ADDENDUM

Strain-level analysis of gut-resident pro-inflammatory viridans group Streptococci suppressed by long-term cotrimoxazole prophylaxis among HIV-positive children in Zimbabwe

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ABSTRACT

Antimicrobials have become a mainstay of healthcare in the past century due to their activity against pathogens. More recently, it has become clear that they can also affect health via their impact on the microbiota and inflammation. This may explain some of their clinical benefits despite global increases in antimicrobial resistance (AMR) and reduced antimicrobial effectiveness. We showed in a randomized controlled trial of stopping versus continuing cotrimoxazole prophylaxis among HIV-positive Zimbabwean children taking antiretroviral therapy (ART), that continuation of cotrimoxazole persistently suppressed gut-resident viridans group streptococcal species (VGS) that were associated with intestinal inflammation. In this addendum, we provide a broader overview of how antibiotics can shape the microbiota and use high read-depth whole metagenome sequencing data from our published study to investigate whether (i) the impact of cotrimoxazole on gut VGS and (ii) VGS associated inflammation, is attributable to strain-level variability. We focus on S. salivarius, the VGS species that was most prevalent in the cohort and for which there was sufficient genome coverage to differentiate strains. We demonstrate that suppression of S. salivarius by cotrimoxazole is not strain specific, nor did stool concentration of the pro-inflammatory mediator myeloperoxidase vary by S. salivarius strain. We also show that gut-resident S. salivarius strains present in this study population are distinct from common oral strains. This is the first analysis of how cotrimoxazole prophylaxis used according to international treatment guidelines for children living with HIV influences the gut microbiome at the strain-level. We also provide a detailed review of the literature on the mechanisms by which suppression of VGS may act synergistically with cotrimoxazole’s anti-inflammatory effects to reduce gut inflammation. A greater understanding of the sub-clinical effects of antibiotics offers new insights into their responsible clinical use.

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Introduction

More than 2000 years ago, Egyptian and Greek civilizations utilized a variety of substances in wound treatment, a handful of which are now known to have antimicrobial properties.1,2 The concept of an antibiotic, however, was not introduced until 1890, when antibiose (an antonym to symbiosis) was used to describe antagonistic interactions between microorganisms.3 Microbes naturally produce antimicrobial agents4 to interfere with the growth of other microbes in inter and intraspecies competition. Mycophenolic acid was the first such naturally produced antibiotic to be discovered and isolated in 1893, although it was not synthesized until 1969 as Salvarsan.5 The discovery of penicillin in 1928, its eventual synthesis in 1957 as penicillin V, and its subsequent success as a clinical therapy,3 led to a surge in antibiotic discovery and synthesis in the 1950–1980s.4

With the increase in diversity and accessibility of antibiotics, their use in people and animals (including misuse and overuse) has increased. The clearest consequence of increasing antibiotic
use has been an alarming rise in antimicrobial resistance (AMR) in recent years, which has made the treatment of many common infections more challenging.\(^5\) It has thus become equally important to understand the longer-term and subclinical effects of antibiotics aside from their role in killing or inhibiting pathogens.

Antibiotics also have the potential to shape the microbiome – the community of microorganisms (bacteria, archaea, fungi, viruses, and protists) that coexist in an intimate relationship with their host. Growing research interest in the composition and function of the microbiome has demonstrated its wide-reaching effects on physiologic development, function, and protection against infections. However, the concentrations at which antibiotics are administered elicit antimicrobial effects that extend beyond targeted pathogen elimination, to impact commensal microbiota members. This can have either detrimental or beneficial effects, depending on the underlying composition and function of an endogenous microbiome.

**Antimicrobial effects of antibiotic use on the microbiome**

Next-generation sequencing has demonstrated that antibiotics induce a reduction in gut bacterial biomass, bacterial taxonomic diversity, and functional diversity.\(^6\) A single antibiotic dose can lead to the replacement of bacteria that are susceptible to its mechanisms of action (antibiotic-sensitive) with the subset of microbes that are not susceptible (antibiotic-tolerant).\(^7\) In some individuals, the microbiota may not return to its initial composition, depending on the class of antibiotic used, the underlying health status of the patient and the use of other medications.\(^8,9\)

