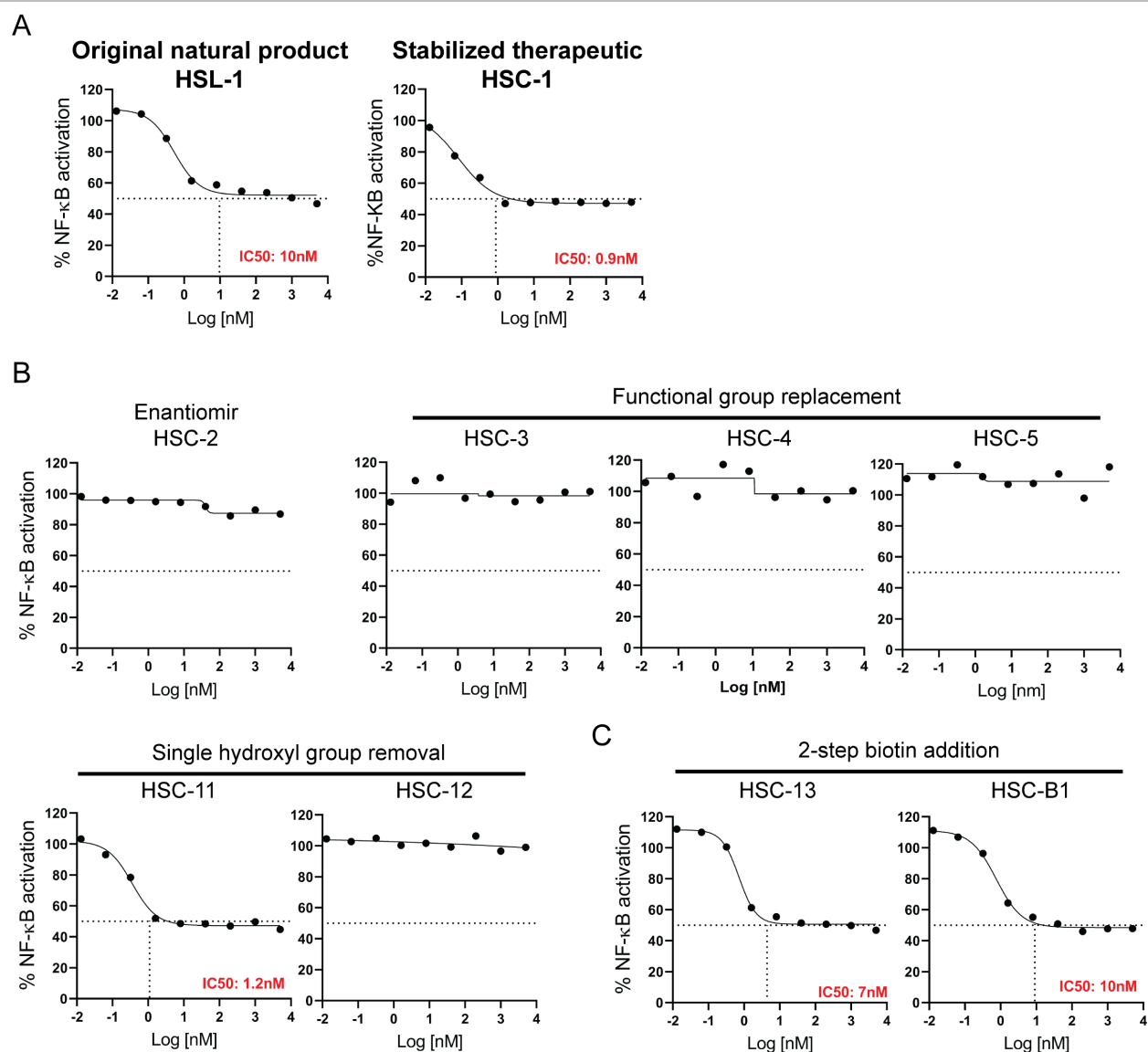


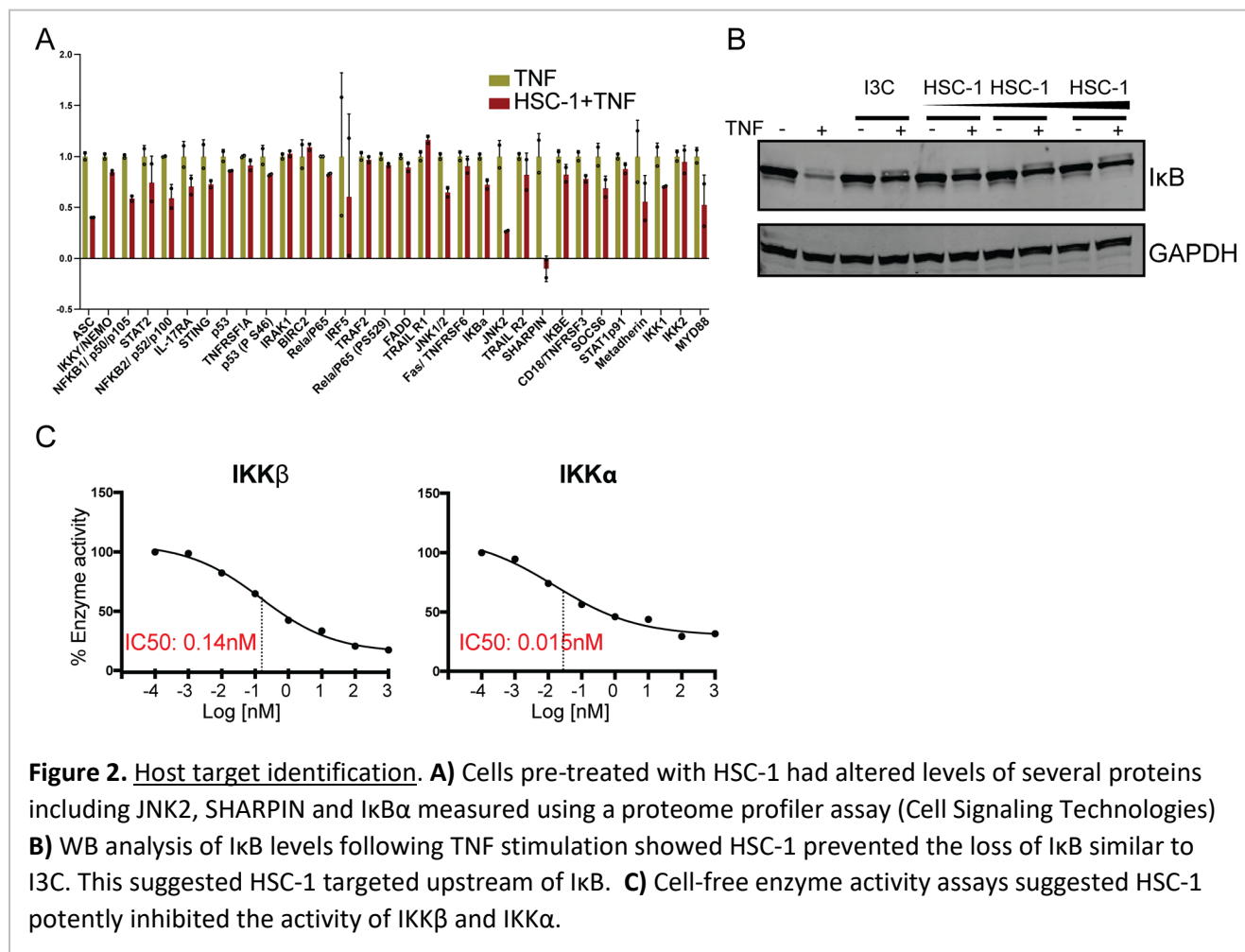
Figure 1. Structure-activity relationship of therapeutically optimized compound HSC. **A)** Synthesis of a putative structure of the natural product (HSL-1) had high NF- κ B inhibitory activity. Modification to stabilize the molecule increased inhibitory activity (HSC-1). **B)** Altering various aspects of the compound abolished activity (HSC-2-5, 12) or had no effect (HSC-11). **C)** Addition of a biotin tag (in a two-step “click” process) reduced NF- κ B activity compared to the untagged molecule but has sufficient activity to be used in target identification assays.

and IKK β in the presence of HSC-1 (**Figure 2C**). We found that HSC-1 was able to inhibit both IKK α and IKK β in the picomolar range.

Thus, we have preliminary evidence that the target of HSC-1 is IKK α and to a lesser extent, IKK β .

To confirm these results, we have generated tools for further proteomic analysis of HSC-protein interactions.

HSC Affinity Probes – To assist in identifying the molecular target we are currently developing a synthetic approach to prepare biotin tagged HSC probes that retain target bioactivity. These would then be used as affinity probes to pull down protein binding partners. To date we have already successfully synthesized an HSC biotin probe that retains activity and is suitable for protein binding studies (**Figure 1C**).



Major Task 1. Subtask 5: Measure microbiota interaction with Hit #1.

How the microbiota is impacted by and interacts with both the natural product (HSL) and our therapeutic compound (HSC) is crucial for understanding the potential long lasting efficacy and off-target effects of the compounds *in vivo*. As the compounds are bacterially derived, it is conceivable they can impact the growth of other bacteria. What this may mean for the microbiota composition when given as a treatment needs investigation. Towards this aim, we have generated tools to be used in stool and mucosal gut microbial

community cultures to examine the metabolism of the compounds by the microbiota and the impact on bacterial activity by the compounds.

HSC/HSL Deuterated Probes. To detect both HSL and HSC chemistry in complex biological samples, we are synthesizing a series of deuterated probes – that differ from the un-deuterated bioactives by 4 atomic mass units. Such probes can be used in *in vivo* animal experiments, human specimens and in microbial cultures to detect and quantify levels of HSC/HSL either naturally occurring or introduced by treatment. We have successfully implemented a synthetic approach that allows us to quickly prepare deuterated analogues of any HSC/HSL structure (**data not shown**).

Major Task 2&3. Subtask 1 – Determine efficacy of Hit #2 and Hit #3 in colitis animal models

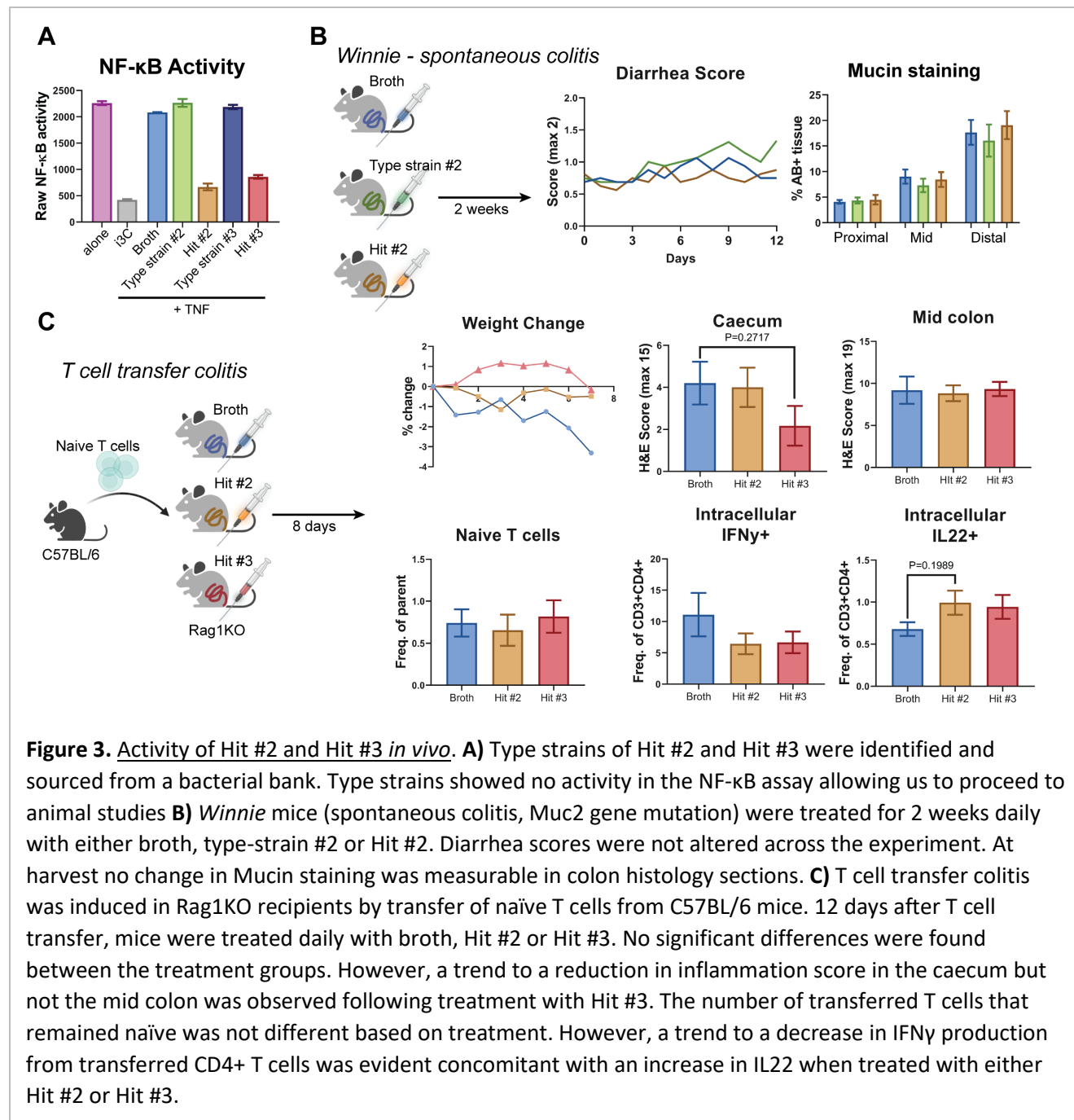


Figure 3. Activity of Hit #2 and Hit #3 *in vivo*. **A)** Type strains of Hit #2 and Hit #3 were identified and sourced from a bacterial bank. Type strains showed no activity in the NF-κB assay allowing us to proceed to animal studies **B)** Winnie mice (spontaneous colitis, Muc2 gene mutation) were treated for 2 weeks daily with either broth, type-strain #2 or Hit #2. Diarrhea scores were not altered across the experiment. At harvest no change in Mucin staining was measurable in colon histology sections. **C)** T cell transfer colitis was induced in Rag1KO recipients by transfer of naïve T cells from C57BL/6 mice. 12 days after T cell transfer, mice were treated daily with broth, Hit #2 or Hit #3. No significant differences were found between the treatment groups. However, a trend to a reduction in inflammation score in the caecum but not the mid colon was observed following treatment with Hit #3. The number of transferred T cells that remained naïve was not different based on treatment. However, a trend to a decrease in IFN γ production from transferred CD4+ T cells was evident concomitant with an increase in IL22 when treated with either Hit #2 or Hit #3.

