

Characterizing Reservoirs of Zoonotic Pathogens and Antibiotic Resistance in
Households in Low- and Middle-Income Countries

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Abstract

Motivation. The global health burden of infectious diseases – which remain a leading cause of death – is exacerbated by the critical threat of antimicrobial resistance. Low- and middle-income countries (LMICs) are disproportionately burdened by this threat, often challenged with addressing issues related to poor regulation of and access to antibiotics; inadequate access to safe drinking water, sanitation, and hygiene (WASH) infrastructure; and insufficient management of human and animal fecal waste. This dissertation examined the role of domesticated animals in community spread of zoonotic pathogens and antibiotic-resistant bacteria. Specifically, it sought to characterize direct human-animal exchange as well as indirect transmission via environmental pathways. **Aims.** In the first aim, evidence for zoonotic exchange of antibiotic resistance in non-occupational settings in LMICs was compiled through a systematic literature review. Exchange of antibiotic-resistant bacteria was found to be widespread between humans and domesticated animals in households and backyard farms, with the environment likely playing an important – but poorly understood – role in that transmission. We therefore leveraged data from a cluster-randomized controlled trial in rural Kenya to identify associations, using regression analyses, between household animal ownership, fecal contamination in domestic environments, and child health outcomes as the second aim. In the third aim, we used 16S rRNA gene and long-read sequencing to understand differences in human-animal overlap in microbiomes and resistomes between rural and urban Bangladeshi communities. We documented genomic evidence, especially in the urban community, of overlapping antibiotic resistance genes (ARGs) between humans, goats, and chickens. Leveraging sample

collection from urban Kenyan households for the final aim, we evaluated optimal genome assembly and ARG detection methods for tracking *Escherichia coli* strains, ARGs, and mobile genetic elements across humans, animals, and environments. We found that hybrid assembly of short and long sequence reads yielded sufficient recovery of reference *E. coli* genomes and accurate ARG hits. **Conclusion.** Collectively, the findings from this work demonstrate widespread opportunities for community spillover of zoonotic pathogens and antibiotic resistance between humans, animals, and the environment in LMICs. This dissertation provides evidence supporting the implementation of One Health surveillance efforts and adoption of high-resolution genomic methods to better understand community transmission mechanisms and pathways. Moving towards a comprehensive understanding of zoonotic exchange dynamics is critical to inform effective intervention strategies to disrupt pathogen and antibiotic resistance transmission for protection of human, animal, and environmental health.

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Table of Contents

<i>Abstract</i>	<i>ii</i>
<i>Acknowledgements</i>	<i>iv</i>
<i>List of Tables</i>	<i>ix</i>
<i>List of Figures</i>	<i>x</i>
<i>Chapter 1: Introduction</i>	<i>2</i>
1.1 <i>Motivation</i>	<i>2</i>
Global Burden of Infectious Disease	<i>2</i>
Zoonotic Pathogen Transmission	<i>2</i>
Global Threat of Antimicrobial Resistance	<i>4</i>
1.2 <i>Research Objectives</i>	<i>6</i>
Research Aim 1	<i>7</i>
Research Aim 2	<i>7</i>
Research Aim 3	<i>7</i>
Research Aim 4	<i>8</i>
<i>Chapter 2: Human colonization with antibiotic-resistant bacteria from non-occupational exposure to domesticated animals in low- and middle-income countries: A critical review</i>	<i>9</i>
2.1 <i>Abstract</i>	<i>9</i>
2.2 <i>Background</i>	<i>10</i>
2.3 <i>Methods</i>	<i>12</i>
Search Strategy	<i>12</i>
Selection of Studies	<i>13</i>
Data Extraction and Synthesis.....	<i>14</i>
2.4 <i>Results</i>	<i>15</i>
Study Selection and Characteristics	<i>15</i>
Evidence of Antibiotic Resistance Exchange Between Humans and Animals.....	<i>17</i>
Differential Risk of Colonization	<i>24</i>
Environmental Transmission Pathways for Zoonotic ARB and ARG Exchange	<i>30</i>
2.5 <i>Discussion</i>	<i>32</i>
Limitations	<i>32</i>
Recommendations for Future Work	<i>34</i>
Conclusion	<i>40</i>
2.6 <i>Acknowledgements</i>	<i>41</i>
2.7 <i>Citation</i>	<i>42</i>
<i>Chapter 3: Addressing fecal contamination in rural Kenyan households: the roles of water treatment and animal ownership</i>	<i>43</i>

3.1 Abstract	43
3.2 Background	44
3.3 Methods	46
Study Design	46
Sample and Data Collection	47
Statistical Analyses	49
3.4 Results	53
Household Characteristics	53
Levels of Environmental Contamination in the Control Group	54
Intervention Effects on Environmental Contamination	55
Associations Between Animal Ownership and Environmental Contamination	56
Associations Between Animal Ownership and Child Health	60
3.5 Discussion	60
Limitations	65
Conclusion	65
3.6 Acknowledgements	66
3.7 Citation	67
<i>Chapter 4: Differential overlap in human and animal fecal microbiomes and resistomes in rural versus urban Bangladesh</i>	68
4.1 Abstract	68
4.2 Background	69
4.3 Methods	72
Study Sites	72
Sample Collection	74
DNA Extraction	76
16S rRNA Gene Amplicon Generation and Sequencing	77
Long-Read Sequencing	79
Data Processing and Analysis	79
Data Availability	82
4.4 Results	83
Similarity in Gut Microbiomes	83
Similarity and Overlap in Resistomes	86
4.5 Discussion	93
Limitations	98
Conclusion	99
4.6 Acknowledgements	100
4.7 Citation	100

<i>Chapter 5: Benchmarking assembly methods for genomic characterization of antibiotic-resistant E. coli strains in humans, animals, and environmental samples</i>	102
5.1 Abstract	102
5.2 Background	103
5.3 Methods	106
Description of Data	106
Benchmarking	107
5.4 Results	109
Assembler Impacts on <i>E. coli</i> Genome Recovery	109
5.5 Discussion	112
Conclusion	114
5.6 Acknowledgements	115
<i>Chapter 6: Summary, Recommendations, and Conclusion</i>	117
6.1 Research Summary	117
Summary of Dissertation Findings	117
6.2 Recommendations and Future Research	120
Existing Knowledge Gaps	120
Recommended Future Research and Actions	123
6.3 Conclusion	126
<i>Chapter 7: Additional Relevant Research</i>	128
7.1 Intervention Impacts on Infectious Diseases	128
7.2 WASH and Nutritional Interventions Against Antibiotic Resistance	129
7.3 Environmental Surveillance for Monitoring Infectious Diseases	130
References	132
Appendices	167
8.1 Appendix A – Chapter 2	167
Supporting Information A-1: Generic search string and search strings adapted for six databases	167
Supporting Information A-2: Methods for categorizing full text publications	170
8.2 Appendix B – Chapter 3	189
Supporting Information B-1: Additional methods details	189
Supporting Information B-2: Additional results	196
8.3 Appendix C – Chapter 4	216
8.4 Appendix D – Chapter 5	226
Supporting Information D-1: Additional methods details	226
Supporting Information D-2: Additional results	230

List of Tables

<i>Table 2-1: Summary of characteristics of included studies (N = 79)</i>	16
<i>Table 2-2: Studies supporting whole bacteria zoonotic transmission of antibiotic-resistant bacteria within and between households/farms</i>	21
<i>Table 2-3: Studies supporting zoonotic horizontal gene transfer of antibiotic resistance genes within and between households/farms</i>	23
<i>Table 2-4: Studies demonstrating differential risk of antibiotic resistance colonization in humans</i>	25
<i>Table 4-1: Plasmids carrying antibiotic resistance genes</i>	90
<i>Table 5-1: Metrics for E. coli isolates included in simulated data set</i>	107
<i>Table 5-2: Metrics for E. coli strains detected in pooled short reads</i>	109
<i>Table A-1: Data extraction codebook</i>	172
<i>Table A-2: PRISMA 2020 checklist</i>	176
<i>Table A-3: Individual study characteristics for included studies (N = 79)</i>	179
<i>Table B-1: E. coli measured at one- and two-year assessments, combined and separately, interventions vs. control.</i>	200
<i>Table B-2: Total coliforms (water and hand samples) or fecal coliforms (toy samples) measured at one- and two-year assessments, combined and separately, interventions vs. control.</i>	201
<i>Table B-3: Fly counts and dirt on hands/fingernails measured at one- and two-year assessments, combined and separately, interventions vs. control</i>	202
<i>Table B-4: E. coli and fly prevalence and concentration measured at one- and two-year assessments, combined and separately, interventions vs. control, subgroup analysis by season.</i>	204
<i>Table C-1: Descriptive statistics for long-read sequencing and assembly</i>	217
<i>Table C-2: Comparisons of normalized abundance of antibiotic resistance genes in humans, chickens, and goats using Wilcoxon rank sum tests</i>	217
<i>Table C-3: Comparisons of normalized abundance of antibiotic resistance genes, paired by drug class, in rural versus urban communities using Wilcoxon signed-rank tests</i>	218
<i>Table C-4: Comparisons of Bray-Curtis dissimilarity indices between resistomes within humans, chickens, and goats using Wilcoxon rank sum tests</i>	218
<i>Table C-5: BLASTN confirmation of potential pathogens classified to the strain or subspecies level and carrying antibiotic resistance genes</i>	218
<i>Table C-6: Quality control parameters for DNA extraction for long-read sequencing.</i>	221
<i>Table C-7: Illumina primers</i>	222
<i>Table D-1: Summary of sequencing statistics for real data from 8 poultry-owning households in Kenya</i>	231

List of Figures

Figure 2-1: Locations of included studies in low- and middle-income countries.	15
Figure 2-2: Research priorities and recommendations for future zoonotic transmission studies	36
Figure 3-1: Data collection and environmental sampling profile in the trial, by study arm and year of measurement.	50
Figure 3-2: Intervention impacts on environmental contamination.....	58
Figure 3-3: Associations between $\log_{10}$ number of animals owned by households and environmental contamination.....	59
Figure 3-4: Associations between $\log_{10}$ number of animals owned by households and child health.....	61
Figure 4-1: Study design flowchart for comparing human and animal fecal composites in a rural and urban community.....	73
Figure 4-2: Relative abundance of 30 most abundant families in all samples from 16S rRNA gene analysis	84
Figure 4-3: Bray-Curtis dissimilarity between resistomes (top and middle; long-read sequencing) and bacterial community compositions (bottom; 16S rRNA gene analysis) in humans, chickens, and goats...85	
Figure 4-4: Abundance of antibiotic resistance genes (ARGs) (identified in long-read sequencing data) as gene copies normalized by Gbp of data classified as bacteria for each fecal host by urbanicity.	86
Figure 4-5: Distinct antibiotic resistance gene (ARG) alleles overlapping between humans and each animal host.....	89
Figure 4-6: Prevalence of potentially pathogenic bacteria species and those carrying antibiotic resistance genes (ARGs) identified in contigs assembled from long reads	92
Figure 5-1: Assembly quality metrics for short-read (SPAdes), short-read-first hybrid (Hybrid SPAdes), and long-read-first hybrid (Flye + Pilon) assemblies, with and without reference-based read binning.....	110
Figure 5-2: Unique antibiotic resistance gene (ARG) A) detection and B) accuracy metrics with raw read (short; long) and assembly (SPAdes; Hybrid SPAdes; Flye + Pilon) inputs	112
Figure A-1: Full-text screening and categorization flow chart.	171
Figure A-2: PRISMA 2020 flow chart.	178
Figure B-1: Directed acyclic graph for <i>E. coli</i> contamination in stored water	197
Figure B-2: Directed acyclic graph for <i>E. coli</i> contamination on child hands	197
Figure B-3: Directed acyclic graph for <i>E. coli</i> contamination on sentinel toys.....	198
Figure B-4: Directed acyclic graph for fly counts	198
Figure B-5: Directed acyclic graph for diarrhea	199
Figure B-6: Directed acyclic graph for length-for-age Z-scores.....	199
Figure B-7: Associations between $\log_{10}$ number of animals (capped at five per species) owned by households and environmental contamination	207
Figure B-8: Associations between binary household animal ownership and environmental contamination	208
Figure B-9: Associations between binary household animal ownership (households with extreme predicted probabilities excluded) and environmental contamination.....	209

Figure B-10: Associations between $\log_{10}$ median number of animals owned by households in the same village and environmental contamination	210
Figure B-11: Associations between $\log_{10}$ number of animals (capped at five per species) owned by households and child health	211
Figure B-12: Associations between binary household animal ownership and child health.....	212
Figure B-13: Associations between binary household animal ownership (households with extreme predicted probabilities excluded) and child health.....	213
Figure B-14: Associations between binary household animal ownership and stunting outcomes, accounting for households that changed animal ownership status between enrollment and year two	214
Figure B-15: Associations between $\log_{10}$ median number of animals owned by households in the same village and child health.....	215
Figure C-1: Relative abundance of the 30 most abundant families in all controls from 16S rRNA gene analysis	216
Figure C-2: Bray-Curtis dissimilarity between bacterial community compositions in humans and chickens (left) and in humans and goats (right) with taxa present in no template control removed	217
Figure C-3: Bray-Curtis dissimilarity between resistomes (top and middle; long-read sequencing) and bacterial community compositions (bottom; 16S rRNA gene analysis) within chickens, goats, and humans	219
Figure C-4: Principal coordinate analyses (PCoAs) of Bray-Curtis dissimilarity between antibiotic resistance gene alleles in humans, chickens, and goats for four most abundant drug classes	220
Figure D-1: Locations of households and primary drinking water sources in the two study communities (Dagoretti South; Kibera) in Nairobi County, Kenya.....	230
Figure D-2: <i>E. coli</i> strain A) multiplicity and B) matching across real samples, as identified from raw Illumina reads by StrainGST	232

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Chapter 1: Introduction

1.1 Motivation

Global Burden of Infectious Disease

Despite substantial efforts encouraged by the Millennium Development Goals and Sustainable Development Goals (SDGs) – and subsequent reductions in under-five child mortality since 2000 – there were still an estimated 5.3 million under-five deaths globally in 2019; Africa and South Asia had the highest child mortality rates of all regions.^{1–3} Infectious disease accounted for 49.2% of these deaths, with 480,000 under-five deaths specifically attributable to diarrheal disease.³ The SDGs have encouraged significant investments to increase access to safe drinking water, sanitation, and hygiene (WASH) and reduce the burden of infectious disease by 2030.² While reviews of predominately observational data suggest WASH interventions can reduce diarrheal disease, recent randomized controlled trials have demonstrated mixed effects of WASH interventions on child health outcomes in low- and middle-income countries (LMICs).^{4–8} The lack of observed effects on diarrhea could partly be explained by insufficient adherence to interventions. An additional explanation is the potential negative health effects resulting from exposure to domesticated animal feces, which has historically been neglected in the design of WASH interventions.^{9–11}

Zoonotic Pathogen Transmission

Animal husbandry is a particularly important source of income to many households in LMICs.¹² Further, animal husbandry is often encouraged in nutritional interventions aiming to improve child growth, as it increases consumption of nutrient-rich animal-sourced foods (e.g., meat; milk; eggs).^{13–15} However, the origination of 60.3% of

emerging infectious diseases has been attributed to zoonotic pathogens from non-human animal sources.¹⁶ While the emergence of 71.8% of these infectious diseases were caused by pathogens originating in wildlife, domesticated animals pose a substantial risk for zoonoses, both directly through close human-animal interactions and indirectly through environmental exposure pathways.¹⁶ Domesticated animals are confirmed reservoirs of many enteric pathogens known to cause diarrheal disease in humans, including pathogenic *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp.^{17–19} As the annual global production of domesticated animal feces is nearly four times that of human feces – and is continually increasing due to projected increases in food animal production – insufficient animal feces management likely contributes to the global burden of infectious disease.^{20,21} Prior research has demonstrated that human exposure to domesticated animals, particularly in the case of cohabitation, is associated with adverse human health outcomes, including diarrheal disease.^{9,22}

Human exposure to enteropathogens primarily occurs in the household environment, particularly for children under five.²³ Previous research has shown that living in close proximity to domesticated animals is associated with increased exposure to fecal contamination in the household environment.^{24–26} Host-specific fecal markers from domesticated animals (dogs; birds; and ruminants) were prevalent in multiple household reservoirs (in soil; on hands; and in drinking water) in rural and urban Bangladesh, rural India, and rural Kenya.^{26–29} In rural Bangladesh, increased concentration of BacCow, an animal-specific fecal marker, was associated with increased prevalence of pathogenic shiga toxin-producing *E. coli* (STEC).²⁷ Human exposure to animal fecal contamination,

which can harbor zoonotic enteropathogens, can therefore put humans at an elevated risk of enteric infections.

Global Threat of Antimicrobial Resistance

Human-animal interactions raise not only the risk of zoonotic disease transmission, but also the risk of the proliferation of antimicrobial resistance (AMR). Antimicrobial-resistant infections (including antibiotic-resistant bacterial infections) are prone to treatment failure, increasing treatment and recovery time, healthcare costs, and mortality rates. In 2019, it was estimated that 1.3 million lives lost globally were attributable to antibiotic-resistant infections alone, with a projected loss of over 10 million lives by 2050 due to antimicrobial-resistant infections generally; the highest burden of these deaths is occurring in LMICs.^{30,31} LMICs face an added challenge of balancing the risks from AMR against inadequate access to antibiotics, as more deaths in LMICs are attributable to limited access to antibiotics than antibiotic-resistant infections.³² AMR, however, still threatens the sustainability of life-saving drugs. Antimicrobials, especially antibiotics, are the basis of modern medicine and are used to treat common illnesses, prevent infections after routine surgeries, and even act as therapeutics for those with compromised immune systems. As bacterial pathogens reproduce and mutate over time, some mutations allow them to develop resistance to combat the antibiotics designed to inactivate them.

Antibiotic resistance can arise through the use, and especially overuse, of antibiotics in humans and domesticated animals, which contributes to the selection of intestinal bacterial strains that have acquired antibiotic resistance.³³ In microbial communities, such as the intestinal microbiota, horizontal gene transfer of antibiotic resistance genes

(ARGs) is facilitated by mobile genetic elements moving within or between DNA molecules (e.g., transposons; integrons) or between bacteria (e.g., plasmids), allowing the exchange of ARGs between benign bacteria and enteric pathogens.^{34,35} The ability of ARGs to transfer indiscriminately between bacteria means that antibiotic-resistant bacteria are not only a concern in clinical settings, but that they can widely circulate between humans, animals, and the environment in community settings as well. A study among six countries – Bangladesh, Bolivia, Ghana, India, Pakistan, and South Africa – found that more than half of community-acquired infections were resistant to antibiotics.³⁶ Spread of antibiotic resistance between humans, animals, and the environment is exacerbated in LMICs due to increasing access to unregulated antibiotics, insufficient human and animal waste management, and limited access to safe drinking water, sanitation, and handwashing infrastructure.^{32,37–41}

The environment is thought to play a substantial role in the proliferation of antibiotic-resistant bacteria.⁴² Previous work has shown that antibiotic-resistant bacteria are prevalent in soil. In rural Bangladesh, >40% of *E. coli* isolated from soil were resistant to at least one antibiotic and >10% were multidrug resistant.⁴³ Antibiotic-resistant bacteria have also been found in drinking water in LMICs such as China, Tanzania, and Peru.^{44–}
⁴⁶ Nearly 50% of *E. coli* isolates tested in northern Tanzania were resistant to at least one antibiotic.⁴⁵ High levels of antibiotic-resistant bacteria in the environment are concerning due to the potential transmission between environmental matrices, humans, and animals. In an investigation of ARG sequences available in databases from soil and human clinical samples, there was a high sequence identity (>99%) between soil ARGs and those found in enteropathogens.⁴⁷ A study of ARGs across multiple environmental

samples in Peru found the same ARGs in different matrices, including soil and water.⁴⁸

While the presence of antibiotic-resistant bacteria and ARGs in the environment is well documented, a comprehensive understanding of which environmental pathways are most important, and how human-animal interactions influence the prevalence of specific antibiotic-resistant bacteria and ARGs in the environment, is still missing.

Understanding the role of the environment in the transmission of antibiotic resistance is crucial to implementing effective intervention strategies.

1.2 Research Objectives

The pressing threats of zoonoses and antibiotic resistance have led to an international consensus that a One Health paradigm should be adopted to develop solutions protective of human, animal, and environmental health.⁴⁹ This dissertation therefore sought to use One Health approaches to understand the exchange of antibiotic resistance and zoonotic enteropathogens between humans and domesticated animals in LMICs, especially within household environments and backyard farms. Specifically this dissertation 1) compiled literature examining zoonotic exchange of antibiotic-resistant bacteria or ARGs within households and backyard farms in LMICs; 2) examined associations between domesticated animal ownership and environmental fecal contamination and child health in rural Kenya; 3) compared similarity and overlap in intestinal resistomes – or the collection of all ARGs – of humans and domesticated animals in rural versus urban Bangladesh; and 4) evaluated optimal genomic methods for matching antibiotic-resistant *E. coli* strains across human, animal, and environmental samples in urban Kenya. Specific research objectives for each of these aims are described below.

Research Aim 1

In the first aim (Chapter 2), *Human colonization with antibiotic-resistant bacteria from non-occupational exposure to domesticated animals in low- and middle-income countries: A critical review*, we systematically compiled evidence of the risk of human colonization with antibiotic-resistant bacteria or ARGs (including antibiotic-resistant infections), resulting from non-occupational exposure to domesticated animals within LMIC households and backyard farms. Secondary objectives included identifying important zoonotic transmission mechanisms (i.e., whole bacteria transmission; horizontal gene transfer), risk factors, and environmental transmission pathways.

Research Aim 2

In the second aim (Chapter 3), *Addressing fecal contamination in rural Kenyan households: the roles of water treatment and animal ownership*, we used generalized linear models to evaluate the impacts of WASH and nutritional interventions on *E. coli* contamination in rural Kenyan households. Further, we determined whether animal ownership was associated with 1) *E. coli* contamination in the domestic environment (stored water; child hand rinses; sentinel toy rinses) and fly presence near food preparation areas; and 2) under-five child health outcomes (diarrhea; stunting).

Research Aim 3

In the third aim (Chapter 4), *Differential overlap in human and animal fecal microbiomes and resistomes in rural versus urban Bangladesh*, we used 16S rRNA gene amplicon and long-read sequencing to characterize similarity and overlap in fecal microbiomes and resistomes between humans and domesticated animals (chickens; goats) in a rural versus urban community in Bangladesh. We also compared ARG abundance by fecal

host type and urbanicity, and identified potentially pathogenic bacterial species and plasmids carrying ARGs.

Research Aim 4

In the fourth aim (Chapter 5), *Benchmarking assembly methods for genomic characterization of antibiotic-resistant E. coli strains in humans, animals, and environmental samples*, we benchmarked metagenome assembly and ARG mapping methods to inform a recommended approach for identifying overlapping *E. coli* strains and ARGs across pooled *E. coli* samples from humans, animals, and environmental samples. Specifically, we compared 1) de novo short-read assemblies and hybrid assemblies; 2) de-novo assemblies and assemblies from reference genome-binned reads; and 3) raw reads mapped to ARGs and assembled contigs mapped to ARGs. Secondary objectives included characterizing strain and ARG diversity – using the recommended approach coming out of the benchmarking objective – across human, animal, and environmental samples collected from urban Kenyan households.

Chapter 2: Human colonization with antibiotic-resistant bacteria from non-occupational exposure to domesticated animals in low- and middle-income countries: A critical review

Jenna M. Swarthout, Elana M. G. Chan, Denise Garcia, Maya L. Nadimpalli, Amy J. Pickering

2.1 Abstract

Data on community-acquired antibiotic-resistant bacterial infections are particularly sparse in low- and middle-income countries (LMICs). Limited surveillance and oversight of antibiotic use in food-producing animals, inadequate access to safe drinking water, and insufficient sanitation and hygiene infrastructure in LMICs could exacerbate the risk of zoonotic antibiotic resistance transmission. This critical review compiles evidence of zoonotic exchange of antibiotic-resistant bacteria (ARB) or antibiotic resistance genes (ARGs) within households and backyard farms in LMICs, as well as assesses transmission mechanisms, risk factors, and environmental transmission pathways. Overall, substantial evidence exists for exchange of antibiotic resistance between domesticated animals and in-contact humans. Whole bacteria transmission and horizontal gene transfer between humans and animals were demonstrated within and between households and backyard farms. Further, we identified water, soil, and animal food products as environmental transmission pathways for exchange of ARB and ARGs between animals and humans, although directionality of transmission is poorly understood. Herein we propose study designs, methods, and topical considerations for priority incorporation into future One Health research to inform effective interventions and policies to disrupt zoonotic antibiotic resistance exchange in low-income communities.

2.2 Background

Animal husbandry is an important source of income and nutrition to many households in low- and middle-income countries (LMICs).^{13,50} Prior research, however, has demonstrated that human exposure to animals, particularly in the case of cohabitation, and animal feces is associated with adverse child health outcomes, including diarrheal disease and impaired growth.^{9,22,51} Interactions between humans and domesticated animals can also encourage dissemination of antibiotic resistance. Antibiotic use as prophylaxis and growth promotion, as opposed to treatment, is common in animal husbandry globally. The subsequent development of antibiotic resistance in animal gut microbiomes and transmission to humans has been demonstrated in intensive farming environments and high-income countries.^{52,53} Data on community-acquired antibiotic-resistant infections in humans, however, are particularly sparse in LMICs, especially in domestic settings and backyard or small-scale farms.⁵⁴ Limited surveillance and oversight of antibiotic use in food-producing animals, lack of safe water access, and inadequate sanitation and hygiene practices in LMICs exacerbate the risk of zoonotic transmission of antibiotic resistance.⁵⁵

Animal feces, which can harbor antibiotic-resistant bacteria (ARB), contributes to fecal contamination in domestic environments, such as in soil near the home, stored drinking water, and food.^{24–28} Additionally, ARB are often prevalent in soil and water.^{45,44,43,46} High levels of ARB in the environment are concerning due to the potential transmission between environmental matrices, humans, and animals. While the presence of ARB in animal and environmental reservoirs is well documented, a comprehensive understanding of which zoonotic transmission pathways are most important in LMIC

households is still missing. Given allowing animals to roam freely for grazing and to cohabitate in human environments is common in low-income households, there are ample opportunities for antibiotic resistance exchange between humans and animals either directly or indirectly via environmental reservoirs.

Zoonotic antibiotic resistance transmission can occur through whole bacteria transmission and through horizontal gene transfer of antibiotic resistance genes (ARGs) within microbial communities, such as intestinal microbiota.⁵⁶ Horizontal gene transfer allows ARGs to exchange between benign bacteria and pathogens as well as to spread between diverse human, animal, and environmental reservoirs.^{34,35} Technological advances in molecular methods, especially in genomics, enables characterization of genomic context around ARGs and differentiation between whole bacteria transmission and exchange via mobile genetic elements.^{57,58} Discerning the relative importance of chromosome- versus mobile genetic element-mediated resistance exchange in zoonotic transmission dynamics will help identify reservoirs and transmission pathways that should be considered when developing interventions for antibiotic resistance spread in communities.

This review was motivated by the need to understand whether domesticated animals are important contributors to human colonization with antibiotic resistance in LMICs. We systematically searched the literature to compile the available evidence on the risk of human colonization with ARB or ARGs (including antibiotic-resistant infections) resulting from non-occupational exposure to animals within LMIC households and backyard farms. Secondary objectives included identifying important zoonotic transmission

mechanisms (i.e., whole bacteria transmission, horizontal gene transfer), risk factors, and environmental transmission pathways.

2.3 Methods

Search Strategy

The protocol for this review can be accessed on the Open Science Framework (<https://osf.io/5wt9b/>). While this review was not registered with PROSPERO, we followed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines. To review the evidence for human colonization (or infections) with ARB or ARGs resulting from non-occupational exposure to animals, we searched peer-reviewed literature published through October 26, 2021 in six electronic databases: CAB Abstracts, Cochrane Library, EMBASE, Global Health, PubMed, and Web of Science Core Collection. We used a generic search string, adapted for the databases listed above, to search titles and abstracts for terms for animals, human exposure to animals, and antimicrobial resistance (Supporting Information A-1: Generic search string and search strings adapted for six databases) While our search results included literature related to all antimicrobial resistance, we included studies focused on bacterial-specific antibiotic resistance. All studies in LMICs were included, regardless of year, language, study design, and publication status. We also searched citations from review articles in our initial search results and personal libraries to identify additional publications for inclusion. We identified citations with relevant titles from traced reviews published in 2010 or later that satisfied our inclusion and exclusion criteria (see Selection of Studies below), aside from requiring original measurement of antibiotic resistance data. If a flagged citation was not in our initial search results, we screened its

abstract according to our inclusion criteria; screened citations were only included if they measured ARB or ARGs in both humans and animals. All search results and included articles from citation searching were exported to a master XML document in EndNote X8 or EndNote 20 to import into Covidence (Melbourne, Australia) for de-duplication, screening, and data extraction.

Selection of Studies

Titles and abstracts were screened separately by four independent reviewers (i.e., each abstract was screened by one reviewer). Prior to independently screening, reviewers duplicated a subset of title and abstract screenings until studies were included and excluded consistently. Publications were included if they met both of the following inclusion criteria: (a) evidence of direct human contact or exposure via environmental pathways to domesticated animals, animal feces, or animal products and (b) measurement of ARB or ARGs in humans or domesticated animals, animal feces, or animal products. Studies were excluded if they (a) measured ARB or ARGs in environmental reservoirs, slaughtered animals/carcasses or animal products without also sampling humans or documenting direct human exposure; (b) only sampled humans primarily exposed to animals through their occupation, with the exception of small-scale or subsistence farmers; (c) focused on aquaculture; or (d) were conducted in high-income countries, as classified by The World Bank Group.⁵⁹

We screened the full text of all included publications and categorized them based on evidence of household- and community-level zoonotic exposures and reported antibiotic resistance data in human and animal domains (Supporting Information A-2: Methods for categorizing full text publications). Specifically, publications were included if they met

both of the following inclusion criteria: (a) evidence of direct human contact or exposure via environmental pathways within household or backyard farm settings to domesticated animals, animal feces, or animal products and (b) measurement of ARB or ARGs in human and domesticated animal (including animal feces and products) samples matched spatially (i.e., collected in the same administrative division within a country) and temporally (i.e., collected in the same month) (Figure A-1). The exclusion criteria applied for the title and abstract screening were also applicable to the full-text screening. We excluded studies for which the full text was not available in English.

