

Influenza A Virus Infection Alters Gut Microbiota Composition in Juvenile Mice

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Abstract

The gut microbiota (GM) stimulates the immune system to facilitate appropriate immune responses at local and distal sites of virus infection. However, the impact of distal virus infections in modulating the composition of GM is less clear. Here, a mouse model was used to examine the effect of influenza a virus (IAV) infection on the composition of GM in mice that did or did not receive oral treatment with broad spectrum antibiotics (ABX). We focused on ABX treatment of 3 weeks old juvenile mice, as this represents a dynamic time in the establishment of GM and immunity, followed by mock- or IAV-infection 2 weeks later. Mice were subsequently assessed for changes in body weight, viral load in the respiratory tract and the composition of GM. As anticipated, oral ABX treatment reduced the diversity of GM and ABX-treated mice showed exacerbated disease, characterized by enhanced weight loss and increased virus titers in the upper and lower airways. Surprisingly, IAV infection also increased the diversity of GM in ABX-treated mice, resulting in a microbial composition qualitatively and quantitatively different to mock-infected ABX-treated mice and IAV-infected mice that did not receive ABX treatment. Specifically, IAV infection increased the relative abundance of eight different bacteria in GM in ABX mice compared to the GM in mock-infected ABX mice. These findings confirm an important role for GM in functional immune defense against IAV infection in juvenile mice, consistent with previous studies in adult animals. Moreover, we report for the first time that distal IAV infection of the airways can enhance the diversity of an ABX-compromised GM.

Keywords: Gut Microbiota; Antibiotic; Influenza A Virus; Mouse Model; *Akkermansia Muciniphila*

Abbreviations: ABX: broad-spectrum antibiotic; CON: control; i.n: intranasal; GM: Gut microbiota; i.t: intratracheal; IAV: influenza A virus; MDCK: Madin-Darby canine kidney; NMDS: Non-metric multidimensional scaling; OUT: Operational Taxonomic Units; p.i: post-infection; PFU: plaque forming units; QIIME: Quantitative Insight In to Microbial Ecology.

Introduction

Gut microbiota (GM) dysbiosis plays an important role in the etiology of an array of diseases, including resistance towards infections caused by Anthrax, et al. [1], Koenigsnecht, et al. [2], Schubert [3], Zainah, et al. [4], parasites e.g. *Toxoplasma gondii* [5] and viruses e.g. influenza A virus (IAV) [6-9], and human immunodeficiency virus (HIV)-1 [10]. A homeostatic GM can contribute to protection against invading pathogens via depletion of nutrients, antimicrobial peptide production and/or priming of immune cells to combat infections at local or distal sites in the body [11,12]. Exposure to oral antibiotics (ABX) can alter GM diversity and this can differ depending on the particular ABX treatment [6]. In mouse models, reduced GM diversity during bacterial or viral challenge is known to exacerbate diseases e verify [2,3,6-8,13-15]. Modulation of GM following pre-treatment of adult mice with ABX resulted in exacerbated disease following subsequent IAV challenge, characterized by delayed virus clearance, reduced titers of virus-specific antibodies and impaired inflammation some-dependent responses by innate and adaptive immune cells [6,7]. Thus, commensal microbes appear to play a critical role in calibrating appropriate immune responses to IAV and other pathogens.

Colonization of the gastrointestinal tract by microorganisms commences at birth, immediately after delivery, and the succession of new bacterial colonizers have been shown to be essential for development of the immune system [16-18]. Not surprisingly, reduced microbiota diversity as a result of ABX treatment or germ-free conditions in early life can affect development of the immune system [19-21]. Making the host more vulnerable to infection. In mice, establishment of intestinal microbiota occurs after weaning between 3-5 weeks post-partum [22]. While the impact of ABX treatment on IAV infection has been established in adult mice, we have investigated the impact of ABX treatment of juvenile animals (3 weeks old) given that this time is critical for the final establishment of GM composition.

Following intranasal (i.n.) or intra tracheal (i.t.) inoculation of mice with seasonal IAV, epithelial cells lining the upper and lower respiratory tract represent the

primary targets for IAV infection and amplification. The expression of particular trypsin-like proteases in the airways is a major factor limiting the systemic spread of seasonal IAV to other sites in the body. Consistent with this, infectious virus is generally not recovered from the intestinal tract other distal organs [23,24]. Nevertheless, experimental infection of mice by the mouse-adapted IAV strain A/Puerto Rico/8/1934 (PR8, (H1N1)) altered GM composition and was associated with increase intestinal injury [9,24]. Furthermore, one of these studies found that use of broad-spectrum ABX prior to and during PR8 infection diminished infection-related intestinal injury. Together, these studies are consistent with an indirect effect of IAV infection on the composition of GM and/or intestinal health in adult mice [9].