Based on results generated in the Discovery project, we prioritized 2 further bacterial strains that produced supernatants with similar NF- κ B inhibitory activity as Hit #1 (whose anti-inflammatory product is HSL/HSC). We chose strains from different bacterial species, as we aimed to decrease the chance of identifying a similar chemical produced by a bacterial relative. We were able to source and import closely related type strains of Hit #2 and Hit #3. When tested in our NF- κ B assay these type strains did not have suppressive activity, making Hit #2 and Hit #3 amenable to mass spec compound identification (**Figure 3A**). Subsequently, we tested these supernatants in two different models of colitis.

Winnie mice have a mutation in their *Muc2* gene which prevents proper protein folding. This reduces the amount of protective mucin secreted by epithelial cells and induces endoplasmic reticulum stress due to presence of mis-folded mucin. By 6-8 weeks of age these mice develop diarrhea with histologic inflammation and crypt hypertrophy in the colon. We previously determined that the supernatant produced by Hit #1 decreased disease severity in this model by reducing diarrhea and increasing the amount of protective mucin staining in colon histology sections. We tested Hit #2 in this model. Despite activity in our NF- κ B bioassay we did not observe significant reductions in diarrhea, or improvements in histological mucin staining after treatment. However, we do notice a trend to reductions in diarrhea and histological mucin staining in females, with no difference observed in males (**Figure 3B**).

T cell transfer (C57BL/6 $\rightarrow$ Rag1KO). In this model naïve T cells are transferred from WT mice into Rag1KO mice which lack both T and B cells. Activated T cells drive inflammation in this model with signs of overt colitis observed within 3 weeks in our facility. We treated mice with supernatants produced by Hit #2 and Hit #3 starting 12 days after T cell transfer for 8 days. Early results from this experiment do not show significant improvement in the mice treated with either active supernatant compared to the control treated mice. However, there are interesting trends in the data that we hope will reach significance with increasing numbers of animals and matching for T cell engraftment. These include a trend of improved histology score in the caecum along with reduced numbers of transferred T cells producing IFN γ and an increase in transferred T cells producing IL-22 (**Figure 3C**).

Major Task 2&3. Subtask 2-5.

Identification, optimization, target identification, pharmacokinetics and microbiota interactions with Hit #2 and Hit #3 all follow on from Subtask 1. As such, we have not begun these investigations. At this stage in the project, we are on schedule.

○ **What opportunities for training and professional development has the project provided?**

Nothing to Report

○ **How were the results disseminated to communities of interest?**

Nothing to Report

○ **What do you plan to do during the next reporting period to accomplish the goals?**

Major Task 1.

In the next year we anticipate continuing our advancements at the pace outlined in the SOW. The biotinylated probes will be used to definitively identify the host target proteins through pull-down and proteomic

assessment. *In vitro* subcellular localization and *in vivo* accumulation in the cellular compartments of organ systems will also be assessed.

Definitive identification of the natural bacterial product (HSL) will continue. This work will inform our assessment of levels in human specimens. It will also allow us to determine how the production of this compound influences bacteria-bacteria interactions in the gut environment.

Microbial responses to the therapeutic compound (HSC) will begin. We have synthesized sufficient quantities of HSC to begin testing in the microbial consortium growth conditions we have previously optimized. Analysis of the impact on bacterial transcriptomics and community composition (metagenomic) is not trivial, and this will extend beyond the next year. As soon as we have verified the structure of the natural product (HSL) we will begin testing the effect of this in the microbial cultures.

Major Task 2&3.

Animal studies to determine efficacy of Hit #2 and Hit #3 will continue. Currently, our results do not conclusively demonstrate efficacy, although with increased numbers to reduce the inherent variability in the models this may improve. If we do not find that the supernatants can convincingly decrease disease severity in the animal models, we will move on to test other bacterial supernatants which were proven to have efficacy in the Discovery project.

4. IMPACT:

○ What was the impact on the development of the principal discipline(s) of the project?

The gut microbiome is an untapped reservoir of bioactive compounds that have the potential to promote homeostasis in the gut, impact distant organ systems and alter or maintain the structural composition of microbiome itself. At the heart of this project is the drive to understand how the microbiota maintains (or fails to maintain) its own balance and a beneficial relationship with the host. Our previous work identified several different strains of bacteria that produce small molecules capable of directly impacting host inflammatory signaling. In this project we aim to develop those molecules for potential use in human clinical trials, but also use them to understand how they may be impacting other members of the microbiota and how this can also drive health or disease in the host.

This project is only five months into its first year of work, and yet has achieved excellent progress to date. Advancements in chemical synthesis methods have allowed us to quickly generate a series of compound derivatives. These derivatives have revealed the crucial structural elements of the compound that confer anti-inflammatory activity. We have also been able to identify non-critical structures that can be modified to produce tagged probes which we will use in all aspects of target identification and *in vivo* pharmacodynamic and kinetic studies. Additionally, the importance of identifying the natural product will be significant. If we can identify this novel compound in human specimens it may correlate with health or disease severity and may prove a useful biomarker.

○ What was the impact on other disciplines?

Nothing to report

○ What was the impact on technology transfer?

Nothing to report

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The start of the project was delayed until 31 January 2022 due to the requirement for legal agreements between the institutions. We were unable to recruit staff until agreements had been reached. However, at this stage (5 months into the funded project) 2 experienced staff members have been recruited, one has already started, and one will start beginning in August. The delay has cost us 7 months, but we are now running at capacity for the project. We anticipate requiring a no-cost extension to the final report to cover the delay in recruitment and start-up caused by the lengthy legal agreements process.

- **Changes that had a significant impact on expenditures**

Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report

- **Significant changes in use or care of human subjects**

Nothing to report

- **Significant changes in use or care of vertebrate animals.**

Nothing to report

- **Significant changes in use of biohazards and/or select agents**

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

- **Journal publications.**

- Results, partly funded by the Discovery project, were recently published. Giri et al., 2022, Cell Reports 39, 110646 (doi.org/10.1016/j.celrep.2022.110646)

- **Books or other non-periodical, one-time publications.**

- Nothing to report

- **Other publications, conference papers, and presentations.**

- Nothing to report

○ **Website(s) or other Internet site(s)**

- At the university level, the *Cell Reports* article was featured in the Faculty of Medicine online newsletter *The Bulletin* (https://comms.uq.edu.au/v/46360/21277616/email.html?k=Dsds_iAcJ7Z_kFS8FYBL5lvkSJX-YOqGFYr3FzHhxRQ).
- Nationally, the *Cell Reports* article was featured on a major news network (7 News) public health news section (<https://7news.com.au/news/public-health/gut-bacteria-breakthrough-for-ibd-patients-c-7396568>) and A/Prof Begun was interviewed for the national news (ABC) on the use of probiotics to treat gut ailments. <https://www.abc.net.au/news/2022-05-25/gut-health-gimmicks-probiotics-versus-prebiotics/100742328>

○ **Technologies or techniques**

Nothing to Report

○ **Inventions, patent applications, and/or licenses**

Nothing to report

○ **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

○ **What individuals have worked on the project?**

Name	A/Prof Jakob Begun
Role	PI/PD
ORCID ID	0000-0001-5256-7672
Nearest person month worked	1 (no salary drawn from project funds – in kind support)
Contribution to project	<i>A/Prof Begun is the coordinator of the project. He has overseen bi-weekly meetings on the project and co-ordinates the collaboration amongst all the participants</i>
Funding support	
Name	Prof Robert Capon
Role	Co-I
ORCID ID	0000-0002-8341-7754
Nearest person month worked	0.5
Contribution to project	<i>Prof Capon co-ordinates and supervises the synthesis of the compounds required for all aspects of the project</i>
Funding support	0.4 FTE UQ, 0.5 FTE MBCRC, 0.1 FTE USDOD IBD
Name	Prof Mark Morrison
Role	Co-I
ORCID ID	0000-0001-9257-9133

Nearest person month worked	0.5
Contribution to project	<i>Prof Morrison co-ordinates and supervises the microbial community assessments.</i>
Funding support	
Name	Dr. Julie Davies
Role	Co-I
ORCID ID	0000-0003-4035-6047
Nearest person month worked	4.5
Contribution to project	<i>Dr Davies co-ordinates the regulatory requirements for the project. She organizes and performs the animal experiments for Hit #2 and Hit #3</i>
Funding support	No change
Name	Waleed Hussein
Role	Senior Research Officer
ORCID ID	0000-0001-5392-1880
Nearest person month worked	2
Contribution to project	<i>Dr Hussein commenced the synthesis of Hit #1 and related analogues to obtain structure activity relationship data, and developed biotin tagged probes to identify the molecular target and mechanism of action. Several analogues have been synthesized.</i>
Funding support	
Name	Rabina Giri
Role	Graduate Student (Ph D)
ORCID ID	0000-0002-6712-6076
Nearest person month worked	3
Contribution to project	<i>Ms Giri has performed all the bioassays for Hit #1, including SAR and tagged compounds as part of her PhD project. RNAseq, proteome profiling and the kinase assays for the target identifications were also performed by Ms. Giri.</i>
Funding support	UQ Research Training Program (RTP) scholarship

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

PI/PD Jakob Begun: no change

Co-I Robert Capon: no change

Co-I Mark Morrison: no change

Co-I Julie Davies: no change

- **What other organizations were involved as partners?**

None

8. SPECIAL REPORTING REQUIREMENTS

○ **COLLABORATIVE AWARDS:**

Not applicable

○ **QUAD CHARTS:**

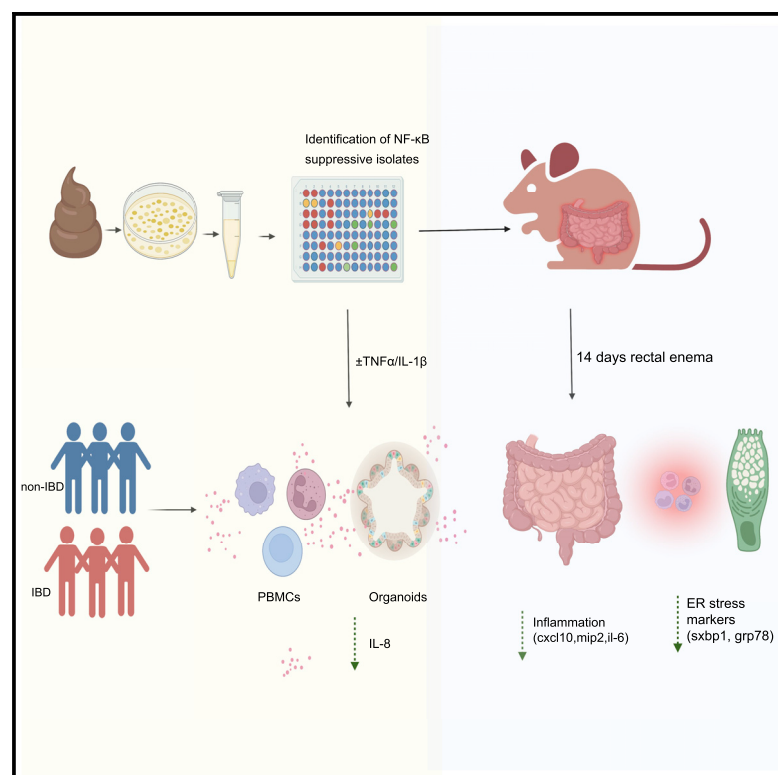
Not applicable

9. APPENDICES:

None

Secreted NF- κ B suppressive microbial metabolites modulate gut inflammation

Graphical abstract



Authors

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In brief

The ability of gut bacteria to modulate inflammation remains cryptic. Using anaerobic cultures, Giri et al. identify multiple Firmicutes-affiliated bacteria capable of reducing NF- κ B-mediated inflammatory signaling in a strain-dependent manner. Cell-free culture supernatants reduce inflammation in patient-derived organoids and in a pre-clinical model of colitis.