Data Extraction and Synthesis

We extracted data from included publications using a standardized form, which was pre-piloted by ensuring all four independent reviewers extracted data consistently from the same five randomly selected studies. Reviewers continued to extract data with 10% record duplication to ensure consistency. Specific data collected from each study included study site details; details about samples from the human, animal, and environmental domains; characterization of zoonotic exposure pathways assessed; methods used for bacterial isolation and identification; and methods used for phenotypic and genotypic measurement of antibiotic resistance (Table A-1). We did not conduct a meta-analysis or standardized risk of bias assessment for this review due to the heterogeneity of methods, exposures, and outcomes used in included studies; a qualitative discussion of the risk of bias in individual studies related to study design and methods is included. We conducted this review in accordance with the PRISMA 2020 statement (Table A-2). Data were prepared for synthesis in Stata SE (v. 14.2); summary statistics and visualizations were generated in R (v. 4.0.4).

2.4 Results

Study Selection and Characteristics

Our search yielded 9,581 unique results, of which the full texts of 847 studies were reviewed. In total, 79 studies met our inclusion criteria (Figure A-2). Individual study characteristics are presented in the supplemental information (Table A-3) and overall study characteristics are summarized in Table 2-1. The World Health Organization (WHO) regions were well-represented by included studies, excluding Europe (Figure 2-1). Studies were fairly evenly distributed between rural (56%, 44/79) and urban (46%, 36/79) settings. A vast majority of studies employed cross-sectional designs (95%, 75/79) and sampled from households/backyard farms (89%, 70/79). Livestock (82%, 65/79) were assessed in studies more often than pets (29%, 23/79). Only 39% (31/79)

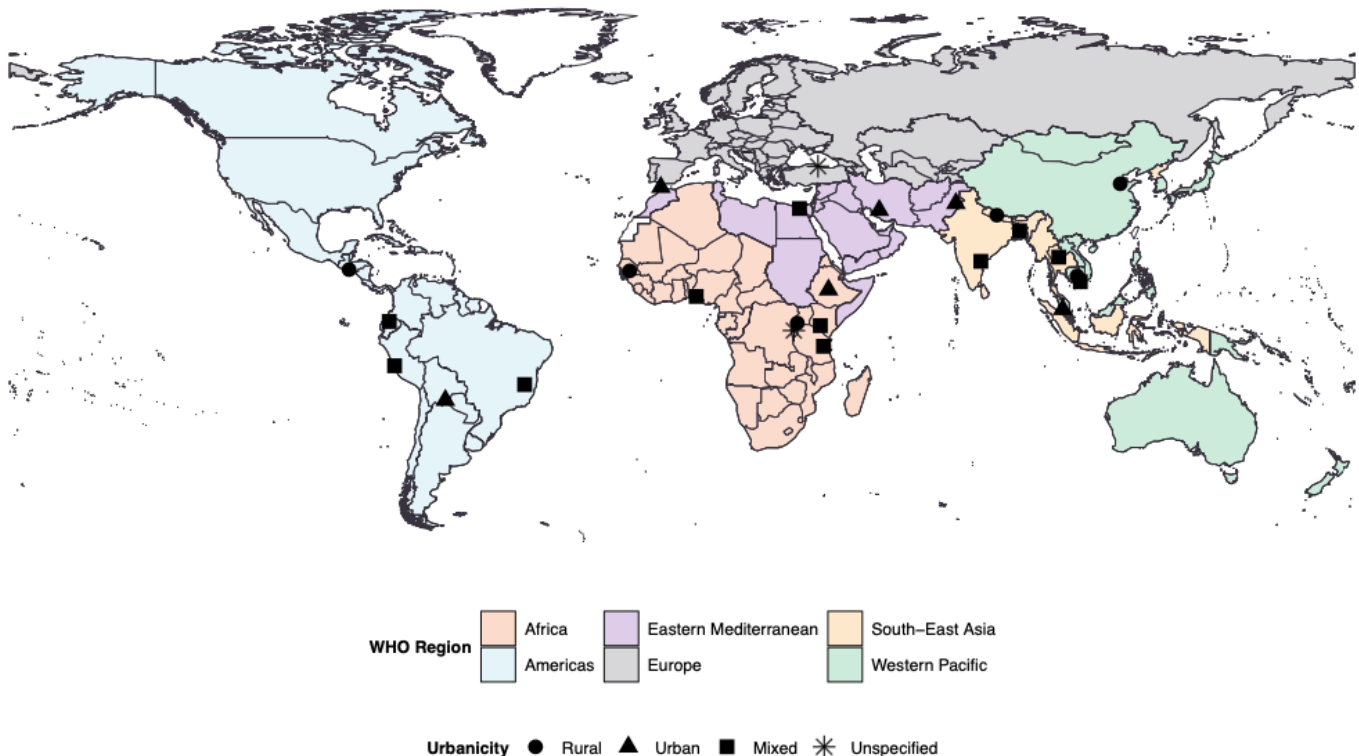


Figure 2-1: Locations of included studies in low- and middle-income countries. Shaded regions are defined by the World Health Organization (<https://www.who.int/countries>). Symbols depict the urbanicity of studies from each country and can represent more than one study in a country.

Table 2-1: Summary of characteristics of included studies (N = 79)

	n (%)		n (%)
Region		Animal Samples^a	
Africa	25 (32)	Feces	42 (53)
South-East Asia	18 (23)	Rectal/Cloacal swabs	28 (35)
Americas	14 (18)	Nasal swabs	8 (10)
Western Pacific	12 (15)	Carcasses	7 (8.9)
Eastern Mediterranean	9 (12)	Dairy	4 (5.1)
Europe	1 (1.3)	Meat	2 (2.5)
Urbanicity^a		Other	9 (11)
Rural	44 (56)	Human Samples^a	
Urban/Peri-Urban	36 (46)	Feces	54 (68)
Not Specified	10 (13)	Nasal swabs	12 (15)
Study Setting^a		Rectal swabs	11 (14)
Household/Backyard farm	70 (89)	Skin	4 (5.1)
Hospital	10 (13)	Blood	2 (2.5)
Market	7 (8.9)	Other	4 (5.1)
Veterinary clinic	6 (7.6)	Environmental Samples^a	
Other	4 (5.1)	Water	20 (25)
Not Specified	1 (1.3)	Soil	10 (13)
Study Design^a		Wastewater	6 (7.6)
Cross-sectional	75 (95)	Surfaces	4 (5.1)
Panel	3 (3.8)	Other	6 (7.6)
Case-control	1 (1.3)	Bacteria Genera^a	
Randomized controlled trial	1 (1.3)	<i>Escherichia</i>	53 (67)
Animal Species^a		<i>Staphylococcus</i>	12 (15)
<i>Livestock</i>	65 (82)	<i>Salmonella</i>	6 (7.6)
Poultry	42 (53)	<i>Klebsiella</i>	4 (5.1)
Ruminants	33 (42)	<i>Campylobacter</i>	2 (2.5)
Pigs	19 (24)	Other	4 (5.1)
Rabbits/Guinea pigs	5 (6.3)	Culture Independent	3 (3.8)
Equines	4 (5.1)	ABR Detection Methods^a	
Camels	2 (2.5)	<i>Culture-based</i>	72 (91)
<i>Pets</i>	23 (29)	<i>Molecular</i>	44 (56)
Dogs	23 (29)	PCR-based	30 (38)
Cats	6 (7.6)	Whole-genome sequencing	14 (18)
<i>Other</i>	10 (13)	Metagenomic sequencing	1 (1.3)
<i>Not Specified</i>	1 (1.3)	Other	6 (7.6)

ABR = antibiotic resistance.

All data are presented as n = number of studies (% of total number of studies).

^a Studies assessing multiple categories are represented for each category assessed

of studies collected environmental samples, most of which collected water (51%, 20/31).

Of studies collecting water, 60% (12/20) collected drinking water meant for human

and/or animal consumption, 30% (6/20) collected surface water that may or may not

have been used for consumption, and 5.0% (4/20) did not specify the water type. The

uses of culture-based and molecular antibiotic resistance detection methods were not

mutually exclusive; 91% (72/79) of studies employed culture-based methods (i.e., agar

and broth dilution assays) to detect phenotypic resistance, and 56% (44/79) of studies used molecular methods to detect ARGs. Specifically, 44% (35/79) of studies only used culture-based methods for antibiotic susceptibility testing, 8.9% (7/79) only used molecular methods to detect ARGs, and 47% (37/79) used culture-based and molecular methods to conduct antibiotic susceptibility testing and ARG detection. Most studies using molecular methods first cultured bacteria (93%, 41/44) and employed PCR-based techniques for ARG detection (68%, 30/44). Only 3.8% (3/79) of studies exclusively used culture-independent approaches to detect ARGs (e.g., PCR of total DNA or metagenomic sequencing).

Evidence of Antibiotic Resistance Exchange Between Humans and Animals

The primary aim of this review was to compile the available evidence for antibiotic resistance exchange between humans and domesticated animals in non-commercial and non-occupational settings. Most studies in our review demonstrated similarities in antibiotic resistance profiles in genetically diverse ARB and plasmids harbored by humans and animals. Though limited, there was evidence of zoonotic whole bacteria transmission of ARB (e.g., indistinguishable pulsed-field gel electrophoresis (PFGE) patterns, shared sequence types (STs), ≤ 11 single nucleotide polymorphism (SNP) differences). As clonal sharing of ARB, and even of plasmids, was rare, horizontal gene transfer was revealed as a likely important mechanism in the complex ARG exchange dynamics between humans, animals, and the environment. Similarities between antibiotic resistance profiles in bacterial isolates from humans and animals could not be assessed in 5.1% (4/79) of studies in this review because isolates demonstrated full

susceptibility to the antibiotics assessed, the bacterial species being investigated were not detected, or animal isolate antibiotic susceptibility results were not reported.^{60–63}

Overlap of Resistance Profiles in Diverse ARB

Several studies included in this review documented overlapping resistance phenotypes and ARG profiles between diverse ARB isolated from humans and domesticated animals. For example, phenotypic resistance profiles were similar for *Escherichia coli* or *Staphylococcus aureus* isolated from humans, animals, and the environment in Tanzania, rural Uganda, rural China, urban Nigeria, and urban Bolivia, but bacterial strains and plasmid replicons were genotypically diverse.^{64–68} Methicillin-resistant *S. pseudintermedius* (MRSP) isolates were obtained from dogs, humans (veterinarians and dog owners), and the environment in urban Thailand, but staphylococcal enterotoxin gene profiles, which are associated with *S. aureus* clonal lineages, varied by source.^{69,70} Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* with multidrug resistance phenotypes were detected across dogs, cats, and pet owners in urban Pakistan, though highly similar clonal groups (based on BOX-PCR) only existed between pets.⁷¹ Phenotypically similar isolates or clinically relevant ARGs were harbored by humans, animals, and/or environmental samples in Northern and Sub-Saharan Africa, South-East Asia, and Eastern Europe, although clonal diversity of ARB was not thoroughly investigated.^{72–83} Similarly, there were no differences between antibiotic susceptibility levels of *S. aureus* isolated from ruminants raised on small farms and their breeders ($p>0.05$ using Fisher's exact or Pearson's chi-square test) in rural Morocco, but genetic diversity of the isolates was not assessed.⁸⁴ Carriage of bacteria harboring the *mcr-1* gene or multidrug-resistant *E. coli* in livestock was associated with

human carriage in Vietnam and rural Uganda, respectively.^{85,86} In rural Ecuador, although children and animals shared antibiotic resistance phenotypes and genes, *E. coli* STs, and plasmid replicons, whole-genome sequencing and SNP analysis revealed strains and plasmids were genetically diverse, drawing into question research that has concluded zoonotic transmission based on matching ARG profiles and STs.⁸⁷

Antibiotic resistance profiles sometimes differed between bacterial isolates from humans and animals. Namely, resistance phenotypes (as well as STs or PFGE patterns) generally differed between *E. coli* isolated from humans and livestock in rural Kenya, rural Egypt, and rural Vietnam.^{88–91} Although 10 cases of recent livestock-human *E. coli* sharing (≤ 10 core genome multilocus sequence typing (MLST) distance) were identified in urban Kenya, resistome sharing (identical ARG profiles) was low among all human and animal isolates in the study.⁹² Although antibiotic-resistant non-typhoidal *Salmonella* isolates from patients, household water, and household soil exhibited similar PFGE patterns, livestock and raw and cooked food were not contributing reservoirs of non-typhoidal *Salmonella* in urban Kenya.⁹³ Additionally, livestock ownership was not associated with antibiotic-resistant *E. coli* carriage in humans (large livestock odds ratio (OR): 0.94, 95% confidence interval (CI): 0.72-1.2; small livestock OR: 1.0, 95% CI: 0.82-1.3) in urban Kenya.⁹⁴

Evidence of Whole Bacteria Transmission of ARB

Clonal relationships (e.g., indistinguishable PFGE patterns, shared STs, ≤ 11 SNP differences) were documented between human and animal ARB isolates using molecular methods, albeit generally in low proportions (Table 2-2). Whole bacteria transmission of ARB within households was especially demonstrated between dogs and

their owners, despite a smaller proportion of studies in this review sampling pets (29%, 23/79). Up to 10% of dog-owner isolate pairs analyzed in rural Thailand, urban Iran, and Brazil were genetically related and had similar resistance phenotypes or ARG profiles.^{95–98} In Rwanda, ESBL-producing *E. coli* ST38 was isolated from a hospitalized human and a related pet; ESBL-producing *E. coli* isolation rates were low in study animals (1.7% 2/120) compared to patients (50.8%, 61/120), indicating likely human-to-animal transmission.⁹⁹ Several studies also revealed evidence of whole bacteria transmission of ARB between backyard or small-scale farmers and their livestock, though cases were rare. In urban Kenya, while human-livestock resistome sharing was limited, 6 pairs of human-livestock *E. coli* isolates from the same farm differed by distances ≤ 10 core genome MLST.⁹² *E. coli* and *Klebsiella pneumoniae* isolated from pigs and farmers on the same farm shared STs and had core genomes differing by ≤ 11 SNPs in Thailand and rural China, though recent clonal relationships represented $< 1\%$ of the total pig-farmer isolate pairs assessed in each study.^{100–102} Similarly, one chicken-farmer isolate pair (0.5% of 204 total isolate pairs) shared STs and core gene sequences differing by only 1 SNP on a Vietnamese farm.¹⁰³ In contrast, 13% (4/31) of *Enterococcus faecalis* isolate pairs from patients' urinary tract infections and close-contact poultry cloacal swabs had shared STs and indistinguishable PFGE patterns in urban Vietnam, suggesting zoonotic whole bacteria transmission of ARB may play a larger role in human infections compared to asymptomatic colonization in apparently healthy humans.¹⁰⁴ While 60% (3/5) of methicillin-resistant *S. aureus* (MRSA) isolate pairs from goats and their handlers shared STs in urban India, only five isolate pairs were subjected to MLST in the study.¹⁰⁵

Table 2-2: Studies supporting whole bacteria zoonotic transmission of antibiotic-resistant bacteria within and between households/farms

Study Identifier	Setting	Domestic Animal Species	Human Population	Bacteria Genera	Antibiotic Resistance Detection Method	Relatedness Assessment Method	Results
Within Households/Farms							
Carvalho 2016	urban Brazil	dogs (134)	dog owners (134), non-dog owners (44)	<i>Escherichia</i>	disk diffusion, PCR	PFGE, phylogrouping	9.5% (4/42) of dog-owner isolate pairs had identical PFGE patterns and similar resistance phenotypes, ESBL gene prevalence, and phylogroups
Derakhshandeh 2018	urban Iran	dogs (31)	dog owners (28), non-dog owners (16)	<i>Escherichia</i>	PCR	RAPD-PCR	7.1% (2/28) of dog-owner isolate pairs had indistinguishable RAPD-PCR profiles, and one of these had identical virulence gene and ARG profiles
Eger 2021	Rwanda (unspecified)	unspecified (120)	patients (120) and their caregivers (120), neighbors (120), and family members (120)	<i>Escherichia</i>	broth dilution assay, WGS	SNP analysis, MLST, plasmid replicon typing	ESBL-producing <i>E. coli</i> ST38 was isolated from a hospitalized human, one of their family members, one of their neighbors, and a related pet
Fabri 2021	Brazil (unspecified)	dogs (241)	dog owners (208)	<i>Staphylococcus</i>	disk diffusion, PCR	rep-PCR	5.8% (4/69) of dog-owner isolate pairs had similar (Dice correlation coefficient ≥ 0.80) fingerprint patterns; 1 <i>mecA</i> -positive dog-human isolate pair (from different households) belonged to the same cluster
Hickman 2021	Thailand (unspecified)	pigs (143)	farmers (90), farmers' family members (54)	<i>Escherichia</i>	broth dilution assay, WGS	SNP analysis, MLST	0.7% (1/143) of farms had pig-farmer isolate pairs with recent clonal relationships (shared ST and core genomes differing by 11 SNPs)
Leangapichart 2021	Thailand (unspecified)	pigs (839)	farmers (158), farmers' family members (114)	<i>Klebsiella</i>	disk diffusion, WGS	SNP analysis, MLST, serotyping	0.6% (1/164) of farms had pig-farmer isolate pairs with recent clonal relationships (shared ST and core genomes differing by 3 SNPs)
Li 2019	rural China	dogs (92), cats (19), cattle (13), goats (10), pigs (417), poultry (312), donkeys (1)	dog owners (735)	<i>Escherichia</i>	disk diffusion; WGS	SNP analysis, MLST, phylogrouping	0.3% (2/735) of pig owner isolates had recent clonal relationships with pig isolates (shared ST and core genomes differing by 3 SNPs)
Muloi 2022	urban Kenya	cattle (61), goats (109), sheep (25), pigs (49), poultry (324), rabbits (38)	household members (333)	<i>Escherichia</i>	WGS	SNP analysis, cgMLST, MLST, phylogrouping	10 livestock-human isolate pairs (6 pairs within farms and 4 pairs between farms) represented recent clonal sharing (≤ 10 cgMLST distance), though livestock-human resistome sharing (identical ARG profiles) was low across all isolates in the study
Poulsen 2012	urban Vietnam	poultry (NA)	UTI patients (31)	<i>Enterococcus</i>	broth dilution assay	PFGE, MLST	23% (7/31) of UTI patient isolates shared STs and identical (13%, 4/31) or closely related (differing by ≤ 3 fragments) (10%, 3/31) PFGE patterns with isolates from the patients' poultry
Trung 2019	rural, urban Vietnam	poultry (204)	farmers (204); non-poultry farmers (306)	<i>Escherichia</i>	disk diffusion, WGS	SNP analysis, MLST	0.5% (1/204) of chicken-farmer isolate pairs were clonal (shared ST, plasmid replicon types, and ESBL genes and core gene sequences differing by 1 SNP)
Venkatvasan 2020	urban India	goats (11)	goat handlers (44)	<i>Staphylococcus</i>	disk diffusion, PCR	MLST	60% (3/5) of goat-handler MRSA isolate pairs analyzed by MLST shared STs
Wu 2020	rural Thailand	dogs (140)	dog owners (140)	<i>Salmonella</i>	disk diffusion	PFGE, SR-FTIR, serotyping	5.2% (2/39) of dog-owner isolate pairs were homologous (shared serotypes, indistinguishable PFGE patterns, and shared SR-FTIR spectra by PCA and SIMCA)
Between Households/Farms							
Gwida 2020	urban Egypt	cattle (50), buffalo (32)	farmers (50)	<i>Escherichia</i>	broth dilution assay	DNA microarray	2.0% (1/50) of farmers had identical hybridization patterns (virulence genes, ARGs, serotypes) to buffalo
Lupindu 2015	urban Tanzania	cattle (446)	cattle owners (100), non-cattle owners (100)	<i>Escherichia</i>	spot-inoculation agar assay	PFGE	38% (3/8) of ampicillin- and tetracycline-resistant <i>E. coli</i> isolate clonal groups, and 6.8% (3/44) of clusters, had cattle and human isolates with identical PFGE patterns
Momoh 2018	rural Nigeria	pigs (300)	pig farmers (64), pig abattoir workers (37)	<i>Staphylococcus</i>	broth dilution assay	<i>spa</i> typing	Clonal complexes CC5, CC15, and CC152, and similar resistance phenotypes, were shared by pig and human isolates
Rwego 2008	rural Uganda	cattle (NA), goats (NA), sheep (NA) ^a	people in contact with gorillas (65)	<i>Escherichia</i>	disk diffusion	rep-PCR	Human and livestock isolates were closely related (short-term genetic distance: 0.027, standard error: 0.17) and exhibited similar prevalence of antibiotic resistance (humans: 35%, 54/153; livestock: 27%, 38/139)
Tansawai 2019	Thailand (unspecified)	poultry (467)	farmers and farmers' family members (32)	<i>Escherichia</i>	disk diffusion, PCR	PFGE, MLST, phylogrouping	1 chicken-human isolate pair (from different farms) had similar PFGE patterns (Dice correlation coefficient ≥ 0.85)

ARG = antibiotic resistance gene. **cgMLST** = core genome multilocus sequence typing. **ESBL** = extended-spectrum beta-lactamase. **MLST** = multilocus sequence typing. **MRSA** = methicillin-resistant *Staphylococcus aureus*. **PCR** = polymerase chain reaction. **PFGE** = pulsed field gel electrophoresis. **RAPD** = random amplified polymorphic DNA. **rep** = repetitive extragenic palindromic. **SIMCA** = soft independent modeling of class analogy. **SNP** = single nucleotide polymorphism. **SR-FTIR** = Synchrotron Fourier transform infrared spectroscopy. **ST** = sequence type. **WGS** = whole-genome sequencing.

^a 48 animal fecal samples were collected in total

Some studies in this review also demonstrated that recent zoonotic transmission of ARB might not only be possible within farms, but also between farms (Table 2-2). For example, *E. coli* isolates with indistinguishable or similar (Dice correlation coefficient ≥ 0.85) PFGE patterns were detected across humans and livestock from different farms in Thailand and urban Tanzania.^{106,107} Human and livestock across various farms also harbored antibiotic-resistant *E. coli* or *S. aureus* with shared serotypes, shared clonal complexes, and small genetic distances between DNA fingerprints in rural Egypt, Nigeria, and Uganda.^{108–110}

Zoonotic Horizontal Gene Transfer of ARGs

As overlapping resistance profiles generally occurred in genetically diverse ARB from humans and animals and zoonotic whole bacteria transmission of ARB was limited, the evidence in this review supported horizontal gene transfer as an important mechanism in human-animal exchange in non-occupational settings (Table 2-3). In urban Nigeria, two dog-human *S. aureus* isolate pairs (one of which included a dog and owner from the same household) had the same sized (kbp) plasmids and resistance phenotypes differing by only one antibiotic each.¹¹¹ ARGs were carried by shared plasmid replicon types across humans and domesticated animals from the same households in rural Bangladesh and urban Ecuador.^{112,113} In rural Ecuador, third-generation cephalosporin-resistant *E. coli* isolates from children, dogs, and chickens in the same households exhibited clonal relationships (differing by <100 SNPs), but often had different resistance phenotypes, ARG profiles, and plasmid replicons, suggesting likely horizontal gene transfer.¹¹⁴

Table 2-3: Studies supporting zoonotic horizontal gene transfer of antibiotic resistance genes within and between households/farms

Study Identifier	Setting	Domestic Animal Species	Human Population	Bacteria Genera	Antibiotic Resistance Detection Method	Relatedness Assessment Method	Results
Within Households/Farms							
Akano 2009	urban Nigeria	dogs (4 ^a)	dog owners (8 ^a), clinical patients (9 ^a)	<i>Staphylococcus</i>	disk diffusion	plasmid molecular weight	2 dog-human isolate pairs (1 was a dog-owner pair) had the same sized plasmids and resistance phenotypes differing by 1 antibiotic each
Loayza-Villa 2020	urban Ecuador	dogs (2), poultry (4), rabbits (2), guinea pigs (2)	male child (1)	<i>Escherichia</i>	broth dilution assay, PCR, whole-genome short- and long-read sequencing	PFGE, MLST, plasmid replicon typing	<i>mcr-1.1</i> gene was carried by IncI2 plasmids (with an average of 90% nucleotide sequence identity to each other) in a human, chicken, and dog isolate, which all had different sequence types
Montealegre 2020	rural Bangladesh	cattle (13 ^a), poultry (14 ^a)	adults aged 18-64 years (14 ^a)	<i>Escherichia</i>	disk diffusion, WGS	SNP analysis, MLST, phylogrouping, plasmid replicon typing	p0111 and IncF plasmids were carried by humans and animals living in the same household in 1 and 6 households, respectively; pairwise SNP differences between human and animal isolates were large (minimum, 189 SNPs)
Salinas 2021	rural Ecuador	dogs (20), poultry (19)	children aged 0.5-4 years (29)	<i>Escherichia</i>	disk diffusion, WGS	SNP analysis, MLST, serotyping, plasmid replicon typing	59% (17/29) of households had human and/or animal isolates with clonal relationships (differing by <100 SNPs), but most isolates with clonal relationships had diverse resistance phenotypes, ARGs, and plasmid types
Between Households/Farms							
Kariuki 1997	rural Kenya	poultry (246)	diarrheic children aged 0.5-2 years (168)	<i>Escherichia</i>	disk diffusion	RED, incompatibility grouping of R-plasmids	71% (83/117) and 79% (38/48) of the R-plasmids from children and chickens, respectively, belonged to the incFII group, but resistance phenotypes and RED-fragment patterns were generally distinct
Li 2019	rural China	dogs (92), cats (19), cattle (13), goats (10), pigs (417), poultry (312), donkeys (1)	pig owners (735)	<i>Escherichia</i>	disk diffusion, WGS	plasmid replicon typing	50 animal and 14 human isolates carrying <i>bla_NDM</i> genes aligned to an IncX3 reference with >99% nucleotide sequence identity
Pehrsson 2016	rural El Salvador; urban Peru	dogs (NA), cattle (NA), poultry (NA), guinea pigs (NA) ^b	household members (115)	Culture-independent	metagenomic sequencing, mass spectrometry	metagenomic sequencing	41% of antibiotic resistance proteins found across human, animal, and environmental domains were always encoded in the same genetic context; e.g., a TetX variant was encoded in the same context in human stool, animal feces, latrine samples, and sewage influent
Salinas 2019	rural Ecuador	dogs (NA), cats (NA), cattle (NA), sheep (NA), pigs (NA), poultry (NA), rabbits (NA), horses (NA), guinea pigs (NA) ^c	children aged <5 years (64)	<i>Escherichia</i>	disk diffusion, WGS	SNP analysis, MLST, plasmid replicon typing	Human and animal isolates had similar resistance phenotypes and ARGs, but were comprised of diverse strains (1 shared ST, which differed by 275 SNPs) and plasmids types

ARG = antibiotic resistance gene. **MLST** = multilocus sequence typing. **PCR** = polymerase chain reaction. **PFGE** = pulsed field gel electrophoresis. **RED** = restriction-endonuclease digestion. **SNP** = single nucleotide polymorphism. **ST** = sequence type. **WGS** = whole-genome sequencing.

^a Sample sizes represent isolates obtained rather than individuals sampled

^b 14 animal fecal samples were collected in total

^c 203 animal fecal samples were collected in total

Similar to whole bacteria ARB transmission, zoonotic exchange of ARGs and mobile genetic elements via horizontal gene transfer was documented across households and farms, highlighting the complexity of exchange dynamics in low-resource settings (Table 2-3). In rural China, both human and animal *E. coli* isolates carrying *bla*_{NDM} aligned to an IncX3 reference plasmid with >99% nucleotide sequence identity.¹⁰² In rural El Salvador and peri-urban Peru, 41% of antibiotic resistance proteins found across human, animal, and environmental compartments were always encoded in the same genetic context.⁴⁸ Similarities in antibiotic resistance profiles were identified in genotypically diverse *E. coli* and distinct plasmids isolated from humans and animals in rural Kenya and Ecuador, suggesting a likely important role of horizontal gene transfer of ARGs through mobile genetic elements smaller than plasmids – such as transposons, integrons, or gene cassettes – although this was not directly investigated.^{87,88}

Differential Risk of Colonization

Based on the scope of this review, we determined that human exposure intensity levels to animals, animal husbandry practices, and demographic factors were found to impact rates of ARB colonization in humans (Table 2-4). Leveraging the data compiled in this review, we also found that geographical factors impacted human ARB colonization and animal ARB colonization rates varied by animal species; these findings are not definitive, however, as the data is limited to what could be extracted from the studies meeting the eligibility criteria of this review.