Strains of human seasonal IAV show marked differences in their ability to infect and induce disease in mice. Historically, serial passage of human IAV in mice has been used to increase their virulence, allowing for studies investigating pathogen city and immune responses elicited to IAV infection. PR8 is a mouse-adapted H1N1 virus that has been widely used to study IAV infection in laboratory mice as it readily infects the lung to induce a viral pneumonia, similar to that seen during severe disease in humans [25]. However, primary viral pneumonia represents the most severe pulmonary manifestation and is relatively uncommon in healthy adults during most seasonal epidemics [26,27]. Moreover, the virulence of the PR8 strain in mice can limit its usefulness, particularly in immune compromised animals or in juvenile mice which also show increased susceptibility to IAV [28]. Herein, we have investigated the ability of HKx31 (H3N2), an IAV strain of moderate virulence in mice compared to PR8 [29,30]. To infect and modulate the composition of GM in juvenile mice. In addition, we have examined the effect of oral ABX treatment prior to and during HKx31 infection on the composition of GM in juvenile mice.

Materials and Methods

Ethics Statement

All experiments with mice were conducted with approval from the University of Melbourne Biochemistry & Molecular Biology, Dental Science, Medicine, Microbiology & Immunology, and Surgery Animal Ethics Committee. Experiments using mice were conducted with approval from the University of Melbourne Biochemistry & Molecular Biology, Dental Science, Medicine, Microbiology & Immunology, and Surgery Animal Ethics Committee (project 1413227.3), in accordance with the National Health and Medical Research Council (NHMRC)

Australian code of practice for the care and use of animals for scientific purposes.

Experiments using 10-day embryonated chicken eggs were conducted with approval from the University of Melbourne Biochemistry & Molecular Biology, Dental Science, Medicine, Microbiology & Immunology, and Surgery Animal Ethics Committee (project 1714213), in accordance with the National Health and Medical Research Council (NHMRC) Australian code of practice for the care and use of animals for scientific purposes. Eggs were obtained from Hy-Line Australia (Bags hot, Victoria, Australia).

Mice and Virus

C57BL/6 male mice were bred and housed in specific pathogen-free facilities at the Department of Microbiology and Immunology, University of Melbourne, Australia. IAV strain HKx31 is a high-yielding reassortant of A/PR/8/34 (PR8, H1N1) and A/Aichi/2/1968 (H3N2), and expresses the H3N2 surface glycoproteins. HKx31 was grown in 10-day embryonated hen's eggs by standard procedures [31]. Viral stock was stored at -80°C. Titers of infectious virus were determined by standard plaque assay on Madin-Darby canine kidney (MDCK, American Type Culture Collection (ATCC)) cells [31]. Expressed as plaque-forming units (PFU)/ml.

Antibiotic Treatment and IAV Infection of Mice

Following weaning at 3 weeks of age, mice received either (i) normal drinking water, or (ii) drinking water supplemented with ampicillin (0.5 mg/ml), gentamicin sulphate (0.5 mg/ml), and metronidazole (0.5 mg/ml) (all from Sigma Aldrich, collectively called ABX). Mice were treated for 3 weeks and ABX water was changed every third day. After 2 weeks ±ABX treatment (i.e. 5 weeks of age), mice were anaesthetized using isoflurane and infected via the intranasal (i.n.) route with 100 PFU of HKx31 in 50 µl PBS. Control mice were mock-infected with 50 µl of PBS alone. Mice were weighed daily and assessed for signs of clinical disease. Animals that had lost >15% of their original body weight and/or displayed evidence of pneumonia were culled. On day 7 p.i., mice were culled and nasal and lung tissues collected from IAV-infected mice were homogenized in 1ml of PBS, clarified by centrifugation and stored at -80°C. Titers of infectious virus were determined by plaque assay MDCK cells [31]. All experiments with mice were conducted with approval from the University of Melbourne Biochemistry & Molecular Biology, Dental Science, Medicine, Microbiology & Immunology, and Surgery Animal Ethics Committee.