Highlights

- Select strains of *Clostridium* from human stool samples can suppress NF- κ B activation
- The immunomodulatory effect of bacteria can be strain- and media-specific
- Inter-patient variation suggests possible development of personalized medicine
- *In vitro* immunosuppression assays using organoids predict *in vivo* activity



Article

Secreted NF- κ B suppressive microbial metabolites modulate gut inflammation

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SUMMARY

Emerging evidence suggests that microbiome-host crosstalk regulates intestinal immune activity and predisposition to inflammatory bowel disease (IBD). NF- κ B is a master regulator of immune function and a validated target for the treatment of IBD. Here, we identify five *Clostridium* strains that suppress immune-mediated NF- κ B activation in epithelial cell lines, PBMCs, and gut epithelial organoids from healthy human subjects and patients with IBD. Cell-free culture supernatant from *Clostridium bolteae* AHG0001 strain, but not the reference *C. bolteae* BAA-613 strain, suppresses inflammatory responses and endoplasmic reticulum stress in gut epithelial organoids derived from Winnie mice. The *in vivo* responses to *Clostridium bolteae* AHG0001 and BAA-613 mirror the *in vitro* activity. Thus, using our *in vitro* screening of bacteria capable of suppressing NF- κ B in the context of IBD and using an *ex vivo* organoid-based approach, we identify a strain capable of alleviating colitis in a relevant pre-clinical animal model of IBD.

INTRODUCTION

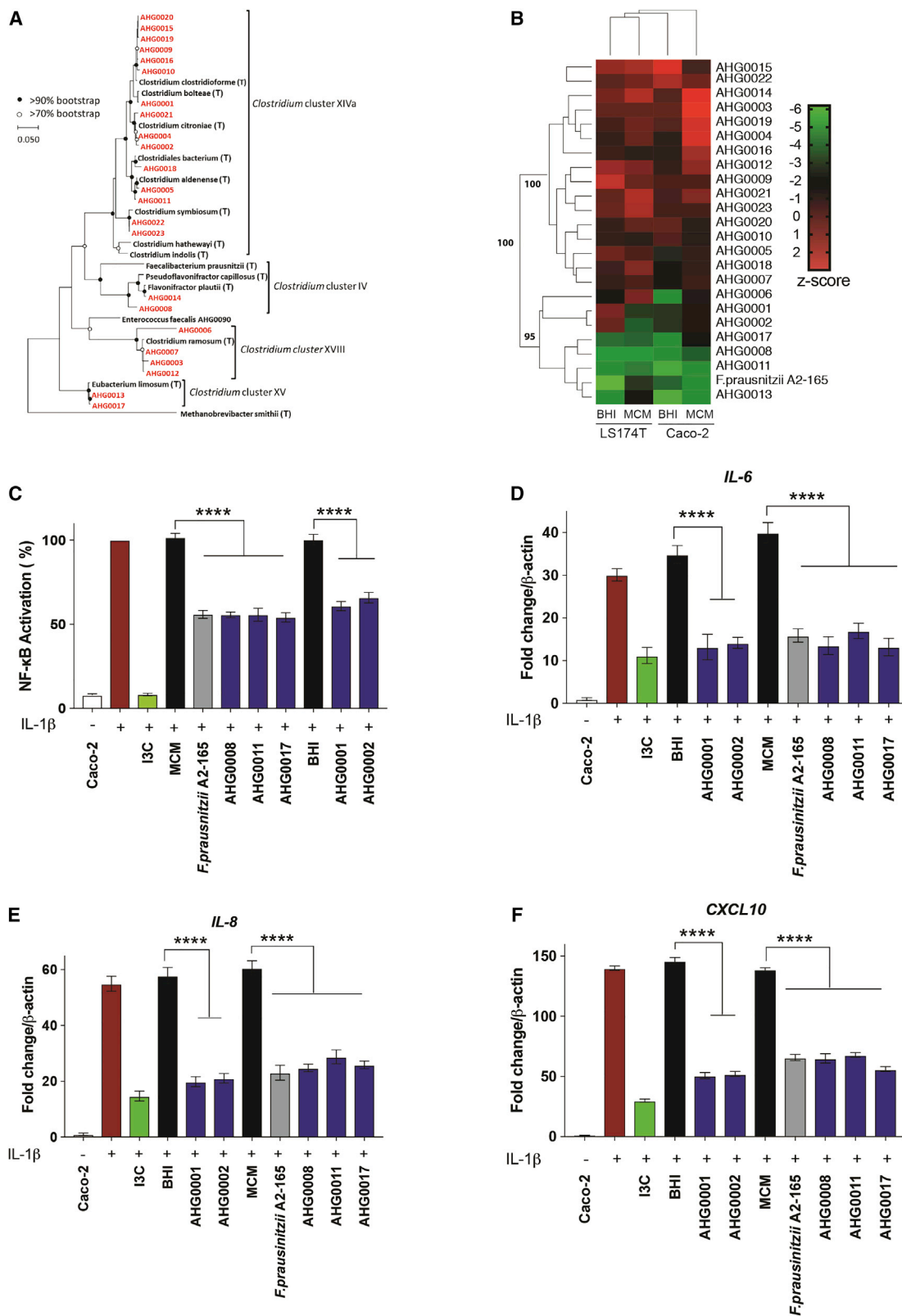
The human gut is the largest immune organ of the body, and gut epithelial cells play a key role in the establishment and maintenance of gut homeostasis, as well as rapid responses to infection (Peterson and Artis, 2014). The gut is colonized by a diverse microbiota that has co-evolved with its host and forms a symbiotic relationship through its modulation of innate and adaptive immune responses (Geva-Zatorsky et al., 2017; Kabat et al., 2014). However, with a few notable exceptions (Mazmanian et al., 2008; Wlodarska et al., 2017), the microbes and microbial determinants of immune homeostasis remain cryptic.

Inflammatory bowel diseases (IBDs) are composed of two predominant subtypes, Crohn disease (CD) and ulcerative colitis (UC), that are characterized by relapsing and remitting gut inflammation. The nuclear factor- κ B (NF- κ B) family of transcription factors are master regulators of gut epithelial integrity and inflammation and activation of antigen-presenting cells and effector leukocytes. Upon activation, NF- κ B dimers translocate to the nucleus, where they regulate transcription of a wide range of genes, including those involved in immune and inflammatory

responses (Wullaert et al., 2011). In the healthy gut, NF- κ B activation is tightly regulated (Renner and Schmitz, 2009), but several IBD genetic risk alleles, including *NOD2*, *TOLLIP*, and *A20*, exert their pathogenic effects at least in part through dysregulated NF- κ B signaling (Zaidi and Wine, 2018). Additionally, macrophages and epithelial cells isolated from the inflamed intestine of patients with IBD show increased activation, and nuclear localization of NF- κ B-p65 (Rogler et al., 1998). NF- κ B signaling contributes significantly to multiple host responses underlying the pathogenesis of IBD.

The gut microbiota is increasingly recognized as an important contributory risk factor for IBD development and activity, as healthy and IBD gut microbiota differ and are characterized by structure and function alterations (Costello et al., 2017; Wilson et al., 2019). Several bacterial taxa are abundant in the healthy gut that can suppress inflammatory responses and alleviate inflammation in animal models of disease (Sokol et al., 2008; Eeckhaut et al., 2012; Takeshita et al., 2016). These “anti-inflammatory” properties are best characterized for the gut bacterium *Faecalibacterium prausnitzii* A2-165, which produces secreted peptides derived from the Mam protein that suppress NF- κ B





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in vitro and *in vivo* via stabilization of the IKK complex (Quevrain et al., 2016). However, although Firmicutes-affiliated Clostridia are among the most abundant and functionally diverse gut bacteria, Mam expression is largely restricted to members of *Faecalibacterium* spp., and much remains to be discovered about the immunomodulatory capacities inherent to other Firmicutes.

Here, we identified five new Firmicutes isolates that are comparable or superior to *F. prausnitzii* A2-165 in their NF- κ B suppressive activity and whose activities are characterized by strain-specific differences. Notably, these bacteria suppressed cytokine-mediated IL-8 secretion in CD and UC gut-derived organoid cultures and peripheral blood mononuclear cells (PBMCs). On the basis of these observations, we demonstrated using two *Clostridium bolteae* strains and *ex vivo* assays that *ex vivo* activity in organoids predicts immunomodulatory bioactivity *in vivo* using the Winnie murine model of spontaneous colitis. Furthermore, using an activity-guided high-performance liquid chromatography (HPLC) purification method, we have shown that non-polar and hydrophobic bacterial supernatant derived fractions from *Clostridium bolteae* can suppress both cytokine and lipopolysaccharide (LPS)-driven chemokine/cytokine expression on human organoids and PBMCs, as well as non-canonical NF- κ B *in vitro* and *in vivo*. These data demonstrate the potential of bioprospecting the human microbiome for novel therapeutic leads targeting immunologically relevant pathways for treating gut inflammation.

RESULTS

Gut clostridia can suppress NF- κ B

We assessed the NF- κ B suppressive capacity of cell-free supernatants (CS) derived from 23 Firmicutes-affiliated gut bacteria previously isolated by metaparental mating (Ó Cuív et al., 2015). The isolates were principally affiliated with *Clostridium* cluster XIVa, with several isolates also affiliated with clusters IV, XV, and XVIII (Figure 1A).

We assessed the ability of individual isolates to suppress NF- κ B activation using LS174T goblet cell-like and Caco-2 enterocyte-like reporter cell lines stimulated with IL-1 β and TNF- α , respectively, which have previously been shown to stimulate NF- κ B activity in these cell-lines (Ó Cuív et al., 2017). A Z factor of 0.805 ± 0.06 (modified clostridial medium [MCM]) and 0.87 ± 0.01 (brain heart infusion [BHI]) was achieved for the LS174T cells, while a Z factor of 0.78 ± 0.057 (MCM) and 0.765 ± 0.02 (BHI) was achieved for the Caco-2 cells. CS prepared from isolates grown to stationary phase in modified clostridial medium

or brain heart infusion medium were assessed for their ability to suppress cytokine-induced NF- κ B activation. The dilutions of CS were chosen on the basis of the dose-response experiment performed on each cell line and the maximal non-suppressive baseline dilution selected (Figures S1A–S1D). In addition to *F. prausnitzii* A2-165, the isolates *C. bolteae* AHG0001, *Clostridium citroniae* AHG0002 *Pseudoflavonifractor* sp. AHG0008, *Clostridium aldenense* AHG0011, *Eubacterium limosum* AHG0013, and *E. limosum* AHG0017 suppressed NF- κ B in both cell lines when grown in MCM and/or BHI (Figure 1B; Z score ≤ -3). The first-pass screen was confirmed for selected isolates following growth in MCM or BHI, with NF- κ B suppression observed in both cell lines (Figures 1C and S2A). Consistent with the reporter assay results, all the isolates suppressed induction of the NF- κ B-regulated genes *IL-6*, *IL-8*, and *CXCL10* in Caco-2 (Figures 1D–1F) and LS174T (Figure S2B) cells following cytokine stimulation. Critically, none of the CS exhibited cytotoxic effects at the dilutions used (Figures S2C and S2D) and did not affect cell proliferation (Figure S2E). Although short-chain fatty acids are posited to suppress gut inflammation, similar to previous reports (Lakhdari et al., 2011), the addition of up to 16 mM of the short-chain fatty acids acetate, butyrate, and propionate actually enhanced cytokine-driven NF- κ B activation (Figures S2F–S2K).