A recent review reported the impact of antibiotics on the gut microbiota and revealed that bacteria belonging to a restricted set of clades (phyla or genera) are most strongly affected by antibiotic use. The breadth of bacterial clades affected was largest for fluoroquinolones (32 phyla), but was as few as a single phylum for several antibiotics.\(^6\) The number of affected bacterial clades varied both between and within antibiotic classes.\(^5\) Suppression of antibiotic-susceptible microbes can disrupt colonization resistance and open an ecological niche for opportunistic or pathogenic bacteria, increasing the host’s susceptibility to post-antibiotic infection.\(^6,7\) Antibiotic use can also produce an increase in antibiotic-tolerant commensal bacteria.\(^10,11\) The impact of antibiotics on the microbiota, therefore, depends on the antibiotic used and the bacterial clades that respond. Sub-species level adaptation of the microbiota to the use of different antibiotic classes is even less well characterized and the clinical relevance of such changes is only beginning to be understood. Such effects may be particularly pronounced and persistent in young children in whom colonization by commensal microbes is ongoing and stable communities have yet to be fully established.

Advances in sequencing and bioinformatics now provide strain-level resolution of bacteria from whole metagenome shotgun sequencing data.\(^12\) Strains are sub-species level genetic variants of a microorganism which reflect adaptation to specific environmental selection pressures. It has become clear that sub-species variation in microbial genomes determines whether a commensal species is beneficial\(^13\) or harmful.\(^14,15\) Strain-level metagenomic analysis allows for more granular investigation of the alterations in microbiota composition and function caused by antibiotic use.

**Non-antimicrobial effects of antibiotic use in vulnerable populations**

Despite the threat of AMR and the impact on the microbiota, antibiotics continue to save lives in vulnerable populations.\(^16,17\) This is particularly relevant to low- and middle-income countries (LMIC) where exposure to pathogens,\(^18\) the prevalence of pathogen carriage,\(^19\) and the occurrence of severe bacterial infections are all high.\(^20\) Specifically, for immunocompromised individuals (e.g. people living with HIV or malnutrition) with limited access to rapid bedside diagnostic testing, the widespread use of prophylactic antibiotics, has become essential to treat and prevent infections. A natural question that follows is how antibiotic treatment continues to reduce morbidity and mortality in the face of high rates of AMR, and selection for resistant bacteria over time.\(^21,22\)

Over six decades ago, researchers first reported that sulfonamide antibiotics had beneficial effects that were not explained by their antibiotic properties.\(^23\)
Other antibiotics are now known to exhibit non-antibacterial effects. Macrolides, for example, modulate pro-inflammatory cytokine secretion, promote phagocytosis of apoptotic cells, reduce neutrophil chemotaxis, enhance granule exocytosis by neutrophils, augment bacridical activity by modulating oxidative bursts, and accelerate neutrophil apoptosis. The tetracycline, minocycline can exert anti-inflammatory and anti-apoptotic effects, inhibit proteolysis, and suppress angiogenesis and tumor metastasis. Cotrimoxazole has also exhibited immunosuppressive properties derived from the same antifolate activity that confers its antimicrobial effects.