Fecal Sample Collection and Microbial DNA Extraction

Fecal samples were collected from mice on the day of infection (day 0 p.i., mice = 5 weeks of age) and immediately prior to euthanasia (day 7 p.i., mice = 6 weeks of age) and stored at -80°C until DNA extraction. Fecal samples were homogenized and DNA was extracted via MoBio Power Soil Kit (Mo Bio Laboratories, QIAGEN) and stored at -80°C prior to analysis.

High Throughput Sequencing of the Gut Microbiota

Fecal microbiota composition of 38 mice was determined using tag-encoded 16S rRNA gene MiSeq-based (Illumina, CA, USA) high throughput sequencing. Cellular DNA extraction, DNA storage and sequencing library preparation steps were conducted as previously described [32]. The following adjustments: the V3 region of the 16S rRNA gene amplified using primers compatible with Nextera Index Kit (Illumina; Nxt_338_F: 5'-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG ACWCTACGGGWWGGCAGCAG-3' and Nxt_518_R: 5'-GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG ATTACCGCGGCTGCTGG-3'). Two samples were excluded from the analysis due to a low read number (<10,000 reads).

The raw data set containing pair-ended reads with corresponding quality scores were merged and trimmed using `fastq_mergepairs` and `fastq_filter` scripts implemented in the UPARSE pipeline [33]. The minimum overlap length of trimmed reads (150 bp) was set to 100 bp. The minimum length of merged reads was 150 bp. The maximum expected error $E = 2.0$, and first truncating position with quality score $N \leq 4$. Purging the data set from chimeric reads and constructing de novo operational taxonomic units (OTU) were conducted using the UPARSE pipeline [33]. Quantitative Insight into Microbial Ecology (QIIME) open source software package [34] (1.7.0 and 1.8.0) was used for subsequent analysis steps. The green genes (13.8) 16S rRNA gene collection was used as a reference database [35].

Unweighted, generalized, and weighted UniFrac distance metrics were calculated from subsampled OTU tables (10,000 reads/sample) and visualized with non-metric multidimensional scaling (NMDS) plots. The differences in ordination between categories were tested using Per mutational Multivariate Analysis of Variance using Distance Matrices, 'Adonis' function from R package 'vegan' [36].

Alpha diversity measures expressed with an observed species (sequence similarity 97% OTUs) value were computed for rarefied OTU tables (10,000 reads/sample) using the alpha rarefaction workflow. Differences in alpha diversity were determined using a t-test-based approach employing the non-parametric (Monte Carlo) method (999 permutations) implemented in the compare alpha diversity workflow. The differences in taxa abundance between categories were estimated with a statistic framework: analysis of composition of microbes (ANCOM)

based on non-normalized species level OTU-table [37].

Results

Oral ABX Treatment Enhances IAV-Associated Disease in Juvenile Mice

Following weaning at 3 weeks of age, mice received normal drinking water (control (CON)) or drinking water supplemented with broad-spectrum antibiotics (ABX, gentamicin, ampicillin, and metronidazole) for 2 weeks.