NF- κ B suppression is strain-specific

Having confirmed their suppressive activity, we next examined the intraspecies variations in NF- κ B suppressive capacity. Isolates *C. bolteae* AHG0001 and American Type Culture Collection (ATCC) BAA-613 (OTU1), *C. citroniae* AHG0002 and AHG0004 (OTU2), and *C. aldenense* AHG0011 and AHG0005 (OTU3) are assigned to the same operational taxonomic units (Figure 1A; $\geq 97\%$ 16S rRNA sequence identity). However, these OTUs were characterized by marked intraspecies differences in their NF- κ B suppressive capacities under standard growth conditions (Figures 2A–2C). We therefore examined the effect of growth medium on the suppressive effects of *C. bolteae* AHG0001 and *C. citroniae* AHG0002. CS prepared from *C. bolteae* AHG0001 and *C. citroniae* AHG0002 following growth in MCM but not BHI suppressed TNF- α -driven mediated NF- κ B activation in LS174T cells (Figure 2D). Conversely, CS prepared from these strains following growth in BHI but not MCM suppressed IL-1 β -driven NF- κ B activation in Caco-2 cells (Figure 2E). Thus, NF- κ B suppressive functionality is strain-specific, and nutritional growth conditions influence bioactive production *in vitro* and by extension may affect the production of anti-inflammatory activity

Figure 1. Gut clostridia can suppress NF- κ B

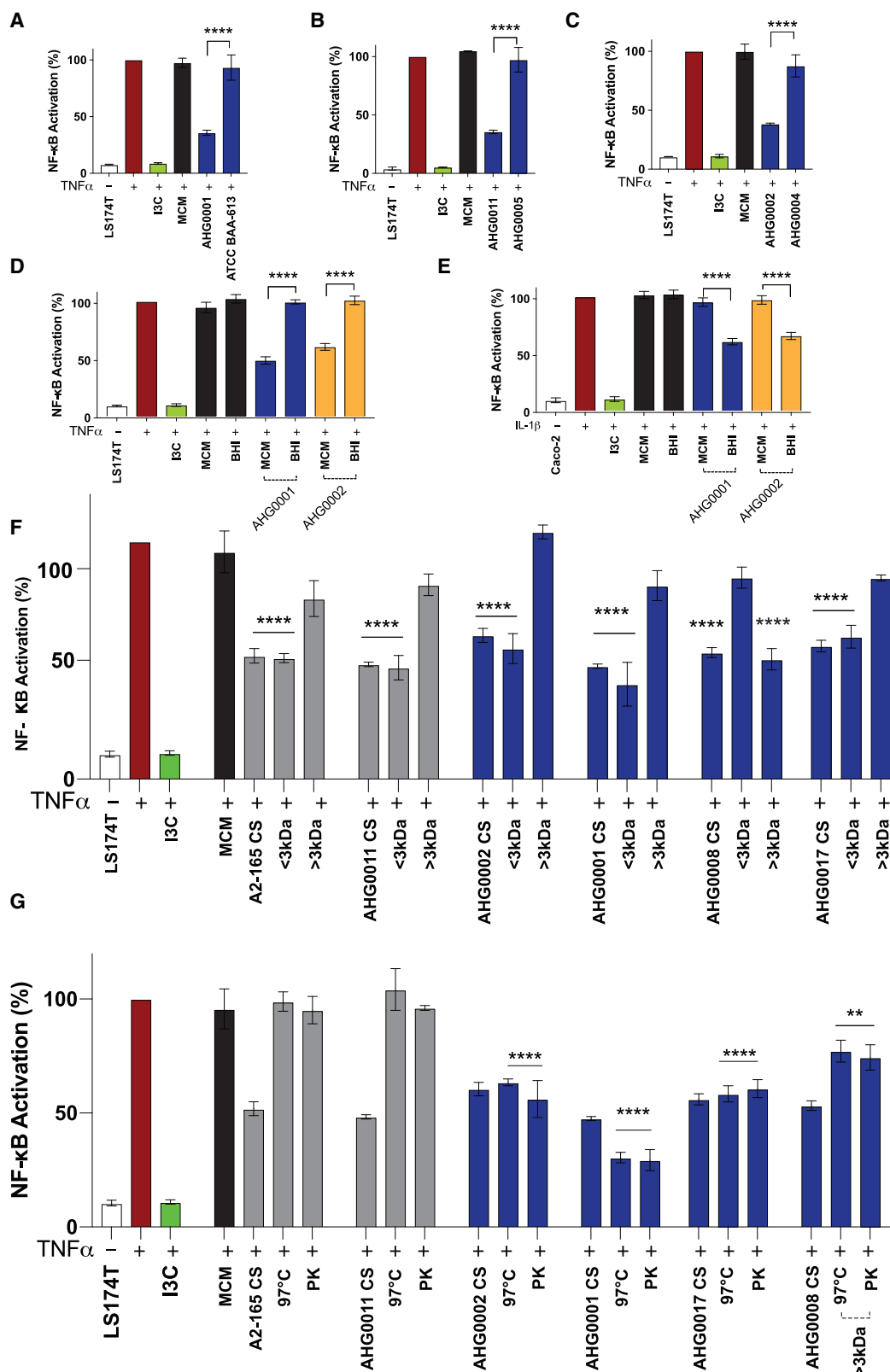
(A) 16S rRNA-based phylogeny of the MPM isolates characterized in this study (red typeface) and representative microbial isolates and reference sequences (bold black typeface).

(B) Heatmap analysis of the NF- κ B suppressive capacity of the bacterial isolates. The ability of CS prepared from bacterial isolates grown in modified clostridia medium (MCM) or brain heart infusion (BHI) to suppress NF- κ B in LS174T or Caco-2 reporter cell lines was assessed twice independently. A subset of strains formed an NF- κ B suppressive cluster with *F. prausnitzii* A2-165.

(C) Caco-2-based confirmatory assay of the hits identified from the first-pass screen. NF- κ B activation was assessed 4 h after IL-1 β stimulation, and the extent of suppression was assessed against sterile medium (mean [SD]). Indole-3-carbinol (I3C), a pharmacological inhibitor of NF- κ B, was used as positive control.

(D–F) Caco-2-based qRT-PCR confirmatory assay of the hits identified from the first-pass screen (mean [SD]). *F. prausnitzii* A2-165 and the validated hits suppress IL-1 β -induced *IL-6*, *IL-8*, and *CXCL10* expression in Caco-2 cells.

n = 2 independent experiment with n = 3 technical replicates each. **p < 0.01 and ****p < 0.0001, as determined using one-way ANOVA with Dunnett's multiple-comparison test.



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Table 1. Biosynthetic gene clusters from strains producing <3 kDa bioactive

Genome designation	CheckM marker lineage (order [GTDB branch])	Number of contigs	Genome size (bp)	GC (%)	CheckM completeness (%)	CheckM contamination (%)	Number of BGCs	NCBI accession number
<i>C. bolteae</i> AHG0001	Clostridiales (UID1342)	96	5,985,600	49.4	98.76	0.56	19	QYRW000000000
<i>C. citroniae</i> AHG0002	Clostridiales (UID1226)	149	6,630,634	48.8	99.37	0	25	QYRX000000000
<i>C. aldenense</i> AHG0011	Clostridiales (UID1226)	263	6,734,822	49.5	99.37	0	24	QYRY000000000
<i>E. limosum</i> AHG0017	Clostridiales (UID1120)	86	4,704,612	47.2	99.3	0.7	31	QYRZ000000000

in situ in the gut. In addition, the context of NF- κ B activation via IL-1 β or TNF- α may determine the suppressive effect of bioactives.

We examined our collection of suppressive CS using a combination of size fractionation, Proteinase K, and heat treatments to determine their biochemical characteristics. Using this approach, we determined that the NF- κ B suppressive activity for all strains except *Pseudoflavonifractor* sp. AHG0008 was predominantly associated with the <3 kDa fraction (Figures 2F, 2S, and 3A–3F). Gut bacteria produce a structurally diverse array of low-molecular weight NF- κ B suppressive bioactives (Quevrain et al., 2016; Ó Cuív et al., 2018; Kaci et al., 2011), and we focused on the <3 kDa fraction. These bioactives could be broadly separated into two classes on the basis of heat and protease sensitivity (e.g., *F. prausnitzii* A2-165 and *C. aldenense* AHG0011) or resilience (e.g., *C. bolteae* AHG0001, *C. citroniae* AHG0002, and *E. limosum* AHG0017), possibly inclusive of peptides and/or thermal and hydrolytically stable small molecules, respectively (Figures 2G and S3A–S3F). Interestingly, the suppressive activity of *C. bolteae* AHG0001 culture supernatant increased after heat and Proteinase K treatment, although this was not significant, which could be a result of degradation of any activating proteins or peptides produced by this strain. We performed genome sequencing of the strains producing <3 kDa bioactives to near completeness to identify candidate bioactive encoding biosynthetic gene clusters (BGCs) (Table 1). Phylogenetic analysis using the Genome Taxonomy Database (GTDB) confirmed the 16S rRNA-based assignments (Figure S4A). We also determined that the strains exhibited a high degree of genome synteny with their near relatives (Figures S4B–S4E) and carried multiple BGCs (Table 1). None of the isolates encoded *F. prausnitzii* Mam-like orthologs, which is consistent with its nar-

row phylogenetic distribution (Quevrain et al., 2016; Martín et al., 2017). No annotated BGCs were identified to explain the observed bioactivity.

CS suppress *ex vivo* IL-8 secretion

To assess whether the suppressive CS could affect epithelial inflammatory responses in primary cells in the context of IBD-associated genetic risk factors, we assessed their ability to prevent IL-1 β -driven IL-8 production in healthy (n = 6), CD (n = 5), and UC (n = 5) derived primary intestinal epithelial organoid cultures. Interestingly, despite removal from the inflammatory environment, there was significantly higher basal IL-8 production by organoids derived from patients with CD compared with those from non-IBD controls and patients with UC (Figure S5A). Following stimulation with IL-1 β , significantly more IL-8 was produced by organoids derived from UC but not CD patients compared with non-IBD subjects (Figure S5B). Treatment with *F. prausnitzii* A2-165 CS significantly suppressed IL-8 secretion compared with the MCM control in non-IBD and CD but not UC subjects (Figures 3A–3C). Treatment with suppressive CS from our identified Firmicutes strains significantly suppressed IL-8 secretion in non-IBD, CD, and UC subjects (Figures 3A–3C). There was a high degree of concordance in the degree of suppression between subjects treated with bacterial CS, although some subject-specific differences were noted (Figure S6A). We did not observe any significant cytotoxic effects from the CS treatments in these primary organoid cultures (Figures S6B–S6D).

In addition to effects on the epithelium, bioactives produced by gut bacteria may also be absorbed and have local or systemic effects on the immune compartment. Therefore, the suppressive effects of the CS on primary immune cells were examined using

Figure 2. NF- κ B suppression is strain-specific

(A–C) Characterization of intraspecies variation in NF- κ B suppressive capacity. The ability of *C. bolteae* AHG0001 and ATCC BAA-613 (A), *C. citroniae* AHG0002 and AHG0004 (B), and *C. aldenense* AHG0011 and AHG0005 (C) to suppress NF- κ B was analyzed using the LS174T reporter cells. NF- κ B activation was assessed 4 h after TNF- α stimulation, and the extent of suppression was assessed against sterile medium (mean [SD]).

(D and E) Characterization of the effect of growth medium on the NF- κ B suppressive capacity of *C. bolteae* AHG0001 and *C. citroniae* AHG0002 in LS174T (D) and Caco-2 (E) reporter cell lines. NF- κ B activation was assessed 4 h after cytokine stimulation, and the extent of suppression was assessed against sterile medium.

(F and G) Characterization of the bioactive factors produced by *F. prausnitzii* A2-165, *C. aldenense* AHG0011, *C. bolteae* AHG0001, *C. citroniae* AHG0002, *E. limosum* AHG0017, and *Pseudoflavonifractor* sp. AHG0008. The cell-free CS were untreated or subjected to size fractionation (F), heat, and/or Proteinase K treatments as appropriate (G). NF- κ B activation was assessed 4 h after TNF- α stimulation, and the extent of suppression was assessed against sterile medium (mean [SD]).

n = 2 independent experiment with n = 3 technical replicates each. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, as determined using one-way ANOVA with Dunnett's multiple-comparison test.