Human Colonization Varies by Contact Levels with Animals

Contact and intensity levels of exposure with domesticated animals affected the prevalence and diversity of ARB harbored by humans in Ecuador, Uganda, Thailand,

Table 2-4: Studies demonstrating differential risk of antibiotic resistance colonization in humans

Risk Factor	Study Identifier	Setting	Comparison Groups	Outcome	Outcome Assessment Methods	Results
Intensity of human exposure to animals	Weil 2020	rural Uganda	(A) Women raising chickens for one year (B) Women receiving chickens after one year	Fecal resistome	qPCR	ARG profiles were more similar between women receiving chickens after one year (control) and their own chickens compared to between women raising broilers for one year prior and control women's chickens (mixed effects model of distance between sample and centroid of chicken samples, $p=0.01$)
	Huber 2021	Thailand (unspecified)	(A) Small-scale pig farmers (B) Humans not in contact with pigs	MDR <i>Escherichia coli</i>	disk diffusion	Small-scale pig farmers had higher rates of MDR <i>E. coli</i> colonization than humans not in contact with pigs (23.9% vs. 15.9%)
	Moser 2018	rural Ecuador	(A) Small-scale poultry producers (B) Household poultry producers	ABR <i>E. coli</i>	disk diffusion	Small-scale poultry producers had higher rates of ABR <i>E. coli</i> colonization for 8 of 12 antibiotics and 4 of 4 MGE markers than household poultry producers
	Lunha 2020	rural Thailand	(A) Medium-scale pig producers (B) Small-scale pig producers	ABR <i>E. coli</i>	disk diffusion	ABR <i>E. coli</i> colonization rates did not significantly differ between medium- and small-scale pig producers
	Rousham 2021	rural, urban Bangladesh	(A) Humans with high poultry exposure (B) Humans with low poultry exposure	ESBL- <i>E. coli</i>	selective media culturing	ESBL- <i>E. coli</i> colonization rates did not significantly differ between humans with high and low poultry exposure
	Hedman 2020	rural Ecuador	(A) Livestock owners (B) Non-livestock owners	ABR <i>E. coli</i>	disk diffusion	Odds of ABR <i>E. coli</i> colonization did not differ between livestock and non-livestock owners (large livestock OR: 0.94, 95% CI: 0.72-1.2; small livestock OR: 1.0, 95% CI: 0.82-1.3)
Animal husbandry practices	Salinas 2021	rural Ecuador	(A) HHs with poor fecal waste management (B) HHs with safe fecal waste management	ABR <i>E. coli</i>	selective media culturing	Most HHs with child-animal ABR <i>E. coli</i> clonal relationships were from HHs with poor fecal waste management practices
	Atterby 2019	rural Cambodia	(A) HHs not removing manure (B) HHs removing manure	ESCE/K	selective media culturing	People in HHs not removing manure from the HH had increased odds of ESCE/K colonization compared to those removing manure (OR: 2.19, 95% CI: 1.07-4.47)
	Muloi 2019	urban Kenya	(A) HHs not removing manure (B) HHs removing manure	Antibiogram length	disk diffusion	People in HHs not removing manure from the HH had higher antibiogram lengths than those removing manure (OR: 1.3, 95% CI: 1.0-1.6)
	Hassell 2019	urban Kenya	(A) HHs not removing rubbish and manure (B) HHs removing rubbish and manure	MDR <i>E. coli</i>	disk diffusion	The probability of MDR carriage in wild birds was higher in HHs not removing rubbish and manure than those removing rubbish and manure
	Li 2019	rural China	(A) HHs using feces as fertilizer (B) HHs not using feces as fertilizer	MDR <i>E. coli</i>	disk diffusion	People in HHs using feces as fertilizer had increased odds of MDR <i>E. coli</i> colonization compared to those not using feces as fertilizer (OR: 2.7, 95% CI: 1.1-6.6)
	Atterby 2019	rural Cambodia	(A) HHs not burning/burying meat waste (B) HHs burning/burying meat waste	ESCE/K	selective media culturing	People in HHs not burning or burying meat waste had lower odds of ESCE/K colonization compared to those burning or burying waste (OR: 0.26, 95% CI: 0.10-0.71)
Demographic factors	Li 2019	rural China	(A) HHs raising additional animal species (B) HHs raising less animal species	MDR <i>E. coli</i>	disk diffusion	People in HHs raising additional numbers of animal species had increased odds of MDR <i>E. coli</i> colonization (OR: 1.5, 95% CI: 1.2-1.7 for each additional species)
	Muloi 2022	urban Kenya	(A) Females (B) Males	ABR <i>E. coli</i>	whole genome sequencing	All livestock-human ABR <i>E. coli</i> sharing (≤ 10 cgMLST distance) pairs ($n=10$) involved male humans (Fisher's exact test, $p=0.003$)
	Atterby 2019	rural Cambodia	(A) Females (B) Males	CPE/K or ESCE/K	selective media culturing	Females had higher rates of CPE/K or ESCE/K colonization than males (25% vs. 11%, Pearson's chi-square test, $p=0.03$)
	Derakhshandeh 2018	urban Iran	(A) Females (B) Males	ABR <i>E. coli</i>	PCR	Dog-owner ABR <i>E. coli</i> RAPD-PCR profile similarities did not differ between females and males (t -test, $p=0.699$)
	Atterby 2019	rural Cambodia	(A) Young (0-5 yrs) children (B) School-aged (6-15 yrs) children	ESCE/K	selective media culturing	Young children had higher rates of ESCE/K colonization than school-aged children (30% vs. 13%, Pearson's chi-square test, $p=0.04$)
	Sahoo 2012	rural India	(A) Non-coastal region (B) Coastal region	ABR <i>E. coli</i>	disk diffusion	Non-coastal HHs had higher prevalence of ABR <i>E. coli</i> in child stool, cow dung, and drinking water than coastal HHs
Geographical factors	Subbiah 2020	rural, urban Tanzania	(A) Rural and peri-urban (B) Urban	ABR <i>Enterobacteriaceae</i>	breakpoint assay	Rural and peri-urban HHs had higher prevalence of ABR <i>Enterobacteriaceae</i> in animal feces than urban HHs

ABR = antibiotic-resistant. ARG = antibiotic resistance gene. cgMLST = core genome multilocus sequence typing. CI = confidence interval. CPE/K = carbapenemase-producing *Escherichia coli/Klebsiella pneumoniae*. ESBL = extended-spectrum beta-lactamase-producing. ESCE/K = extended-spectrum cephalosporinase-producing *E. coli/K. pneumoniae*. HH = household. MDR = multidrug-resistant. MGE = mobile genetic element. NTS = non-typhoidal *Salmonella*. OR = odds ratio. PCR = polymerase chain reaction. qPCR = quantitative polymerase chain reaction. RAPD = random amplified polymorphic DNA.

and Malaysia.^{115–117} Specifically, humans in direct contact with pigs had higher multidrug-resistant *E. coli* (resistance to ≥ 3 antibiotics) levels than non-contact humans (contact: 23.9%, 27/113; non-contact: 15.9%, 18/113) in small-scale farms in Thailand, and MRSA was isolated more often from pet owners than non-pet owners (owners: 30%, 9/30; non-owners: 20%, 6/30) in urban Malaysia.^{117,118} ARG profiles, detected by quantitative polymerase chain reaction (qPCR) and assessed using the Jaccard dissimilarity index, slightly differed between women without chickens and those who had been raising broiler chicks for a year as part of a randomized controlled trial intervention ($p=0.09$ using PERMANOVA) in rural Uganda.¹¹⁶ Small-scale poultry production, compared to backyard production, increased the risk of human antibiotic-resistant *E. coli* colonization in rural Ecuador.¹¹⁵ However, human ABR colonization rates did not significantly differ based on levels of exposure to poultry in Bangladesh or whether they worked on small- versus medium-scale pig farms in Thailand.^{119,120} Similarly, the prevalence of non-typhoidal *Salmonella* and resistance phenotypes did not substantially differ between backyard chicken farmers and humans not exposed to poultry in Vietnam.¹²¹

Animal Husbandry Practices Influence Human Colonization Rates

Several animal husbandry practices – such as poor fecal waste management, poor meat waste management, and allowing animals to roam freely – were associated with human antibiotic resistance colonization. In most rural Ecuadorian households exhibiting clonal relationships (i.e., isolate core genomes differing by <100 SNPs) between third-generation cephalosporin-resistant *E. coli* isolated from children and

animals, animal fecal waste management and handling practices were poor; animal feces were left in the yard or used as fertilizer in 82.4% (14/17) of households with clonal relationships, and children had contact with animals in 88.2% (15/17) of households.¹¹⁴ At the same time, water, sanitation, and hygiene (WASH)-related and occupational risks were low.¹¹⁴ Not removing animal manure from the household perimeter was associated with increased odds of human colonization with extended-spectrum cephalosporinase-producing *E. coli* and *K. pneumoniae* (ESCE/K) (OR: 2.19, 95% CI: 1.07-4.47) in rural Cambodia and the total number of antibiotic classes to which an *E. coli* isolate was phenotypically resistant (OR: 1.3, 95% CI: 1.0-1.6) in urban Kenya.^{94,122} The probability of multidrug resistance carriage in wild birds sampled at households increased when human rubbish and manure were kept within the household perimeter in urban Kenya, suggesting human and animal waste were channels for antibiotic resistance exchange between humans, livestock, and wildlife.¹²³ In rural China, using human or animal feces as fertilizer (OR: 2.7, 95% CI: 1.1-6.6) and the number of species of backyard animals (dogs, cats, chickens, ducks, cattle, goats, pigs, donkeys) raised in a household (OR: 1.5, 95% CI: 1.2-1.7) were significant risk factors for multidrug-resistant *E. coli* colonization in humans.¹⁰² In rural Cambodia, not burning or burying meat waste was associated with decreased odds of ESCE/K colonization (OR: 0.26, 95% CI: 0.10-0.71).¹²² Large spatial ranges of free-roaming backyard chickens might have facilitated antibiotic resistance spillover from separately housed broiler chicks to backyard chickens and children in rural Ecuador.^{124,125}

Human Colonization may Vary by Gender and Age

Demographic factors, such as gender and age, can influence zoonotic antibiotic resistance exchange through differences in animal caretaking responsibilities or exposure levels. The combined detection frequencies of carbapenemase-producing *E. coli* and *K. pneumoniae* (CPE/K) and ESCE/K were significantly different in adult females, who are primarily responsible for caring for poultry and pigs with high levels of ESCE/K, and males, who are primarily responsible for caring for ruminants (low-level ESCE/K colonization), (females: 25% (34/135), males: 11% (6/54), $p=0.03$ using Pearson's chi-square test) in rural Cambodia.^{122,126} In addition, ESCE/K detection was higher in young children (0-5 years), who spend much of their time in their households, than their older school-aged siblings (6-15 years) (young: 30% (10/33), school-aged: 13% (11/85), $p=0.04$ using Pearson's chi-square test). Since women are also generally the primary caretakers of young children with high levels of ESCE/K colonization, mother-child interactions might also partially explain higher ESCE/K colonization rates in women than men. In contrast, all 10 *E. coli* isolate sharing pairs identified between humans and livestock in urban Kenya involved human males ($p=0.003$ using Fisher's exact test).⁹² In urban Iran, however, gender of dog owners did not affect the RAPD-PCR profile similarity between antibiotic-resistant *E. coli* isolates from dogs and their owners ($p=0.699$ using t-test).⁹⁶

Geographical Factors Influence ARB Prevalence in Humans, Animals, and Environments

Among the studies included in this review, we observed spatial variations in ARB prevalence across all three domains within LMICs. In rural India, the prevalence of

antibiotic-resistant *E. coli* in children's stool, cow dung, and drinking water was higher in a non-coastal than coastal region, suggesting potentially important interactions between human and animal fecal microbiota and the environment.¹²⁷ The mean prevalence of antibiotic-resistant *Enterobacteriaceae* in dog, ruminant, and chickens feces was generally higher in rural and peri-urban, compared to urban, Tanzania, perhaps from farmers keeping larger herds, drawing animals from a wider range of sources (markets, livestock exchange partners), and purchasing younger animals; resistance in water, however, was generally higher in urban and peri-urban households.⁶⁴

Colonization is Often Higher in Poultry than Ruminants

Based on differential prevalence data that could be drawn from studies included in this review, higher proportions of ARB were consistently isolated from poultry compared to other livestock species. ESCE/K isolates were more often obtained from pigs (46%, 18/39) and poultry (28%, 39/128) relative to ruminants (7%, 7/103) in rural Cambodia ($p < 0.0001$ using Pearson's chi-square test for pigs versus ruminants and poultry versus ruminants).¹²² In urban Kenya, all non-typhoidal *Salmonella* isolates from household-raised pigs and dairy cows were fully susceptible to 11 tested antibiotics, while 39% (15/39) of chicken isolates from commercial farms were multidrug resistant and 100% (5/5) of chicken isolates from small-scale farms were resistant to tetracycline; human isolates, however, were genetically distinct from animal and environmental (surface water, raw and cooked food) isolates according to PFGE patterns.^{93,128} Phenotypic resistance was more often observed in *E. coli* isolates from chickens (56.9%, 29/51) and humans (54.0%, 27/50) compared to cattle (15.7%, 8/51) in rural Bangladesh.⁴³

Environmental Transmission Pathways for Zoonotic ARB and ARG Exchange

We identified water ($n = 11$ studies supporting this pathway), soil ($n = 6$ studies), and animal food products ($n = 4$ studies) to be potential pathways for the exchange of antibiotic resistance between humans and animals, though directionality of transmission has not been established. Notably, the evidence compiled here is focused on environmental transmission pathways between humans and animals, as the evidence is from studies measuring ARB and ARGs in humans and animals.

Water

There is widespread evidence of drinking water contamination with ARB originating from humans and animals. Household drinking water samples were contaminated with *E. coli* serogroups, *E. coli* PFGE patterns, and ARGs detected in humans and animals in rural Vietnam, urban Tanzania, and Egypt.^{72,77,107} *E. coli* with similar resistance profiles or indistinguishable PFGE patterns were isolated from animal feces and water in Thailand, rural Peru, and rural China.^{66,106,129} When phylogenetically comparing *tet(O)* gene sequences obtained from *Campylobacter jejuni* isolated from humans, chickens, and drinking water from the same farm in urban Egypt, researchers found that human and chicken *tet(O)* genes were more similar to drinking water *tet(O)* genes than to each other, suggesting an important role of water in the transmission between humans and chickens.¹³⁰ Several studies detected ABR across humans, animals, and water samples and concluded that human-to-animal was the likely direction of transmission. For example, multidrug-resistant *E. coli* or *bla_{CTX-M-1}* genes were detected in humans, animals, and drinking water in India, though the prevalence was highest in

humans.^{131,132} Carbapenem-resistant *Pseudomonas aeruginosa* isolates clonally related to the hospital-associated lineage ST233 were isolated from dog owners, dogs, and a sofa, balcony surface, and water cooler in a Brazilian household. Additionally, humans, livestock, and water (drinking water or river) harbored *Enterobacteriaceae* resistant to imipenem and ciprofloxacin in India and rural Brazil, respectively, despite imipenem and ciprofloxacin not being widely used in agriculture in the respective regions.^{133–135}

Soil

Soil has also been documented as an important reservoir of ARB. Antibiotic-resistant *E. coli* with closely related PFGE patterns were isolated from humans, livestock, soil, and water in Thailand and urban Tanzania.^{106,107} Chicken feces were identified as a contributing source of ARGs in soil in rural El Salvador, and human fecal microbiota and soil collected near chicken coops shared 80 antibiotic resistance proteins at >99% identity.⁴⁸ *E. coli* resistance patterns were similar between isolates from humans, animals, and soil in rural Peru and Bangladesh, though the Bangladeshi isolates exhibited great genomic diversity.^{43,112,129} The prevalence of *E. coli* resistance to third-generation cephalosporins was slightly higher in soil than humans and animals in Bangladesh, highlighting the potential for antibiotic selection to occur in environmental reservoirs.⁴³

Animal Food Products

Antibiotic resistance might be transferred to humans through contaminated milk and meat. Raw milk consumption was a risk factor for antibiotic-resistant *E. coli* carriage in human stool in rural and peri-urban Tanzania; the odds of detecting multidrug

resistance increased from 1.5 (95% CI: 1.2-2.0) to 10.4 (7.8-13.7) when comparing 1.1-L household milk consumption to 6.0-L consumption.¹³⁶ The authors hypothesized that whole bacteria transmission of ARB – as opposed to acquisition of milk-borne illness and subsequent antibiotic use, and/or presence of veterinary antibiotic residues in milk – was the likely mechanism explaining the associations between antibiotic-resistant *E. coli* carriage in humans and raw milk consumption.¹³⁶ Similarly, raw milk and cheese consumption has been linked to outbreaks of enteric illnesses in the United States.¹³⁷ Additionally, the odds of detecting antibiotic-resistant *Enterobacteriaceae* in dogs was lower in households that boiled milk (OR: 0.30-0.43 for ampicillin, tetracycline, or streptomycin resistance) in Tanzania.⁶⁴ Phenotypic resistance levels of *Campylobacter* spp. isolates were higher from pork and chicken meat than from pig rectal swabs/carcasses or chicken cloacal swabs/carcasses in Thailand, suggesting market practices were an important factor in exposing consumers to food contaminated with ARB.¹³⁸ In Peru, living in a zone where a larger proportion of households served home-raised chickens (primarily raised without antibiotics) rather than market-purchased chickens (often fed commercial feed with antibiotics) protected against *E. coli* ampicillin, sulfamethoxazole, and multidrug resistance in children (prevalence risk ratio: 0.46-0.60, $p \leq 0.05$ for all).¹³⁹

2.5 Discussion

Limitations

We identified several limitations of the current evidence base. While numerous studies identified overlap in ARB and ARGs between humans and animals, a vast majority

(95%, 75/79) used cross-sectional study designs with limited samples sizes and could not discern transmission directionalities. There was widespread heterogeneity in outcomes assessed (e.g., antibiotic susceptibility profiles, presence/absence of specific ARGs, resistomes), antibiotic resistance measurement methods (i.e., culture-based, molecular), outcome metrics utilized (e.g., proportions, ORs, risk ratios, Jaccard dissimilarity indices, SNP differences, nucleotide sequence identity), and statistical methods used for comparisons (e.g., Pearson's chi-square test, *t*-test, regression analysis, PERMANOVA). We were therefore unable to conduct a meta-analysis. Only 3.8% (3/79) detected genotypic resistance using culture-independent methods (i.e., PCR, metagenomics), thus limiting most studies to assessing human-animal exchange of specific bacteria or plasmids derived from those organisms. Further, studies relying on culture-based methods most often obtained single isolates from sampled humans and animals, which may partially explain why the collective evidence of zoonotic whole bacteria transmission was limited. Even fewer studies (2.5%, 2/79) to date have incorporated long-read sequencing to detect identical plasmids across sample types or ARGs situated within bacterial genomes. Finally, the scope of this review is limited to strong supporting or contradicting evidence directly relevant to human antibiotic resistance colonization associated with domesticated animal exposure; individual studies should be referenced for a thorough description of non-significant results, such as lack of resistance observed in certain assessed animal species or for specific antibiotics. The studies included in this review are likely subjected to publication biases originating from researchers choosing which results to report and from journals tending to accept publications with conclusive results; our findings might therefore not represent

an exhaustive synthesis of evidence for zoonotic exchange of antibiotic resistance.¹⁴⁰ Additional factors in zoonotic exchange in non-occupational settings were beyond the scope of this review, including wildlife and insects as vectors or sinks for resistance, antibiotic consumption in humans and animals, and virulence of resistant pathogens.

Recommendations for Future Work

Overall, this review revealed that although antibiotic use and resistance colonization may be lower in backyard animals compared to intensively raised animals or even small-scale production animals, exchange of antibiotic resistance with humans is widespread in LMIC households and backyard farms. Whole bacteria transmission and horizontal gene transfer have both been documented as zoonotic transmission mechanisms in non-occupational settings, though their relative importance is poorly understood and likely varies in different contexts. For example, whole bacteria transmission may play a larger role in ARB transmission between dogs and their owners due high-intensity interaction levels, while horizontal gene transfer might be important in exchanging ARGs between humans, animals, and environmental reservoirs within and between backyard farms. Complicated zoonotic exchange dynamics emphasize the potentially important role of environmental transmission pathways in transferring ARB and ARGs between humans and animals. While a limited numbers of studies included in this review evaluated environmental pathways, there is a large body of literature (outside the scope of this review) documenting ARB and ARGs in environmental reservoirs with which human and animal populations regularly have contact.^{141,142}

Further, directionality of transmission between humans and animals is poorly understood.⁴¹

The large amount of evidence for zoonotic transfer of ARB and ARGs compiled in this review highlights a need for research on biosecurity and WASH-related interventions to disrupt antibiotic resistance transmission in LMIC communities^{41,143,144} While specific research priorities should be set in consultation with stakeholders from LMICs, exploring the following research questions could generate valuable insights to inform effective interventions and policies for controlling zoonotic antibiotic resistance exchange in non-commercial and non-occupational settings:

- What social, behavioral, and biological characteristics of human and animal hosts influence whether or not zoonotic exchange of ARB occurs and how frequently?
- What is the relative importance of direct human-animal transmission versus indirect transmission via environmental pathways in facilitating zoonotic antibiotic resistance transmission?
- What is the likely directionality of zoonotic transmission in varying contexts?

We propose several study design, methods, and topical recommendations for future zoonotic transmission studies to address these gaps in knowledge (Figure 2-2).

Implementing Longitudinal One Health Study Designs

One Health study designs are essential for future research to substantially impact our understanding of zoonotic antibiotic resistance transmission. Established One Health reporting frameworks should be followed to guarantee human, animal, and

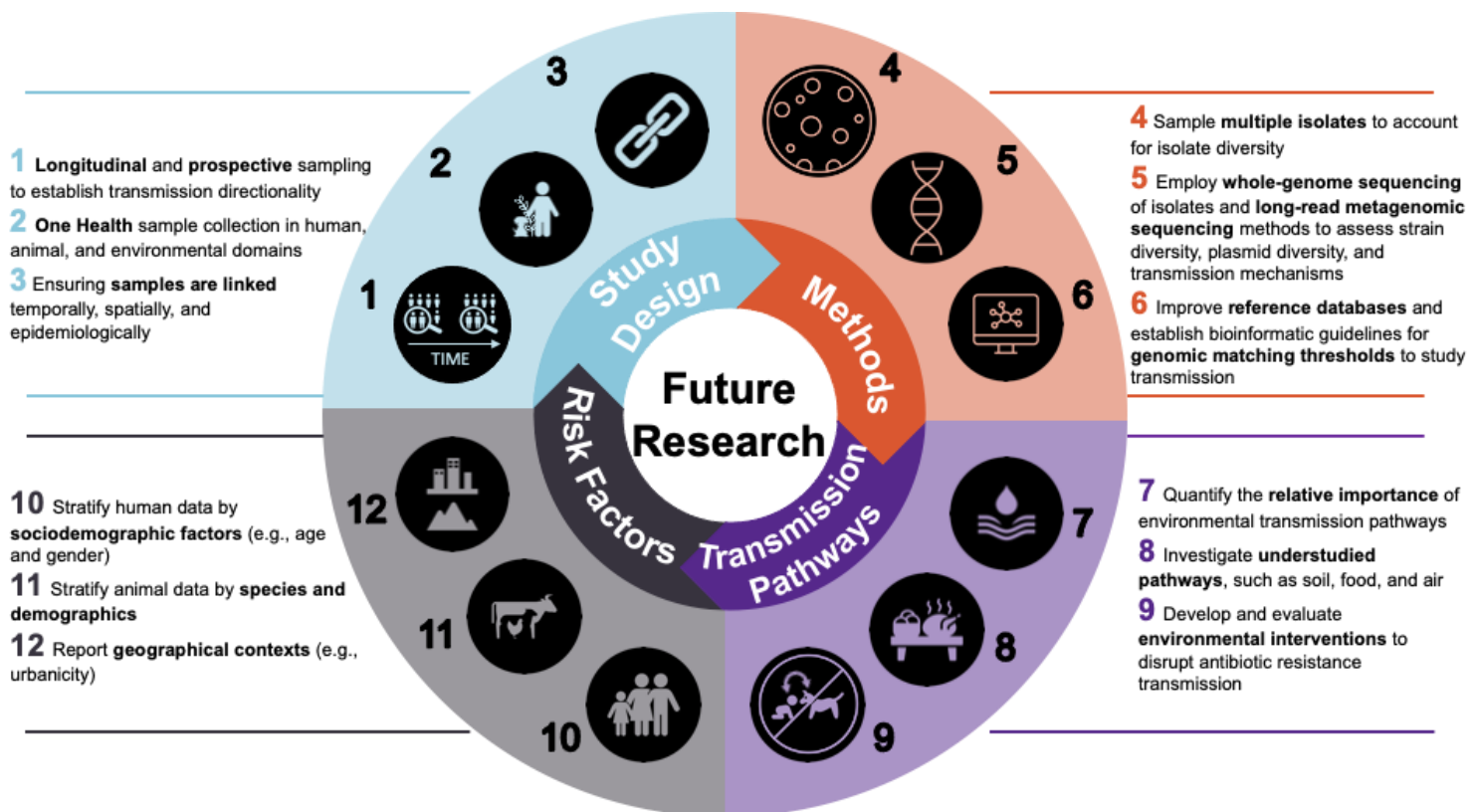


Figure 2-2: Research priorities and recommendations for future zoonotic transmission studies

environmental domains are comprehensively integrated in future antibiotic resistance transmission research and to ensure study reporting is of high quality and consistent across diverse disciplines.^{145–147} There was significant heterogeneity in antibiotic resistance outcome measures, metrics, and statistical methods used in studies included in this review, limiting comparability of studies. It would be of considerable value to convene an interdisciplinary panel of experts to establish guidelines for standardizing analysis approaches and reporting elements for One Health antibiotic resistance studies, similar to what has previously been done for reporting on antibiotic resistance in water, qPCR/digital PCR for environmental microbiology applications, and One Health studies generally.^{146–148} Temporal, spatial, and epidemiological (i.e., documenting direct or indirect exposure between linked samples) matching of human, animal, and

environmental samples is necessary to draw stronger conclusions regarding antibiotic resistance outcomes across sample types. Ideally, transmission studies should include longitudinal designs with repeated prospective sampling of animal and environmental samples prior to repeated collection of human stool samples, both to better establish directionality of resistance exchange and capture short-term dissemination events.⁴¹

Employing Genomic Methods

Technologically advanced genomic methods can greatly enhance our understanding of zoonotic resistance transmission.^{41,57} Most of the studies in this review relied on single cultured *E. coli* isolates from humans and animals to assess zoonotic exchange, which does not account for within-host isolate diversity. For example, Muloi et al. (2021, preprint) demonstrated that a 10-fold increase in the number of isolates picked from a host impacted the number of recent sharing pairs that could be identified between human and animal study populations.¹⁴⁹ Studies in this review employing whole-genome sequencing methods revealed that bacteria may be genetically diverse despite exhibiting similar resistance phenotypes, ARG profiles, or identical STs. This finding emphasizes the necessity for strain sequencing instead of relying on lower resolution methods, such as MLST and PFGE, to establish recent zoonotic transmission. Further, there is a need to better define standards and thresholds for whole bacteria transmission in community settings. When applying SNP analysis, for example, to assess clonality in One Health studies, it is important to consider that thresholds for recent transmission might differ from outbreak settings, particularly when the impacts of environmental factors on strain diversification are largely unknown.¹¹²

Due to the complications described above and the complexity of antibiotic resistance exchange dynamics, metagenomics can help elucidate the broader picture regarding zoonotic resistance transmission in communities. While metagenomic methods might reduce sensitivity to detect ARGs in complex microbiomes, targeted sequencing methods can be employed to enrich for specific ARGs. Further, long-read sequencing can reveal the genomic context around detected ARGs to discern between whole bacteria transmission and horizontal gene transfer. As the field of metagenomics advances, our previous call for standardization of reporting outcomes, measurement metrics, and statistical methods becomes increasingly critical.

Any genomic methods are only as reliable as the databases used to detect bacterial strains, ARGs, and mobile genetic elements. Most databases are biased toward human pathogen genomes and common commensal bacteria that are easily cultured, particularly from high-income countries. Reference databases would benefit from studies sequencing animal pathogens, environmental bacteria from different reservoirs (e.g., water, soil, air), and more samples from LMICs.

Comprehensively Evaluating Transmission Pathways

While multiple studies have documented ARB and ARGs in environmental media that humans and animals might encounter, few have concurrently assessed subsequent transmission to humans and/or animals.^{141,142} Less than half of the studies in this review (which required ARB or ARG measurements in human samples to be eligible for inclusion) collected environmental samples, and of those, most studies relied on water samples. This review included evidence for water, soil, animal food products, and

surfaces to serve as potential reservoirs of antibiotic resistance. Air is a potentially underappreciated zoonotic transmission pathway for ARB and ARGs in LMICs. In high-income settings, bioaerosols containing enteric bacteria have been widely detected near concentrated animal feeding operations, and ARGs have even been detected in ambient air surrounding residential areas.^{150–154} Environmental transmission pathways and their relative importance under various contexts (e.g., urbanicity, availability of infrastructure, socioeconomic status) should be investigated to design and prioritize potential interventions. There is also a scarcity of research that has been conducted on potential environmental interventions to disrupt antibiotic resistance transmission in community settings, particularly in LMICs.¹⁴³

Determining Risk Factors

This review documented variation in human antibiotic resistance colonization associated with domesticated animals dependent on sociodemographic factors, animal species, and environmental factors. Even still, many of the included studies aggregated data from humans across ages and genders, from multiple animal species, and even across human and animal hosts. Comprehensive knowledge can be obtained from future studies that present antibiotic resistance outcomes stratified by individual-level factors for humans and animals, such as age, gender, and species, rather than aggregating all humans and all animals together. Additionally, contextual definitions (e.g., “small-scale”, urbanicity) were often heterogeneous or unreported in studies included in this review. Consistent reporting of fine-scale contexts, such as urbanicity, would aid in identifying environmental factors impacting zoonotic antibiotic resistance transmission.¹⁴⁷

Our review also demonstrated that dogs might be a particularly understudied animal species; only 29% (23/79) of studies in this review sampled dogs. In addition to the strong potential for dog-human whole bacteria transmission of ARB and horizontal gene transfer of ARGs presented in this review, free-roaming dogs commonly contaminate domestic and public environments with their feces and may be especially important spreaders of ARB and ARGs in LMICs.^{9,155–157} Carbapenem-resistant *Enterobacteriaceae* (CRE) are an increasing problem in high-income countries following recent outbreaks associated with companion animals, including dogs.¹⁵⁸ Considering a 2018 systematic review found that CRE prevalence was <1% among livestock and companion animals in Europe, but 2-26% in Africa and 1-15% in Asia, CRE transmission between dogs and their owners might be an emerging public health concern in LMICs.¹⁵⁹

Conclusion

In response to the increasing threat of antibiotic resistance, the WHO recommends all countries develop national action plans and strategies, including vaccinations and effective WASH, to tackle antibiotic resistance across multiple sectors using a One Health approach.¹⁶⁰ Especially in LMICs where ARB prevalence is high, we must better understand directionality and transmission pathways of ARB exchange to develop feasible and cost-effective strategies for antibiotic resistance mitigation. As most emerging pathogens can be transmitted zoonotically, there is an international consensus that One Health surveillance systems and research priorities should be developed to encourage collaboration across human, animal, and environmental health

sectors.¹⁶¹ This review indicates that exchange of antibiotic resistance between domesticated animals and in-contact humans is widespread in LMIC households and backyard farms. The relative contribution of zoonotic exchange in non-occupational and non-commercial settings to the overall burden of community-acquired antibiotic resistance in low-resource settings is unclear, but merits further investigation given projected increases in food animal production and antibiotic resistance levels in LMICs, as well as growing interest in promoting animal husbandry to improve livelihoods and nutrition in low-income households.^{14,15,21,162} Many recommendations derived from this review (e.g., longitudinal designs, reliance on genomic methods) increase logistical challenges and costs associated with conducting zoonotic transmission studies. It is therefore imperative to leverage community samples for multiple efforts, such as evaluating interventions to mitigate the health impacts of emerging zoonotic pathogens and antibiotic resistance while ensuring the clear benefits of animal husbandry for human nutritional improvements are still realized.

2.6 Acknowledgements

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2.7 Citation

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Chapter 3: Addressing fecal contamination in rural Kenyan households: the roles of water treatment and animal ownership

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3.1 Abstract

Combined water, sanitation, and handwashing (WSH) interventions have the potential to reduce fecal pathogens along more transmission pathways than single interventions alone. We measured *Escherichia coli* levels in 3909 drinking water samples, 2691 child hand rinses, and 2422 toy ball rinses collected from households enrolled in a two-year cluster-randomized controlled trial evaluating single and combined WSH interventions. Water treatment alone reduced *E. coli* in drinking water. A combined WSH intervention improved water quality by the same magnitude but did not affect levels of fecal indicator bacteria on child hands or toy balls. One explanation for limited impact of the sanitation intervention is failure to address fecal contamination from domesticated animals. Animal ownership, especially ruminant ownership, was associated with increased *E. coli* contamination in stored water and on child hands in study households. However, livestock ownership was also protective against child stunting and not associated with child diarrhea. Our results have important implications for WSH program design; future interventions must not introduce barriers to household animal ownership which has clear economic and child nutritional benefits.