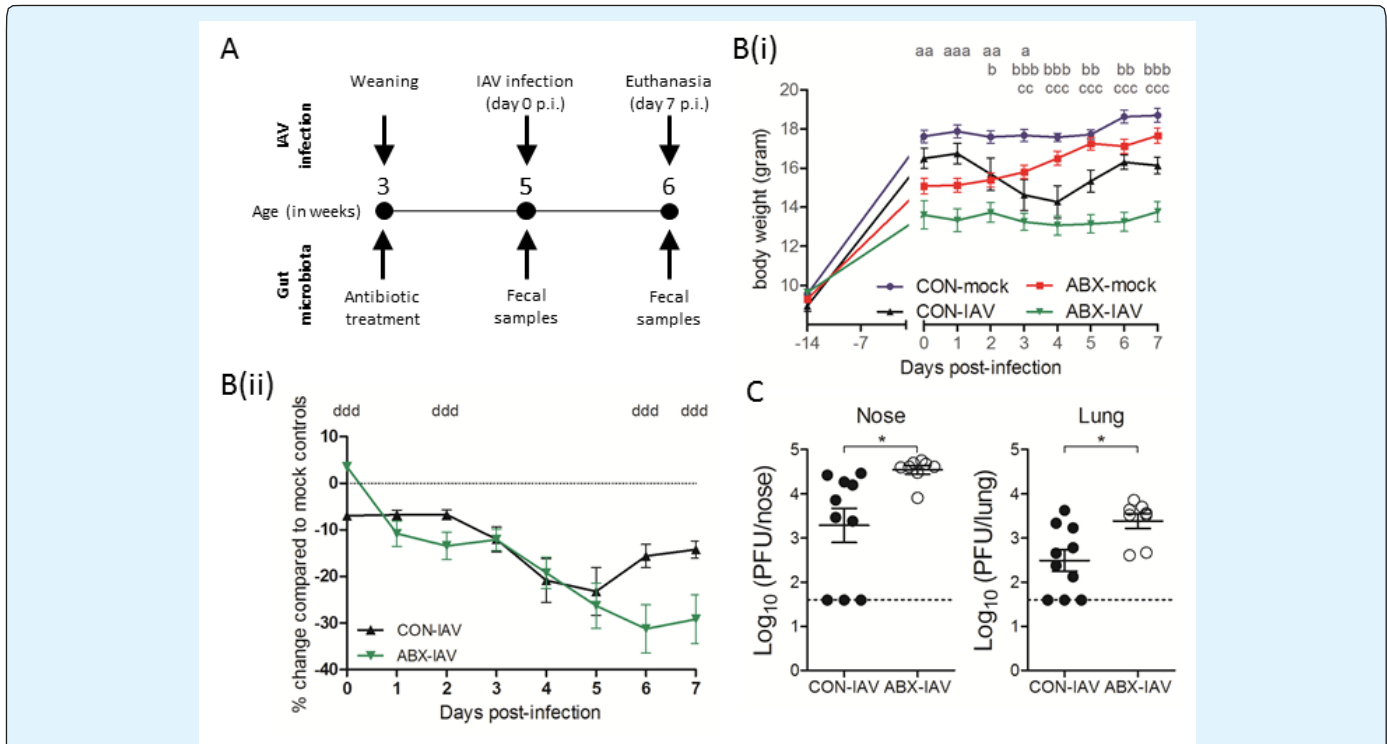


Figure 1: Oral broad-spectrum ABX treatment prior to and during IAV infection enhances IAV-induced disease in juvenile mice. (A) Experimental model. Following weaning of male mice at 3 weeks of age, half were immediately given drinking water supplemented with broad-spectrum antibiotics (ABX: ampicillin, gentamycin and metronidazole, 0.5mg/ml) for 2 weeks and half of the mice received normal drinking water alone (control (CON)). At 5 weeks of age, half of the ABX and CON mice were intranasally infected with 100 PFU HKx31 (ABX-IAV and CON-IAV mice, respectively) and half were mock-infected with virus diluent (ABX-mock and CON-mock mice, respectively). ABX were maintained in the drinking water of ABX-mock and ABX-IAV mice throughout the course of infection (7 days). All mice were euthanized for analysis at day 7 post-infection (p.i.). Fecal samples were collected for analysis of microbiota at day 0 and day 7 p.i. (B) Body weight changes measured as (i) grams, (ii) percentage of original body weight on day 0 relative to infection (CON-mock, blue, n = 10; CON-IAV, black, n = 10; ABX-mock, red, n = 10; ABX-IAV, green, n = 8), and (iii) percentage weight change relative to appropriate mock-infected controls (based on body weight in grams; CON-IAV, open black; ABX-IAV, open green). Data show the mean \pm SEM for groups of 10 mice, pooled from two independent experiments and were analyzed by two-way ANOVA followed by Bonferroni's post-test. a = CON-mock vs. ABX-mock, b = CON-mock vs. CON-IAV, c = ABX-mock vs. ABX-IAV, d = CON-IAV vs. ABX-IAV where $p < 0.001$, $p < 0.01$ and $p < 0.05$ are denoted by 3, 2 or 1 letters, respectively. (C) Virus titers were determined in clarified homogenates prepared from lung and nasal tissues of IAV-infected mice. Titers from individual animals are shown and horizontal bars represent the mean \pm SD for each group. The dashed line shows the detection limit of the assay. Data are pooled from two independent experiments. Data were analyzed using Student's t-test. * $p < 0.05$.