The gut microbiome and antibiotic synergy

Tension between the benefits and potential harms of widespread antimicrobial use is well illustrated by cotrimoxazole – a broad-spectrum antibiotic that is now recommended long term for people living with HIV in areas where malaria or severe bacterial infections are highly prevalent. This recommendation arises from multiple studies that have demonstrated reductions in morbidity and mortality among HIV-positive adults and children taking cotrimoxazole prophylaxis, regardless of HIV disease stage, degree of immunosuppression or use of antiretroviral therapy (ART). However, there have been understandable concerns about the benefits to morbidity and mortality versus the risks of AMR posed by increasing the coverage and duration of cotrimoxazole use in LMIC. Interestingly, cotrimoxazole retains its clinical benefits despite a high preexisting prevalence of AMR among common pathogens, for reasons that are unclear. We recently reported findings from a subgroup of HIV-positive children (median [IQR] age, 8.9 [5.7,11.1] years) in Zimbabwe who were on ART and randomized within the Anti-Retroviral Research for Watoto trial (ARROW; Trial registration: ISRCTN24791884) to continue or stop taking prophylactic cotrimoxazole after an average of two years of daily use. We found that continuing daily cotrimoxazole reduced levels of biomarkers associated with systemic (plasma C-reactive protein, CRP, and interleukin-6, IL-6) and intestinal (stool myeloperoxidase) inflammation compared to stopping cotrimoxazole. This finding was not simply due to typical antimicrobial effects on pathogenic organisms, since the relative abundance of gastrointestinal Enterobacteriaceae detected in stool and the reported frequency of infection symptoms were unaffected. Instead, we found that cotrimoxazole decreased inflammation through three pathways: (1) suppressing viridans group streptococci (VGS), whose relative abundance was associated with increased intestinal neutrophil activity; (2) directly reducing activation of innate immune cells, which produce pro-inflammatory cytokines; and (3) suppressing production of the neutrophil chemoattractant IL-8 by gut epithelial cells (Figure 1). Importantly, the continued use of daily cotrimoxazole at prophylactic doses for an additional two years of follow-up did not affect measures of microbiota alpha or beta diversity, suggesting that the persistent alterations in gut microbiome composition and function caused by long-term use in this target population were narrow. We proposed that synergy between the antibiotic effects on gastrointestinal VGS and direct inhibition of pro-inflammatory mediator production may contribute to the sustained morbidity and mortality benefits of cotrimoxazole among people living with HIV. These observations were made despite high levels of resistance to cotrimoxazole in the ARROW trial target population.

The role of bacterial species in intestinal immune regulation

We also identified a potential gut VGS-dependent mechanism for this beneficial antimicrobial-anti-inflammatory synergy (Figure 1). Continued use of cotrimoxazole decreased the abundance of bacterial genes that encode mevalonate metabolism. These genes predominantly mapped to VGS and were positively associated with fecal myeloperoxidase concentrations (a marker of neutrophil activation and accumulation in the gut). The mevalonate pathway is one of two metabolic pathways utilized by bacteria to produce isoprenoids. Isoprenoids are precursors to eukaryote cholesterol and prokaryote cell wall peptidoglycan production. Mevalonate pathway activity has been shown to promote leukocyte recruitment and pro-inflammatory cytokine responses in vitro. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), the rate-limiting enzyme in mevalonate production. Treating neutrophils with pravastatin or
cerivastatin to block HMG-CoA reductase activity significantly decreased neutrophil chemotaxis and endothelial transmigration in vitro. The addition of exogenous mevalonate restored neutrophil chemotaxis.

The effect of mevalonate on chemotaxis and neutrophil migration may involve inhibition of protein prenylation by isoprenoids. Protein prenylation by isoprenoids is a key step in cell membrane receptor binding, activation of cell membrane trafficking, cytoskeletal organization, growth, apoptosis, and differentiation. Isoprenoids are responsible for the prenylation of several small GTPase molecules such as Rho. In neutrophils, Rho activity is required for neutrophil migration. Pravastatin, simvastatin, and atorvastatin were effective at reducing neutrophil transendothelial migration through a reduction in Rho activity. Neutrophil migration was restored by exogenous isoprenoids. Inhibition of isoprenoid synthesis also reduced neutrophil priming by IL-8 through reduction of protein prenylation; while inhibition of mevalonate pathway isoprenoid synthesis reduced IL-6 and IL-8 production by monocytes. VGS species also stimulate IL-8 secretion by monocyte-derived dendritic cells. Our original publication demonstrated that the production of IL-8 by gut epithelial cells activated with the pro-inflammatory cytokine IL-1β in vitro was also reduced by pre-treatment with cotrimoxazole. Streptococcus sp. are susceptible to cotrimoxazole, and the mevalonate pathway they encode is the less common of the two pathways for isoprenoid synthesis found in bacterial genomes. Together, our results support the notion that suppression of VGS, specifically, by cotrimoxazole in the gut of HIV-positive children, has a synergistic effect on suppressing molecular triggers for neutrophil chemotaxis to the gut.