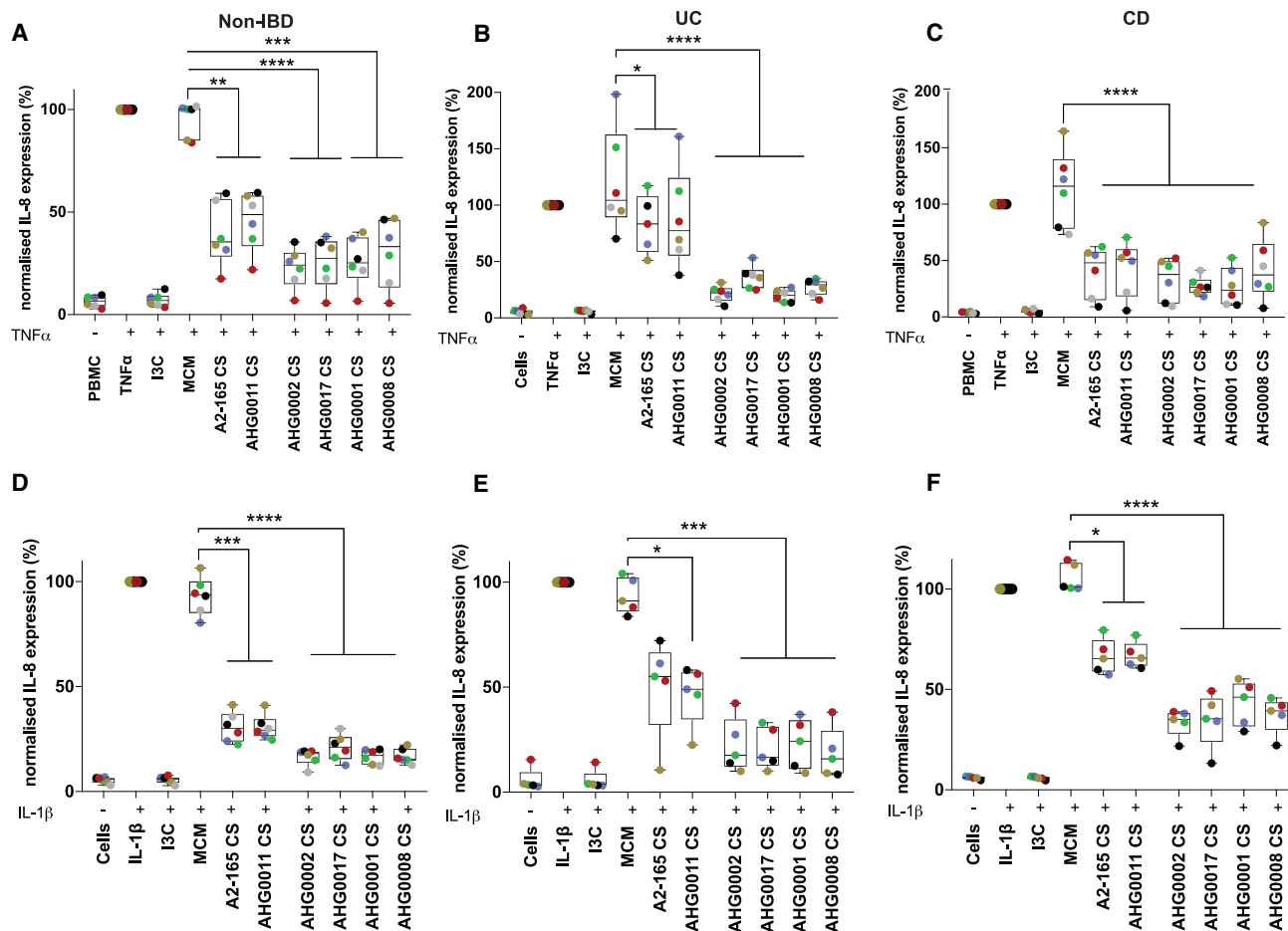


Figure 3. CS suppress ex vivo IL-8 secretion

(A–C) Analysis of the ability of *F. prausnitzii* A2-165, *C. aldenense* AHG0011, *C. citroniae* AHG0002, *E. limosum* AHG0017, *C. bolteae* AHG0001, and *Pseudo-flavonifactor* sp. AHG0008 to suppress IL-8 secretion in non-IBD (A), UC (B), and CD (C) subject-derived gut epithelial organoids. IL-8 secretion was assessed 24 h after cytokine stimulation and compared against the sterile medium (mean [SD]).

(D–F) Analysis of the ability of *F. prausnitzii* A2-165, *C. aldenense* AHG0011, *C. citroniae* AHG0002, *E. limosum* AHG0017, *C. bolteae* AHG0001, and *Pseudo-flavonifactor* sp. AHG0008 to suppress IL-8 secretion in non-IBD (D), UC (E), and CD (F) subject-derived PBMCs. IL-8 secretion was assessed 24 h after cytokine stimulation and compared against the sterile medium (mean [SD]).

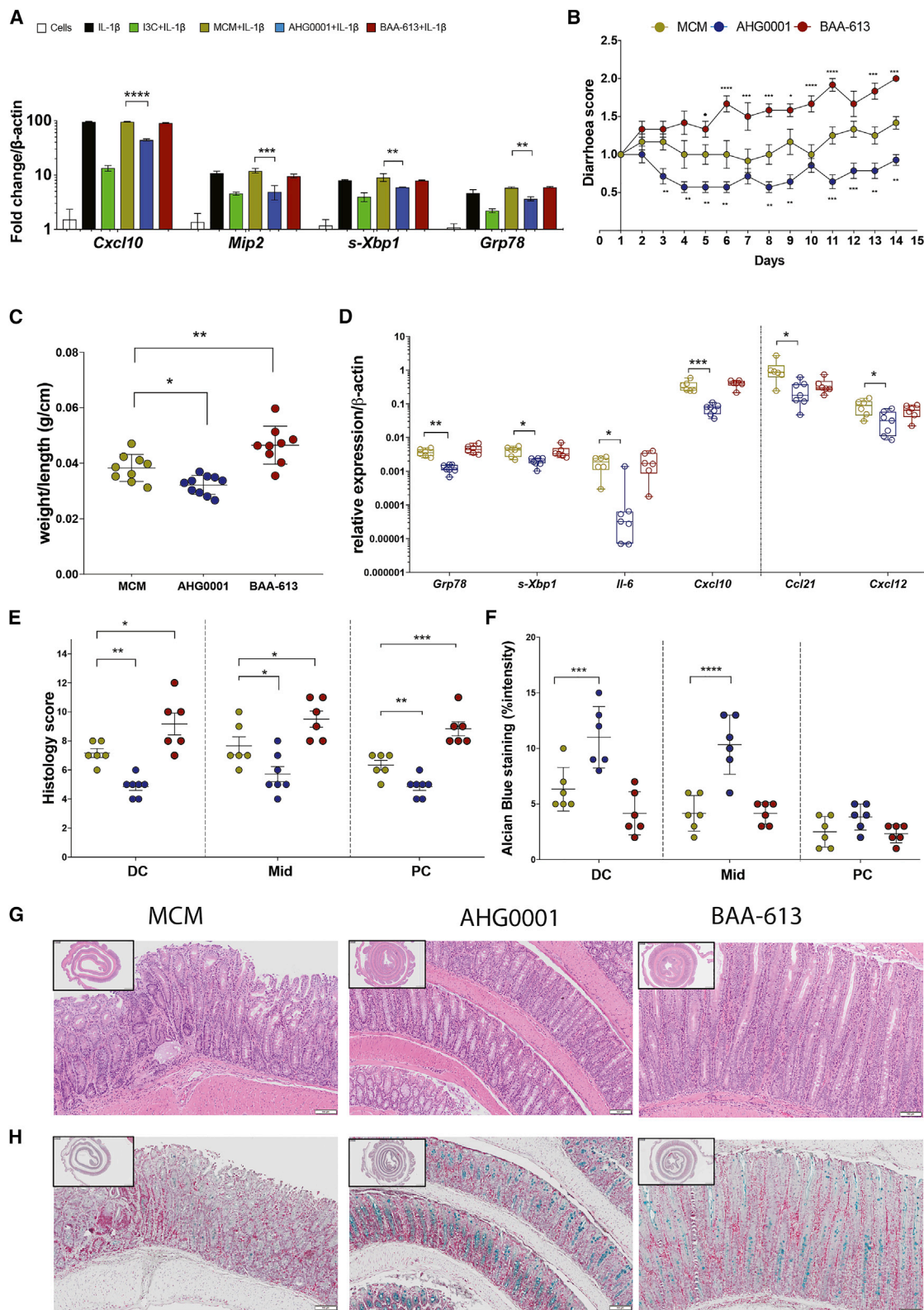
Each dot represents an individual subject. n = 3 technical replicates per subject. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, as determined using one-way ANOVA with Dunnett's multiple-comparison test.

PBMCs collected from non-IBD, CD, and UC (n = 6 per group) subjects. Although the basal concentrations of IL-8 released by PBMC from all three groups were not significantly different (Figure S5C), stimulation with TNF- α resulted in more IL-8 released from the PBMCs of the CD group in comparison with the non-IBD or UC group (Figure S5D). As expected, IL-8 secretion by PBMCs from non-IBD, CD, and UC subjects was significantly inhibited by I3C and *F. prausnitzii* A2-165 CS (Figures 3D–3F). Similarly, IL-8 secretion by PBMCs from non-IBD, CD, and UC subjects was suppressed by treatment with suppressive CS at least as effectively as *F. prausnitzii* A2-165 (Figures 3D–3F). There was limited variation in the response to CS within the non-IBD, CD, and UC subject groups, although there were some subjects that showed varying levels of suppression with individual CS (Figure S7A). We also did not observe any significant cytotoxic effects from the CS treatments on PBMCs

(Figures S7B and S7C). Collectively, these results show that the suppressive CS can blunt cytokine-mediated inflammatory responses in the gut epithelial and immune compartments in both an IBD and non-IBD genetic background without significant cellular toxicity.

Effectiveness of culture supernatants in murine colitis

The NF- κ B pathway is highly conserved in mammals, and we next examined the ability of the CS to suppress IL-1 β -induced expression of the NF- κ B-regulated chemokine genes *Mip-2* and *Cxcl-10* in murine colonic organoids derived from C57BL/6 mice. All the CS tested suppressed chemokine induction, suggesting that the bioactives likely act through conserved mammalian cell targets (Figures S8A and S8B). We also examined the ability of the CS to suppress expression of *Mip-2* and *Cxcl-10* in organoids derived from Winnie mice (Figures S8C and S8D).



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Winnie mice carry a missense mutation in *Muc2* that results in protein misfolding, increased endoplasmic reticulum (ER) stress, and defects in gut barrier function. These mice develop a spontaneous colitis characteristic of UC and are an excellent pre-clinical model for human treatments (Oancea et al., 2017; Das et al., 2013; Wang et al., 2015). We found that the majority of CS significantly suppressed IL-1 β -induced expression of *Mip-2* and *Cxcl-10* on *Winnie*-derived organoids. However, in contrast to the findings in wild-type organoids, CS from *C. aldenense* AHG0011 and *F. prausnitzii* A2-165 did not suppress *Mip-2* and *Cxcl-10* (Figures S8C and S8D). Furthermore, using *Winnie*-derived gut epithelial organoids, we determined that *C. bolteae* AHG0001 but not *C. bolteae* ATCC BAA-613 CS suppressed induction of *Mip-2* and *Cxcl-10* expression, confirming the strain-specific differences observed in the reporter cell lines (Figure 4A). Interestingly, *C. bolteae* AHG0001 but not *C. bolteae* ATCC BAA-613 CS suppressed induction of the ER stress markers, *Grp78* and *sXbp1*, in *Winnie* organoids (Figure 4A), which is believed to be the upstream driver of intestinal inflammation in this model.

We hypothesized that functional capacity rather than phylogeny would be the principle determinant of therapeutic efficacy and that primary organoid cultures could be used to predict *in vivo* host responses to select CS. To test this, CS prepared from *C. bolteae* AHG0001 and ATCC BAA-613 were administered intrarectally for 14 days to 6-week-old *Winnie* mice with established colitis. *C. bolteae* AHG0001 CS significantly reduced diarrhea scores over the course of the experiment compared with media alone (MCM), whereas *C. bolteae* ATCC BAA-613 CS increased diarrhea (Figure 4B). Furthermore, 2 weeks of rectal administration of CS from *C. bolteae* AHG0001 significantly reduced colonic inflammation as determined by a decreased colon weight-to-length ratio (Figure 4C), reduced histology scores (Figures 4E and 4G), and less immune cell infiltration (Figures S8E and S8F). Moreover, *Winnie* mice treated with *C. bolteae* AHG0001 demonstrated improved mucin production in the distal and mid colon as determined by Alcian blue staining (Figures 4F and 4H), indicative of reduced ER stress and histologic healing. Consistent with reduced colitis, there was a significant reduction in colonic expression of the inflammatory genes *Il-6*, *Ccl21*, *Cxcl12*, and *Cxcl10* and the ER stress markers *spliced-Xbp1* and *Grp78* in the colon (Figure 4D). Together, these results showed that topically administered CS from

C. bolteae AHG0001 but not ATCC BAA-613 suppresses colitis in a pre-clinical animal model and that *ex vivo* efficacy of CS in suppressing NF- κ B-dependent chemokine production correlates with *in vivo* effects.

Suppressive supernatants affect both canonical and non-canonical NF- κ B pathway

As the CS from *C. bolteae* AHG0001 could suppress NF- κ B in both epithelial and immune cell compartments, we used mouse derived splenocytes and bone marrow-derived macrophages to elucidate the effect on immune cell types. CS from *C. bolteae* AHG0001 reduced PMA/ionomycin-mediated *Tnf α* and *Ifn γ* secretion from T cell lymphocytes (Figures 5A, 5B, and S9). In addition to the effects on the T cells, CS from *C. bolteae* AHG0001 also reduced LPS-mediated *Il-6* and *Mip-2* from bone marrow-derived macrophages (Figure 5C). Additionally, mice treated with CS from *C. bolteae* AHG0001 had reduced phosphorylation of NF- κ B p65 in colonic epithelial cells, consistent with reduced NF- κ B activation (Figure 5D) and reduced nuclear NF- κ B p65 translocation (Figure 5E), suggesting inhibition of canonical NF- κ B signaling. As both canonical and non-canonical NF- κ B pathway activation occurs in *Winnie* mice, we used colonic explants from *Winnie* mice to test whether non-canonical NF- κ B activation is affected by CS treatment. We found that the expression of genes downstream of non-canonical NF- κ B activation, including *Cxcl13* and *Cxcl21*, are reduced after treatment with CS from *C. bolteae* AHG0001, with associated reduction in nuclear Rel-B translocation (Figures 5F and 5G). Together, these results demonstrate that treatment with CS from *C. bolteae* AHG0001 affects both canonical and non-canonical NF- κ B signaling in various cell types.