3.2 Background

Diarrheal disease remains a leading cause of under-five child mortality in low- and middle-income countries (LMICs).¹⁶³ Chronic diarrhea is also associated with stunting (length-for-age Z-scores >2 standard deviations below the median of the World Health Organization (WHO) growth standard for age and gender); stunting affected an estimated 144 million children under five years globally in 2020.^{164,165} The Sustainable Development Goals have encouraged significant investments to increase access to safe drinking water, sanitation, and hygiene (WSH) and reduce the burden of infectious disease by 2030.¹⁶⁶ WSH interventions are hypothesized to interrupt transmission of fecal pathogens in the household environment, thereby reducing the risk of diarrhea.¹⁶⁷ Previous studies have predominately used observational data to study associations between fecal indicator bacteria in the household environment (e.g., in water; on hands; in soil; on surfaces), child health outcomes (e.g., diarrhea; stunting), and the quality of WSH infrastructure.^{4,168–172} However, observational studies are not ideal for inferring causal effects.

A large number of randomized controlled trials (RCTs) have assessed the effect of water treatment interventions on fecal indicator bacteria levels in drinking water, but few sanitation and handwashing RCTs have measured environmental indicators of fecal contamination.^{5,173} We identified two RCTs evaluating the effect of handwashing interventions on hand contamination, neither of which reduced bacteria levels on hands.^{174,175} A 2016 systematic review concluded there was no evidence that improved sanitation reduces fecal contamination levels in water, on hands, on sentinel toys, on

household surfaces, or in soil, and recommended that future evaluations of sanitation interventions include environmental fecal assessments.¹⁷⁶ Recent RCTs have also demonstrated mixed effects of WSH interventions on child health outcomes.^{6–8} The lack of observed effects on diarrhea and stunting could partially be explained by insufficient adherence to interventions, while an additional explanation is the potential negative health effects of exposure to animal feces, which has historically been neglected in the design of WSH interventions.^{9–11}

Previous research has shown that animal feces contributes to fecal contamination in the domestic environment. A study in rural Bangladesh (Bangladesh WASH Benefits study) found that *E. coli* concentrations were higher in soil, stored water, and food sampled from compounds with animals compared to those without animals.²⁴ Host-specific fecal markers from animals (dogs; poultry; ruminants) were detected in multiple household reservoirs (soil; hands; stored water) in rural and urban Bangladesh, rural India, and rural Kenya.^{24–29} In all sample types tested, animal fecal markers were more prevalent than human fecal markers. In rural Bangladesh, increased concentration of an animal-specific fecal marker (BacCow) was associated with increased prevalence of pathogenic *E. coli*.²⁷ Animal ownership can therefore put humans at elevated risk of exposure to animal fecal contamination, which can harbor zoonotic enteropathogens. As animal exposure has been linked to child health outcomes, including diarrheal disease and growth faltering, there is a need to better understand through which pathways children are exposed to animal feces to inform effective interventions.^{22,51}

The Kenya WASH Benefits study was a cluster-randomized controlled trial designed to test the effects of water, sanitation, handwashing, and nutritional interventions, alone and in combination, on child diarrhea prevalence, linear growth, parasite infections, biomarkers of environmental enteric dysfunction (EED), and child development.^{7,177–180}

The trial's primary outcomes have been reported: WSH interventions, whether separately or in combination, did not reduce child diarrhea or improve child growth during the trial.⁷ To assess the extent to which the interventions may have reduced child exposure to fecal contamination – a key intermediate step to health outcomes – we nested environmental sample collection within a subset of enrolled households in selected trial arms. Our aim was to determine if the interventions reduced levels of fecal indicator bacteria in the domestic environment along likely exposure pathways for young children. Further, we leveraged data from the study to determine whether household-level animal ownership was associated with 1) higher *E. coli* prevalence and concentrations in the domestic environment (stored water; child hand rinses; sentinel toy rinses) and fly prevalence and densities near food preparation areas; and 2) under-five child health outcomes (diarrhea; stunting). We also assessed whether community-level (village-level) animal ownership was associated with the environmental contamination and child health outcomes.

3.3 Methods

Study Design

The Kenya WASH Benefits trial enrolled pregnant women in Kakamega, Bungoma, and Vihiga counties of Western Kenya and followed the children born to these pregnancies

(including twins) for two years. Study design details for the trial, including details regarding the six intervention arms (chlorinated drinking water; improved sanitation through pit latrine upgrades, child potties, and scoops for removing human and animal feces from households; handwashing with soap; combined WSH interventions; improved nutrition through infant and young child feeding counselling and lipid-based nutrient supplementation; combined WSH and nutritional (WSHN) interventions) and behavior change messaging for the Kenyan study, and accompanying trial in Bangladesh, were previously published.^{6,7,177}

Sample and Data Collection

Approximately one year and two years after intervention delivery (timed to match the collection of the trial's primary child health outcomes), we assessed environmental contamination in a subset of approximately 1500 households that were enrolled in the EED cohort in the trial (approximately 375 children from each of the control; nutrition; combined WSH; and combined WSHN arms that participated in EED biomarker measurement). The intensive environmental contamination assessment was conducted in the EED subgroup because of the added benefit of being able to assess relationships between environmental contamination and EED biomarkers, as well as leverage the data collection infrastructure in the cohort. We also measured selected indicators of environmental contamination in similar sized subsets of households enrolled in the single water, sanitation, and handwashing arms. Considering improved nutrition would not be expected to influence environmental contamination, for models evaluating intervention impacts and associations between household animal ownership and

environmental contamination, we grouped measurements collected from the WSH and WSHN arms together (WSH/WSHN) as well as measurements collected from the nutrition and control arms together (C/N). Stored drinking water samples were collected from the C/N, WSH/WSHN, water, and handwashing arms (Figure 3-1). Stored drinking water was sampled in the handwashing arm because previous evidence suggests that caregiver hand and stored water contamination are highly correlated, and stored drinking water fecal bacteria levels are typically less variable than hand rinse fecal bacteria levels.^{170,181,182} Child hand and sentinel toy ball (after 24 hours of play) rinse samples were collected from the C/N and WSH/WSHN arms as proxy indicators of overall environmental fecal contamination in the household, and because they represent likely exposure pathways for children under two years old.¹⁸³ Fly densities near food preparation areas and latrines were measured in the C/N, WSH/WSHN, and sanitation arms. Visible hand cleanliness for mothers and children were measured in the C/N and WSH/WSHN arms.

All environmental samples (stored water; child hand rinses; sentinel toy (plastic ball) rinses) were collected according to previously published protocols and analyzed by membrane filtration with MI media to enumerate *Escherichia coli*.^{170,184} Fly densities (counts) were measured at food preparation areas and latrines using the scudder fly grill method; flies were classified as house, bottle, flesh, or “other” species. Field staff observed visible cleanliness of mothers’ and children’s hands (palms; fingerpads; underneath fingernails). Additional sample collection and processing details are provided in the supporting information (Supporting Information B-1).

Household animal ownership data, including binary ownership and number of animals owned, were recorded at the time of enrollment and two years after intervention delivery (Figure 3-1). Data on child health outcomes (diarrhea; linear growth) were also collected two years after intervention delivery. The passive control arm was purposefully excluded from year two stool collection activities in the parent trial, during which animal ownership and diarrhea data were collected; data from the passive control arm were still leveraged to increase statistical power when assessing associations between baseline animal ownership and child growth. Caregiver-reported diarrhea was defined as three or more watery stools in 24 hours or a single episode of blood in the stool. Anthropometric measurements were collected following standard protocols.^{185,186} Z-scores were calculated for length-for-age using WHO child growth standards; outliers were excluded following WHO recommendations.¹⁸⁷

Statistical Analyses

We published a pre-specified statistical analysis plan for evaluating WSH intervention impacts on fecal contamination in household environments (<https://osf.io/eq2rc/>). All intervention impact statistical analyses were independently replicated by two different authors (AJP; JS). Outcomes measured in the single intervention arms (water; sanitation; handwashing) and pooled combined arms (WSH/WSHN) were compared to outcomes in the pooled control (control/nutrition) arms. We estimated unadjusted and adjusted intention-to-treat effects between arms, relying on the unadjusted analysis as our primary analysis. We estimated log reductions, prevalence ratios, and prevalence differences using generalized linear models with robust standard errors. We used

		Control/ Nutrition	Water	Sanitation	Handwashing	WSH/ WSHN	Passive Control
One-Year Assessment	Enrollment	 Animal ownership <i>n</i> =1578	Animal ownership <i>n</i> =523	Animal ownership <i>n</i> =506	Animal ownership <i>n</i> =529	Animal ownership <i>n</i> =1051	Animal ownership <i>n</i> =936
	 	Stored water <i>n</i> =550 Child hands <i>n</i> =518 Sentinel toys <i>n</i> =687 Flies (food prep) <i>n</i> =562 Flies (latrine) <i>n</i> =535 Caregiver hands <i>n</i> =1494 Child hands <i>n</i> =1289	Stored water <i>n</i> =251	Flies (food prep) <i>n</i> =395 Flies (latrine) <i>n</i> =384	Stored water <i>n</i> =275	Stored water <i>n</i> =504 Child hands <i>n</i> =520 Sentinel toys <i>n</i> =628 Flies (food prep) <i>n</i> =590 Flies (latrine) <i>n</i> =578 Caregiver hands <i>n</i> =1027 Child hands <i>n</i> =884	
	Two-Year Assessment	   	Animal ownership <i>n</i> =524 Stored water <i>n</i> =384	Animal ownership <i>n</i> =506 Flies (food prep) <i>n</i> =606 Flies (latrine) <i>n</i> =587 Diarrhea <i>n</i> =729 Stunting <i>n</i> =739	Animal ownership <i>n</i> =529 Stored water <i>n</i> =392 Diarrhea <i>n</i> =692 Stunting <i>n</i> =702	Animal ownership <i>n</i> =1052 Stored water <i>n</i> =727 Child hands <i>n</i> =793 Sentinel toys <i>n</i> =518 Flies (food prep) <i>n</i> =796 Flies (latrine) <i>n</i> =776 Caregiver hands <i>n</i> =1415 Child hands <i>n</i> =1406 Diarrhea <i>n</i> =1466 Stunting <i>n</i> =1480	Stunting <i>n</i> =716

Figure 3-1: Data collection and environmental sampling profile in the trial, by study arm and year of measurement. Chicken indicates animal ownership data collection; bacteria plate (circle with dots) indicates *E. coli* measurements; eye indicates rapid observations by field staff; heart indicates child health data collection. The majority of measurements were conducted in the control and nutrition (C/N) and combined water, sanitation, and handwashing (WSH/WSHN) study arms.

modified Poisson regression for binary outcomes.^{188,189} All models included fixed effects for randomization block (to take advantage of the pair-matched design). In adjusted analyses, we included pre-specified variables strongly associated with the outcome to potentially improve the precision of our estimates (Supporting Information B-1: Additional methods details). We conducted subgroup analyses by year of data collection (year one versus year two).

As ad hoc analyses, we estimated unadjusted and adjusted associations between household animal ownership and household environmental contamination and child health outcomes, relying on the analyses adjusted for relevant covariates (Figures B-1 – B-6) as our primary analyses. The exposures for the primary analyses were the log₁₀-transformed number of animals owned overall and by species (cattle; goats; sheep; poultry; dogs); results from analyses with binary animal ownership as exposures are presented in the SI. We evaluated associations between cross-sectional (year two) animal ownership and environmental contamination outcomes (*E. coli* in water, hand rinses, and toy rinses; flies at food preparation areas) and diarrhea; we leveraged prospective (at time of enrollment) animal ownership data to assess associations with linear growth outcomes (stunting; continuous length-for-age Z-scores). Sheep ownership data was not collected at enrollment. Covariates, including intervention arm, were pre-screened for their association with each outcome (likelihood ratio test *p*-value < 0.2) and variation (prevalence ≥ 5%). Nutritional intervention arms were pooled as above (WSH/WSHN; C/N) for environmental contamination outcomes, and all intervention arms were kept separate for child health outcomes. We used generalized

linear models with robust standard errors to account for village-level clustering in the parent trial. We used modified Poisson regressions with log links to estimate prevalence ratios and Gaussian regressions to estimate prevalence differences for binary outcomes; we also used Gaussian regressions to estimate differences in log-transformed bacterial counts for *E. coli* concentration and fly counts for fly density. For *E. coli* prevalence and concentration in stored water analyses, we excluded households with detectable free chlorine in their stored drinking water at year two.

To account for the larger number of poultry owned by households compared to other species (cattle; goats; sheep; dogs), we capped the number of each animal species owned by each household at five before calculating the overall number of animals owned as a sensitivity analysis. When assessing binary animal ownership as an exposure, we also excluded observations with extreme predicted probabilities of animal ownership (within 1% of the minimum and maximum predicted probability across all households). SuperLearner was used to estimate the predicted probability of animal ownership for all covariates considered, and to discover and prevent positivity violations (lack of variation in animal ownership in a specific covariate stratum).¹⁹⁰ Finally, for the prospective analyses, we also excluded households that changed animal ownership status between enrollment and two years after intervention delivery, and reclassified households that obtained animals during the study as animal-owning households.

As a secondary analysis, we estimated unadjusted and adjusted associations between community-level animal ownership and all outcomes. Animal ownership census data was not available for the Kenya WASH Benefits study sites, so community-level animal

ownership was defined as the median number of animals owned by study households living in the same village.

3.4 Results

Household Characteristics

We previously reported indicators of intervention uptake.⁷ In water intervention arms, the proportion of households with detectable chlorine residual in their stored drinking water ranged from 39-43% at year one and from 19-23% at year two. Among households in the sanitation intervention arms, the proportion with access to an improved latrine was 89-90% at year one and 78-82% at year two. In handwashing intervention arms, soap and water was present at a handwashing location at 76-78% of households at year one and at 19-23% at year two.

We collected and measured levels of fecal indicator bacteria in 3909 drinking water samples, 2691 child hand rinses, and 2422 child toy ball rinses (including both the one- and two-year assessments; Figure 3-1). We collected 6047 caregiver and 5684 child hand cleanliness observations in the C/N and WSH/WSHN arms. Flies were counted at 4269 food preparation areas and 4118 latrines. Diarrhea data were collected from 5795 children at year two, and length-for-age data from 6587 children.

Animal ownership was high in our study population, with 90.2% (3777/4187) and 90.6% (3796/4192) of households owning any animal at enrollment and year two, respectively. By species, household poultry ownership was most common (enrollment, year two (%): 85.3, 84.7), followed by cattle (47.2, 51.4), dogs (20.0, 14.9), goats (12.5, 16.4), and

sheep (NA, 5.56) ownership. Among animal-owning households, households owned an average of 9 animals (standard deviation (SD): 9) at enrollment and year two. By species at enrollment and year two, animal-owning households owned the greatest number of poultry (mean [SD]: 8 [8]), followed by cattle (2 [2]), goats (2 [2]), sheep (2 [2]), and dogs (2 [1]). Between enrollment and year two, 15.1% (633/4192) of households changed ownership status for any type of animal. The greatest number of households changed cattle ownership status (30.4%), followed by poultry (23.0%), dogs (20.8%), and goats (19.4%); sheep ownership data was not collected at enrollment.

Levels of Environmental Contamination in the Control Group

When combining the one- and two-year assessments, 94% of stored drinking water samples were contaminated with *E. coli* (log₁₀ mean: 1.48 colony forming units [CFU]/100 ml), 90% of child hands were contaminated with *E. coli* (log₁₀ mean: 1.74 CFU/two hands), and 73% of toys were contaminated with *E. coli* (log₁₀ mean: 0.58 CFU/toy) in the control group (C/N). One quarter (26%) of caregivers had visible dirt observed on their palms or fingerpads, and over half (54%) had dirt observed underneath their fingernails. Approximately one third (36%) of children had visible dirt observed on their palms or fingerpads, while two thirds (67%) had dirt observed underneath their fingernails. Flies were present at 62% of food preparation areas (mean: 3.4 flies, SD: 6.2) and at 67% of latrines (mean: 3.7 flies, SD: 5.5). House flies were more common at food preparation areas (60% prevalence) than latrines (30%); bottle flies were observed at 3% of food preparation areas but were observed at more

than half of latrines (55% prevalence); flesh flies were observed at <1% of food preparation areas and 3% of latrines.

Intervention Effects on Environmental Contamination

Water Quality

Water treatment reduced the prevalence of *E. coli* in drinking water by 34% (Prevalence Ratio [PR]: 0.66, 95% Confidence Interval [CI] 0.58, 0.76) after one year of intervention exposure and by 24% (PR: 0.76, 95% CI: 0.72, 0.81) two years after interventions began (Figure 3-2, Table B-1). The combined WSH intervention showed similar effects on water quality to water treatment alone (45% reduction in *E. coli* at year one; 19% reduction at year two). Handwashing with soap slightly reduced the prevalence of *E. coli* contamination in stored drinking water at year two (PR: 0.95, 95% CI: 0.92, 0.99), but not at year one.

Child Hand and Toy Contamination

The combined WSH interventions did not affect the presence or levels of fecal indicator bacteria on child hands or toy balls at any time point (Figure 3-2, Table B-1). The combined WSH interventions reduced the prevalence of visible dirt on caregiver hands (PR averaged over both measurements: 0.86, 95% CI: 0.78, 0.95) and under caregiver fingernails (PR averaged over both measurements: 0.90, 95% CI: 0.85, 0.96), but did not affect visible dirt on child hands or underneath child fingernails (Table B-3). These data suggest handwashing frequency marginally increased in the WSH arms, but these increases were not sufficient to reduce *E. coli* contamination on hands.

Fly Prevalence and Densities

The combined WSH intervention reduced the prevalence and density of flies near the latrine at year one (PR: 0.88, 95% CI: 0.79, 0.97; -0.6 less flies counted, 95% CI: -1.2, 0.0), but not at year two (Figure 3-2, Table B-3). No interventions affected the prevalence or density of flies at the food preparation area. We also did not detect any differences in prevalence of fly species between the study arms (data not shown).

Associations Between Animal Ownership and Environmental Contamination

Water Quality

For every additional log₁₀ number of sheep owned by a household, the prevalence of *E. coli* in stored water was 10% greater (PR: 1.10, 95% CI: 1.04, 1.15) at year two (Figure 3-3, Figure B-7). The prevalence of *E. coli* in water was also 6% (PR: 1.06, 95% CI: 1.02, 1.10) higher in sheep-owning households than in households without sheep (Figures B-8 – B-9). There were no other associations between household animal ownership and drinking water quality. There were even stronger associations between community-level sheep ownership and *E. coli* in water (PR: 1.16, 95% CI: 1.08, 1.26; 0.88 log₁₀ CFU/100 mL increase, 95% CI: 0.28-1.47 for every additional log₁₀ median number of sheep owned in the same village) (Figure B-10). Higher numbers of goats, poultry, and any animal species owned in the community were also associated with higher *E. coli* concentrations in water.

Child Hand and Toy Contamination

A 1 log₁₀ increase in the number of goats owned by a household was associated with 7% (PR: 1.07, 95% CI: 1.01, 1.14) greater *E. coli* prevalence and 0.22 log₁₀ CFU/100 mL (difference: 0.22, 95% CI: 0.09 2.29) higher *E. coli* concentration on child hands

(Figure 3-3, Figure B-7). Binary household goat ownership was also associated with higher *E. coli* prevalence and concentration on hands (Figures B-8 – B-9). Sensitivity analyses revealed potential associations between cattle, poultry, and any animal ownership and higher *E. coli* concentration on hands, but there were no other associations between household animal ownership and hand contamination (Figures B-7 – B-9). Community-level goat ownership showed slightly stronger positive associations with *E. coli* prevalence and concentration on child hands (PR: 1.16, 95% CI: 1.08, 1.25; log₁₀ CFU/100 mL difference: 0.50, 95% CI: 0.04, 0.95) (Figure B-10).

Household animal ownership was not associated with *E. coli* prevalence or concentration on sentinel toys (Figure 3-3; Figures B-7 – B-9). An increase of 1 log₁₀ village-median number of goats owned, however, was associated with 34% higher prevalence of *E. coli* in toy ball rinses (PR: 1.34, 95% CI: 1.00, 1.80) (Figure B-10).

Fly Prevalence and Densities

Increased numbers of ruminants (cattle, goats, sheep) and any animal species owned by households were associated with higher fly prevalence and densities (excluding sheep ownership) at food preparation areas (Figure 3-3; Figure B-7). Similarly, binary cattle and goat ownership were associated with 11-18% greater fly prevalence and 0.69-1.28 more flies counted at food preparation areas (Figures B-8 – B-9). Community-level ownership of goats and any animal species were also associated with greater fly prevalence and densities at household food preparation areas (Figure B-10).

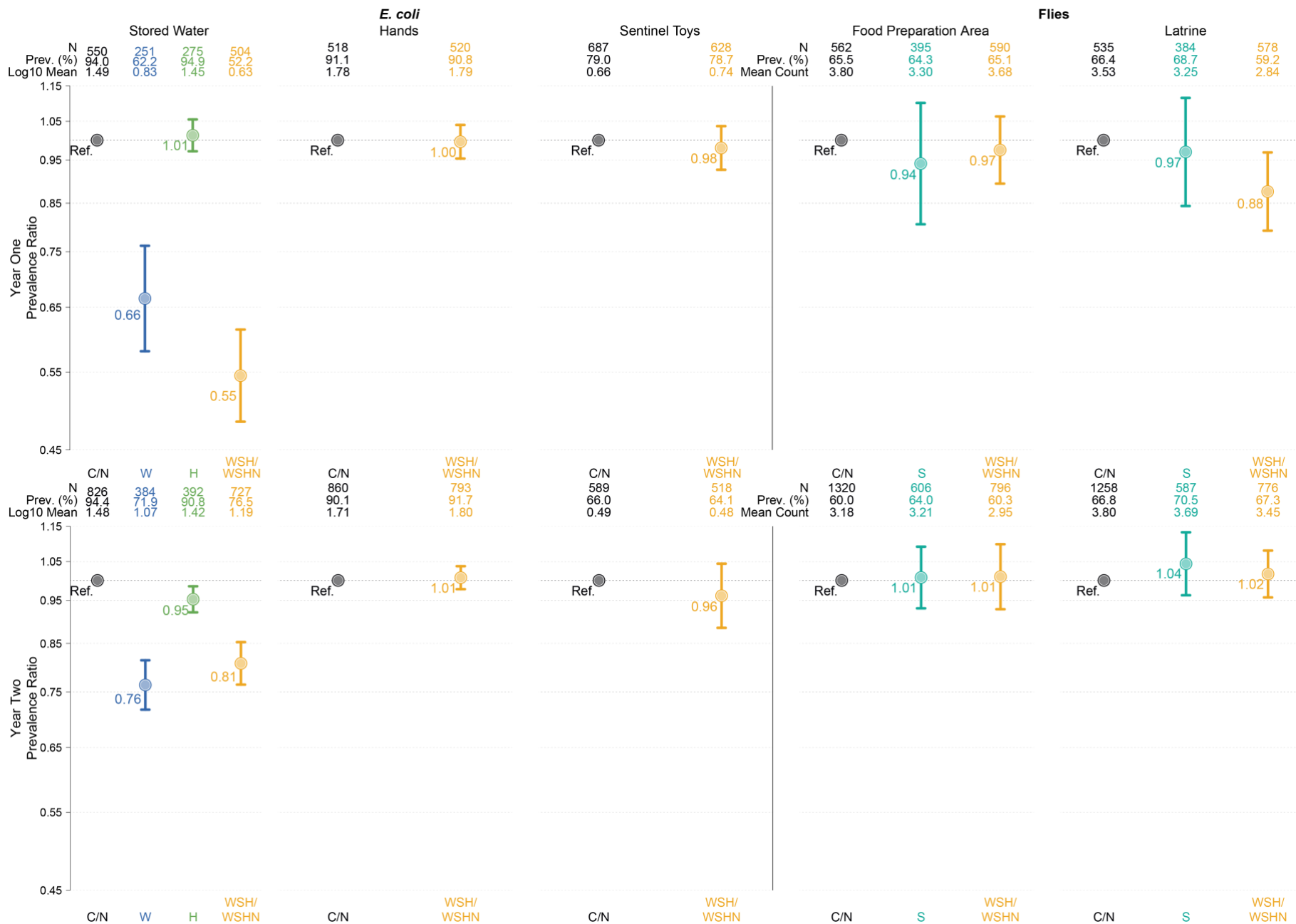


Figure 3-2: Intervention impacts on environmental contamination. Prevalence ratios of *E. coli* in stored water, on child hands, and on toys by intervention arm compared to the control group (C/N) (left); prevalence ratios of flies measured at food preparation areas and latrines by intervention arm compared to the control group (right)

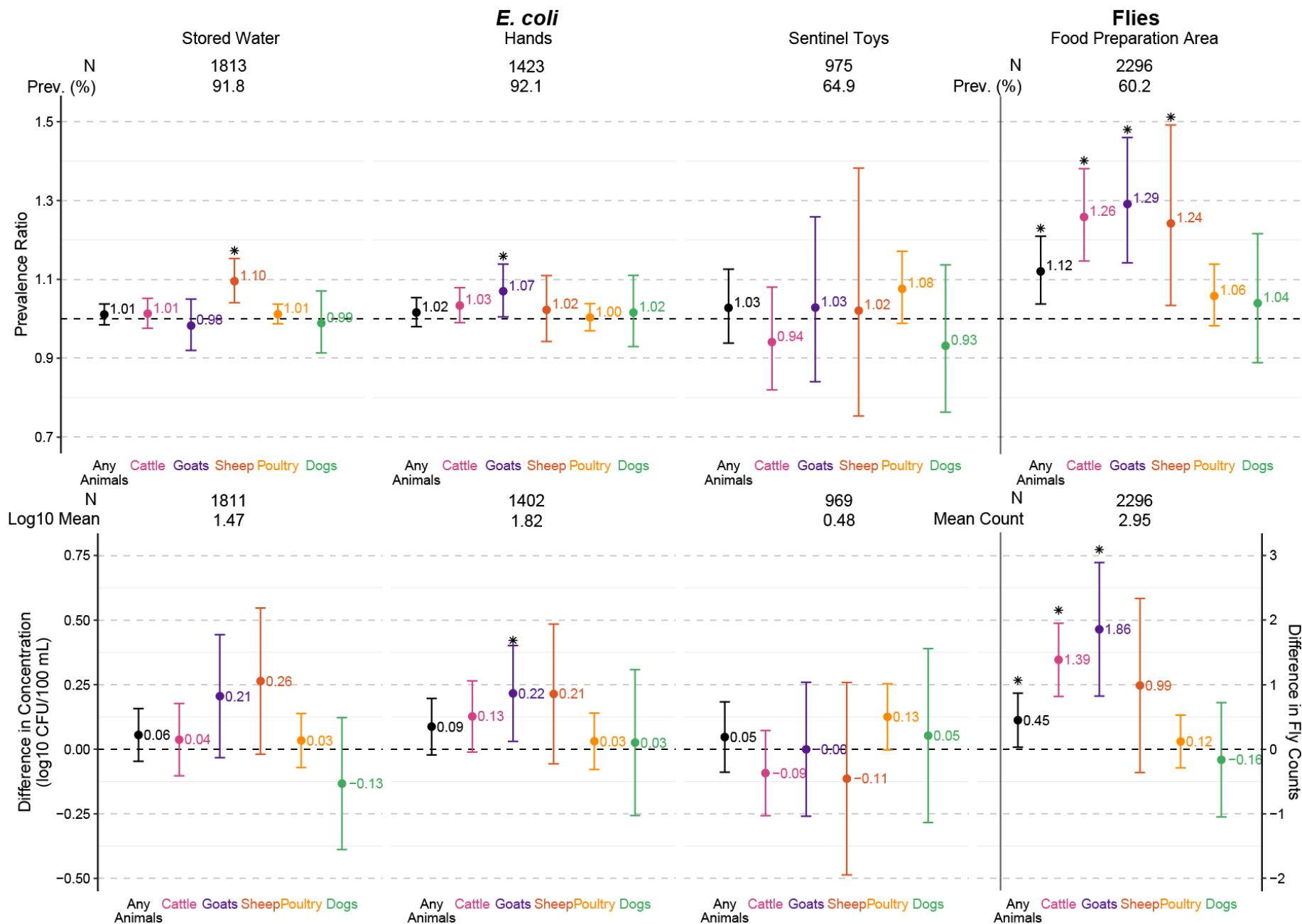


Figure 3-3: Associations between $\log_{10}$ number of animals owned by households and environmental contamination. Prevalence ratios (top) and differences in $\log_{10}$ concentrations (bottom) for *E. coli* in stored water, on child hands, and on toys for each additional $\log_{10}$ animal owned (left); prevalence ratios (top) and differences in fly counts for flies at food preparation areas for each additional $\log_{10}$ animal owned (right). Asterisks indicate significance at a significance level of 0.05.

Associations Between Animal Ownership and Child Health

Neither household nor community-level animal ownership of any species were associated with the prevalence of diarrhea in young children (Figure 3-4; Figures B-11 – B-13; Figure B-15). Greater numbers of cattle, poultry, or any animal species owned by a household were associated with 0.10-0.24 higher length-for-age Z-scores, and consequently 15-33% lower prevalence of stunting (PR: 0.67-0.85), among under-five children (Figure 3-4; Figure B-11). The prevalence of stunting was also 24% (PR: 0.76, 95% CI: 0.69, 0.85) lower in cattle-owning households compared to non-cattle owning households (Figures B-12 – B-13). Community-level ownership of any animal species overall was associated with decreased stunting (PR: 0.83, 95% CI: 0.70-0.98) in young children (Figure B-15).