After this time, mice were infected via the intranasal (i.n.) route with 100 plaque-forming units (PFU) of IAV strain HKx31 (IAV) or mock infected with an equivalent volume of virus diluent (mock). IAV- and mock-infected mice were monitored for a further 7 days with ABX present or absent from the drinking water (for experimental design (Figure 1A). Mice were weighed daily after commencement of ABX treatment and following IAV infection. Note that no clinical signs of disease were evident throughout the monitoring period (data not shown). Compared to CON mice, two weeks of ABX treatment resulted in a significant weight reduction on the day of infection (Figure 1B(i)), day -14 to 0 post-infection (p.i.). These findings are consistent with previous studies showing that ABX treatment of juvenile mice is associated with delayed weight gain [38]. Next, we assessed the impact of IAV infection on weight loss in ABX- and CON-treated mice by examining body weight in grams (Figure 1B(i)) or as the percentage change in each of the IAV-infected groups relative to their appropriate mock-infected control (Figure 1B (ii)). The mock-infected mice, CON-mock animals showed minimal weight gains between day 0-7 p.i. whereas ABX-mock mice continued to gain weight during this time (Figure 1B (i)). These findings are consistent with studies showing that ABX treatment of adult mice is associated with increased weight gain [39,40]. Interestingly, when examining the percentage weight change compared to the appropriate mock-infected control, it was evident that maximum

relative weight loss for CON-IAV animals occurred at days 4-5 p.i., whereas ABX-IAV continued to lose weight and maximum relative weight loss was recorded at days 6-7 p.i. (Figure 1C (ii)). While IAV infection of both CON and ABX mice induced significant weight loss compared to respective mock-infected animals, viral replication was enhanced in both the upper (nose) and lower (lungs) respiratory tract of ABX-IAV mice compared to CON-IAV mice (Figure 1D). Thus, while HKx31 infection of juvenile CON mice was associated with weight loss and viral replication in the airways, ABX treatment prior to and during infection resulted in prolonged weight loss (indicative of disease) and enhanced virus replication.

***Parabacteroides Distasonis* was the Dominating Taxa after Oral Antibiotic Treatment at the Time of IAV Infection**

To determine the effects of ABX treatment on GM composition at the time of IAV infection, we examined the microbial composition in fecal samples collected on the day of infection (i.e. day 0 p.i., after two weeks of ABX treatment) and displayed all taxa with an abundance threshold of 0.1%. Compared to CON mice, ABX mice showed an overall reduction in the relative abundance of most types of bacteria and the GM of ABX mice was dominated by *Parabacteroides distasonis* (Bacteroidetes) at levels of 87.5% in ABX mice compared to 7.4% in CON mice (Figure 2).

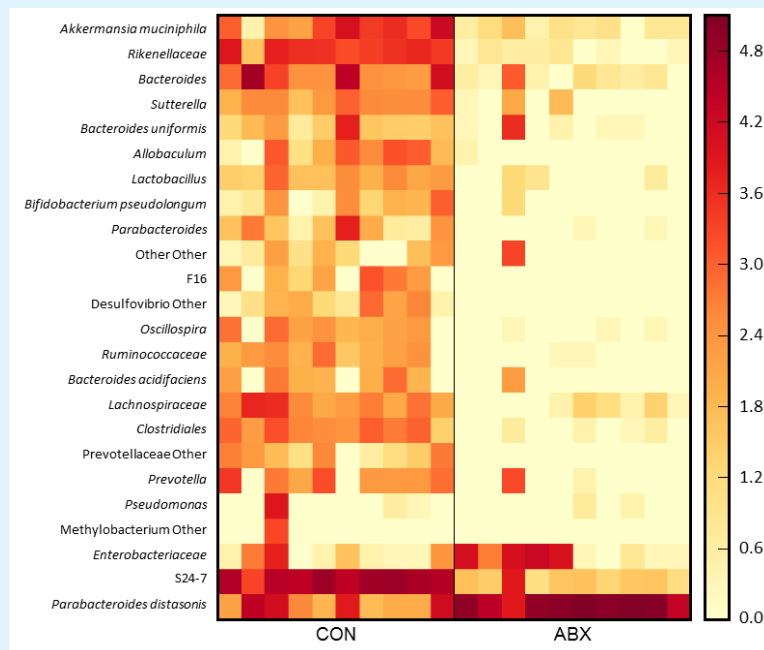


Figure 2: Oral broad-spectrum ABX treatment changes gut microbiota diversity in juvenile mice just prior to IAV infection.

Heat map depicting taxa relative abundance reported as significantly different (ANCOM, $p < 0.05$) between CON ($n = 10$) and ABX ($n = 10$) mice on day 0 p.i., just prior to IAV infection. Analyses were performed using non-normalized, summarized to the species level OTU-table with abundance threshold 0.1%. Instead, members of S24-7 represented the dominant phyla detected in the GM of CON mice at 58.1% compared to just 2% in ABX mice. The decrease in relative abundance of most bacteria was paralleled by a strong reduction in the measurement of

observed species, showing a marked reduction in the diversity of GM in ABX mice compared to CON mice.