Finally, we hypothesized that intraspecies variations in NF- κ B suppressive capacity, together with the influence of culture media on bioactive production, could facilitate identification of bioactive molecules produced by *C. bolteae* using comparative genomics or metabolomics. Comparative genomic analyses revealed that *C. bolteae* AHG0001 carries 19 predicted BGCs, of which 14 are either highly or partially conserved in *C. bolteae* ATCC BAA-613 (Figures 6A and S10A). However, as the biosynthesis of bioactives by gut bacteria may be driven principally through minor modifications of common primary metabolites (Cohen et al., 2017; Colosimo et al., 2019) we considered it likely that other biosynthetic pathways would be overlooked by *in*

Figure 4. Effectiveness of culture supernatants in murine colitis

(A–D) Effects of bioactives on pro-inflammatory gene expression using murine-derived organoids from *Winnie* mice. Colonic organoids were treated with CS for 30 min and then stimulated as appropriate with mL-1 β for 6 h. (A) *Winnie* organoid-based qRT-PCR quantification of *cxcl10*, *mip-2*, *sxbp1*, and *grp78* expression following treatment with *C. bolteae* AHG0001 or *C. bolteae* BAA-613. (B) Effect of daily administration of MCM, *C. bolteae* AHG0001 CS, or *C. bolteae* BAA-613 CS on diarrhea score. (C) Changes in colon weight/length ratio following treatment with MCM, *C. bolteae* AHG0001 CS, or *C. bolteae* BAA-613 CS. (D) Relative gene expression of ER stress markers (*Grp78* and *Sxbp1*) and pro-inflammatory (*Il-6*, *Cxcl10*, *Ccl21*, and *Cxcl12*) genes in colonic tissue sections as analyzed using qRT-PCR.

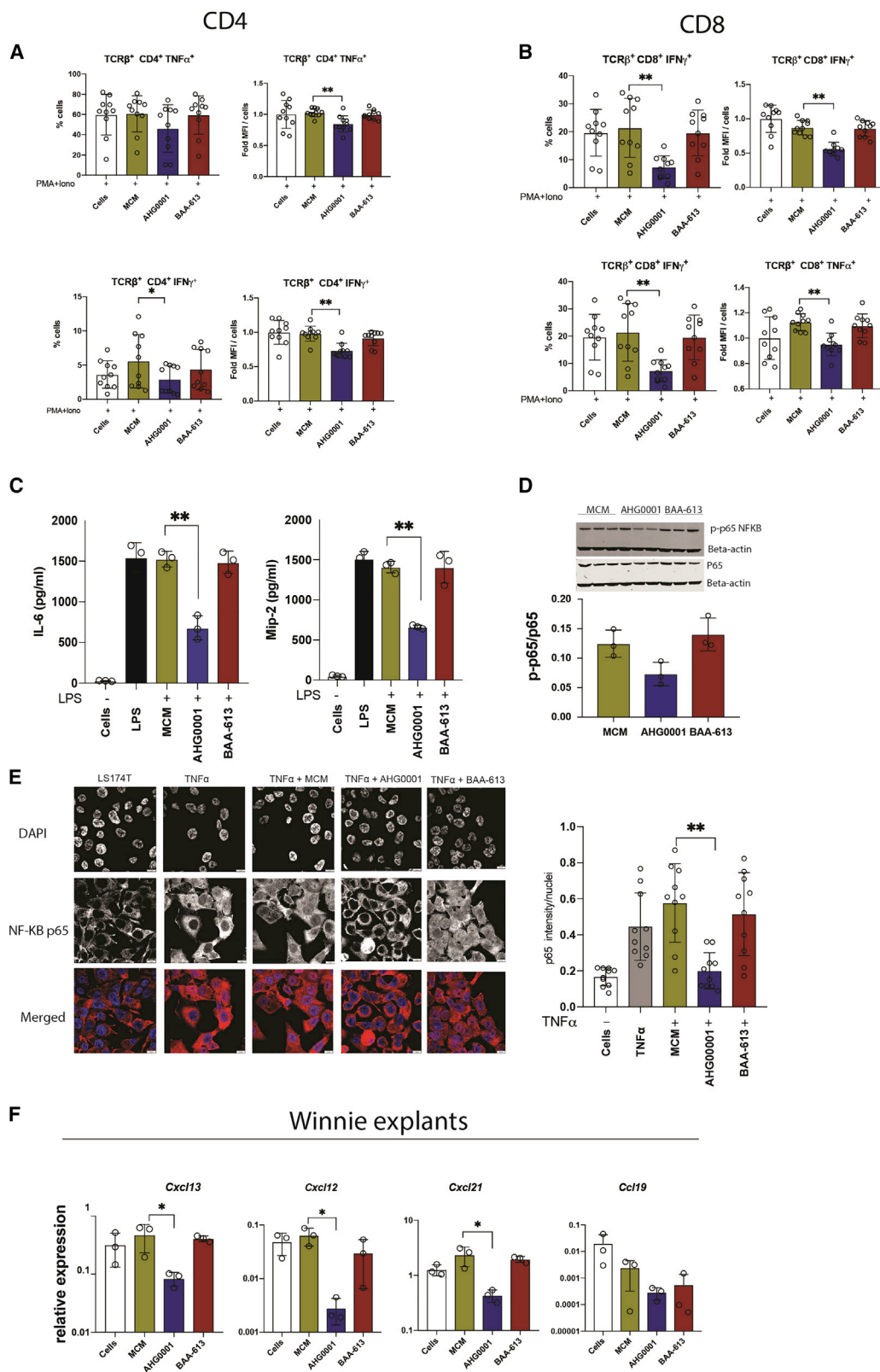
(E) Blinded histology scores following treatment with MCM, *C. bolteae* AHG0001 CS, or *C. bolteae* BAA-613 CS.

(F) Alcian blue quantification of mucin production in *Winnie*-derived colon sections.

(G) Representative H&E images from distal colon.

(H) Representative Alcian blue images.

n = 2 independent experiment with n = 3 technical replicates for organoids experiments. Animal experiments were performed in two batches with n = 3 or 4 per group. Findings from both experiments were pooled for the data presented: n = 6 for MCM, n = 7 for AHG0002, and n = 6 for BAA-613. Each dot represents an individual mouse. ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. The significance for diarrhea was determined by comparison with MCM using one-way ANOVA with Dunnett's multiple-comparison test. Sidak's multiple-comparison tests were used for A₁(C–F).



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silico analyses. We consequently applied a process of bioassay-guided solvent extractions and filtrations, followed by high-performance liquid chromatography to fractionate the bioactivity further. After ethanol acetate (EtOAc) extraction of *C. bolteae* AHG0001 culture supernatants, followed by drying and reconstitution in cell culture media, full suppressive activity was observed (Figures 6B–6D). Fractionation of the EtOAc extract using a reversed-phase analytical HPLC column revealed that the NF- κ B suppressive activity was found in the late eluting non-polar fractions, highlighting the non-polarity and hydrophobicity of the putative bioactive(s) (Figures 6B–6D, S11).

In the future, an integrated approach of combining bacterial isolation, functional screens, and comparative metabolomics might be beneficial in identification of novel bacterial secreted molecules and to expedite bioactive discovery.

DISCUSSION

Firmicutes-affiliated bacteria are among the most abundant gut microbes, and these taxa are widely recognized to possess immunomodulatory capacities (Atarashi et al., 2011, 2013; Sokol et al., 2008). However, they are poorly represented in culture collections, and their ability to modulate immune responses remains largely undefined. In this study, we identified five gut bacterial strains affiliated with *Clostridium* clusters IV, XIVa, and XV that have NF- κ B suppressive activity comparable or superior to the well-characterized *F. prausnitzii* A2-165 strain. The NF- κ B suppressive bioactivities were characterized by significant biochemical and intraspecies variations suggesting that NF- κ B suppressive capacity may be more prevalent than previously appreciated and not adequately captured in existing genetic-based studies of the gut microbiome. This is consistent with Geva-Zatorsky et al. (Geva-Zatorsky et al., 2017), who determined that as few as 53 isolates were associated with more than 24,000 immune phenotypes that include functionalities relevant to IBD such as Treg induction. Modulating host immune responses may support the ability of gut bacteria to colonize and persist in the gut environment. Furthermore, the ability of the microbiota to act as an extrinsic regulator of host immunity may underpin immune homeostasis, and therefore alterations in the gut microbial composition may contribute to disease risk in genetically susceptible individuals.

IBD is characterized by a dysregulated immune response with select genetic susceptibilities affecting therapeutic responsiveness (Niess et al., 2012; Barber et al., 2016). Given this variability

in IBD, we used patient-derived gut epithelial organoids and immune cells to confirm bacterial capability to suppress cytokine-mediated inflammatory responses. The heat- and Proteinase K-resilient bioactives showed strong suppression of IL-8 secretion in organoids and immune cells from non-IBD, CD, and UC subjects. Interestingly, the proteinase sensitive bioactives produced by *F. prausnitzii* A2-165 and *C. aldenense* AHG0011 appeared less suppressive in UC-derived organoids and PBMCs, and in CD organoids, compared with organoids derived from non-IBD controls, which may be reflective of the increased endogenous protease activity observed in IBD (Vergnolle, 2016). Our *in vitro* and *ex vivo* data also suggested that functional capacity rather than phylogeny may be the key determinant of biologic effects. To explore this hypothesis, we capitalized on the *C. bolteae* intraspecies differences in activity and demonstrated that *ex vivo* effects observed in Winnie organoids, where CS from *C. bolteae* AHG0001 but not ATCC BAA-613 suppressed immune responses, predicted *in vivo* efficacy in ameliorating established colitis in Winnie mice. Notably, treatment with *C. bolteae* AHG0001 CS was associated with a rapid onset of action with improvement in diarrhea, alleviation of inflammation and ER stress, as well as restoration of mucin production. Mucosal and histologic healing are among the best predictors of long-term outcomes in IBD (Turner et al., 2021) and taken together, our data suggest a precision medicine approach using *ex vivo* patient-derived organoids could be applied to select effective microbiome derived IBD treatments.

The NF- κ B suppressive strains we have identified carry multiple BGCs, many of whose products remain cryptic, underlining the inherent challenges in applying genomic-based approaches to map genotype with phenotype. In addition, the biosynthesis of bioactives by gut bacteria may be driven principally through modest modifications of common primary metabolites that are underpinned by small BGCs (Cohen et al., 2015, 2017). As the medium dependent effects on NF- κ B suppression may affect the therapeutic efficacy of live biotherapeutics for IBD, we used a bioassay-guided ethyl acetate extraction coupled with reversed-phase analytical high-performance liquid chromatography fractionation to further characterize low-molecular weight non-polar bioactive associated with the NF- κ B suppressive activity of *C. bolteae* AHG0001. Consistent with other microbial bioactives, the *C. bolteae* AHG0001 bioactive acts independently of the bacterial cell and suppresses the inflammatory response in animals.

In summary, our functional approach provides new opportunities to rationally bioprospect the gut microbiota for precision

Figure 5. CS affects both canonical and non-canonical NF- κ B signaling

Effect of CS in various cell types.

(A and B) PMA/ionomycin-mediated *Tnfa* and *Ifn γ* secretion in CD4⁺ and CD8⁺ T cells.

(C) LPS-mediated IL-6 and *Mip-2* secretion in BMDMs.

(D) Changes in phosphorylated NF- κ B p65 expression levels following 1 h treatment with MCM, *C. bolteae* AHG0001 CS, or *C. bolteae* BAA-613 CS on colonic epithelial cells from Winnie mice as determined using western blot against total and phospho-P65 using colonic tissue lysates.

(E) Representative immunofluorescence (IF) images of P-65 nuclear translocation on LS174T cells following 30 min treatment with CS and TNF- α . Nuclear translocation was quantified using ImageJ.

(F) Colonic tissues from Winnie mice were treated with CS for 6 h and tissues analyzed using qRT-PCR.

n = 10 mice were used for flow cytometry analysis; each circle represents an individual mouse. Data were pooled from two independent experiment. n = 3 mice were used for *ex vivo* assays. n = 2 biological replicates with technical triplicates were used for IF analysis. Ten different representative fields were used for intensity measurement.