3.5 Discussion

We found limited impacts of WSH interventions, alone and in combination, on *E. coli* contamination in rural Kenyan households. Our findings confirm that chlorination is an effective method to improve drinking water microbial quality in low-income settings.¹⁷³ If adoption of chlorine had been higher in the parent trial, the reductions in stored water *E. coli* contamination likely would have been larger.^{191,192} The effectiveness of the water intervention in improving water quality is consistent with observed reductions in roundworm (*Ascaris lumbricoides*) infection prevalence among children receiving the water intervention in the trial.¹⁷⁸ *Ascaris* infection prevalence was reduced by 18-22% in the intervention arms that included a water treatment component (water; WSH; and WSHN arms), but not in other intervention arms. Further, water chlorination may be an

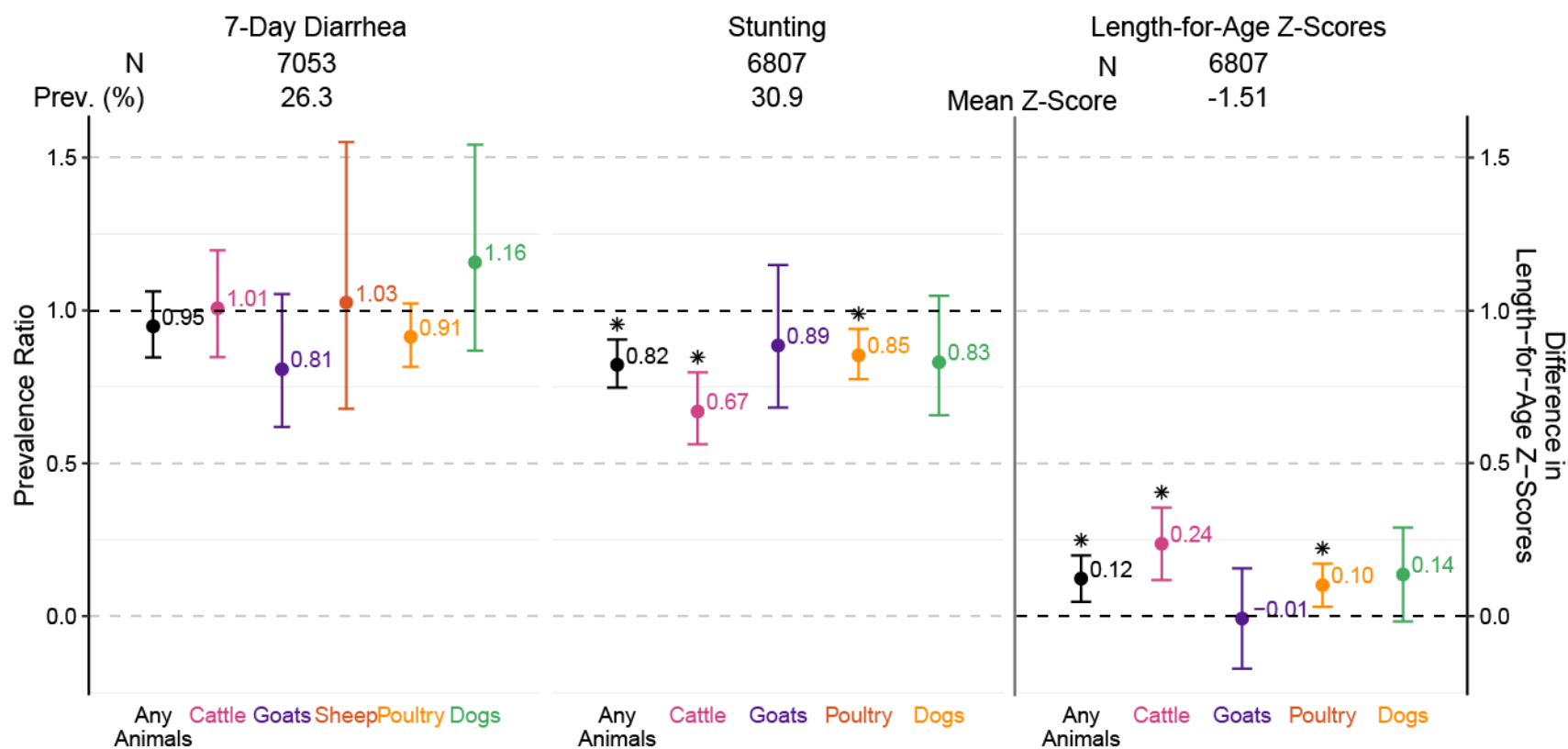


Figure 3-4: Associations between $\log_{10}$ number of animals owned by households and child health. Prevalence ratios for diarrhea and stunting for each additional $\log_{10}$ animal owned (left); differences in length-for-age Z-scores for each additional $\log_{10}$ animal owned (right). Asterisks indicate significance at a significance level of 0.05.

important strategy for protecting household members from zoonotic enteropathogens, as sheep ownership was associated with increased *E. coli* contamination in drinking water. Similarly, previous work in the same study area found that 89% (40/45) of household stored water and 27% (12/45) of source water samples were contaminated with the ruminant-specific fecal marker, BacR.²⁹ While most households (> 68%) enrolled in the WASH Benefits Kenya trial accessed protected springs or wells as their primary drinking water source, roughly a quarter of households relied on unprotected water sources (e.g., unprotected springs; dug wells; surface water) that are prone to contamination by animal feces.^{9,178} Our findings are consistent with previous work in rural India that found that higher village sheep populations increased the odds of detecting higher *Cryptosporidium* spp. concentrations in ponds.¹⁹³

A previous study in Tanzania found that the level of fecal indicator bacteria on caregiver hands was the strongest predictor of the level of fecal indicator bacteria in stored drinking water.¹⁷⁰ Our finding that handwashing marginally reduced *E. coli* concentration in water supports a link between hand contamination and stored drinking water quality; however, the magnitude of the improvement in water quality was small. Further trials that achieve high handwashing with soap rates could be useful to better quantify the effect of increased frequency of handwashing with soap on drinking water quality.

The lack of effect on fecal indicator bacteria measured on hands and toys has several possible explanations, including inconsistent compliance of the targeted hygiene and sanitation behaviors, failure of these specific types of WSH interventions to reduce human fecal contamination on child hands and toys, or animal fecal contamination in the household environment.^{9,24,26} Our findings are consistent with the parallel WASH

Benefits trial in Bangladesh, which also reported no reduction in child hand or toy *E. coli* contamination in the WSH intervention arms.¹⁹⁴ Notably, our ad hoc analyses revealed that child hand contamination was higher in households owning greater numbers of goats. Similarly, ruminant fecal markers were prevalent in child hand rinse samples in urban and rural Bangladesh.^{25,26} Hand contamination can be an especially important risk factor for transmission of zoonotic pathogens to young children, as hand- and object-mouthing is frequent in children from low-income settings.¹⁸³

Flies are recognized vectors for human pathogens, and fly control programs have been found effective in reducing diarrheal illness.^{195–197} We observed marginal fly reductions at latrines from the combined WSH intervention and no fly reductions at food preparation areas. A trial evaluating community-led total sanitation in Mali detected a reduction in fly presence at latrines, while other trials in rural India and The Gambia found no reduction.^{198–200} Fly reductions in this trial may have been limited because the intervention was delivered at the compound rather than the community level (neighboring compounds to the study compound without a pregnant woman did not receive upgraded pit latrines with covers). Additionally, the scoop provided in the sanitation intervention may have been insufficient for removing animal feces from household living areas. We found that animal ownership, particularly ruminant ownership, was associated with higher fly prevalence and densities near food preparation areas. The WASH Benefits trial in Bangladesh found that a higher concentration of *E. coli* on flies caught in the food preparation area was associated with greater *E. coli* contamination of food, though the presence of animal feces was not associated with *E. coli* levels on flies.²⁴

In this study, we found that village-level ownership of ruminants, particularly sheep and goats, was associated with fly presence near household food preparation areas, as well as *E. coli* contamination in household drinking water, on child hands, and on sentinel toys. Allowing animals to freely roam and graze is a common animal husbandry practice in many low-income communities. Non-animal-owning households might therefore also be at high risk of exposure to fecal contamination from animal feces. Previous work in Bangladesh has documented ruminant-specific fecal markers on child hands or floor sponge samples in both goat-owning and non-ruminant-owning compounds.²⁵ In combination, these results emphasize the importance of implementing future interventions for interrupting community-wide enteropathogen transmission as opposed to intervening at the individual household level.

Although animal ownership was associated with increased fecal contamination in our study households, it did not appear to be a risk factor for under-five child diarrhea or growth faltering. On the contrary, household cattle and poultry ownership was associated with higher length-for-age Z-scores and lower prevalence of stunting. Livestock husbandry is often promoted as a means to improve livelihoods and incur nutritional benefits, through increased consumption of animal-sourced food, for low-income households.^{14,15} Our work is consistent with a growing body of evidence that owning domesticated animals may reduce stunting among young children.^{51,201,202} The true relationship between animal exposure and linear growth is likely dependent on specific animal husbandry practices within the household. In rural Ethiopia, for example, poultry ownership was associated with increased length-for-age Z-scores, but corralling poultry inside the home overnight was negatively associated with child growth.⁵¹ Key

behaviors affecting human exposure to animal fecal contamination under varying contexts (e.g., rural versus urban; domestic versus community) are poorly understood and should be addressed in future research.⁹

Limitations

There are some limitations in this study. First, fecal indicator bacteria can be a poor proxy for enteric pathogens, and thus may exhibit limited associations with health outcomes in individual studies.²⁰³ Future evaluations should consider measuring specific human pathogens in water and in the environment to better understand how WSH interventions affect transmission pathways. Second, while we adjusted for relevant covariates in the association between animal ownership and other outcomes, including intervention arm, we were unable to account for all potential confounders or draw conclusions about causal relationships. Further, animal ownership is likely correlated with some covariates, such as household asset ownership as measures of socioeconomic status. We discovered and prevented positivity violations by excluding households with extreme predictive probabilities of animal ownership as sensitivity analyses for models with binary animal ownership as the exposures.

Conclusion

Promotion of chlorine improved drinking water quality, but adoption of the water treatment intervention was much lower than expected by the end of the study. This emphasizes the difficulty of achieving sustained and consistent usage of household water treatment products with monthly, or less frequent, behavior promotion visits.^{204,205} We found no evidence that combining water, sanitation, and handwashing interventions led to larger reductions in fecal contamination in the household environment than single

interventions, a finding consistent with no additive benefit on health outcomes measured in this trial or in other studies.^{6,7,206–209} Our results indicate that the intensive WSH interventions implemented in this study did not reduce levels of fecal indicator bacteria on child hands or toys, while they slightly reduced fly presence near latrines and marginally improved visible hand cleanliness of caregivers. The failure of the interventions to reduce fecal contamination along important exposure pathways in the household suggests that WSH programs that aim to improve child health may need to consider interventions that cost more but also more comprehensively reduce fecal contamination in the household setting; our findings provide additional support for transformative WSH interventions.^{9,10}

Improved animal feces management is one such consideration that has been called for in transformative WSH solutions.¹¹ Our findings demonstrated that while domesticated animals did increase fecal contamination in the household environment, they did not negatively impact under-five child health – and may have even protected against stunting – in rural Kenya. As there are two growing and opposing bodies of literature on the risks and benefits of animal husbandry on health in LMICs, it is imperative that future interventions avoid jeopardizing child growth benefits by promoting practices that increase the cost or burden of household animal rearing.

3.6 Acknowledgements

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3.7 Citation

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Chapter 4: Differential overlap in human and animal fecal microbiomes and resistomes in rural versus urban Bangladesh

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4.1 Abstract

Low- and middle-income countries (LMICs) bear the largest mortality burden of antibiotic-resistant infections. Small-scale animal production and free-roaming domestic animals are common in many LMICs, yet data on zoonotic exchange of gut bacteria and antibiotic resistance genes (ARGs) in low-income communities are sparse. Differences between rural and urban communities in population density, antibiotic use, and cohabitation with animals likely influence the frequency of transmission of gut bacterial communities and ARGs between humans and animals. Here, we determined the similarity in gut microbiomes, using 16S rRNA gene amplicon sequencing, and resistomes, using long-read metagenomics, between humans, chickens, and goats in a rural compared to urban community in Bangladesh. Gut microbiomes were more similar between humans and chickens in the rural (where cohabitation is more common) compared to urban community, but there was no difference for humans and goats in the rural versus urban community. Human and goat resistomes were more similar in the urban community, and ARG abundance was higher in urban animals compared to rural animals. We identified substantial overlap of ARG alleles in humans and animals in both settings. Humans and chickens had more overlapping ARG alleles than humans and goats. All fecal hosts from the urban community and rural humans carried ARGs on chromosomal contigs classified as potentially pathogenic bacteria – including

Escherichia coli, *Campylobacter jejuni*, *Clostridioides difficile*, and *Klebsiella pneumoniae*. These findings provide insight into the breadth of ARGs circulating within human and animal populations in a rural compared to urban community in Bangladesh.

4.2 Background

In low- and middle-income countries (LMICs), animal husbandry is an important source of household income and nutrition.^{12,210} Human exposure to livestock – particularly through cohabitation – is associated with diarrheal disease and impaired child growth^{211,212}. Sixty-nine percent (20/29) of studies included in a systematic review and meta-analysis found a significant positive association between human exposures to domestic animals and diarrhea.²¹¹ An observational analysis in rural Ethiopia determined that corralling poultry inside households overnight was associated with a 0.25 decrease (standard error = 0.118) in child height-for-age Z-scores.²¹² Domestic animals are confirmed reservoirs of enteropathogens, including pathogenic *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp.^{17–19} Living in close proximity to animals, as is common in LMICs, is associated with increased presence of animal feces, which can harbor enteropathogens, in households.^{24–26} Host-specific fecal markers from animals (dogs, birds, and ruminants) were detected in multiple household reservoirs (soil, hands, and stored drinking water) in rural and urban Bangladesh and rural India.^{26–28} In rural Bangladesh, increased concentration of an animal-specific fecal marker, BacCow, was associated with increased prevalence of shiga toxin-producing *E. coli* on hands.²⁷

Cohabitation between humans and domestic animals may also promote dissemination of antibiotic resistance. Globally, by 2050, more than 10 million deaths due to antibiotic-

resistant infections are estimated to occur annually.³⁰ LMICs are predicted to bear the largest burden of these deaths, partially from poor regulation of antibiotic use in humans and animals, management of human and animal excreta, and access to safe drinking water, sanitation infrastructure, and hygiene.³⁰ Antibiotic resistance arises through antibiotic use, accelerated by overuse and misuse of antibiotics (e.g., prophylaxis, incomplete courses, growth promotion) in humans and domestic animals, which contributes to the selection of intestinal bacterial strains that have acquired resistance.³³ Further, in microbial communities, such as the intestinal microbiota, bacteria can exchange antibiotic resistance genes (ARGs) through horizontal gene transfer, allowing the exchange of ARGs between benign bacteria and pathogens, as well as between distinct microbial communities (humans, animals, environmental matrices).^{34,35} The proliferation of antibiotic resistance in animal gut microbiomes in response to antibiotic use as prophylaxis or growth promotion and subsequent transmission to humans, particularly farm workers, has been demonstrated in intensive farming environments and high-income countries.²¹³ Particularly in LMICs, data on antibiotic-resistant infections acquired in domestic environments, however, are limited. A study among six LMICs – Bangladesh, Bolivia, Ghana, India, Pakistan, and South Africa – found that more than half of community-acquired infections in children were resistant to antibiotics.³⁶ It is crucial to determine whether domestic animals raised through small-scale animal production and backyard farming are important reservoirs of antibiotic resistance in LMICs to determine if community-level interventions – in addition to national-level regulation of antibiotic use in agriculture, veterinary medicine, and human medicine – could reduce the burden of antibiotic-resistant infections.

While less than 30 % of urban Bangladeshi households own domestic animals, compared to the more than 70 % of rural households, there is a greater ratio of backyard farms (< 2.5 acres of land) to medium- and large-scale farms in urban compared to rural communities.^{214,215} Although humans might be exposed to a greater number of animals in rural areas, limited space in urban areas makes it more difficult to separate animals and animal feces from humans living in the community. It is unclear how these differing levels of human exposure to animals might affect similarity in gut bacterial communities or intestinal resistomes (collections of all ARGs) between humans and animals in rural versus urban communities. Urban communities have been hypothesized as potential hotspots of antibiotic resistance transmission.²¹⁶ Previous work has shown there is a higher proportion of antibiotic-resistant infections among children from urban compared to rural communities.^{217–219} As rural residents continue to migrate to urban areas, urban populations are expected to rise.^{220,221} Thus, it is increasingly important to characterize transmission pathways of antibiotic resistance – and how they differ in rural versus urban settings – to design targeted and effective interventions.

While prior research in LMICs demonstrates the potential for gut bacteria and ARGs to be shared between humans and domestic animals, metagenomic studies examining resistomes have primarily relied on fragmented assemblies produced with short-read sequencing technologies, limiting their ability to link ARGs to specific bacterial species or locate ARGs on chromosomes or mobile genetic elements (e.g., plasmids, transposons). A study in rural Kenya found greater similarities in gut microbiota composition between children and cattle sharing a household compared to those from

different households, though the results were not statistically significant.²²² A study in rural and peri-urban Peru found that 41 % of antibiotic resistance proteins shared by human, animal, and environmental samples were encoded in the same genetic context (neighboring core genes), indicating past horizontal gene transfer, though the short-read assemblies revealed that 11 % of unique antibiotic resistance proteins were encoded in multiple contexts.²²³ Recent work, limited to the study of *E. coli* cultured from children and domestic animals in Ecuador, has shown that while clonal relationships exist, horizontal gene transfer via diverse plasmids may present a greater challenge in the dissemination of antibiotic resistance.^{87,224}

Technological advances in molecular methods, such as long-read sequencing, enable improved assemblies of complex metagenomes over those produced with short-read technologies, thus facilitating a deeper understanding of how antibiotic resistance circulates in communities. In this study, we used a combination of 16S rRNA gene amplicon and long-read sequencing to characterize differential overlap in fecal microbiomes and resistomes between humans and common domestic animals (chickens and goats) in a rural versus urban community in Bangladesh (Figure 4-1). We also compared ARG abundance by fecal host type and urbanicity and identified potentially pathogenic bacterial species and plasmids carrying ARGs.

4.3 Methods

Study Sites

We collected human and animal fecal samples from one rural and one urban community in Bangladesh. The rural community, Mymensingh district, is located roughly 120 km

north of Dhaka, the capital city of Bangladesh. Rural households in Mymensingh are typically organized into compounds that share a common courtyard, sometimes including a water source and/or latrine. Mymensingh compounds are characterized by high levels of animal ownership (94 %), poor drinking water quality (77 % of stored water samples had detectable *E. coli*), limited latrine ownership (54 %), and limited access to handwashing stations with soap (21 %).^{6,24} The urban community, Tongi sub-district, is 15 km north of Dhaka. It is densely populated, with many families living in single-room homes and sharing a common stove, water source, and/or latrine with up to 15 other families. While less than 30 % of urban households own animals, a greater

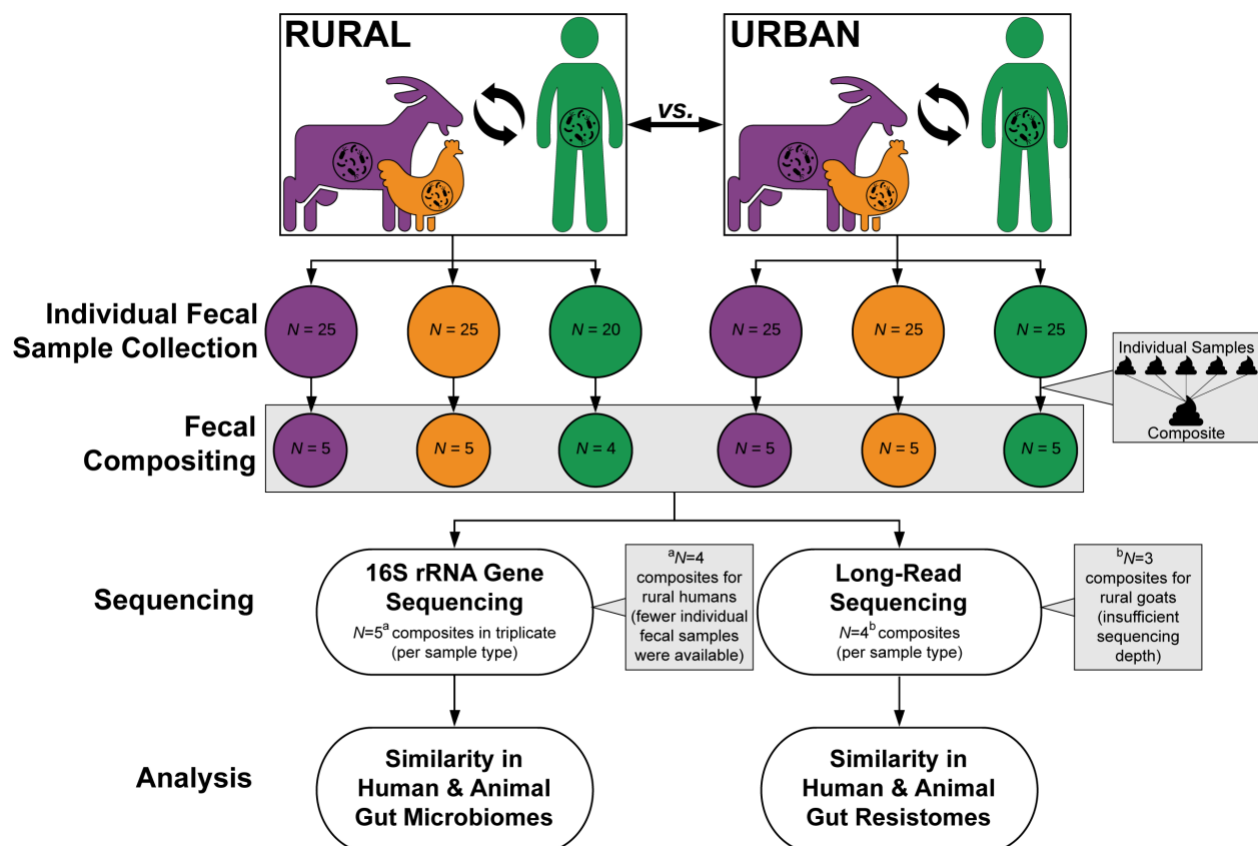


Figure 4-1: Study design flowchart for comparing human and animal fecal composites in a rural and urban community

proportion of animal-owning households have less than 2.5 acres of land in urban compared to rural areas.^{25,214,215} Similar to rural areas, low-income households in urban Bangladesh are characterized by poor drinking quality (70 % of stored water samples had detectable *E. coli* in Tongi) and insufficient sanitation facilities.^{225,226} Household asset ownership is higher among Tongi (T) compared to Mymensingh (M) residents for televisions (T: 84 %, M: 51 %), mobile phones (T: 94 %, M: 86 %), wardrobes (T: 63 %, M: 17 %), and refrigerators (T: 36 %, M: 8.0 %).^{6,226} According to recent studies, reported household antibiotic use among children under 5 years is fairly similar in rural (39 %) and urban (44 %) communities in Bangladesh^{226,227}

Sample Collection

Between June and August 2015, 50 individual chicken and 50 individual goat fecal samples were identified by observation in public and domestic areas in Bangladesh, half of which were from Mymensingh (rural) and half from Tongi (urban). Using sterile scoops, fresh feces were scooped from the center and top of feces piles to avoid soil contamination. Samples were stored on ice and transported to a field laboratory (Mymensingh) or International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b; Tongi) within six hours. Trained icddr,b staff carried out the animal fecal sample collection.

Twenty-five human stool samples were collected from adult (>18 years) residents of different households in Tongi between April and October 2013; participants were asymptomatic household members of cholera patients presenting with diarrhea to the Tongi Sub-district Hospital or icddr,b Dhaka Hospital. The samples were transported in Cary-Blair media to icddr,b and frozen at -80 °C. Between June and September 2015,

20 human stool samples were collected from adult (>18 years) residents of different households in Mymensingh; participants were primary caregivers of target children enrolled in the parent trial.⁶ The samples were transported to the field laboratory and frozen at -80 °C before being shipped to icddr,b on dry ice. Written informed consent was obtained from all study participants. The studies were approved by the icddr,b Ethical Review Committee (PR-11063, PR-13004), Johns Hopkins University Institutional Review Board (IRB) (00004795), University of California, Berkeley Committee for the Protection of Human Subjects (2011-09-3652), and Stanford University IRB (25863).

Animal fecal samples were immediately processed after arrival to the laboratories, and human stool samples were thawed from -80 °C prior to further processing. The samples were combined into fecal composites for each fecal host (chicken, goat, human) from the urban or rural community, respectively by measuring 0.2 g of five individual samples from corresponding locations into 50-mL centrifuge tubes using ethanol and flame-sterilized spatulas. Samples were composited due to resource constraints and allowed us to reduce the number of samples while preserving information from all collected samples. We have used this approach previously when working in resource-constrained environments.^{25,229} After mixing the samples, 1-g composites were transferred to 1.5-mL microcentrifuge tubes. 1 mL of RNALater™ solution (Invitrogen, Carlsbad, CA) was mixed into each composite and left to sit for five minutes. Five composites were made for each fecal host in each community (rural, urban), with the exception of rural human stool; as each composite contained equal masses of five individual fecal samples and there were only 20 total rural human stool samples, only four composites were possible

for rural human stool. Chicken fecal composites were treated in a hot water bath (100 °C) for 20 minutes in accordance with our United States Department of Agriculture (USDA) import permit (128693). All composites were stored at -80 °C before shipment at room temperature to Stanford University, where samples were stored at -80 °C upon arrival. Aliquots for the 16S rRNA gene analysis were stored at -80 °C (stored 2 years and 9 months before processing) at Stanford University. After storage at Stanford University, aliquots for the long-read sequencing analysis were shipped on dry ice to Tufts University and stored at -80 °C (stored an additional 4 months before processing).

DNA Extraction

For 16S rRNA gene analysis, DNA was extracted from 0.25 g of each fecal composite using the PowerSoil® extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. We extracted one blank sample per day of DNA extractions (three extraction controls total) to control for contamination during the extraction process. *E. coli* K-12 (ATCC 10798) was used as a positive control. The culture was grown on tryptic soy agar and a single colony was suspended in nuclease-free water (Thermo Fisher Scientific, Waltham, MA). The suspension was centrifuged at 10,000 x g for 5 minutes, and the pellet was resuspended in nuclease-free water, heated to 100 °C for 10 minutes, cooled at 20 °C for 10 minutes, and centrifuged at 14,000 x g for 5 minutes. The supernatant was used as template in polymerase chain reactions (PCRs).

Due to budget constraints, only four of the five fecal composites from each fecal host were processed with long-read sequencing. For long-read sequencing, DNA was extracted from 0.25 g of each fecal composite using the DNeasy® PowerSoil® Pro

extraction kit (QIAGEN, Germantown, MD). We followed the manufacturer's instructions, with the exceptions of using an alternate lysis method (heating samples at 70 °C for 10 minutes followed by vortexing at full speed for 10 minutes, adapted from Earth Microbiome Project's DNA extraction protocol) and eluting in Tris-EDTA buffer (Integrated DNA Technologies, Inc., Coralville, IA) to prevent degradation.²³⁰ We extracted one blank extraction control per day of DNA extractions (three extraction controls total). DNA purity was quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA). For extraction controls, nucleic acid concentrations were measured using a Qubit™ 4.0 fluorometer (1X dsDNA HS assay kit; Thermo Fisher Scientific, Waltham, MA). For fecal composite extracts, nucleic acid concentration and fragment size distributions were assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Tufts University Core Facility (Boston, MA). Quality control parameters are presented in Table C-6.

16S rRNA Gene Amplicon Generation and Sequencing

We used a previously described 16S rRNA gene sequencing Illumina amplicon protocol with 515f and 805r primers (GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT, respectively) that targeted the V4 region of the 16S rRNA gene.^{231–236} Golay barcodes were embedded in each forward primer (Table C-7). Previously described PCR protocols were used to generate 16S rRNA gene amplicons from all samples, including the negative and positive controls: 25 µL PCRs consisted of 2x QIAGEN HotStarTaq® Plus mastermix (Germantown, MD), 10 µM of forward primer, 10 µM of reverse primer, and 1 µL of template DNA. PCRs were run in triplicate using the following thermocycler conditions: 94 °C for 3 minutes; then 35 cycles of 94 °C for

45 seconds, 50 °C for 60 seconds, 72 °C for 90 seconds; followed by 72 °C for 10 minutes and a hold at 4 °C.^{231–235} A no template control reaction was included in every PCR run. After PCR, we visualized product size using electrophoresis on 1.5 % agarose gels containing ethidium bromide. All fecal composites and positive controls showed a band of appropriate size (~390 bp). The PCR triplicates each received unique barcodes during PCR (i.e., each sample was sequenced in triplicate). We did not observe any bands in the lanes of the gel containing the negative controls, including extraction and no template controls.

A Qubit™ 2.0 fluorometer (dsDNA HS assay kit; Thermo Fisher Scientific, Waltham, MA) was used to quantify the nucleic acid concentration in PCR products. Based on those concentrations, amplicons from the samples and the positive control were multiplexed and pooled in equimolar proportions (10 µM) for sequencing. Additionally, we multiplexed and pooled extraction and no template controls (negative controls) for sequencing. 10 µL of each of the negative control pools were added to the total pool before purification. The total pool was purified using the UltraClean® PCR clean-up kit (MoBio, Carlsbad, CA) before sequencing. Sequencing was performed using an Illumina MiSeq at the Stanford Functional Genomics Center (Stanford, CA) ²³⁶. We used 250-bp paired-end reads (2x250) and spiked the total pool with 10 % phiX before sequencing. We sent index primers

(AATGATACGGCGACCACCGAGATCTACACGCT), read 1 primers

(TATGGTAATTGTGTGYCAGCMGCCGCGGTAA), and read 2 primers

(AGTCAGCCCAGCCGGACTACNVGGGTWTCTAAT) with the total pool.^{231–235}

Long-Read Sequencing

Long-read sequences were obtained from DNA extracts using Oxford Nanopore Technologies' (ONT's) MinION™. Library preparation was conducted using the SQK-LSK109 ligation sequencing kit (ONT, Oxford, United Kingdom) according to the manufacturer's instructions. When preparing libraries for goat and human samples, the L Fragment Buffer (LFB) was used to enrich for DNA fragments of 3 kbp or longer. Since DNA concentrations were low in chicken fecal composite extracts (Table C-1), when preparing libraries for chicken samples, the S Fragment Buffer (SFB) was used to retain DNA fragments shorter than 3 kbp. Two fecal composite libraries were multiplexed per flow cell (FLO-MIN106; ONT, Oxford United Kingdom) in equimolar proportions (except for chicken samples, for which all DNA was retained to account for the lower DNA concentrations) using the EXP-NBD103 native barcoding kit (ONT, Oxford, United Kingdom).