Neutrophils kill pathogens via oxidative bursts involving myeloperoxidase. Fungal and bacterial pathogens, including group A and B streptococci, are effectively killed by myeloperoxidase in vitro. The bactericidal effect of myeloperoxidase on VGS,
by contrast, is very limited at low myeloperoxidase concentrations. Hydrogen peroxide (H$_2$O$_2$) is a key substrate in the bactericidal activity of myeloperoxidase. Streptococcus sp. produce H$_2$O$_2$ as a metabolic by-product, and the bactericidal activity of myeloperoxidase is enhanced in the presence of VGS-produced H$_2$O$_2$ in vitro. Thus, VGS appear to benefit from a competitive advantage over other pathogens and phenotypically related group A and B streptococci that are more effectively killed by myeloperoxidase in their presence. However, myeloperoxidase activity may also contribute to host tissue damage. The gut is a key site of inflammation in HIV-positive people (HIV enteropathy). Suppression of VGS may thus limit the detrimental effects of inflammation on the host gut, by reducing myeloperoxidase activity. This could have additive benefits during HIV infection.

**Intestinal VGS strain diversity in the ARROW cohort**

VGS are a heterogenous group of 30 streptococci species that have been classified as part of the core oral microbiome, but have also frequently been isolated from the small intestine. We previously reported that compared to the children randomized to stop cotrimoxazole treatment in the ARROW trial, children who continued cotrimoxazole had a lower relative abundance of the VGS species S. salivarius, S. parasanguinis, S. mutans, and S. vestibularis, all of which were positively associated with fecal myeloperoxidase concentration post-randomization. The most commonly identified species was S. salivarius.

A recent publication proposed that oropharyngeal bacteria, such as VGS, may be unrecognized drivers of intestinal inflammation, with important implications for treatment. However, distinguishing between VGS originating from the oral cavity versus other body sites requires strain-level rather than species-level analysis. If the long-term clinical benefits of continuing cotrimoxazole that we previously reported are partly driven by suppression of VGS, it is important to determine whether cotrimoxazole’s antimicrobial effects and VGS driven gut inflammation are associated with sub-species characteristics of VGS, since strain-level variation can determine pathogenic properties.

We initially analyzed microbial taxon abundance profiles generated using MetaPhlAn2 to demonstrate suppression of gastrointestinal VGS species among HIV-positive children randomized to continue taking cotrimoxazole relative to those who stopped. We further applied Pangenome-based Phylogenomic Analysis (PanPhlAn) to confirm the species identity of the streptococci that were suppressed. With PanPhlAn, the pangenome for a species is first obtained by compiling available, sequenced strain genomes into a single set of all genes that the species is known or predicted to carry. In addition to providing greater specificity for species identification, sub-species variants can be identified by PanPhlAn when bacterial genes with a similar depth of coverage are identified that map to a distinctive subset of the genes in that particular species’ pangenome. Identified gene sets can then be compared to determine differences in the functional capacity of unique strains across specimens.

In our original publication, 140 stool samples were collected at 84 (n = 36 continue and n = 36 stop) and 96 weeks (n = 33 continue and n = 35 stop) after randomization to stop or continue cotrimoxazole. Of these, PanPhlAn identified stool samples from six children who continued and 14 children who stopped cotrimoxazole that were positive for VGS (S. salivarius, S. parasanguinis, S. vestibularis, and/or S. mutans) at both follow-up time points, confirming VGS as the Streptococcal species suppressed by the continuation of cotrimoxazole. Here, we build on these observations by further exploring strain-level patterns within the VGS identified as being suppressed by long-term cotrimoxazole prophylaxis. S. salivarius was the most prevalent VGS species identified and was the only VGS species with sufficient genome coverage to undertake strain-level analyses. We used PanPhlAn to further characterize S. salivarius sub-species variants in order to determine whether: 1) S. salivarius strains in HIV-positive children on ART who continued cotrimoxazole use are different from those who stopped; 2) stool myeloperoxidase concentration differs by S. salivarius strain in ARROW samples; 3) S. salivarius strains in ARROW children are functionally distinct from S. salivarius reference strain genomes, most of which are derived from the oral cavity.
We used non-metric multi-dimensional scaling (NMDS) of the Jaccard distance between strain gene presence/absence profiles as an ordination technique. NMDS illustrates that the genetic composition of *S. salivarius* strains identified in ARROW samples did not differ between continue and stop groups (*p* = .310 by permutation test) (Figure 2a). In light of our original findings that VGS abundance was reduced in children who continued cotrimoxazole use, the strain-level similarity of *S. salivarius* between treatment groups indicates that subspecies variation in *S. salivarius* between children does not explain the ability of cotrimoxazole to suppress this VGS species, nor did continue cotrimoxazole favor expansion of specific strains.