IAV Infection Modulates the Composition of Gut Microbiota in ABX Mice

The non-metric multidimensional scaling (NMDS) plots based on unweighted, generalized and weighted UniFrac distance matrices showed significant (Adonis $p < 0.01$) separation between the 4 groups, indicating clear qualitative and quantitative differences in microbial composition (Figure 3).

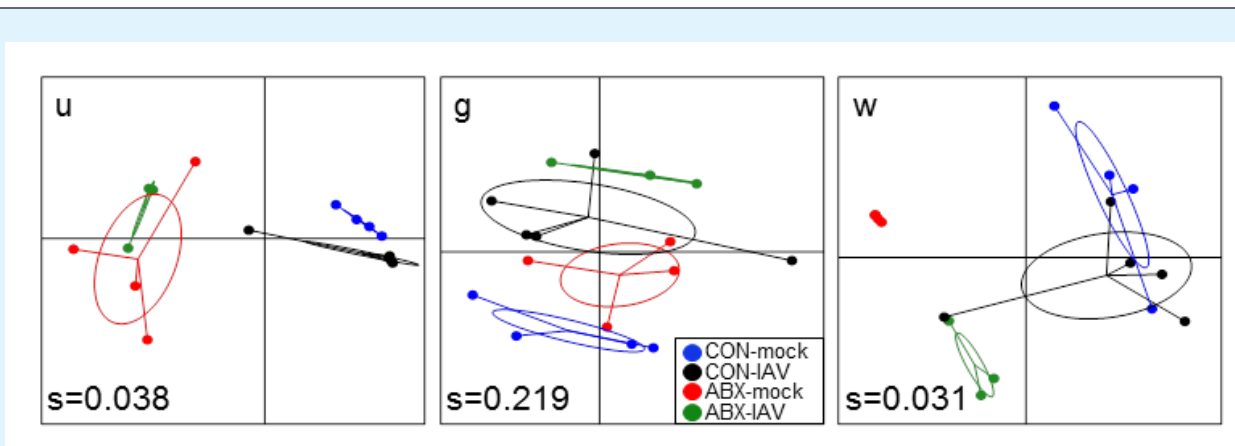


Figure 3: IAV infection enhances the diversity of the compromised gut microbiota in ABX mice. Nonmetric multidirectional scaling (NMDS) plots based on unweighted (u), generalized (g), and weighted (w) UniFrac distance matrices. Clear separation between all 4 treatment groups on unweighted (adonis $R^2 = 0.593$; $p < 0.01^{**}$) and generalized (adonis $R^2 = 0.675$; $p < 0.01^{**}$). NMDS plots indicate that main differences are to be expected within low and semi-abundant taxa. The NMDS plot based on the weighted UniFrac distance matrix indicates differences in high abundant taxonomic group within ABX-IAV group (adonis $R^2 = 0.687$; $p < 0.01^{**}$) (w). S values represent the stress of each NMDS ordination. (CON-mock (blue) $n = 4$, CON-IAV (black) $n = 5$, ABX-mock (red) $n = 4$, ABX-IAV (green) $n = 3$).

The bacterial diversity (observed species index) tended to increase in both of the infected groups (CON-IAV and ABX-IAV), however this was only significant between ABX-mock and ABX-IAV mice (Figure 4A). Infection of CON mice (CON-IAV) significantly reduced the relative abundance of *Allobaculum* (Firmicutes) (Figure 4B). Interestingly, infection of ABX mice resulted in a significant increase in relative abundance of several taxa, including *Akkermansia muciniphila* (Verrucomicrobia), *Enterobacteriaceae* and *Sutterella* (Proteobacteria),

Bacteroides, Candidate phyla S24-7, *Bacteroides uniformis*, *Bacteroides acidifaciens* and *Bacteroidales* (all Bacteroidetes), (Figure 4C).

While the number of animals, and hence fecal samples analyzed in the present study was relatively low, it was sufficient to provide statically confidence in differences in the diversity of GM. No doubt that the quality and statistical power of this data would be improved by additional sampling.