Data Processing and Analysis

16S rRNA Gene Amplicon Analysis

The DADA2 pipeline was used to process forward and reverse reads.²³⁷ Forward reads were truncated to 173 nucleotides and reverse reads were truncated to 162 nucleotides, after which the quality score dropped. Paired-end reads were merged to yield 250 bp sequences, and chimeras were removed. Taxonomy was assigned in DADA2 using a Naïve Bayes classifier that was trained on the Silva v132 database.^{238,239} Species-level identification was based on 100% identity between the reference database and amplicon sequence variants (ASVs).²⁴⁰

Data were analyzed using *phyloseq* (version 1.26.1) in R (v3.5.0).²⁴¹ All ASVs associated with eukaryotic organelles – chloroplasts and mitochondria – were removed. ASVs were normalized using the inverse hyperbolic sine transformation.²⁴² ASVs identified in the three technical replicates (from the PCR step of library preparation) were merged by summing, and the merged data were used for all subsequent analyses. To determine the similarity of bacterial communities between humans and animals, we calculated pairwise Bray-Curtis dissimilarity indices between humans and goats and between humans and chickens in rural and urban areas. A Wilcoxon rank sum test ($p < 0.05$) was used to determine if the differences in Bray-Curtis indices were statistically significant in urban versus rural areas. Variables contributing to differences between communities, such as fecal host and location, were identified by PERMANOVA or pairwise PERMANOVA with Bray-Curtis dissimilarity using *vegan* (v2.5.6) and *pairwiseAdonis* (v0.4) in R (v3.5.0).²⁴³

To determine if sample processing contamination impacted the results, a post-hoc sensitivity analysis was conducted where ASVs classified as the 140 taxa present in the no template control (Figure C-1) were removed from all samples and Bray-Curtis dissimilarities were recalculated.

Long-Read Sequencing Analysis

Long-read sequences were basecalled, demultiplexed, and trimmed using ONT's toolkit, Guppy (v3.3.0). To identify ARGs, reads were aligned to the ResFinder database (duplicates removed) using Minimap2 (v2.15).^{244,245} Only primary ARG alignments with ≥ 90 % similarity and ≥ 100 bp alignment lengths were retained for the analysis. To quantify data classified as bacteria, raw reads were aligned to the National Center for

Biotechnology Information's (NCBI's) RefSeq database (plasmid sequences removed) using a least common ancestor approach in Centrifuge (v1.0.4).²⁴⁶ In accordance with a previously published normalization method, ARG gene copies (sum of alignment length over reference read length for all relevant ARG hits) were normalized by gigabase pairs (Gbp) of data classified as bacteria for each sample type and each ARG.²⁴⁷ Wilcoxon matched-pairs signed-rank tests ($p < 0.05$) were used to test for statistical differences in the normalized abundance of ARGs, paired by drug class, in urban and rural samples by fecal host. Wilcoxon rank sum tests ($p < 0.05$) were used to test for differences in the normalized abundance of ARGs in human, chicken, and goat samples by urbanicity. To test the hypothesis that human and animal resistomes were more similar in the rural versus urban community, Bray-Curtis dissimilarities were calculated for all pairwise comparisons, and a Wilcoxon rank sum test ($p < 0.05$) was used to determine statistical significance. We identified overlapping distinct resistance gene alleles between humans and each animal within the urban and within the rural community by generating ARG overlap matrices and using two proportion Z-tests ($p < 0.05$) to determine statistical significance in the rural versus urban community.

To identify chromosome- or plasmid-associated ARGs, we determined whether long reads aligning to ARG reference sequences were also classified as potential pathogens or plasmids. Specifically, to identify potential pathogens, reads were assembled using Flye (v2.6) (with the meta option) and polished using ONT's tool for sequence correction, medaka (v0.11.0).²⁴⁸ Polished contigs were aligned to the modified RefSeq database using Centrifuge and modified ResFinder database using Minimap2 (v2.15).^{244–246} We focused our analysis on the 12 bacterial pathogens identified as

antibiotic-resistant threats in the Centers for Disease Control and Prevention (CDC) 2019 Antibiotic Resistance Threats in the United States report: *Acinetobacter baumannii*, *Campylobacter coli*, *Campylobacter jejuni*, *C. difficile*, *Enterococcus faecium*, *E. coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella enterica*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.²⁴⁹ The reliability of pathogen classifications at the sub-species and strain level carrying resistance genes was assessed by aligning potential pathogen sequences against their respective classifications in NCBI's RefSeq database using BLASTN; an *E*-value that rounded to 0 (BLASTN uses double-precision floating-point format, therefore the smallest *E*-value reported is about 1e-179) and percent identity ≥ 98 % were the thresholds used to confirm whether a pathogen classification was reliable.²⁵⁰ Plasmids were identified from the contigs using the MOB-recon tool with default parameters to determine whether identified ARGs were chromosome- or plasmid-associated.²⁵¹ Long-read data from the same type of fecal host and location were pooled together for presentation of the results. Statistical analyses and data visualization were conducted using Stata SE (v14.2) and R (v3.6.3).

Data Availability

Metagenomic sequence reads and 16S rRNA amplicon sequences are available in the sequence read archive (SRA) under accession numbers SRR13059261- SRR13059374 and BioProject PRJNA678365. Human sequences were removed from all samples. Relevant analysis scripts and files are available in GitHub (https://github.com/jennaswa/arg_bd).

4.4 Results

Similarity in Gut Microbiomes

We obtained a total of 11.7 million reads from the 16S rRNA gene amplicon sequencing of DNA extracts from chicken, goat, and human fecal composites. The average sequencing depth per sample was 97,000 reads in chickens, 134,000 in goats, 144,000 in humans, 72,000 in the positive control (*E. coli* K-12 (ATCC 10798)), 180 in the extraction blank, and 6,600 in the PCR no template control (nuclease-free water in place of template DNA). On average, 79 % of the input reads remained after quality filtering, merging forward and reverse reads, and chimera removal. Sixty-six percent and 8 % of reads were classified at the genus and species level, respectively. In total, 1,528 ASVs were present in chicken fecal composites, 3,270 in goat composites, and 655 in human composites. The relative abundance of the 30 most abundant families in all sample types are presented in Figure 4-2. There were 140 ASVs identified in the no template control (Figure C-1) indicating potential contamination during sample processing. We performed a sensitivity analysis in which the ASVs present in the no template control were removed from all samples (Figure C2). Due to the absence of standardized methods to account for negative controls and our finding that the primary study conclusions remained unchanged in the sensitivity analysis, we did not adjust fecal sample ASV counts in the analysis presented here.

The bacterial community compositions of human, chicken, and goat feces were significantly different by fecal host (pairwise PERMANOVA chicken vs. human $R^2=0.46$, chicken vs. goat $R^2=0.34$, human vs. goat $R^2=0.50$, $p_{\text{adjusted}}=0.003$ for all) (Figure 4-3B). Urbanicity was an important determinant for the gut bacterial community composition in

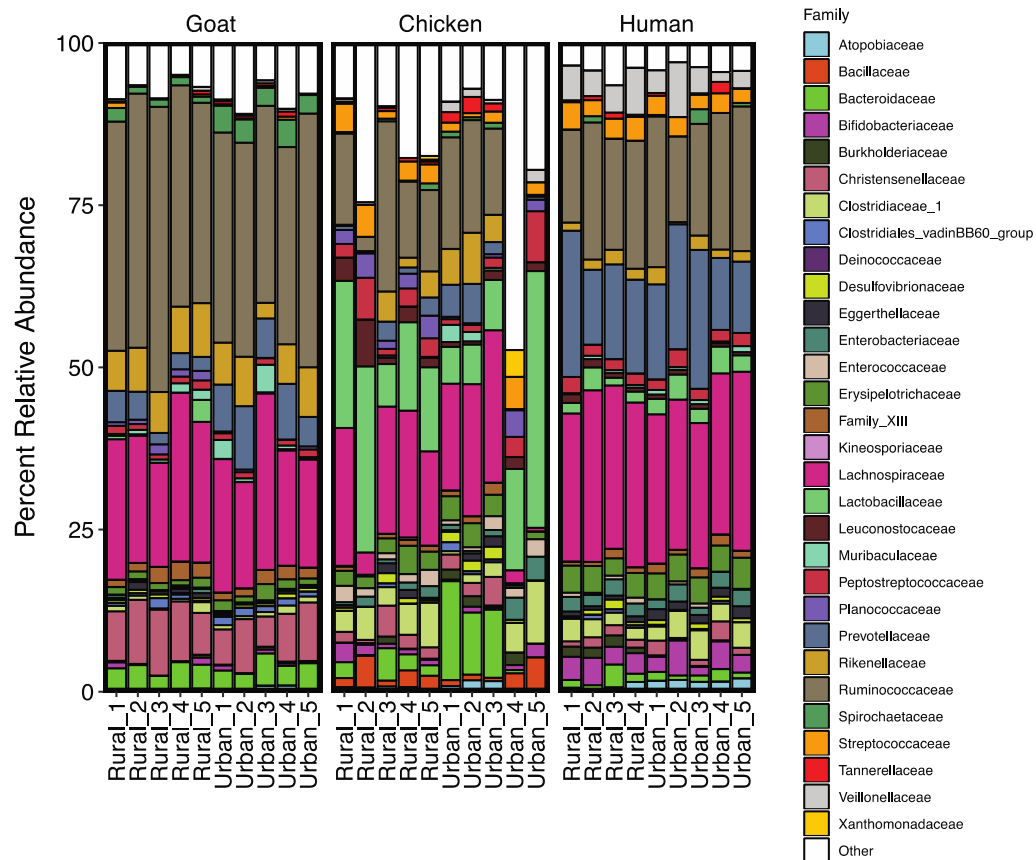


Figure 4-2: Relative abundance of 30 most abundant families in all samples from 16S rRNA gene analysis. All other taxa are grouped into “Other”.

humans and in goats, but not in chickens. Specifically, within each fecal host, rural and urban bacterial community compositions were significantly different in goats (PERMANOVA $R^2=0.24$, $p=0.01$) and humans ($R^2=0.20$, $p=0.02$) but not in chickens ($R^2=0.14$, $p=0.19$). While microbiomes of humans and animals were distinct, human microbiomes were more similar to those in chickens than in goats (Wilcoxon rank sum for human-chicken vs. human-goat microbiomes $p<0.001$). The similarity of human and chicken microbiomes was greater in rural versus urban fecal samples (Bray-Curtis dissimilarity index median [IQR] rural=0.92 [0.03], urban=0.94 [0.01]; Wilcoxon rank sum $p<0.001$) (Figure 4-3A). The similarity between human and goat fecal bacterial community compositions, however, did not differ in the rural versus urban community (rural=0.97 [0.04], urban=0.95 [0.03]; Wilcoxon rank sum $p=0.30$).

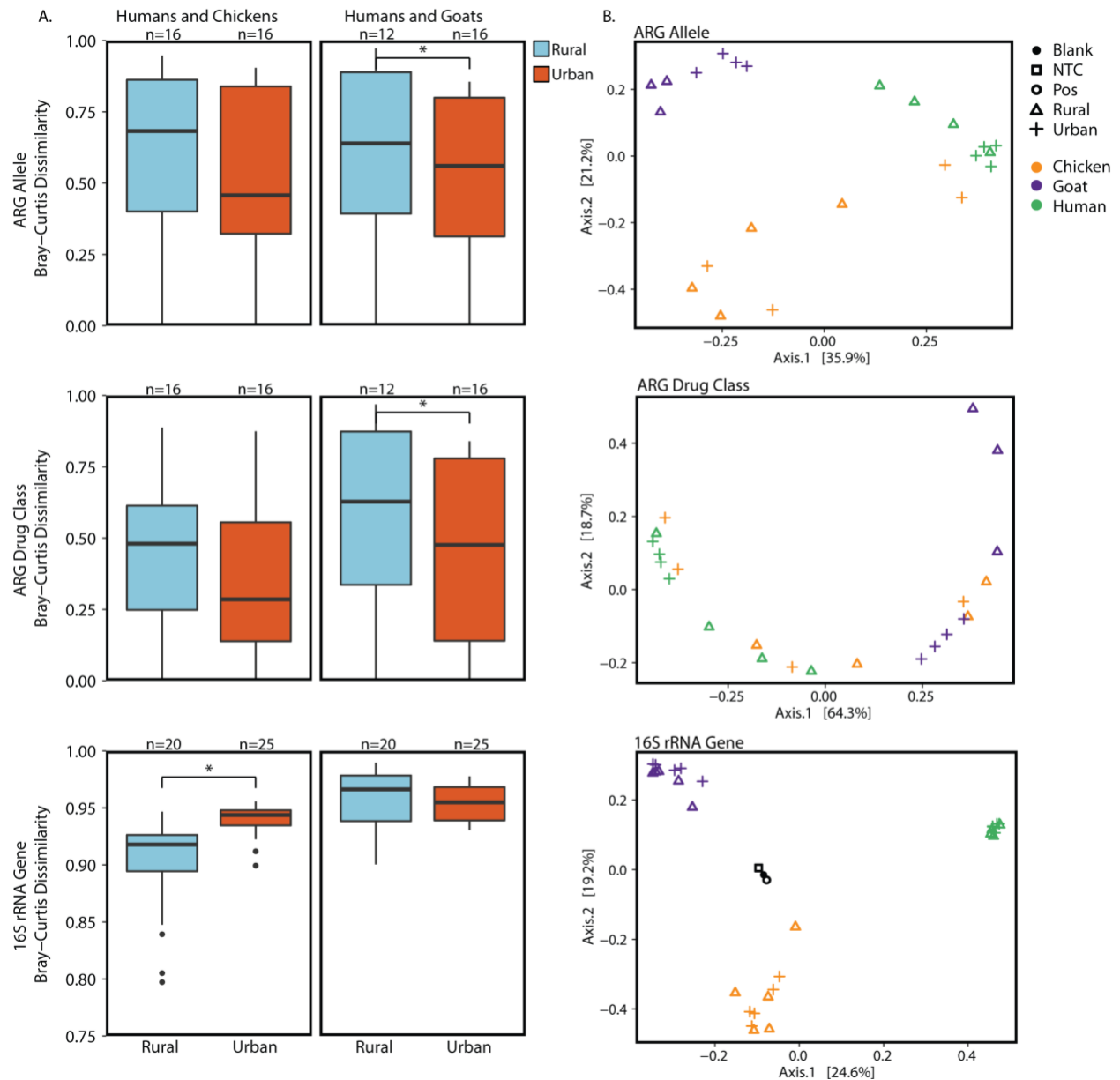


Figure 4-3: Bray-Curtis dissimilarity between resistomes (top and middle; long-read sequencing) and bacterial community compositions (bottom; 16S rRNA gene analysis) in humans, chickens, and goats. A. Box-and-whisker plots illustrating Bray-Curtis dissimilarity between humans and chickens (left) and humans and goats (right). The line through each box denotes the median Bray-Curtis dissimilarity index, the lower and upper box boundaries denote the first and third quartiles, respectively, and the whiskers extend to the minimum and maximum indices up to 1.5 times the interquartile range, with outliers plotted as dots. A Bray-Curtis dissimilarity index of 0.00 indicates identical community compositions, and an index closer to 1.00 indicates more dissimilarity between community compositions. **B.** Principal-coordinate analyses of Bray-Curtis dissimilarity. Color indicates fecal hosts, and symbols indicate rural, urban, and control samples. NTC, no-template control. *, significant at a significance level of 0.05 using a Wilcoxon rank sum test.

Similarity and Overlap in Resistomes

The long-read sequencing of DNA extracts from the fecal composites yielded, on average, 8.8 million reads per sample type (fecal host by urbanicity) with an average read length of 1,872 bp (standard deviation: 2,546) (Table C-1). We obtained a total of 99 Gbp of data, 71 % of which was classified as bacteria. Assembly yielded an N50 of 50 kbp, on average, and a median contig length of 25 kbp.

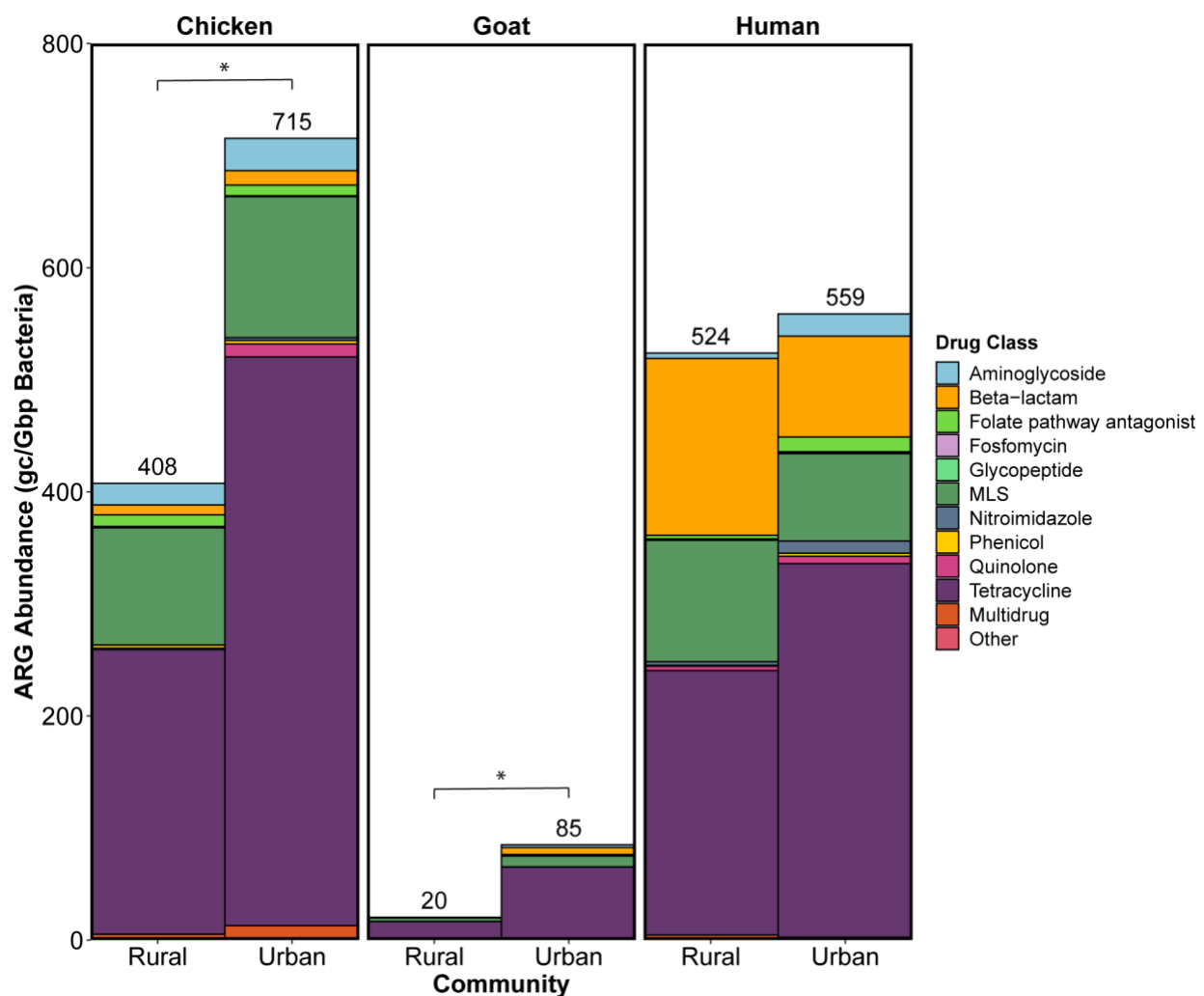


Figure 4-4: Abundance of ARGs (identified in long-read sequencing data) as gene copies (gc) normalized by gigabase pairs of data classified as bacteria for each fecal host by urbanicity. MLS represents macrolide, lincosamide, and streptogramin B resistance genes. *, significant at a significance level of 0.05 using a Wilcoxon rank sum test.

We identified a total of 44,077 ARG hits and 497 distinct ARG alleles. Tetracycline, beta-lactam, and macrolide, lincosamide, and streptogramin B (MLS) resistance genes dominated human and animal resistomes (Figure 4-4). ARGs were widespread, especially in chicken and human feces. The abundance of ARGs (gene copies normalized by Gbp of data classified as bacteria for each fecal host) was greater in chickens (Wilcoxon rank sum test, rural: $p=0.034$, urban: $p=0.021$) and in humans (Wilcoxon rank sum test, rural: $p=0.034$, urban: $p=0.021$) compared to goats (Table C-2). The abundance of ARGs was higher in urban versus rural animals, but not significantly different by urbanicity for human guts. Specifically, within each fecal host, ARG abundance (paired by drug class) was greater in urban compared to rural samples, though results were only statistically significant for chickens and goats (Wilcoxon signed-rank test, chickens: $p=0.016$, goats: $p=0.017$, humans: $p=0.40$; Figure 4-4, Table C-3). Similarity between chicken hosts' resistomes did not differ by urbanicity, though between-goat and between-human resistomes were more similar in the urban than rural community (Figure C-3, Table C-4). In the rural versus urban community, the similarity between human and chicken resistomes at the allele level (Bray-Curtis dissimilarity index median [IQR] rural=0.68 [0.46], urban=0.46 [0.52]; Wilcoxon rank sum $p=0.09$) and grouped by drug class (rural=0.48 [0.37], urban=0.29 [0.42]; Wilcoxon rank sum $p=0.07$) did not differ (Figure 4-3A). Human and goat resistomes, however, were more similar in the urban compared to rural community at the allele level (rural=0.64 [0.45], urban=0.56 [0.49]; $p=0.01$) and grouped by drug class (rural=0.63 [0.54], urban=0.48 [0.64]; $p=0.02$) (Figure 4-3A).

Overlap of distinct ARG alleles was greater between humans and goats (Z-test $p < 0.001$) in the urban compared to rural community, though not significantly different between humans and chickens (Z-test $p = 0.95$) in urban versus rural samples (Figure 4-5A). In the rural and urban communities, the overlap of ARGs in humans and chickens was greater than that in humans and goats (Z-test $p < 0.001$). Among genes overlapping between urban chickens and urban humans, three extended-spectrum beta-lactamase (ESBL) genes, *bla_{TEM}-126*, *bla_{TEM}-132*, and *bla_{TEM}-207*, and three beta-lactamase inhibitor (BLI) resistance genes, *bla_{TEM}-32*, *bla_{TEM}-76*, and *bla_{TEM}-122*, were identified (Figure 4-5C). In addition, *bla_{TEM}-76* overlapped in rural chickens and rural humans. While not overlapping between fecal hosts living in the same community, additional ESBL genes, *bla_{SHV}-12* and *bla_{TEM}-147*, were detected in chicken and human hosts.

Contigs from the urban chicken, rural human, and urban human samples were classified as plasmids using the MOB recon tool (Table 4-1) ²⁵¹. One plasmid cluster was shared by humans and chickens, but it could not be typed. A total of seven unique plasmid types (ColRNAI, Inc18, IncFIB, IncR, IncFII, IncFIIA, IncQ1) were present in the rural and urban human samples, and none of the plasmids identified in urban chicken samples could be typed. *bla_{TEM}* genes conferring resistance to beta-lactams were carried by IncF-type and ColRNAI plasmids in human stool.

Of the contigs assembled from the long reads, 87.5 % (99,823/114,093) were classified as bacteria. Eighty-nine percent (89,228/99,823) of the bacterial contigs were classified to the strain, subspecies, or species level, of which 2.37 % (2,112/89,228) were classified as potentially pathogenic species highlighted in the CDC 2019 Antibiotic Resistance Threats report ²⁴⁹. We determined that several important potentially

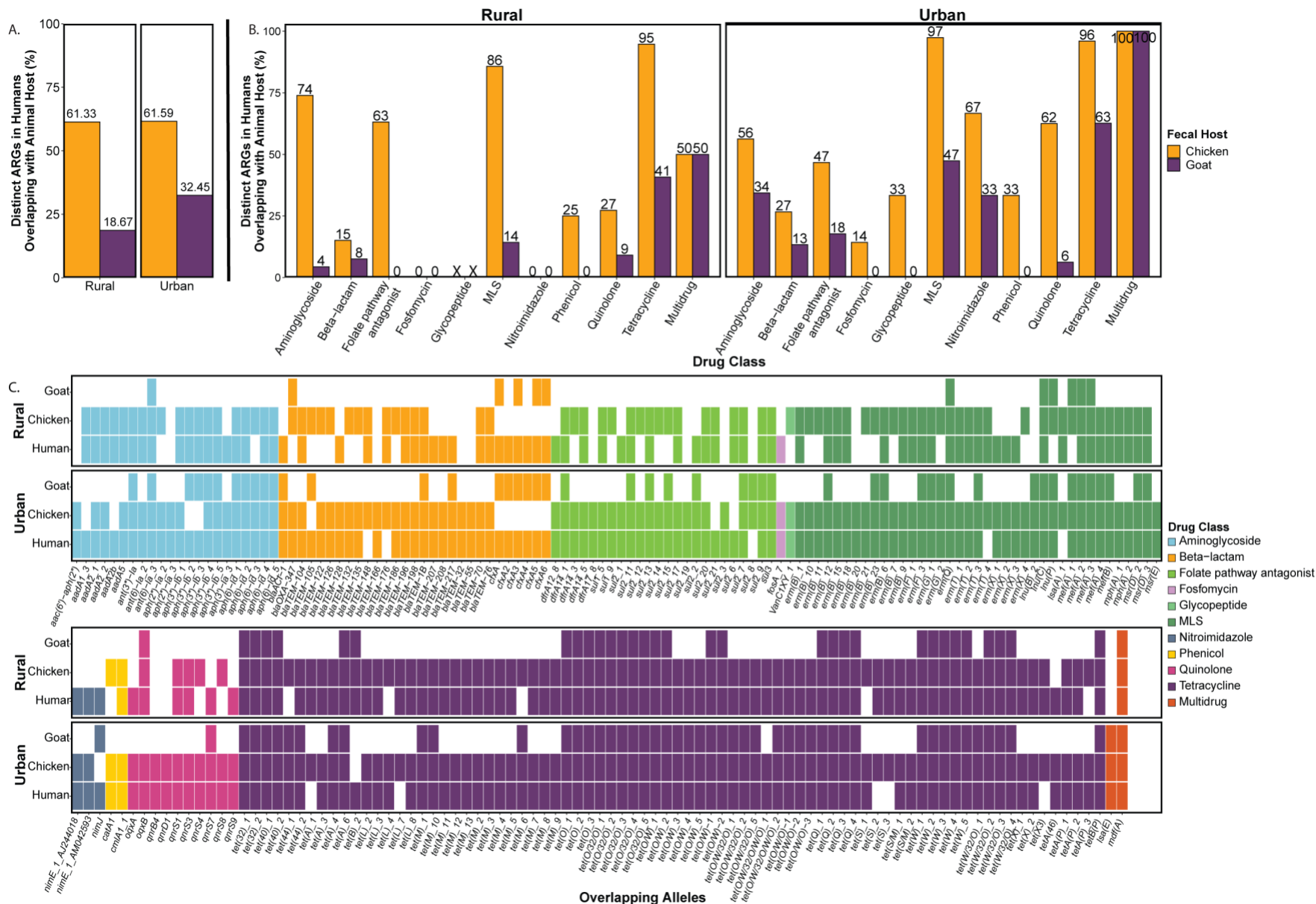


Figure 4-5: Distinct ARG alleles overlapping between humans and each animal host. **A.** Percentage of distinct ARGs identified (using long-read sequencing) in humans that were also found in each animal host. **B.** Percentage of distinct ARGs, by drug class, identified (using long-read sequencing) in humans that were also found in each animal host. An 'X' indicates ARGs from that drug class were not detected in humans from that community. **C.** ARG alleles overlapping between at least two fecal hosts living in the same community.

Table 4-1: Plasmids carrying antibiotic resistance genes. Some fecal hosts (rural chicken, rural goat, urban goat) are not shown, because no plasmids carrying antibiotic resistance genes were detected in their respective samples. NA, not applicable.

Fecal Host	Cluster ID	Replicon Type	ARG Alleles Carried by Plasmid	ARG Drug Classes
Rural Human				
	545	IncFII	blaTEM-105, tet(A)_6	Beta-lactam, Tetracycline
	545	IncFII, IncFIIA	blaTEM-1B, mph(A)_2	Beta-lactam, MLS
	3619	rep_cluster_1079	nimE_1_AM042593	Nitroimidazole
	Novel	rep_cluster_475	tet(O/32/O)_4	Tetracycline
	Novel	rep_cluster_1085	erm(F)_3	MLS
Urban Human				
	156	NA	qnrS1	Quinolone
	487	NA	tet(B)_2	Tetracycline
	730	rep_cluster_1418	aph(6)-Id_1, blaTEM-1B, mef(B), qnrS1, sul3	Aminoglycoside, Beta-lactam, Folate pathway antagonist, MLS, Quinolone
	1095	NA	aadA2_1, dfrA12_8, mph(A)_1, sul1_5	Aminoglycoside, Folate pathway antagonist, MLS
	1222	IncFIB, IncR	ant(3'')-Ia, aph(3'')-Ib_5, aph(6)-Id_4, dfrA14_1, qnrS1, sul2_2	Aminoglycoside, Folate pathway antagonist, Quinolone
	1423	NA	tetA(P)_1, tetB(P)	Tetracycline
	1552	IncFIIA, IncFII, IncFIB	erm(B)_18, mph(A)_1	MLS
	1553	NA	aadA2_1, ant(3'')-Ia, dfrA12_8, mef(B), sul3	Aminoglycoside, Folate pathway antagonist, MLS
	1553	NA	catA1	Phenicol
	1553	ColRNAI_rep_cluster_1987	blaTEM-1B, tet(A)_6	Beta-lactam, Tetracycline
	3393	NA	erm(B)_18	MLS
	3619	rep_cluster_1079	nimE_1_AM042593	Nitroimidazole
	3619	rep_cluster_1079	erm(F)_3, tet(X)_2	MLS, Tetracycline
	Novel	NA	aac(3)-IId, aadA2_1, ant(3'')-Ia, blaTEM-1B, dfrA1_9, mph(A)_2, sul1_5, tet(A)_6	Aminoglycoside, Beta-lactam, Folate pathway antagonist, MLS, Tetracycline
	Novel	IncFIIA, IncFII	aac(3)-IId, blaTEM-1B	Aminoglycoside, Beta-lactam
	Novel	IncQ1	aadA1_3, catA1, sul2_3, tet(A)_6	Aminoglycoside, Folate pathway antagonist, Phenicol, Tetracycline
	Novel	rep_cluster_1079	erm(F)_1, erm(F)_3, tet(X)_2	MLS, Tetracycline
	Novel	Inc18	aac(6')-aph(2''), erm(B)_23, str_1, tet(L)_2, tet(O/W/32/O)_2	Aminoglycoside, MLS, Tetracycline
Urban Chicken				
	2351	NA	tet(L)_8	Tetracycline
	3619	rep_cluster_1079	erm(F)_3, tet(X)_2	MLS, Tetracycline
	Novel	rep_cluster_1079	erm(F)_3, nimE_1_AM042593, tet(X)_2	MLS, Nitroimidazole, Tetracycline
	Novel	rep_cluster_54	tet(L)_8, tet(M)_4, tet(W)_4	Tetracycline

pathogenic bacteria were likely present and carried ARGs, particularly in the urban community (Figure 4-6). Most (95.7 %, 45/47) of the ARG-carrying contigs aligning to potential pathogen reference sequences were also determined to be chromosomal using the MOB-recon tool; two ARG-carrying contigs aligning to potential pathogens were classified as plasmids: 1) a *C. jejuni*-aligning contig carrying *tet(O/32/O)* in a rural human sample and 2) an *E. coli*-aligning contig carrying *qnrS1*, *sul3*, *aph(6)-Id*, *bla_{TEM-1B}*, and *mef(B)* in an urban human sample. In total, 29 distinct ARGs, representing 10 drug classes (aminoglycoside; beta-lactam; fluoroquinolone; folate pathway antagonist; fosfomycin; macrolide, lincosamide, and streptogramin B; phenicol; quinolone; rifampicin; and tetracycline), were carried by potential pathogens. No ARGs were carried by potential pathogens in rural chickens or rural goats. Of the six bacteria species carrying ARGs in humans, two were also present in urban animal hosts. *Clostridioides difficile* organisms carrying tetracycline (*tet(W)*, *tet(O)*, *tet(40)*) and macrolide (*mef(A)*) resistance genes were present in humans and urban goats, and *C. difficile* organisms carrying aminoglycoside (*ant(6)-Ia*) resistance genes were present in urban humans and urban chickens. Multidrug (*mdf(A)*) resistance genes carried by *E. coli* were prevalent in humans, urban chickens, and urban goats. *E. coli* carrying beta-lactam (*bla_{TEM}*) resistance genes were identified in urban chickens and urban humans. Only three contigs carrying ARGs were classified as potential pathogens to the strain or subspecies level. *E. coli* PCN033 carrying a multidrug (*mdf(A)*) resistance gene was identified in a rural human sample, and *C. difficile* M120 carrying macrolide (*mef(A)*) and tetracycline (*tet(44)*) resistance genes were present in an urban goat and urban human sample, respectively. Though the potential pathogen classifications were confirmed

through BLASTN with low *E*-values and high percent identity (Table C-5), we noted off-target hits with similar *E*-values and percent identities for the *E. coli* PCN033 and *C. difficile* M120 classifications.