To investigate whether gut neutrophil activity depends on *S. salivarius* strains, we compared gene presence/absence profiles of *S. salivarius* strains by stool myeloperoxidase concentration with permutation testing. Strain genetic composition was not associated with stool myeloperoxidase concentration (*p* = .298 by permutation test) (Figure 2b). These analyses provide evidence that the increase in stool myeloperoxidase with increasing *S. salivarius* abundance observed in our original study was not driven by sub-species genetic variants. Thus, our original findings demonstrated that continued cotrimoxazole use suppressed *S. salivarius* and that *S. salivarius* abundance was associated with greater gut neutrophil activity. Here we further demonstrate that suppression of *S. salivarius* by continued cotrimoxazole confers these anti-inflammatory benefits regardless of strain-level genetic variation between individual patients. It is unclear whether this broad suppression of *S. salivarius* by long-term cotrimoxazole use and the associated reduction in intestinal inflammation is specific to children living with HIV in Zimbabwe or a more universal feature of cotrimoxazole treatment.

Next, we investigated whether the *S. salivarius* strains identified in stool samples from ARROW were distinct from the *S. salivarius* reference strains utilized by PanPhlAn, which were predominantly isolated from the oral cavities of subjects from high-income countries. We found that the genetic composition of ARROW *S. salivarius*

![Figure 2](image-url). Comparison of gene profiles from *S. salivarius* strains in stool samples from ART-treated HIV-positive children randomized to continue versus stop cotrimoxazole prophylaxis. (a) Nonmetric multidimensional scaling ordination plot of *S. salivarius* gene presence/absence profiles in ARROW strains identified in ART-treated HIV-positive Zimbabwean children randomized to stop cotrimoxazole (orange) or continue cotrimoxazole (green), and the PanPhlAn reference strain genomes (black). The blue circle represents the minimal area occupied by ARROW strains. (b) Nonmetric multidimensional scaling ordination plot of *S. salivarius* gene presence/absence in ARROW strains colored from lowest stool myeloperoxidase concentration (light blue) to highest stool myeloperoxidase concentration (dark blue). (c) Boxplots showing the distance of ARROW *S. salivarius* strains (blue) and PanPhlAn reference strains (white) from their respective group centroids.
strains was more similar to each other than to the genetic composition of S. salivarius reference strain genomes used by PanPhlAn ($p = .001$ by permutation test) (Figure 2c). In high-income settings, VGS are considered to predominantly reside in the oral cavity and to form part of the core oral microbiome; our data do not support that this is the case for S. salivarius strains identified in the stool of ART-treated children living with HIV in Zimbabwe.

Finally, to identify gene families that uniquely identify ARROW strains, we used Fisher’s Exact test to explore whether individual gene families were more likely to be present or absent in ARROW S. salivarius strains, pooled across treatment groups, compared to PanPhlAn S. salivarius reference strains. If the probability of observing a greater (or lower) proportion of strains with a specific gene in the ARROW group, compared to the reference group, was <0.05 after false discovery rate correction, the gene family was reported as enriched (or depleted) in the ARROW group. Forty-eight gene families were more likely to be present in ARROW S. salivarius strains and two were less likely to be present compared to the PanPhlAn reference strain genomes. Gene families disproportionately present in the ARROW S. salivarius strain genomes predominantly encoded cell membrane components, membrane biosynthesis, transmembrane transporters, transmembrane channels, membrane-bound surface-exposed environmental sensors, and enzymes involved in gene translation (Table 1). ARROW strains are therefore more likely than the reference strains to encode mechanisms to sense and respond to changing conditions in their environment, to survive in harsh environmental conditions, for nutrient acquisition and bacterial communication.

We did not have stool samples from HIV-uninfected, ART-naïve or cotrimoxazole-naïve children to determine whether S. salivarius strains differ by HIV or treatment status. However, it is possible that these are useful genetic attributes for bacterial strains residing in an intestinal milieu characterized by enteropathy, inflammation, and increased susceptibility to enteric infection. This finding may also reflect a lack of representative reference genomes for this species of VGS from children in LMICs. We also did not have oral samples from the same children to more directly explore the similarities between oral and intestinal strains. However, we have shown that strain-level analysis is crucial to the determination of niche-specific microbe adaptations of potential clinical relevance. Since our stool samples were collected at 84- and 96-weeks post-randomization to stop versus continue cotrimoxazole, we do not know the impact of stopping or continuing cotrimoxazole on S. salivarius strain diversity at earlier time-points. We did not have sufficient coverage of other VGS genomes to determine whether our findings also apply to those species.