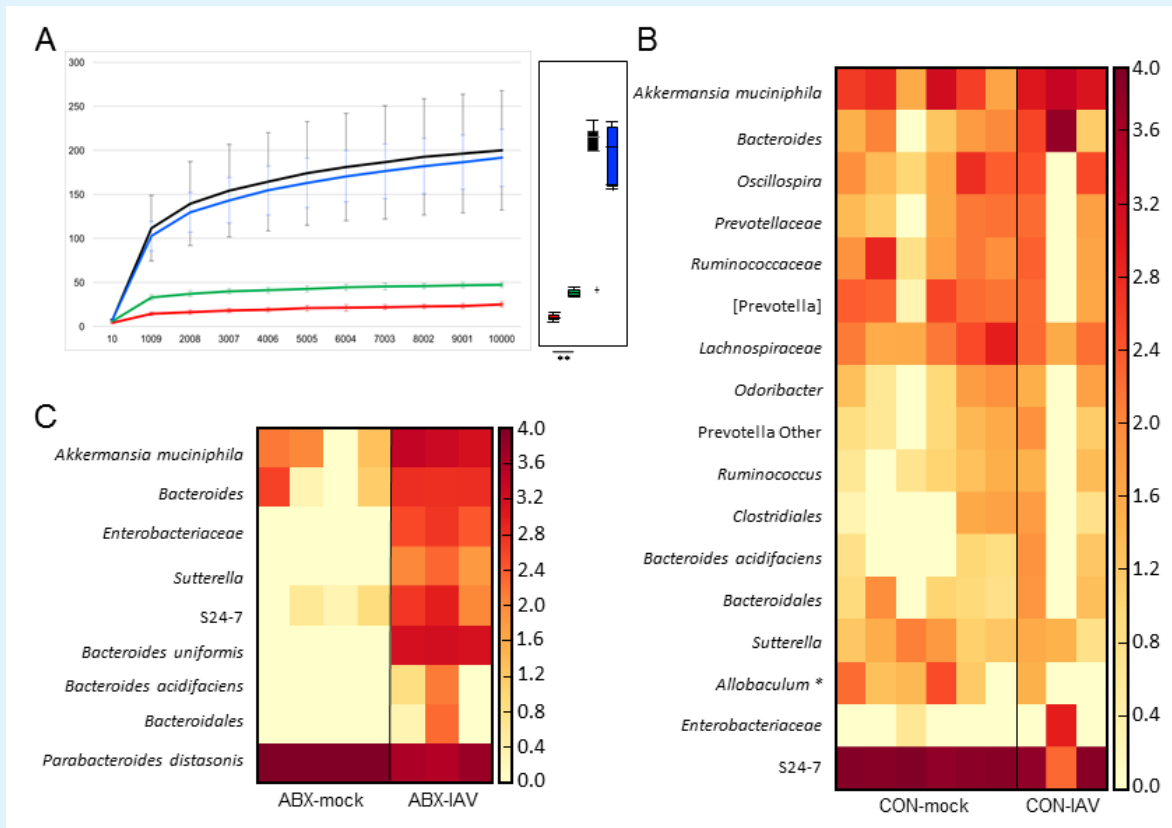


Figure 4: IAV infection increases the relative abundance of five different bacteria species in ABX mice. (A) Alpha rarefaction curves showing significant differences in observed species index between ABX-mock (red, n=4) and ABX-IAV (green, n=3) ($p=0.018$) but not between CON-mock (blue, n=4) and CON-IAV (black, n=5). (B) Heat map showing species-level taxa above 0.1% of abundance. Taxa that reached the significant difference (ANCOM, $p<0.05$) between CON-mock and CON-IAV are designated with *. (C) Heat map showing all taxa selected with ANCOM to be significantly different ($p<0.05$) between ABX-mock (n=4) and ABX-IAV (n=3).

Discussion

Herein, we have investigated how ABX treatment of juvenile mice impacts on the course of acute IAV infection and how IAV infection and ABX treatment modulate GM composition. An important feature of our experimental design was to focus on juvenile mice, commencing ABX treatment immediately after weaning at a time known to be important for establishment of the GM [22]. Moreover, given that the majority of IAV infections in healthy humans do not result in viral pneumonia and severe disease, we utilized the HKx31 strain which is of moderate virulence in mice [27,29]. To more accurately recapitulate the severity of typical human infections in the mouse model.