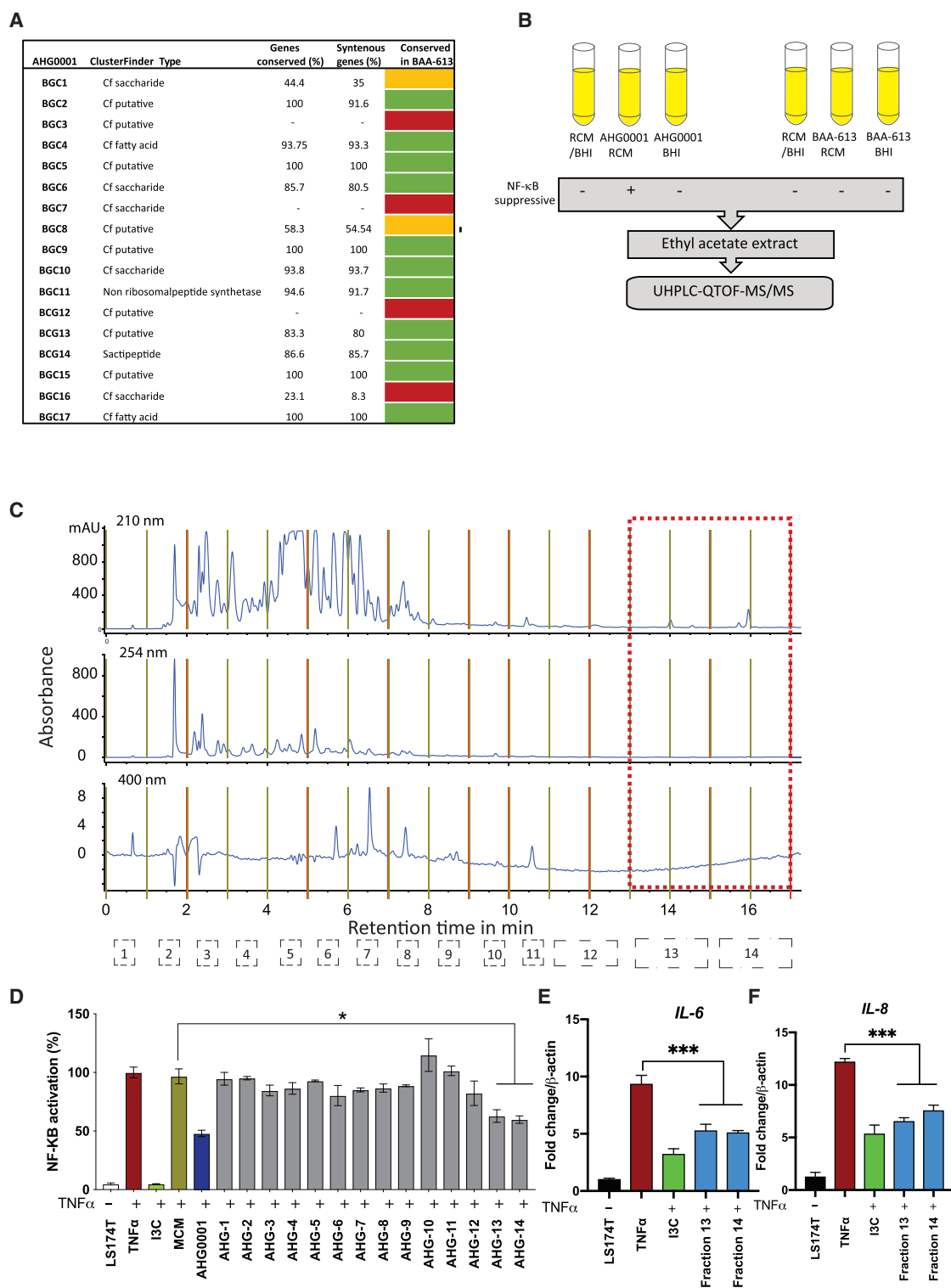


Figure 6. Comparative genome and chemical analysis of *C. bolteae* AHG0001 and *C. bolteae* ATCC BAA-613

(A) Determination of the extent of *C. bolteae* AHG0001 BGC conservation in *C. bolteae* ATCC BAA-613. The extent of protein (genes conserved) and syntenic gene pair (syntenous pairs) conservation were assessed. *C. bolteae* AHG0001 BGCs were classed as being conserved (green), partially conserved (orange), or not conserved (red).

(B) An overview of the experimental approach used to characterize the bioactives associated with the NF κ B suppressive activity of *C. bolteae* AHG0001.

(legend continued on next page)

live biotherapeutic strains and/or bioactives that could be used to expedite the development of safer and more efficacious therapeutics in IBD.

Limitations of the study

In this study, we showed that *Clostridium* species isolated from a single individual have NF- κ B suppressive ability, but the immunomodulatory properties are likely not limited to *Clostridium*. Although we identified multiple strains with secreted immunomodulatory activity, it remains undetermined if the immunomodulatory properties of *Clostridium* can be generalized to other species of bacteria. Similarly, we used one mouse model of colitis to validate the *in vitro* activity of culture supernatants, although the activity was validated in multiple cell lines as well as patient-derived organoids. Future studies should use other models of murine colitis.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.110646>.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.G., R.J.C., M.M., T.H.F., M.A.M., P.Ó.C., and J.B.; Methodology, R.G., S.K., A.A.S., A.-S.B., R.T., V.S., M.M., R.J.C., P.Ó.C., and J.B.; Investigation, R.G., P.Ó.C., S.K., A.A.S., A.-S.B., V.S., E.C.H., M.M., R.J.C., and J.B.; Resources, R.T., T.H.F., P.Ó.C., M.M., R.J.C., M.A.M., and J.B.; Visualization, R.G., P.Ó.C., S.K., E.C.H., and J.B.; Data Curation, R.G., E.C.H., S.K., A.A.S., A.-S.B., M.M., R.J.C., P.Ó.C., and J.B.; Funding Acquisition, R.G., J.B., P.Ó.C., M.M., and R.J.C.; Project Administration, V.S., J.B., and P.Ó.C.; Supervision, J.B., P.Ó.C., M.M., and R.J.C.; Writing – Original Draft, R.G., P.Ó.C., and J.B.; Writing – Review & Editing, R.G., P.Ó.C., S.K., A.A.S., A.-S.B., V.S., E.C.H., T.H.F., R.T., M.M., R.J.C., M.A.M., and J.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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SUPPORTING CITATIONS

The following references appear in the Supplemental information: Gulhane et al. (2016); Hasnain et al. (2014); Heazlewood et al. (2008); Maudet et al. (2014).

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(C) Elution profiles after semi-preparative HPLC fractionation of the EtOAc extract of *C. bolteae* AHG0001 cultured in MCM. The collected fractions are indicated with black dashed boxes, and the red dashed box represents the NF- κ B suppressive fractions.

(D) The presence of the suppressive activity after EtOAc extraction in the various fractions was determined using the LS174T reporter cell assay.

(E) Effects of active fractions 13 and 14 on pro-inflammatory chemokines/cytokines. Cells were stimulated for 6 h and analyzed using qRT-PCR.

n = 2 biological independent experiments with technical triplicates for cell based assays.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
NF-κB p65 (D14E12) XP® Rabbit mAb	Cell Signalling	Cat# 8242; RRID:AB_10859369
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb	Cell Signalling	Cat# 3033; RRID:AB_331284
Beta-actin monoclonal antibody	Novus Biologicals	Cat# NB 600-501; RRID:AB_343280
Alexa Fluor® 488 anti-mouse IFN-γ Antibody	BioLegend	Cat# 505813; RRID:AB_493312
Brilliant Violet 421™ anti-mouse TNF-α Antibody	BioLegend	Cat# 506327; RRID:AB_10900823
Brilliant Violet 605™ anti-mouse CD8a Antibody	BioLegend	Cat# 100743; RRID:AB_2561352
BUV395 Rat Anti-Mouse CD4	BD Biosciences	Cat# 563790; RRID:AB_2738426
Brilliant Violet 605™ anti-mouse CD8a Antibody	BioLegend	Cat# 100743; RRID:AB_2561352
Brilliant Violet 785™ anti-mouse TCR β chain Antibody	BioLegend	Cat# 109249; RRID:AB_2810347
RelB Antibody (C-19)	Santa Cruz	Cat# sc-226; RRID:AB_632341
Anti-NFκB p65 Antibody (F-6)	Santra Cruz	Cat# sc-8008; RRID:AB_628017
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	Cat# A-21235; RRID:AB_2535804
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	Cat# A-21245; RRID:AB_141775
Bacterial and virus strains		
AHG0001-AHG0023	This paper	N/A
<i>F.prausnitzii</i> A2-165	This paper	N/A
<i>C.boiteae</i> BAA-613	ATCC	N/A
<i>L. rhamnosus</i> GG (LGG)	This Paper	N/A
<i>L. casei</i> Shirota (LCS)	This Paper	N/A
<i>E. coli</i> Nissle 1917 (Nissle)	This Paper	N/A
<i>B. animalis subsp. lactis</i> BB-12 (Bifido)	This Paper	N/A
Biological samples		
Human biopsies	This paper	N/A
Human PBMCs	This paper	N/A
Chemicals, peptides, and recombinant proteins		
DMEM, high glucose, no glutamine	Gibco	Cat# 11960069
GlutaMAX™ Supplement	Gibco	Cat# 35050061
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat# 15140122
TrypLE™ Express Enzyme (1X), no phenol red	Gibco	Cat# 12604021
DPBS	Sigma-Aldrich	Cat# D8537
FBS QUALIFIED, USA ORIGIN	Gibco	Cat# 26140079
Advanced DMEM/F12	Gibco	Cat# 12634010
Collagenase Type I	Gibco	Cat # 17018029
Gentamicin	Gibco	Cat# 15710064
Cultrex PathClear BME, Type 2	R&D systems	Cat# 3532-005-02
Ficoll® Paque Plus	Sigma-Aldrich	Cat# GE17-1440-03
CHIR 99021	Tocris	Cat# 4423
Y-27632 2HCl	Selleckchem	Cat# S1049
SB431542	Selleckchem	Cat# S1067
Recombinant Mouse TNF-α (carrier-free)	Biolegend	Cat# 575204
Recombinant Human TNF-alpha	R&D systems	Cat # 210-TA-005
Recombinant Human IL-1 beta/IL-1F2 Protein	R&D systems	Cat # 201-LB-005/CF

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Indole-3-carbinol	Sigma-Aldrich	Cat # I7256
Confix NBF Conc.x5 Purple (Formalin)	Australian Biostain	Cat# ACFP.5L
TRIzol TM Reagent	Invitrogen	Cat# 15596018
SensiFAST SYBR Lo-Rox Kit	Bioline	Cat# BIO-94050
EDTA disodium salt dihydrate	Sigma-Aldrich	Cat# E5134
LPS-SM Ultrapure	Invivogen	Cat# tlrl-smmps
Lab-Lemco	Oxoid	Cat# LP0029; CAS: 91079-38-8
Peptone	Oxoid	Cat# LP0049; CAS: 93394-33-9
Yeast extract	Sigma-Aldrich	Cat# LP0021B; CAS: 801 3-01-2
Cysteine-HCL	Sigma-Aldrich	Cat# C7477; CAS: 52-89-1
Resazurin sodium salt	Sigma-Aldrich	Cat# 195481; CAS: 62758-13-8
Sodium Bicarbonate	Sigma-Aldrich	Cat# S6014; CAS: 144-55-8
Starch	Sigma-Aldrich	Cat# S9765; CAS: 9005-84-9
Glucose	CSA Scientific	Cat# GA018; CAS: 50-99-7
BHI	Oxoid	Cat# CM1135
Proteinase K solution	Invitrogen	Cat# 25530049

Critical commercial assays

ELISA MAX TM Standard Set Human IL-8	Biolegend	Cat# 431501
Pierce TM Firefly Luc One-Step Glow Assay Kit	Thermo Scientific	Cat# 16197
CytoTox 96 [®] Non-Radioactive Cytotoxicity Assay	Promega	Cat# G1780
ISOLATE II RNA Mini Kit	Bioline	Cat# 52073
SensiFAST cDNA Synthesis Kit	Bioline	Cat# 65054
R&D Systems Mouse IL-6 Duo Set ELISA, 5 Plate, Per Set	R&D systems	Cat# DY40605
R&D Systems Mouse CXCL2/MIP-2 DuoSet ELISA, 5 Plate, Per Set	R&D systems	Cat# DY45205

Deposited data

Whole genome of isolates	DDBJ/EMBL/GenBank	QYRW000000000
Whole genome of isolates	DDBJ/EMBL/GenBank	QYRX000000000
Whole genome of isolates	DDBJ/EMBL/GenBank	QYRY000000000
Whole genome of isolates	DDBJ/EMBL/GenBank	QYRZ000000000

Experimental models: Cell lines

L-WRN cells	Kind gift from Prof Thaddeus Stappenbeck	
LS174T	ATCC	Cat# CL-188
Caco-2	ATCC	Cat# HTB-37
L929	Kind gift from Prof Antje Blumenthal	
LCLs	Kind gift from Prof Maher Gandhi	

Experimental models: Organisms/strains

Mouse: C57BL/6	ARC	
Winnie	In house	

Oligonucleotides

Primers for mouse and human genes, see Table S2	This Paper	N/A
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Software and algorithms

SILVA		
MEGAX		
Cytoscape v. 3.8.2		https://cytoscape.org/
Graphpad Prism v 8.0.1	GraphPad	www.graphpad.com

Other

Graphical Abstract	Created with BioRender.com	https://biorender.com/
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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, A/Prof Jakob Begun (jakob.begun@mater.uq.edu.au).

Materials availability

This study didn't generate new unique reagents.

Data and code availability

- Genome of the isolates have been deposited at **DDBJ/EMBL/GenBank** and accession numbers are listed in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the [lead contact](#) upon request

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patient samples

All patient samples were collected in accordance with the recommendations of the Mater Health Services Human Research Ethics Committee (HREC 2016001782 & HREC/14/MHS/125) for the Mater Inflammatory Bowel Disease Biobank.