**Future directions**

This is the first analysis of how cotrimoxazole prophylaxis used according to international treatment guidelines for children living with HIV influences the gut microbiome at the strain-level. We characterized S. salivarius strain gene compositions in stool samples from the ARROW trial and found that, in HIV-positive Zimbabwean children on ART, S. salivarius strains were distinct from the predominantly oral reference strains, and may be adapted to the gut milieu induced by chronic HIV-infection. There was no evidence that suppression of this VGS species in the gut by the continuation of cotrimoxazole prophylaxis, or its association with stool levels of myeloperoxidase, is dependent on sub-species characteristics. This suggests that broad suppression of VGS by cotrimoxazole is sufficient to confer these synergistic antimicrobial-anti-inflammatory benefits.

Our current understanding of how the microbiota contributes to human health is focused on composition and function at the level of species or, more commonly, higher-order taxa. New analytical tools allow us to characterize the microbiome at the strain-level, which provides new insights into the impact of antibiotics. This may be of particular importance for chronic infections, like HIV, where long-term cotrimoxazole prophylaxis is recommended, but the basis for the sustained clinical benefit of long-term therapy is not well understood. Furthermore, AMR to cotrimoxazole is already high in LMIC and several studies demonstrate that the impact of treatment on
Table 1. Gene families that were more likely (enriched) or less likely (depleted) to be present in ARROW sub-study *S. salivarius* strains based on PanPhlan strain profiling.