Consistent with studies in adult mice using the virulent PR8 strain [9,24], ABX treatment of juvenile mice

was associated with enhanced severity of HKx31-induced disease and this was characterized by prolonged weight loss and enhanced virus growth in the airways. HKx31 infection induced only minor changes in the GM of control mice as mock- and IAV-infected CON mice displayed similar GM diversity. However, HKx31 infection of ABX-treated juvenile mice was associated with a significant increase in the GM diversity compared to mock-infected ABX-treated mice (ABX-IAV vs. ABX-mock: $p = 0.018$; CON-IAV vs. CON-mock: $p = 1.0$). Specifically, the relative abundance of eight taxa was significantly increased in ABX-IAV mice compared to ABX-mock mice. ABX treatment alone eradicated most bacteria except *Parabacteroides distasonis*, which is known to be resistant to β -lactam antibiotics through production of β -lactamases, however the abundance of these bacteria was further increased during IAV infection of ABX-treated mice. We speculate that this could relate to the focus of

the immune system, which must adjust from GM maintenance towards limiting IAV infection thereby facilitating uncontrolled growth of *Parabacteroides distasonis*. Thus, our studies suggest that in the context of ABX treatment, a moderate IAV infection represents an additional factor that may modulate GM diversity in a juvenile population.

Two studies reported that IAV infection of adult SPF mice induced changes in the GM composition [9,24]. This was associated with increased susceptibility to secondary bacterial infections [24]. In these studies, infection of adult mice (6-10 weeks of age) with sub-lethal doses of PR8 resulted in weight loss, disease and immunopathological lung injury [9,24]. PR8 infection was also associated with recruitment of T helper 17 (Th17) cells and local interferon- γ production to modulate the intestinal microbiota (including an increase in *Enterobacteriaceae*), thereby contributing to intestinal injury [9]. Furthermore, eradicating most of the GM with a broad spectrum ABX or specifically targeting *Escherichia coli* with streptomycin prior to PR8 infection reduced pathological injury of the small intestine [9]. In the present study, infection of juvenile mice with HKx31 did not affect the relative abundance of *Enterobacteriaceae* in CON-IAV mice compared to CON-mock mice, despite clear evidence of IAV-induced disease (as evidenced by virus replication and weight loss over time). However, ABX treatment of mock-infected animals increased the relative abundance of *Enterobacteriaceae* in the gut, which was further increased in ABX-IAV mice. Thus, HKx31 infection of juvenile mice does not exert the same indirect immune modulatory effects as PR8 infection of adult mice with regard to *Enterobacteriaceae*. Nonetheless, the combination of ABX-treatment and HKx31 infection did increase the relative abundance of *Enterobacteriaceae*. In future studies, it will be of interest to determine if the abundance of *Enterobacteriaceae* observed in ABX-IAV mice is also associated with enhanced intestinal injury in these animals.

In our study, ABX treatment alone reduced overall GM diversity while HKx31 infection of ABX-treated mice resulted in increased diversity, specifically in regard to the relative abundance of eight different bacteria. Five of these belonged to the phylum of Bacteroidetes (*Bacteroides*, *Bacteroides uniformis*, *Bacteroides acidifaciens*, *Bacteroidales* and Candidate phyla S24-7), as well as two Proteobacteria (*Sutterella* and *Enterobacteriaceae*) and one Verrucomicrobia (*Akkermansia muciniphila*). *Akkermansia muciniphila* uses gastrointestinal mucin as its carbon and nitrogen source [41]. The increased presence in ABX-IAV mice suggests that viral infection may promote its growth, possibly via

enhancing mucin production in the gut. This appears plausible since (i) it is established that IAV infection in the airways can induce immunomodulatory effects in the intestines and (ii) that a range of bioactive molecules, including cytokines and growth factors, can modulate mucin secretion in the gut [42]. In future studies, it would be of interest to investigate the relationship between growth of *Akkermansia muciniphila* and the ability of IAV infection to induce mucin secretion in the gut of ABX-treated and control animals.

Despite the increase in GM diversity between ABX-IAV and ABX-mock animals, ABX treatment prior to and during IAV infection was associated with exacerbated weight loss as well as enhanced virus replication in the airways. In future studies it will be of interest to investigate mechanisms underlying the ability of a moderate IAV infection to promote enhanced growth of specific gut bacteria in ABX-compromised mice. Further, it will be important to clarify the role of the enhanced GM diversity as it did not ameliorate disease severity in IAV-infected animals. Whether IAV-induced changes in ABX-compromised GM play any role in modulating disease severity should be the focus of future studies.

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Author Contributions Statement

EF, HF and PCR designed research; EF, LK and PCR performed research, EF and LK analyzed data, and EF, LK, DSN, HF and PCR wrote the paper.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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