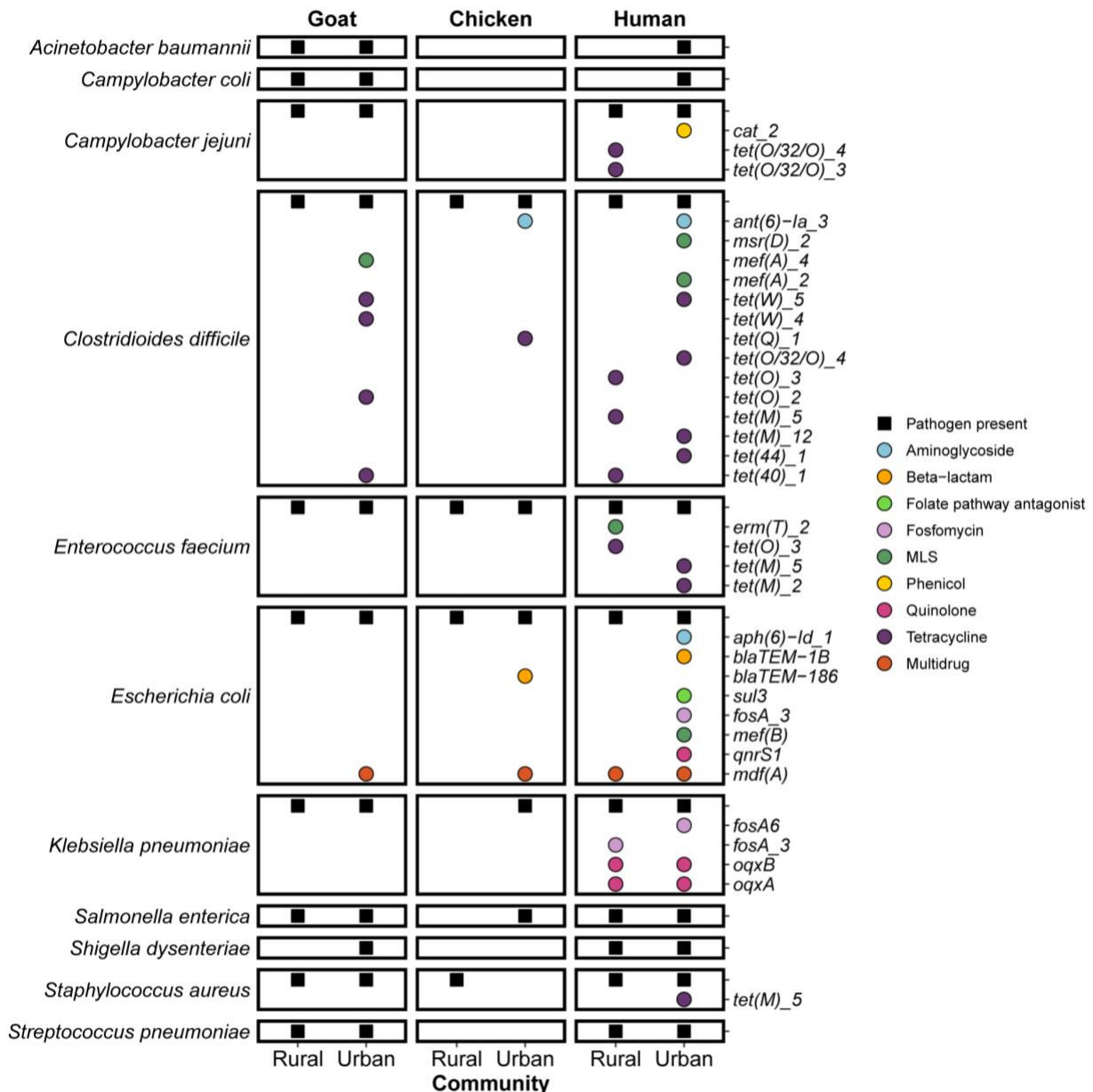


Figure 4-6: Prevalence of potentially pathogenic bacteria species and those carrying antibiotic resistance genes (ARGs) identified in contigs assembled from long reads. Black squares indicate at least one contig for the respective fecal host was classified as the bacteria species on the left. Colored dots indicate ARGs (by drug class) identified on contigs classified as the bacteria species listed on the left. Bacteria species represent those identified in the CDC's 2019 Antibiotic Resistance Threats in the United States report (31). MLS represents macrolide, lincosamide, and streptogramin B resistance genes

4.5 Discussion

We found greater similarity in bacterial communities between humans and chickens in a rural versus urban community in Bangladesh, though human and animal microbiomes were generally distinct (Bray-Curtis dissimilarity indices >0.93) in both communities. Human-chicken similarities in the rural community could partially be explained by a higher prevalence of animal ownership in the rural site. While 94 % of households owned livestock in the rural community, only 52 % owned animals in Dhaka, Bangladesh.^{24,214} As our study did not specifically collect human stool from animal-owning households, it is possible human participants from the urban community had limited exposure to animals. Future research purposely sampling human stool from animal-owning households may help elucidate whether rearing animals nearer to human living quarters impacts human-animal microbiome similarities. Additionally, goats are ruminants, which have complex digestive systems that make their gut microbiomes diverse and highly dissimilar from those of nonruminants.^{252,253} Further, human microbiomes are generally more diverse in urban compared to rural areas.²⁵⁴ Findings from previous studies have shown that gut microbiota are influenced by geography and diet.^{223,254–257} People living in urban Bangladesh tend to have diverse diets consisting of most food groups (i.e., starches, pulses, fish, eggs, meat, vegetables, fruit, milks, oil, spices, sweets, beverages), while those in rural areas are more likely to have limited diets.²⁵⁸ As free-roaming animals are common in Bangladesh, similar human and chicken gut microbiomes in the rural community might be explained by chickens scavenging through human rubbish and/or being fed scraps and leftovers from human meals.^{24,259,260} It is also possible the differences in bacterial communities between rural

and urban goats could be attributed to grazing on differing food sources in the rural and urban communities.

On the other hand, there was greater similarity in resistomes between humans and goats (and within goats and within humans) in an urban versus rural community in Bangladesh. Urban settings might serve as hotspots of zoonotic antibiotic resistance transmission, partially due to poor human and animal fecal management.²¹⁶ In Dhaka, Bangladesh, for example, an estimated 2 % of human fecal waste is safely managed while the remaining 98 % directly enters the environment.²²⁵ There is therefore ample opportunity for grazing animals to consume food and water contaminated with human feces.

Our findings highlight domestic chickens as important reservoirs of antibiotic resistance. Similarly, a comparison of resistomes in chickens, pigs, and humans in China found ARG abundance in chicken feces to be the highest.²⁶¹ Another study found that publicly available human stool bacterial genomes shared a greater number of mobile ARGs with chicken gut microbiomes than with pig and cattle microbiomes.²⁶² The high abundance of ARGs in chicken feces and resistome sharing between chickens and humans is consistent with existing literature, much of which has focused on occupational exposures in commercial settings. For example, recent work in Nigeria identified occupational exposure to chickens as a risk factor for multidrug-resistant *E. coli* colonization among poultry workers and identified similar resistance patterns and identical plasmid replicons in *E. coli* recovered from chickens and poultry workers.^{263–265} While previous work in Ecuador found that higher levels of antibiotic-resistant bacteria were isolated from commercially raised poultry than backyard poultry, our work uniquely

demonstrates the important role of poultry in antibiotic resistance circulation in an urban community.²⁶⁶

Tetracycline resistance genes dominated the resistomes of humans, chickens, and goats. Tetracyclines have been widely used for prophylaxis and to treat a variety of Gram-positive and Gram-negative bacterial infections in humans and domestic animals; tetracyclines have also been used as growth promoters in animal husbandry.²⁶⁷ Our results mirror previous work that found tetracycline resistance genes to be among the most abundant ARGs in human and animal feces.^{261,268–270} In layer farms in Bangladesh, tetracycline was one of the most prevalent antibiotic residues, in addition to ciprofloxacin and enrofloxacin (fluoroquinolones), in the eggs, tissue, and internal organs of chickens.^{271,272} Genes conferring resistance to MLSs were also abundant in our study, particularly in humans and chickens. Similar to tetracyclines, MLSs have a long history of prophylactic and treatment use in human and veterinary medicine.²⁷³ Additionally, beta-lactam resistance genes, including ESBL and BLI resistance genes, were prevalent in our study samples, especially in chickens and humans. Beta-lactams, particularly cephalosporins, were one of the most widely prescribed antibiotics in multiple studies surveying pharmacies, outpatients, and hospitals in rural and urban Bangladesh.^{274–277} ESBL-producing *Enterobacteriaceae* infections are a global public health concern and have limited treatment options, often requiring last-resort treatment with carbapenems.²⁷⁸ In addition to other Gram-negative bacterial infections, ESBL-producing *Enterobacteriaceae* infections are often treated by co-administering beta-lactams and beta-lactamase inhibitors.²⁷⁹ Further, beta-lactamase enzymes are widely plasmid-mediated, allowing for ease of horizontal gene transfer between bacterial hosts

and between humans, animals, and the environment.^{34,280,35,281} Although human and animal, especially chicken, resistomes were dominated by genes conferring resistance to similar antibiotic classes, it is not possible to assume directionality of exchange. While this is partially due to antibiotic classes being used across both human and veterinary medicine and animal husbandry, widespread human and animal fecal contamination in shared living environments further complicates exchange dynamics. Particularly when allowing animals to roam and graze freely is common, animals are likely at risk of acquiring ARGs originating from humans through the environment, emphasizing how close proximities between humans and domestic animals propagates antibiotic resistance, despite whether humans or animals are the primary sources.

We detected *bla*_{TEM} genes (beta-lactam resistance) located on long-read assembled contigs classified as IncF plasmids in rural and urban human stool and on a contig classified as a ColRNAI plasmid, a plasmid group known to carry beta-lactam resistance in *Enterobacteriaceae*, in urban human stool.^{280,282–285} IncF plasmids are particularly concerning in the spread of antibiotic resistance, as they often carry multiple replicons, or regions of the genome where replication is initiated, allowing for rapid evolution and great adaptability to promote acquisition by various bacterial host species.²⁸⁴ Although there is a recognized importance of mobile genetic elements (e.g., plasmids) in horizontal gene transfer and the spread of antibiotic resistance, we detected few plasmids in our long-read sequencing data. Accurate identification of plasmids using the MOB-suite tool, as well as other plasmid detection tools, relies on high quality genome assemblies as input.²⁵¹ Despite advances in the accuracy of long-read sequencing technology, sequencing errors and insufficient coverage make plasmid detection difficult

in complex metagenomes using long reads alone. Deeper sequencing and hybrid assemblies of short- and long-read data may improve the ability to characterize the role of plasmids in human and animal resistomes. Further, MOB-suite uses a database-based approach, and performance is reduced when classifying novel plasmids not available in NCBI's RefSeq and GenBank databases.²⁵¹ Due to overrepresentation of plasmids from human clinical isolates from high-income countries in databases, it is possible that we were limited in our ability to detect plasmids in chickens and goats or specific to Bangladesh using a database approach.

We leveraged long sequence reads to identify bacterial hosts of ARGs, focusing on species identified as antibiotic resistance threats by the CDC.²⁴⁹ While these species often present as commensals in human and animal guts, some strains are pathogens or opportunistic pathogens. Importantly, ARG-carrying commensals can still pose a health risk as horizontal gene transfer can facilitate exchange of ARGs between benign and pathogenic bacteria in intestinal microbiomes.^{34,35} *C. difficile* carried a wide range of ARGs across various drug classes (aminoglycoside, MLS, and tetracycline) in humans and animals. Spore-forming *C. difficile* can colonize the gut without causing illness, particularly in infants; asymptomatic colonization in adults is rare, but possible.^{286–288} Historically, *C. difficile* infections have been known to arise after taking antibiotics and account for the highest morbidity and mortality of hospital-acquired infections in high-income countries, though the burden of *C. difficile* infections in LMICs is poorly understood.^{289–292} There is, however, increasing recognition of *C. difficile* as a community-acquired infection, in addition to hospital-acquired infection, and potential zoonosis.^{293,294} We also identified *E. coli* carrying *bla*_{TEM}, a gene family containing

alleles conferring resistance to beta-lactams, in urban chickens and urban humans. Previous work has documented poultry as reservoirs of ESBL-producing *Enterobacteriaceae*.^{295,296} Our results therefore stress the importance of understanding and targeting transmission pathways of ESBL-producing *Enterobacteriaceae* in curbing antibiotic resistance, particularly in an urban community where humans and animals live in close proximity and share environments.

Limitations

This study has some limitations. In leveraging human stool collection from previous studies, urban human samples were collected two years prior to urban animal samples while rural human and animal samples were simultaneously collected. Temporal differences in sample collection between the two communities might partially explain the greater microbiome similarity observed between rural humans and chickens compared to urban humans and chickens. Due to this temporal issue, the compositing of fecal samples, and the use of 16S rRNA gene sequencing as opposed to bacterial strain detection, we are unable to draw conclusions about whether zoonotic transmission of gut bacteria or ARGs occurred (or its directionality) in our study communities. As chicken and goat feces were collected from the ground, the findings presented here may reflect fecal host and environmental microbiomes and resistomes; however, trained sample collectors took care to only sample from the center and top of fresh feces piles to limit soil contamination. In addition, few barcodes were detected in the long-read sequence data for one of the rural goat samples, so we were not able to detect any ARGs, plasmids, or potential pathogens in that sample; however, as all long-read sequencing analyses (excluding the Bray-Curtis dissimilarity analysis) relied on pooled

data from each fecal host in a particular setting, we do not anticipate the missing sample should influence the interpretation of our results. Also, we obtained less long sequence reads, as well as more fragmented reads, from chicken DNA extracts than human and goat DNA, potentially due to the heterogeneity, heat treatment (required by our USDA import permit), or high uric acid concentration of chicken fecal matter. It is therefore likely that chicken metagenome coverage was low, biasing plasmid and potential pathogen detection; we do not believe this limitation drastically impacted ARG detection, as the normalized abundance of ARGs was higher in chickens than in humans and goats. Finally, our results may not be generalizable across all rural and urban areas in Bangladesh or other LMICs, as we sampled only one rural and one urban community; there may be factors other than urbanicity that we were unable to measure confounding our findings.

Conclusion

This community-level study demonstrates similarities in resistance profiles, as well as reveals potential pathogens carrying resistance genes, in humans and domestic animals living in the same community in rural and urban Bangladesh. Our results contribute to a gap in knowledge regarding the role of small-scale animal production in antibiotic resistance dissemination within resource-constrained communities. While domestic goats and chickens harbored potentially pathogenic hosts of ARGs, our results suggest that compared to goats, backyard poultry might be an especially important reservoir for ARGs that are also present in the human gut. In addition, our findings provide some evidence that ARGs are more widespread in an urban compared to rural community. While directionality for ARG exchange between humans and

animals in community settings is still unclear, substantial sharing of ARGs in humans and animals in both rural and urban communities in this study highlights the importance of developing interventions and antibiotic stewardship strategies considering shared environments between human and animal reservoirs.

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Jenna M. Swarthout and Erica R. Fuhrmeister share the first author position; JMS led the long-read sequencing analysis and drafted the manuscript, while ERF led the 16S rRNA gene amplicon sequencing analysis.

Chapter 5: Benchmarking assembly methods for genomic characterization of antibiotic-resistant *E. coli* strains in humans, animals, and environmental samples

5.1 Abstract

Although zoonotic transmission of antibiotic-resistant bacteria is widely suspected in community settings, particularly in low-income communities, traditional methods have been limited in their abilities to conclusively document strain and antibiotic resistance gene (ARG) sharing events. Emerging genomic methods, especially those related to metagenomic and long-read sequencing, provide new opportunities to better contextualize ARGs detected across human, animal, and environmental reservoirs. Between June and August 2019, we collected matched sample sets from 120 urban Kenyan households, including soil; water; poultry cloacal swabs; dog feces; and human stool. Here, we benchmark assembly and ARG mapping methods to inform an optimal sequencing and bioinformatic approach for tracking strains, ARGs, and mobile genetic elements within and between the study households. Specifically, we used a simulated data set of five pooled *E. coli* isolates (that had both short- and long-read sequencing data available) to assess whether 1) short-read-only, short-read-first hybrid, or long-read-first hybrid assembly improves recovery of reference *E. coli* genomes; 2) reference genome-based read binning could improve assembly quality; and 3) raw short reads, raw long reads, or assembled contigs as input yield more sensitive detection of ARGs across their entire reference gene length with 100% identity. Performance across these metrics was highest when assembling contigs from long reads and polishing with short-read data (without reference-based binning of input reads). However, we recommend additional benchmarking to explore impacts of varied sequencing depth (for short and

long reads) and to evaluate detection of ARGs within bacterial genomes or on mobile genetic elements. These results inform the most appropriate approach for sequencing and analyzing metagenomes comprised of closely related bacterial strains.

5.2 Background

Animal husbandry is a common source of income and nutrition in many low- and middle-income countries (LMICs).^{12,13} Particularly in urban communities where animal husbandry is practiced, the close proximity between humans and domesticated animals can result in adverse human health outcomes, including diarrheal illness and growth faltering in children.^{22,51} While previous research, including Chapter 3, has shown that living in close proximity to animals is associated with increased exposure to fecal contamination in domestic environments, a mechanistic understanding of zoonotic pathogen transmission is still missing.^{24–29}

Cohabitation between humans and animals can also enhance zoonotic exchange of antibiotic-resistant bacteria and antibiotic resistance genes (ARGs). Zoonotic exchange of antibiotic resistance has been suggested in previous research by documenting similarities between antibiotic-resistant strains of bacteria, such as *Escherichia coli*, isolated from humans and domesticated animals (see Chapter 2). Previous work has also shown that antibiotic-resistant bacteria are prevalent in soil and drinking water in LMICs.^{43,45,46} However, most previous studies have employed traditional methods (e.g., multilocus sequence typing), which are not always able to differentiate between highly similar strains and rely on current knowledge of known strains, or investigated only one isolate per sample type. Better capturing the within-host diversity of bacteria may

increase the ability to detect zoonotic sharing pairs in the community.^{297,149} Plate sweep metagenomics has emerged as a cost-effective approach when sequencing bacterial isolates is insufficient for the intended analysis; all cultured colonies are swept from an agar plate prior to sequencing, falling between whole-genome sequencing of isolates and culture-independent metagenomics.²⁹⁸

Emerging technologies and tools also enable new high-resolution perspectives on the relationships between bacterial strains and ARGs in complex microbial communities. Oxford Nanopore Technologies' (ONT) MinION is capable of routinely producing up to 50 times longer reads (>5,000 base pairs (bp)) compared to short-read sequencing technologies (<300 base pairs); long-read sequences, however, are less accurate than sequences produced by commonly used short-read sequencing technologies (e.g., Illumina).^{299–301} Although short reads can be assembled into bacterial genome sequences, de novo (reference-independent) short-read-only assembly tools for metagenomic samples (e.g., metaSPAdes; IDBA-UD; MEGAHIT) often struggle to overcome challenges with uneven genome abundance and ambiguity associated with closely related strains or sequencing errors.^{302–306} Reference genome-based binning of reads prior to assembly is one approach for parsing between highly similar bacterial strains.³⁰⁷ Another strategy is combining ONT long reads with Illumina short reads through hybrid assembly to yield highly accurate scaffolds (i.e., oriented and ordered contigs separated by gaps), enabling comparisons of organism genomes and ARG contexts across sample types and between households. Current methods for hybrid assemblies of metagenomes usually create short-read-first assemblies corrected with long reads (e.g., hybridSPAdes; Unicycler; OPERA-MS), or create long-read-first

assemblies polished with short reads (e.g., metaFlye; Canu; or Miniasm, all followed by Pilon polishing).^{248,308–313} As long-read sequencing remains more costly than Illumina sequencing, the added utility of obtaining long-read sequence data (in addition to short read sequences) for a specific research objective – and the optimal bioinformatic approaches – should be considered when designing sequencing-based experiments.³⁰¹

Between June and August 2019, we collected matched sample sets (including human stool, domesticated animal feces, and environmental samples) from 120 urban Kenyan households. For each sample collected, we pooled together up to five cultured *E. coli* colonies for sequencing analysis. The primary objective of this chapter was to benchmark metagenome assembly and ARG mapping methods – and evaluate the added benefits of performing long-read in addition to short-read sequencing – to inform a recommended approach for identifying overlapping *E. coli* strains and ARGs across the human, animal, and environmental samples. We utilized three commonly used metagenome assembler tools and pipelines to compare short-read-only (i.e., metaSPAdes), short-read-first hybrid (i.e., hybridSPAdes), and long-read-first hybrid (i.e., metaFlye and Pilon) assemblies.^{248,303,304,308,309,314,315} Specifically, a simulated pooled *E. coli* sample (comprised of five well-characterized *E. coli* genomes that were both long- and short-read sequenced) was used to answer the following research questions:

1. Do assemblies from metaSPAdes, hybridSPAdes, or metaFlye and Pilon yield (in contigs ≥ 1 kbp) higher i) contig N50s, ii) coverage for specific *E. coli* genomes, and iii) percent identities of specific *E. coli* assemblies mapped against their relevant reference genomes?

2. Compared to de novo assembly as described in objective 1, does adding a reference genome-based binning step yield (in contigs ≥ 1 kbp) higher i) contig N50s, ii) coverage for specific *E. coli* genomes, and iii) percent identities of specific *E. coli* assemblies mapped against their relevant reference genomes?
3. Does mapping of raw short reads, raw long reads, or assembled contigs (using de novo assembly as described in research question 1) yield more accurate detection of ARGs (alignment along entire reference gene length; 100% identity)?

5.3 Methods

Description of Data

As a first step, we characterized *E. coli* strains (using the Strain Genome Search tool (StrainGST)) in a subset of pooled *E. coli* samples from urban Kenya for which we obtained short- and long-read sequence data ($n = 48$); all *E. coli* genomes were downloaded from the National Center for Biotechnology Information (NCBI) on September 23, 2021 for creating the StrainGST reference database. To create a simulated data set for benchmarking assembly and ARG mapping methods, the Broad Institute provided data from five *E. coli* isolates – chosen to mirror prevalent phylogroup patterns observed in the samples from urban Kenya (Figure D-2). The provided isolates were extended spectrum beta-lactamase-producing or colistin-resistant *E. coli* (carrying *bla*_{CTX-M-15} or *mcr-1* genes), representing clinically relevant antibiotic-resistant bacteria, and carried several additional ARGs as well. Isolates carrying ARGs were selected for which Illumina sequence, ONT sequence, and complete assembly data were available (Table 5-1). Additional sample processing information for the urban Kenyan samples

and isolates selected for simulation are provided as supplemental information (Supporting Information D-1: Additional methods details).

Table 5-1: Metrics for *E. coli* isolates included in simulated data set

Identifier	StrainGST Identification	Phylotype	Sequence Type	Number of Unique ARGs	Number of Scaffolds	Mobsuite Plasmid Clusters
ISO1	Esch_coli_WP8-S17-ESBL-12	D	69	49	6	189; 469; 586; 841; 11
ISO2	Esch_coli_NCTC9087	A	10	40	2	1553
ISO3	Esch_coli_NCTC9080	A	226	38	3	538; 841
ISO4	Esch_coli_14EC033	E	7377	39	15	30; 475; 487; 546; 825; 1366
ISO5	Esch_coli_2011C-3911	B1	1727	39	2	218

Benchmarking

To create a simulated sample data set, an equal number of short (Illumina) reads were pooled (using seqtk; <https://github.com/lh3/seqtk>) from each of the five *E. coli* isolates such that the total summed to the maximum number of reads obtained from a real sample (Supporting Information D-2). We also used seqtk to pool an equal number of long-read (ONT) base pairs for each isolate such that the total summed to the maximum sequencing length obtained from the real data (Supporting Information D-2: Additional results). StrainGST was used to identify *E. coli* strains from the pooled short reads.³¹⁶ We mapped the pooled short (using bbmap; sourceforge.net/projects/bbmap/) and pooled long (using minimap2) reads to the reference genomes of strains identified by StrainGST, using the alignments to perform reference-based binning of the reads. The ClermonTyping tool was used to assign phylotypes to the detected strains, and the mlst software (<https://github.com/tseemann/mlst>) was used for sequence typing.^{317,318}

We selected three assemblers for benchmarking, using as input: 1) the unbinned pooled reads and 2) the binned pooled reads. The short reads were assembled using metaSPAdes.³⁰⁴ metaSPAdes was also used to perform a short-read-first hybrid assembly, by assembling short reads and correcting the assembly with long reads.³⁰⁴ Finally, we performed a long-read-first assembly using metaFlye (corrected with the same long reads using ONT's medaka), followed by short-read polishing using Pilon; pooled short reads were aligned to metaFlye contigs (using bbmap; sourceforge.net/projects/bbmap/) and the subsequent alignments were supplied as the Pilon input.^{248,309} Assembly quality metrics were computed and compared using MetaQUAST, with the polished genome assemblies provided by the Broad Institute specified as reference genomes.³¹⁹ To determine which of the Broad-provided reference genomes to designate as a reference genome in MetaQUAST for each set of binned reads (Table 5-2), we compared StrainGST identifications of the pooled data against the StrainGST identifications of the source isolates (Table 5-1).

Finally, we detected ARGs in the simulated data (pooled short reads; pooled long reads; and de novo metaSPAdes, hybridSPAdes, and metaFlye with Pilon assemblies) by aligning reads (using DIAMOND) to the CARD, using a minimum match length of 25 amino acids and identity of 95%.^{320,321} To account for errors and lengths spanning several genes in the pooled long read and assembled data, we set the DIAMOND frameshift penalty to 15 and enabled hit culling with respect to the query range, in line with the DIAMOND developers' recommendations.

5.4 Results

Assembler Impacts on *E. coli* Genome Recovery

StrainGST categorized 90.6% of the simulated data (pooled short reads) as *E. coli* strains; in total, 4 unique *E. coli* strains were detected (Table 5-2). While StrainGST was able to detect *E. coli* NCTC9080 when using the ISO3 reads prior to pooling as input (Table 5-1), it did not differentiate the highly similar ISO2 and ISO3 reads when pooled together; we therefore computed quality metrics (using MetaQUAST) for assemblies from *E. coli* NCTC9087 (SIM2)-binned reads twice: once supplying ISO2 as the reference genome and once supplying ISO3 as the reference.

Table 5-2: Metrics for *E. coli* strains detected in pooled short reads

Identifier	StrainGST Identification	Phylotype	Sequence Type	Relative Abundance	Assumed Reference Genome(s)
SIM1	Esch_coli_WP8-S17-ESBL-12	D	69	25.0%	ISO1
SIM2	Esch_coli_NCTC9087	A	10	15.5%	ISO2; ISO3
SIM3	Esch_coli_14EC033	E	2064	27.7%	ISO4
SIM4	Esch_coli_2011C-3911	B1	1727	31.8%	ISO5

We compared assembly quality metrics, calculated from independent, non-competitive contig alignments to each reference genome, across the various assembly approaches (Figure 5-1). On average, total assembly length relative to the combined reference length was 27.8% across all assemblies. Overall, N50s were highest for long-read-first assemblies. When mapped against relevant reference genomes and retaining contigs ≥ 1000 bp in length, long-read-first assembly (metaFlye and Pilon) yielded the highest coverage and contiguity (longest alignment length between assembly and reference relative to the reference length). On the other hand, short-read (SPAdes) and short-read-first hybrid (hybridSPAdes) assemblies yielded higher average percent identities

(filtering out those <95%) between contigs ≥ 1000 bp aligning to relevant reference genomes.