| Cluster ID    | Cluster name                                                                 | Description                                                                                           | Enriched/Depleted |
|---------------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|-------------------|
| UniRef90_F2BP82 | Cluster: PTS family glucitol/sorbitol porter, IIC component                  | Biological process: Uptake and metabolism of carbohydrates                                             | Enriched          |
| UniRef90_E9DL83 | Cluster: Alanine racemase                                                      | Biological process: Alanine biosynthesis                                                              | Enriched          |
| UniRef90_J7T771 | Cluster: Alanyltransferase/seryltransferase                                   | Biological process: Cell wall synthesis                                                               | Enriched          |
| UniRef90_J7TUD0 | Cluster: Glycosyltransferase involved in cell wall biogenesis                | Biological process: Cell wall synthesis                                                               | Enriched          |
| UniRef90_A3CKP1 | Cluster: Leucine–tRNA ligase                                                  | Biological process: Coupling Leucine to its cognate tRNA in translation                               | Enriched          |
| UniRef90_V889Y3 | Cluster: Phage_integrase domain-containing protein                            | Biological process: DNA integration and recombination                                                | Enriched          |
| UniRef90_QSM2T6 | Cluster: ATP-dependent helicase/deoxyribonuclease subunit B                  | Biological process: DNA repair                                                                      | Enriched          |
| UniRef90_A0A3R9S70 | Cluster: Glucose-6-phosphate isomerase                                        | Biological process: Gluconeogenesis and glycolysis                                                   | Enriched          |
| UniRef90_Q03K75 | Cluster: Histidinol-phosphate aminotransfer                                   | Biological process: Histidine biosynthesis                                                           | Enriched          |
| UniRef90_A0A2X3UHU0 | Cluster: Response regulator                                                   | Biological process: Histidine kinase sensor autophosphorylation and transcription regulation         | Enriched          |
| UniRef90_A0A3S5E1K2 | Cluster: DNA-binding response regulator                                        | Biological process: Histidine kinase sensor autophosphorylation and transcription regulation         | Enriched          |
| UniRef90_F8LH13  | Cluster: Sensor protein                                                        | Biological process: Histidine kinase sensor autophosphorylation and transcription regulation         | Enriched          |
| UniRef90_A0A3S5E1E5 | Cluster: Accessory Sec system protein translocase subunit SecY2             | Biological process: Intracellular protein transport                                                   | Enriched          |
| UniRef90_F8LR08  | Cluster: Accessory Sec system protein translocase subunit SecY2              | Biological process: Intracellular protein transport                                                   | Enriched          |
| UniRef90_QSM376  | Cluster: UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2                 | Biological process: Peptidoglycan biosynthesis                                                        | Enriched          |
| UniRef90_A0A3E4XAO5 | Cluster: Accessory Sec system protein translocase subunit SecY2             | Biological process: Protein transport                                                                | Enriched          |
| UniRef90_J0K77   | Cluster: 30S ribosomal protein S16                                             | Biological process: Translation                                                                      | Enriched          |
| UniRef90_A0A3S4MD22 | Cluster: ABC transporter permease                                              | Biological process: Transmembrane transport                                                          | Enriched          |
| UniRef90_E3CN82  | Cluster: Oligopeptide-binding protein AmiA                                    | Biological process: Transmembrane transport                                                           | Enriched          |
| UniRef90_W3XS6    | Cluster: Oligopeptide-binding protein AmiA                                    | Biological process: Transmembrane transport                                                           | Enriched          |
| UniRef90_A0A1C7CB97 | Cluster: ABC transporter, permease protein                                     | Biological process: Transmembrane transport                                                           | Enriched          |
| UniRef90_C2LQC3   | Cluster: DUF4173 domain-containing protein                                     | Domain: Membrane component                                                                             | Enriched          |
| UniRef90_C2LVA7  | Cluster: Gram-positive signal peptide protein, YSIRK family (Fragment)       | Domain: Membrane component                                                                             | Enriched          |
| UniRef90_F8LGT8  | Cluster: Putative ABC transporter, integral membrane protein                  | Domain: Membrane component                                                                             | Enriched          |
| UniRef90_Q5M5D7  | Cluster: Conserved hypothetical, predicted membrane protein (TMSS)             | Domain: Membrane component                                                                             | Enriched          |
| UniRef90_A0A0Y1UI35 | Cluster: Drug/metabolite transporter permease                                 | Domain: Transmembrane                                                                                  | Depleted          |
| UniRef90_E9DMC8  | Cluster: ABC-2 type transporter                                                | Domain: Transmembrane                                                                                  | Enriched          |
| UniRef90_F8LRX0  | Cluster: Transmembrane protein, ortholog TM1408                                | Domain: Transmembrane                                                                                  | Enriched          |
| UniRef90_E9DMC5  | Cluster: HATPase_c_5 domain-containing protein                                | Domain: Transmembrane (histidine kinase sensing)                                                     | Enriched          |
| UniRef90_A0A074IU53 | Cluster: Accessory secretory protein Asp4                                    | Domain: Transmembrane helical protein: SecA-SecY accessory                                            | Enriched          |
| UniRef90_A0A2U2MF2 | Cluster: ABC transporter ATP-binding protein                                   | Molecular function: Catalyze ATP to ADP                                                                | Enriched          |
| UniRef90_F8LGT7  | Cluster: Nod factor export ATP-binding protein I (Nodulation ATP-binding protein) | Molecular function: Catalyze ATP to ADP                                                                | Enriched          |
| UniRef90_F8LL21  | Cluster: Beta-lactamase domain-containing protein                             | Molecular function: Catalyze beta-lactam deactivation                                                 | Enriched          |
| UniRef90_F8LI15  | Cluster: Glucosyltransferase-I (GTF-I) (Dextranucrase) (Sucrose 6-glucosyltransferase) | Molecular function: Catalyze fructose production from glucose                                        | Depleted          |

(Continued)
the microbiome may be limited, as few differences in the alpha- or beta-diversity of microbial species or metabolic functions have been observed. As the proportion of HIV-infected children taking cotrimoxazole expands in-line with international guidelines, investigating changes in microbiome strain membership and functions may become increasingly important for promoting the health of these children. Our work and that of others demonstrates that continued cotrimoxazole use reduces systemic inflammation associated with poor clinical outcomes for children and adults living with HIV in sub-Saharan Africa. These additional analyses suggest that this beneficial effect is independent of sub-species genetic variants. Current and future treatment of infectious diseases will benefit from a better understanding of immunopathology; the role of the microbiota in inflammation; the sub-clinical, non-antibiotic effects of different antimicrobials; and the effect of antimicrobials on strain-specific host–microbiota interactions. It is important to better understand the risks and benefits of widespread antibiotic use such as cotrimoxazole, to inform policy around their use, to balance population health and antimicrobial stewardship.

Disclosure of Potential Conflicts of Interest

The authors report no conflict of interest.

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