Human organoid culturing and immunomodulatory assays

Colonic biopsies (6 × 3 mm pinch biopsies) were collected from n (n = 6), CD (n = 5) and UC (n = 5) patients ([Table S1](#)). The colonic biopsies were processed and cultured as previously described. Briefly, the biopsies were washed with PBS and digested with collagenase type I (2 mg.ml⁻¹) supplemented with gentamicin (50 µg.ml⁻¹) for 15-20 minutes at 37°C. The isolated crypts were washed with DMEM/F12 medium and centrifuged at 50 × g for 5 mins at 4°C. The pellets were then suspended in Basement Membrane Extract (BME, Invitrogen) in a 1:1 ratio. Then, 20 µl of the mixture was plated in a 24 well tissue culture plate and cultured in 50% L-WRN conditioned medium. The crypts were expanded by serial culture until sufficient numbers were obtained for experimentation. To assess the ability of the CS to suppress IL-8 secretion the organoids were seeded in a 48 well plate and grown for 48 hours. Then, organoids were treated with 10% v/v of select CS in 50% L-WRN conditioned medium for 30 min and subsequently stimulated with rhIL-1β (50 ng.ml⁻¹) for 24 hours before quantifying IL-8 in the supernatant. Cytotoxicity was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay.

Peripheral blood mononuclear cell (PBMC) isolation and immunomodulatory assays

Human peripheral blood was obtained from healthy controls and patients with CD and UC (n = 6 each) from the Mater Inflammatory Bowel Disease biobank. PBMCs were isolated by Ficoll gradient density centrifugation. Briefly, 20 ml of freshly drawn blood was diluted in phosphate buffered saline (1:2) and well mixed. The diluted blood was then carefully layered over Ficoll paque. The tubes were centrifuged without brakes at 400 g for 20 minutes at 20°C. The interphase containing mononuclear cells were transferred into a new tube and washed twice in PBS. Prepared cells were stored in liquid nitrogen until required. For the treatments, 500,000 cells per well were plated on a 96-well plate and treated with 10% v/v of CS in RPMI medium for 30 minutes, followed by stimulation with rhTNFα (50 ng/ml). IL-8 secretion and cytotoxicity was assessed as previously described.

Animal experiments

All animal experiments were approved by the University of Queensland Animal Ethics Committee. *Winnie* mice were bred in-house in a pathogen-free animal facility. 6 weeks old male and female mice were intrarectally gavaged with 50 µl of CS from *C. bolteae* AHG0001 and ATCC BAA-613 for 14 days. MCM medium processed in the same manner as the CS was used as the vehicle control. Disease activity was assessed using established protocols. Briefly, the body weights of the mice as well as diarrhoea and rectal bleeding were monitored and recorded daily. Diarrhoea scoring was interpreted as follows: 0 = no diarrhoea, solid stool; 0.5 = very mild diarrhoea, moist but formed stool; 1 = mild diarrhoea, formed but easily bisected by pressure applied with pipette tips; 1.5 = diarrhoea, no fully formed stools, and; 2 = severe, watery diarrhoea with minimal solid present. For histology scoring, the whole colon was rolled, fixed in 10% neutral buffered formalin, and paraffin embedded and sectioned and stained with Haematoxylin and Eosin (H&E) and Alcian blue. Blind assessment of histologic inflammation (increased leukocyte infiltration, neutrophil counts, depletion of goblet cells, crypt abscesses, aberrant crypt architecture, increased crypt length, and epithelial cell damage and ulceration) for proximal, mid, and distal colon was performed as previously described. To quantify *in vivo* gene expression, the distal colon was snap frozen and homogenised in TRIzol. RNA was extracted using the Bioline RNA extraction kit according to manufacturer's instructions. RNA concentration was measured using a Nanodrop 1000 spectrophotometer, followed by cDNA synthesis using 1 µg of RNA and the iScript cDNA synthesis kit (BioRad). The expression of genes of interest ([Table S2](#)) were analysed using quantitative real time PCR

(qRT-PCR) according to the manufacturer's instructions. C_t values were generated, and relative quantitation was determined by the ΔC_t method.

Murine organoid culturing and immunomodulatory assays

Colonic tissues from C57BL/6 and Winnie mice ($n = 2$) were segmented and the crypts were isolated and cultured. Briefly, the tissues were segmented and washed with PBS, followed by EDTA (8 mM) digestion for 1 hour at 4°C and further digested with collagenase type I (2 mg.mL⁻¹) (Thermo Fisher Scientific) supplemented with gentamicin (50 µg.mL⁻¹) for 15-20 minutes at 37°C. The isolated crypts were washed with complete F12 medium (identical to complete media except DMEM/F12 was used instead of DMEM) and centrifuged at 50 x g for 5 mins at 4°C. The pellets were then suspended in BME in a 1:1 ratio. Then, 20 µL of the mixture was plated in a 24 well tissue culture plate and cultured in 50% L-WRN conditioned medium. The crypts were expanded by serial culture until sufficient numbers were obtained for experimentation. For the treatments, the organoids were first seeded in a 24 well plate and grown for 48 hours. The organoids were then pre-treated with 10% v/v of select CS for 30 mins and then stimulated with 50 ng/ml mL-1β for 6 hours. The cells were lysed and used for mRNA expression.

For explants, colonic tissue from 8-weeks old Winnie mice were cultured in DMEM complete media with 10% v/v CS and medium control for 6 hours. Tissues were then lysed and used for mRNA expression.

METHOD DETAILS

Measurement of immunomodulatory activities

The LS174T-NF-κB_{luc} or Caco-2-NF-κB_{luc} reporter cell lines were adapted for high-throughput screening using the criterion defined by Zhang et al. (Zhang et al., 1999), where a Z factor ≥ 0.5 represents an excellent assay, thereby providing a sensitive and specific approach to assess the NF-κB suppressive capacity of the isolates. The Z factor for each assay was determined and only assays achieving a Z factor ≥ 0.5 were processed for further analysis. The high-throughput assays were performed in 96-well microtiter plates as previously described (Ó Cuív et al., 2018) except that the LS174T reporter cells were stimulated with 50 ng.mL⁻¹ TNFα and the Caco-2 cell lines were treated with 7.5% v/v CS in complete DMEM medium. NF-κB driven luciferase expression was assessed using the Pierce™ Firefly Luc One-Step Glow Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The NF-κB suppressive isolates were scored and ranked on their Z-score (Junker and Clardy, 2007; Malo et al., 2006).

Bacterial strains, culture conditions and analyses

Anaerobic Firmicutes affiliated bacteria were cultured in anoxic MCM (Lab-Lemco 10 g.L⁻¹, Peptone P 10 g.L⁻¹, Yeast extract 3 g.L⁻¹, Glucose 5 g.L⁻¹, Starch 2 g.L⁻¹, Sodium chloride 5 g.L⁻¹, Sodium bicarbonate 15 g.L⁻¹, Resazurin 1 mg.L⁻¹, Cysteine-HCl 1 g.L⁻¹) or BHI supplemented with salt solutions 2 and 3 (McSweeney et al., 2005). *F. prausnitzii* A2-165 was grown as previously described (Ó Cuív et al., 2018). A Coy vinyl anaerobic chamber with an anoxic atmosphere (85% N₂:10% CO₂:5% H₂) was used to process the anaerobic Firmicutes cultures. Bacterial cultures were incubated at 37°C for up to 48 hours. Bacterial growth was measured by spectrophotometry (OD_{600nm}) using a SPECTRONIC 20D + Spectrophotometer (ThermoFisher, Sydney).

Analytical fractionation of NF-κB suppressive extract

An EtOAc extract (3 mg) of *C. boltea* AHG0001 cultivated on MCM medium was subjected to analytical HPLC (Agilent Zorbax SB-C8, 5 µm, 4.6 mm x 150 mm column, gradient elution at 1 mL/min from 10% MeCN/ H₂O to 100% MeCN over 15 min followed by 2 min wash with 100% MeCN, without TFA modifier) to yield 17 fractions. Only fractions 14-17 demonstrated an ability to suppress NF-κB activity.

Production of bone-marrow derived macrophages and immunomodulatory assays

Bone marrow was isolated from the femurs and tibias of C57BL/6 mice (6 weeks old). Briefly, bone marrow was grown for 7 days at 37°C in macrophage differentiation medium (10% FBS, 1% L-Glutamine, 1% sodium pyruvate, 1% HEPES in DMEM) containing 20% L929-conditioned medium. On day 7, the cells lifted with ice-cold EDTA (1 mM) in PBS, replated, and stimulated with 10 µg/ml LPS in the presence of select CS (10% v/v) for 24 hours. IL-6 and MIP-2 level was quantified using ELISA (R&D).

Nuclear translocation immunofluorescence assays

A 12-well plate was seeded with 20,000 LS174T cells per well on glass coverslips and cultured overnight at 37°C. Cell-free supernatants harvested from isolates were added (10% v/v) to the cells for 30 minutes and then stimulated with 25 ng/ml rhTNF-α for 1 hour. Cells were fixed with 4% paraformaldehyde for 15 min at 4°C followed by permeabilisation using 0.1% Triton X-100 for 15 mins. Cells were then stained with mouse anti-NF-κB p65 antibody (santa-cruz) for 1 hour, followed by Alexa Fluor 594 anti-mouse secondary antibody. The cells were mounted onto glass slides using Fluoroshield with DAPI (Sigma-Aldrich) and visualised using an Olympus Confocal Microscope at 60x magnification.

For LCLs, 100,000 cells were seeded and stimulated with CS for 30mins. Cells were fixed and permeabilised as above and stained with mouse anti-RelB antibody (santa-cruz) for 1 hour, followed by appropriate secondary antibody. Cells were stained mounted on slides using cytospin and stained with DAPI and visualised at 60 x magnification.

PMA/ionomycin stimulation of spleenocytes

Spleens were mechanically passed through 70 μ m cell strainer and resuspended in FACS buffer ((2% FBS, 0.5 mM EDTA in PBS) and centrifuged at 1400 rpm for 6 mins. Cell pellets were then lysed with ACK lysis buffer for 1 min at room temperature. Cells were counted and 1×10^5 cells resuspended in complete media (RPMI supplemented with 10% FBS, 1% PS and 1% sodium pyruvate) and plated in 96-well plate. Cells were stimulated with 10 ng/ml PMA and 250 ng/ml ionomycin in the presence of Brefeldin A for 4 hours at 37°C.

Flow cytometry

Single cell suspensions were incubated with live/dead Fixable aqua for 10mins at room temperature. Cells were then incubated with 50 μ l of a cocktail of antibodies reactive against surface molecules for 30 min. For intra-cellular staining, cells were washed, fixed, and permeabilised with eBioscience FoxP3/Transcription Factor staining buffer kit and stained with 50 μ l of a cocktail of antibodies reactive against intracellular molecules for 30mins at room temperature. Samples were acquired on a BD LSRFortessa and analysed on FlowJO Version 10.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis for each figures was performed using Graphpad Prism V8.01. Details of the statistical tests and methodology for quantification are provided in the figure legends.

Bacterial genetics comparative analyses

Phylogenetic trees were constructed by aligning the 16S rRNA gene sequences using the SILVA database(Quast et al., 2013) and the alignment was then imported into MEGAX(Kumar et al., 2018). The alignment was refined, and a maximum-likelihood phylogenetic tree constructed displaying the isolate and select reference sequences. The stability of the maximum-likelihood tree was evaluated by 1000 bootstrap replications and Kimura 2-parameter modelling. Where necessary, select isolates were subject to whole cell protein profiling to determine intraspecies variations(Ismail et al., 2012; Mahendran et al., 2013). High molecular weight DNA was prepared as previously described(Ó Cuív et al., 2011). The SPAdes assembler v 3.11.0 was used to quality check, filter, and then *de novo* assemble the sequence data(Bankevich et al., 2012). CheckM(Parks et al., 2015) was used to evaluate the genome sequencing quality by estimating the completeness and contamination based on the phylogenetic assignment of a broad set of marker genes. The *C. bolteae* AHG0001, *C. citroniae* AHG0002, *C. aldenense* AHG0011 and *E. limosum* AHG0017 contigs were ordered using Mauve(Darling et al., 2010) with the *C. bolteae* ATCC BAA-613, *C. citroniae* WAL-17108, Clostridiales bacterium 1_7_47FAA and *E. limosum* ATCC 8486 genome sequences respectively as references. Genome based phylogeny was determined using GTDB(Parks et al., 2018) as previously described(Ó Cuív et al., 2018). Candidate BGC were identified using the antiSMASH webserver(Weber et al., 2015) with the ClusterFinder Detection Strictness settings set to “loose” and the Extra Features turned on. Similar candidate BGC were identified in select genomes or the Genbank Database using MultiGeneBlast(Medema et al., 2013) in homology search mode. BGCs were considered highly conserved if (i) $\geq 80\%$ of the genes in an *C. bolteae* AHG0001 BGC were conserved in *C. bolteae* ATCC BAA-613, with genes defined as being conserved if the query exhibited $\geq 80\%$ sequence identity over $\geq 80\%$ of the query length, and; (ii) $\geq 70\%$ of the potential syntenic genes in a *C. bolteae* AHG0001 BGC were conserved in a *C. bolteae* ATCC BAA-613 BGC (calculated as ((MultiGeneBlast Total score – No. of Blast hits)/0.5)/(No. of syntenic genes in *C. bolteae* AHG0001 BGC)). BGC were considered partially conserved if $\geq 40\%$ of both the genes and potential syntenic genes were conserved.

Normalization of IL-8 expression for PBMCs and organoids

The absorbance readings recorded for IL-8 ELISA expression for each condition were subtracted from the cells only control. The absorbance values were normalized using the IL- 1β control and converted to percentage.