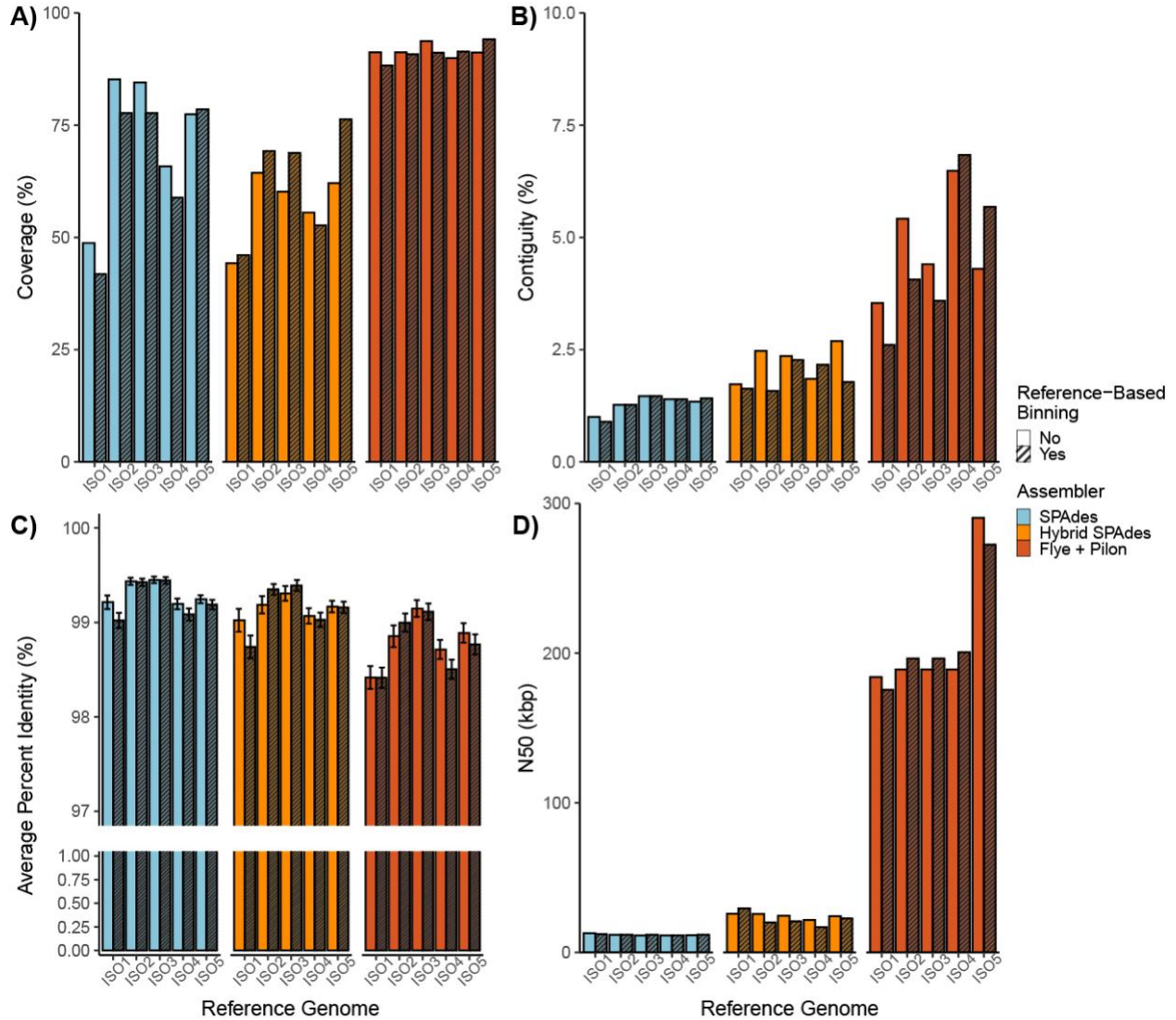


Figure 5-1: Assembly quality metrics for short-read (SPAdes), short-read-first hybrid (Hybrid SPAdes), and long-read-first hybrid (Flye + Pilon) assemblies, with and without reference-based read binning. A) Assembly coverage of reference genome (%), captured in contigs ≥ 1000 bp. B) Contiguity (%), or the longest alignment between assembly and reference genome relative to the reference length, captured in contigs ≥ 1000 bp. C) Average percent sequence identity (%) between assembly and reference alignments, captured in contigs ≥ 1000 bp. Error bars represent 95% confidence intervals. To zoom in on the small differences between percent identities across assembly methods, the R package, ggbreak, was used to create the broken y-axis.³²² D) N50 of contig lengths (kbp).

Adding a reference-based read binning step to all three assembly methods had mixed impacts on the quality metrics (Figure 5-1). Read binning had negligible effects on

coverage overall but trended towards increasing reference genome coverage by the short-read-first hybrid assemblies. Although contiguity varied between assemblies with and without binning, especially for long-read-first hybrid assemblies, these differences were small in magnitude as contiguity was similar across assembly approaches. The impacts of read binning on N50s and percent identities were limited.

Assembler Impacts on ARG Detection

We evaluated whether raw reads (short and long) or assembled contigs (short-read; short-read-first hybrid; and long-read-first hybrid) yielded more accurate detection of ARGs (Figure 5-2). Raw short reads yielded a greater number of unique ARG hits than all other inputs. However, after retaining only the ARG hits with 100% identity and the maximum alignment length for each unique ARG, the average coverage of reference genes (alignment length relative to reference gene length) was low (15.9%), as expected given the short read lengths; no short reads aligned to an entire reference gene at 100% identity (hereby designated as “accurate” hits). Similarly, raw long reads as input did not yield any accurate ARG hits. All three assembly approaches (retaining ARG hits with 100% identity and the maximum alignment length for each unique ARG) yielded average coverages of reference genes greater than 75%, with long-read-first hybrid assembly achieving an average coverage of 96.9%. The short-read and short-read-first hybrid assemblies detected 31 accurate ARG hits each, while the long-read-first hybrid assembly detected 30 accurate hits. For comparison, 36 unique, accurate ARG hits were obtained in total from the isolate reference genomes (prior to subsampling and pooling).

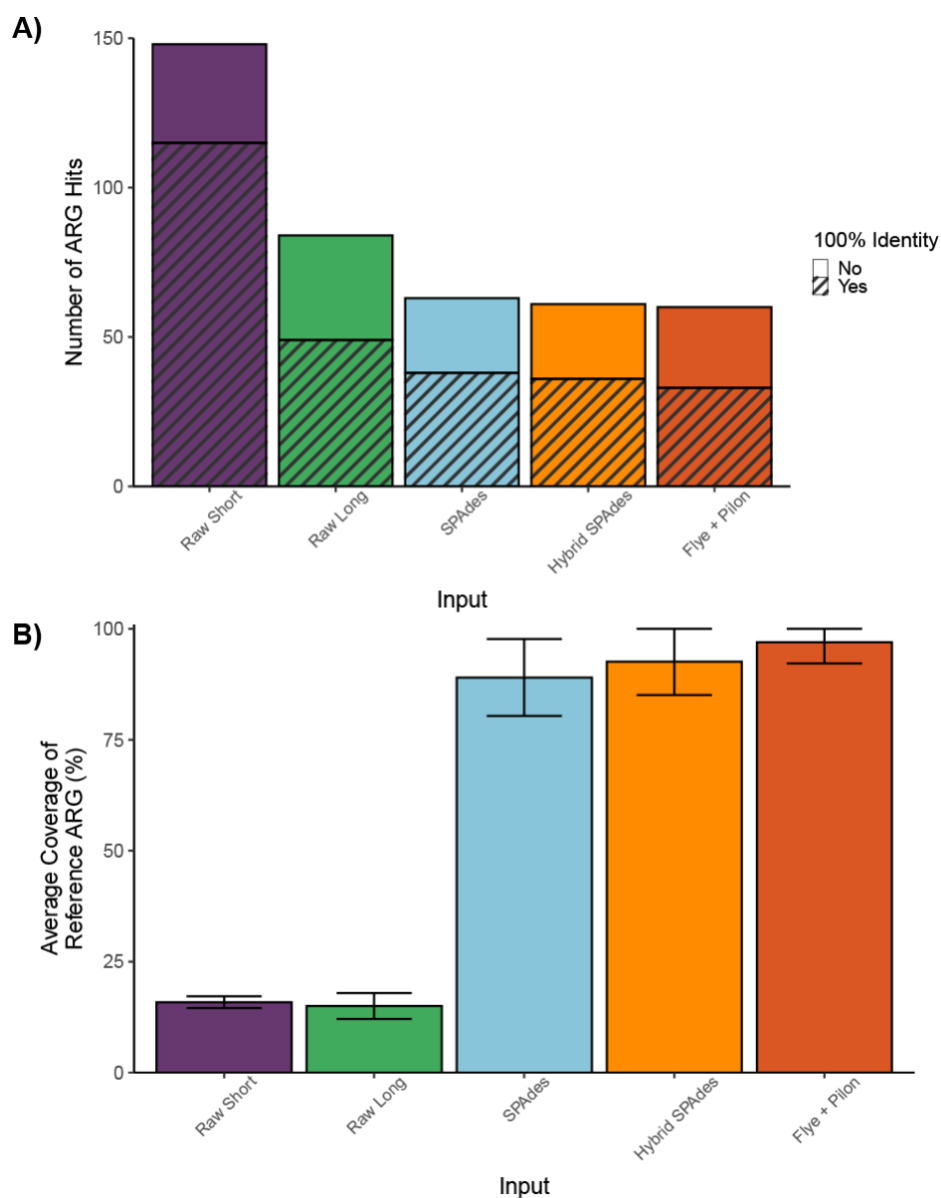


Figure 5-2: Unique antibiotic resistance gene (ARG) A) detection and B) accuracy metrics with raw read (short; long) and assembly (SPAdes; Hybrid SPAdes; Flye + Pilon) inputs. A) Number of unique ARG hits with ≥ 25 bp alignment length and $\geq 95\%$ identity, with number of unique ARG hits with 100% identity depicted with stripes. B) Average alignment length relative to reference gene length for unique ARG hits with 100% identity. Error bars represent 95% confidence intervals.

5.5 Discussion

In this analysis, we found that long-read-first hybrid assembly with short read polishing performed better than short-read-only or short-read-first hybrid assembly in terms of recovering *E. coli* genomes, and similarly regarding accurate ARG sequence detection, in a pooled *E. coli* sample. Similarly, previous work has recommended hybrid assembly

as the most reliable approach for contextualizing ARGs in environmental metagenomes.³⁰³ Our results are consistent with previous work that has found long-read-first hybrid assembly to produce more accurate contigs than short-read-first assembly.³¹⁵ However, these findings are sensitive to the sequencing depths achieved from both short- and long-read sequencing.^{303,315} Due to the higher error rates, it is especially important to achieve sufficient sequencing depth of long reads, which varies depending on specific research objectives. However, as long-read sequencing is still more costly than commonly used short-read sequencing technologies, budget and research objectives need to be considered – in addition to commonly reported assembly quality metrics – when choosing the most appropriate approach for a genomic experiment.³²³

Here, we reported measures of reference genome and accurate ARG sequence recovery as our primary evaluation metrics. There are several additional metrics that are important considerations in recommending a final approach for detecting shared *E. coli* strains and ARGs between humans, domesticated animals, and environmental samples. For example, while 100% identical ARGs may be identified across sample types, the genomic context around ARGs must be characterized to draw conclusions about whether transmission occurred between hosts and reservoirs. We demonstrated that while assembly-based resistome (or collection of all ARGs) analyses may yield a smaller number of ARG hits, they likely ensure more accurate results (i.e., fewer false positive hits) and can produce alignments along entire reference gene lengths. As a result, assemblies can yield better predictions of whether ARGs are carried within bacterial chromosomes or on mobile genetic elements.

We did not find convincing evidence that reference-based binning of raw reads prior to assembly improves assembly quality. This may partially be explained by persistent challenges associated with differentiating between highly similar strains of bacteria in metagenomic samples.³²⁴ Once short reads were pooled, StrainGST was unable to separate out ISO2 and ISO3. In addition, many reads aligned to multiple reference genomes of the StrainGST identifications, and reference-based read bins were not mutually exclusive. To improve assembly quality by leveraging reference genome sequences, it may be necessary to use assemblers with unique approaches to reference binning, such as OPERA-MS.³¹¹ OPERA-MS differs from many traditional binning algorithms, in that it does not merge repeat contigs from low coverage genomes into bins for high coverage genomes.³¹¹ Other researchers, however, have found that OPERA-MS yielded frequent misassemblies for environmental metagenomes; consensus assemblies, achieved through the use of multiple tools, may therefore be valuable in applications requiring highly accurate genome assemblies.^{303,315} Further, reference-based assembly approaches may not perform as well for microbial communities poorly characterized in common reference databases. The utility of reference-based binning for improving assemblies may therefore be limited for the real *E. coli* samples we collected from urban Kenya, as these microbiota may include under sequenced bacteria from animal and environmental microbiomes, as well as gut microbiota from humans in LMICs.

Conclusion

While closely related strains could not be parsed out, we demonstrated the ability of StrainGST to capture strain diversity in the pooled *E. coli* samples. Analysis of the real

pooled *E. coli* samples from humans, domesticated animals, and the environment is therefore likely to improve sensitivity for detecting strain sharing events over previous studies that have relied on single isolates. Based solely on the primary metrics reported for this analysis, reference *E. coli* genome recovery and ARG hit accuracy, metaFlye long-read assembly (without reference-based read binning) followed by short-read polishing with Pilon is the recommended approach for evaluating strains and ARGs in pooled *E. coli* samples. Considering long-read sequencing costs, however, short-read-only assembly – which yielded similar values for reference genome coverage and percent identity, as well as accurate ARG detection – may be a sufficient alternative. This is particularly the case when using variant-based tools that utilize short reads, such as the variant calling counterpart to StrainGST, Strain Genome Recovery (StrainGR). As a comprehensive understanding of genomic context around ARGs is necessary for inferring zoonotic transmission dynamics in community settings, future work should benchmark additional metrics – such as strain-level taxonomic identification and complete assembly of mobile genetic elements – to inform the optimal approach.

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Chapter 6: Summary, Recommendations, and Conclusion

6.1 Research Summary

Summary of Dissertation Findings

This dissertation sought to understand whether substantial zoonotic exchange of enteropathogens and antibiotic-resistant bacteria (or ARGs) occurs between humans and domesticated animals in households and backyard farms in LMICs. Further, a variety of methods were employed (e.g., literature review; regression analysis; genomics) to elucidate potential mechanisms, environmental transmission pathways, and contextual factors influencing the occurrence of zoonotic exchange.

In Chapter 2, we conducted a systematic literature review to compile 79 studies that 1) documented evidence of human exposure to domesticated animals within households or backyard farms; and 2) measured antibiotic-resistant bacteria or ARGs in human and domesticated animal samples that were spatially and temporally linked. We found that a substantial number of studies demonstrated zoonotic exchange of antibiotic resistance in these settings. While there were examples supporting whole antibiotic-resistant bacteria transmission between humans and domesticated animals, most studies in the review demonstrated overlapping antibiotic resistance profiles across genetically diverse bacteria; horizontal gene transfer is thus likely an important mechanism for zoonotic exchange in non-occupational settings. Our review revealed that several contextual factors – such as the intensity of human exposure to and contact with domesticated animals, and human gender and age – impact levels of human colonization with antibiotic-resistant bacteria associated with exposure to animals. Further, Chapter 2 highlighted the potentially important role of environmental

transmission pathways – particularly drinking water, soil, and animal food products (i.e., milk and meat) – in zoonotic exchange dynamics within households and backyard farms.

Chapter 3 therefore set out to understand the associations between animal ownership and fecal contamination in the domestic environment – namely in drinking water, on child hands, and on sentinel toy balls – in more than 4,000 rural Kenyan households. Overall, we found that *E. coli* contamination on child hands and flies near food preparation areas were positively associated with the number of ruminants owned by a household. Community-level ruminant ownership was also associated with *E. coli* contamination on child hands, in addition to contamination in household drinking water and toy ball rinses. The common practice of allowing domesticated animals to roam freely and graze therefore likely puts even members of non-animal-owning households at increased risk of exposure to fecal contamination. Through this work, we also found that chlorination was significantly effective, and handwashing was moderately effective, at reducing *E. coli* contamination in stored drinking water; WASH interventions as delivered in the WASH Benefits Kenya trial, however, were generally insufficient for reducing contamination on child hands and toy balls. Despite the positive associations observed between animal ownership and environmental fecal contamination, animal ownership was not associated with under-five child diarrhea, and was associated with healthier child growth. This finding emphasizes the importance of understanding which animal husbandry practices (e.g., housing animals in human sleeping quarters) and contexts (e.g., rural versus urban settings) influence the relationships between human-animal interactions and health outcomes. Such work is necessary to ensure intervention

strategies protect against any potential risks while realizing the nutritional benefits of animal ownership.

We thus aimed to determine whether behavioral and contextual differences between rural and urban communities might impact zoonotic exchange of gut bacteria and ARGs in Chapter 4. We utilized 16S rRNA gene amplicon sequencing and novel long-read metagenomics to compare overlapping fecal microbiomes and antibiotic resistomes from humans, chickens, and goats in a rural compared to urban community in Bangladesh. Human and chicken gut microbiomes were more similar in the rural community, potentially due to greater prevalence of animal ownership in the rural community, or less diversity in rural human microbiomes (compared to urban human microbiomes) due to geography or diet. On the contrary, human and goat resistomes were more similar in the urban community. ARG abundance generally was also higher in urban compared to rural animals, likely due to poor human and animal fecal waste management and shared environments between humans and animals in urban Bangladesh. Chapter 4 also highlighted chickens as particularly important reservoirs of ARG alleles overlapping with those found in humans from both communities. Further, leveraging long-read sequences revealed ARGs circulating on chromosomal contigs classified as potentially pathogenic bacteria in fecal samples from rural humans and urban humans and animals. While this work elucidated the breadth and overlap of ARGs circulating within humans and domesticated animals in Bangladeshi communities, the use of fecal composites and samples not matched epidemiologically or temporally prevented us from conclusively determining whether transmission (or its directionality) of gut bacteria or ARGs occurred in our study communities.

The study presented in Chapter 5 therefore intends to fill this gap in knowledge by identifying overlapping *E. coli* strains, ARGs, and mobile genetic elements in matched household sets of human stool, domesticated animal feces, and environmental samples (i.e., soil and water) from urban Kenya. The use of genomics will enable us to determine whether zoonotic strain sharing occurred within and between households and backyard farms, as well as to provide evidence of whole bacteria transmission versus horizontal gene transfer of ARGs between human, animal, and environmental samples. As we pooled *E. coli* isolates to account for *E. coli* diversity within sample types, Chapter 5 focused on optimizing bioinformatic methods (using a simulated data set) for assembling short and/or long sequence reads to differentiate between highly similar organisms in a metagenome. While quality metrics were generally comparable across approaches, we found that long-read-first hybrid assembly was better than short-read-only or short-read-first hybrid assembly at recovering *E. coli* reference genomes and detection 100% identical ARGs across their entire reference gene lengths. However, we recommend further exploration of impacts of different bioinformatic strategies on the ability to contextualize ARGs (i.e., predict their locations on chromosomes or mobile genetic elements), and weighing of other logistical considerations (e.g., sequencing depth and costs), to inform an optimal approach.

6.2 Recommendations and Future Research

Existing Knowledge Gaps

This dissertation demonstrates that there are widespread opportunities for spillover of zoonotic pathogens and antibiotic resistance between humans, domesticated animals, and the environment in LMICs. Food animal production systems are increasingly

recognized as important players in the rising antibiotic resistance crisis.^{41,162} Use of antibiotics for prophylaxis, metaphylaxis, and growth promotion is common in animal husbandry globally, particularly in intensive farming environments.⁵⁴ While intensification of farming is growing globally, along with demand for meat and animal-sourced foods, diverse food production systems (e.g., wet markets; informal livestock trading; and subsistence farming) remain common in LMICs.^{41,325,326} This dissertation (focusing on subsistence farming and household animal ownership) provides evidence of humans and domesticated animals carrying overlapping bacteria, antibiotic resistance profiles, and ARGs in their guts, as well as associations between animal ownership and fecal contamination in domestic environments. However, the use of cross-sectional study designs throughout this work prevents establishment of directionality of zoonotic transmission. In addition, further molecular and genomic work, such as that presented in Chapter 5, would be necessary to confirm the associations between animal presence and environmental fecal contamination observed in Chapter 3. These limitations leave the following important questions, for example, unanswered:

1. *What is the directionality of zoonotic pathogen and antibiotic resistance transmission in spillover events between humans, animals, and the environment?*

While many studies are designed to examine bacterial transmission from animals or environmental media to humans, many of these exchange events are likely occurring from humans to animals and/or the environment, or even occurring in circular patterns between all three domains.⁴¹

2. *What is the relative importance of direct versus indirect transmission via various environmental pathways in facilitating zoonotic pathogen and antibiotic*

resistance transmission? This dissertation demonstrates a likely important role of whole bacteria transmission versus horizontal gene transfer under varying contexts; however, the relative role of these transmission mechanisms is poorly understood, and gene transfer via mobile genetic elements is challenging to conclusively establish.¹⁰⁰ Further, few studies have taken a truly One Health approach to detect antibiotic-resistant bacteria in the human, animal, and environmental domains, so environmental reservoirs contributing to zoonotic exchange are not fully understood.⁵⁴

3. *What is the role of the household and backyard farm setting, relative to food animal value chains, in facilitating zoonotic pathogen and antibiotic resistance transmission to the broader community outside the clinical setting?* The proliferation of antibiotic resistance in human gut microbiomes resulting from antibiotic overuse and subsequent transmission to humans – particularly farm workers – has been demonstrated in intensive farming environments and high-income countries.⁵² The public health significance of zoonotic antibiotic resistance transmission associated with household animal ownership and subsistence farming, however, remains unclear.
4. *How can zoonotic risks of human exposure to domesticated animals be mitigated while harnessing the economic and nutritional benefits associated with household animal ownership?* In Chapter 3, we presented data paralleling other literature that show animal ownership can be associated with improved linear growth in young children.⁵¹ Exposure to domesticated animals and particular animal husbandry practices (e.g., corralling poultry inside the household

overnight), however, have also been associated with stunting, diarrheal disease, and fecal carriage of antibiotic-resistant bacteria.^{22,51,52} There is a need to better understand the social, behavioral, and biological characteristics of human and animal hosts that influence whether or not (and how frequently) zoonotic exchange of antibiotic-resistant bacteria occurs.

There is currently a lack of research on biosecurity and WASH-related interventions to disrupt zoonotic antibiotic resistance transmission in LMIC communities.^{41,143,216} Given recent large-scale randomized controlled trials of WASH interventions that yielded limited beneficial impacts on child health, it is imperative to understand why and how zoonotic antibiotic resistance transmission occurs to inform effective and comprehensive intervention strategies.^{6–8,10,11}

Recommended Future Research and Actions

Expanding on the recommendations arising from the systematic review presented in Chapter 2, three priority areas for action are recommended to address the knowledge gaps around community-level zoonotic antibiotic resistance transmission in LMICs: implementing One Health surveillance; employing genomic methods; and developing multidimensional intervention strategies.

One Health Surveillance

As more than 60% of emerging infectious diseases are attributable to zoonotic pathogens, there is an international consensus that One Health surveillance systems should be developed to encourage collaboration and data sharing across human, animal, and environmental health sectors.^{16,49,160} Specifically, longitudinal monitoring

across a variety of contexts (e.g., clinical; community; food production value chains), sample types (e.g., humans; domesticated animals; wildlife; environment), and demographics (e.g., age; gender; socioeconomic status) would help elucidate the knowledge gaps presented above. Specifically, longitudinal surveillance would help overcome cross-sectional studies' limited abilities to detect short-term colonization of human and animal hosts with antibiotic-resistant organisms.^{41,327} Additionally, ensuring diversity among the sampled populations would aid in illuminating potential risk factors, human-animal interactions, animal species, vectors, and environmental pathways important in community zoonotic exchange dynamics. Widespread surveillance may also assist with improving the availability of antibiotic usage data in LMICs. Human antibiotic usage data are limited in LMICs and primarily focus on knowledge, attributes, and practices rather than consumption.³²⁸ Sales and usage of veterinary antibiotics are even more sparse, especially in Africa.^{41,329}

Genomic Methods

Culture-based methods and molecular methods, such as PCR, have been used to demonstrate overlapping resistance profiles and potentially pathogenic bacteria between humans, animals, and environmental samples. Genomics, however, are necessary to confirm whether zoonotic whole bacteria transmission of specific strains, or horizontal gene transfer of identical mobile resistance elements, has occurred. Likewise, genomics can inform whether zoonotic transmission occurred directly between humans and animals, or indirectly via environmental reservoirs. As sequencing costs continue to decrease, and technological and bioinformatic advances are made for long-read sequencing and metagenomics, genomic methods are becoming more

accessible tools for studying One Health transmission dynamics in LMICs.^{41,57} Even so, sequencing and relevant reagent costs remain high in many resource-constrained settings. This inhibits the ability of LMIC stakeholders to widely adopt genomic methods, as was especially evidenced by global disparities in SARS-CoV-2 genomic surveillance during the COVID-19 pandemic.³³⁰ It is imperative that local capacity from within LMIC institutions and stakeholders expands alongside access to advanced genomic methods, ensuring the advantages of sequencing data for understanding transmission dynamics can be harnessed equitably.³³¹

Multidimensional Intervention Strategies

As evidenced herein, zoonotic pathogen and antibiotic resistance transmission dynamics are complex and intertwined between human, animal, and environmental domains in LMIC communities. Interdisciplinary and multidimensional intervention strategies should be developed and tested to disrupt these transmission patterns. Antibiotic stewardship programs are already widely adopted throughout human and veterinary hospitals globally. However, as unregulated antibiotic use is common in LMICs, there is a need to explore ways stewardship efforts could be adapted outside of clinical settings as well.⁴¹ There are many human medical and veterinary advances that could be made in addition to widespread calls for antibiotic stewardship, including vaccines, improved diagnostics, alternate therapeutics, and alternate growth promoters.⁴¹ Vaccines could reduce bacterial infections and subsequent needs for antibacterial therapeutics, while improved diagnostics could reduce misuse and overuse of antibiotics. Although developing suitable therapeutic and growth-promoting alternatives to antibiotics would reduce antibiotic use, it is important that efforts to

incentivize new antibiotic development – in response to the broken antibiotic market – continue.³³² Despite the overwhelming threat of antibiotic-resistant infections, LMICs also continue to battle issues with access to life-saving drugs.³²

In addition to medical interventions, WASH and behavioral strategies might be effective in disrupting zoonotic transmission of enteric pathogens and antibiotic resistance. Limited studies have evaluated interventions specifically designed to limit human exposure to animal feces, and even fewer have assessed intervention impacts on reducing antibiotic resistance in LMICs.^{9,143} As supported by Chapter 3 of this dissertation – in addition to other previous work – water treatment might be an effective strategy for limiting human exposure to human and animal fecal contamination, reducing the burden of enteric infections, and limiting human antibiotic use.^{178,226} In addition, behavioral and infrastructure interventions – such as finished household flooring or animal corralling – have the ability to disrupt enteric pathogen and antibiotic resistance transmission pathways.^{143,333} The potential for WASH interventions to disrupt community spread of zoonoses and antibiotic resistance might be particularly great in hotspot settings, such as urban slums and wet markets.^{216,334}

6.3 Conclusion

Domesticated animals are recognized reservoirs for zoonotic pathogens and antibiotic resistance, and increasing focus has been given on food production systems for their roles in the clinical burden of disease. Despite intensive human-animal interactions and widespread participation in subsistence farming in LMICs, zoonotic spread of antibiotic-resistant bacteria through the community is poorly understood. This dissertation

demonstrates widespread exchange of gut bacteria and ARGs between humans and animals in household and backyard farm settings and highlights the potentially important role of environmental media in that exchange. We applied diverse methods to explore factors contributing to zoonotic exchange dynamics and exposed contextual considerations that should be considered when assessing community transmission networks. Future research in line with this work should be continued and advanced to inform the design and evaluation of One Health intervention strategies capable of protecting humans, animals, and the environment from the threats of zoonoses and antibiotic resistance. Although beyond the scope of this dissertation, there are several players in addition to humans, domesticated animals, and domestic environments that should be considered for a comprehensive understanding of exchange dynamics, including but not limited to: wildlife; insects; crop agriculture; aquaculture; and community environments (e.g., markets). Wholistic integration of these many factors, and relevant disciplines, is necessary to truly adopt the One Health framework for addressing global burdens of disease and complex human, animal, and environmental health issues.

Chapter 7: Additional Relevant Research

While Chapters 2-5 comprise the projects forming this dissertation and inform its conclusions, I have collaborated on other related research projects during the course of my PhD. These research works focused on the understanding of infectious diseases and antibiotic resistance, as well as addressed potential intervention strategies. Brief findings from each of these relevant projects are presented below.

7.1 Intervention Impacts on Infectious Diseases

The Kenya WASH Benefits study was a cluster-randomized controlled trial designed to test the effects of WASH and nutritional interventions not only on its primary outcomes (diarrhea and child growth) and environmental contamination as presented in Chapter 3, but on several other important health outcomes as well. Namely, we examined the intervention impacts on respiratory, soil-transmitted helminth (STH), and *Giardia* infections in young children.^{178,335} WASH interventions and behavior change messaging did not reduce symptoms of acute respiratory infections (cough, panting, wheezing, or difficulty breathing) in children younger than three years, and nutrition counseling and lipid-based nutrient supplementation moderately reduced acute respiratory infection symptoms after one year.³³⁵ Water chlorination, integrated WASH interventions, and combined WASH and nutritional interventions, two years after delivery, reduced *Ascaris* prevalence in children aged 2-15 years; however, none of the interventions reduced *Giardia* prevalence.¹⁷⁸ These findings revealed water to be a potentially underappreciated transmission pathway for *Ascaris*. In a separate prospective cohort analysis, we also found that household finished flooring was associated with lower prevalence of *Ascaris*, any STH (i.e., *Ascaris lumbricoides*; *Necator americanus*;

Trichuris trichiura), and *Giardia* in Kenyan and Bangladeshi children, as well as with lower prevalence of hookworm (i.e., *N. americanus*) in Bangladeshi children.³³³

7.2 WASH and Nutritional Interventions Against Antibiotic Resistance

The work presented in Chapters 2, 4, and 5 of this dissertation focused on understanding which transmission mechanisms and pathways are important for zoonotic exchange of antibiotic resistance in LMICs to help inform the design of effective intervention strategies. Following a cluster-randomized trial in urban Bangladesh that found automated water chlorination of shared taps reduced child diarrhea and antibiotic use, we leveraged stool samples collected from a subset of children enrolled in the trial to examine how water chlorination impacted children's gut microbiomes.^{226,336} We found that while chlorination was associated with increased abundance of some bacterial genera linked to healthy human guts, there were no differences between the overall richness or diversity of taxa in treatment versus control children; some clinically relevant ARGs, however, were more abundant in treatment children, potentially explained by increases in *Enterobacteriaceae*.³³⁶ Overall, water chlorination did not substantially impact child gut microbiome development in our study community.

I have also had the opportunity to collaborate on research to identify associations between water and sanitation infrastructure and human resistomes, as well as between breastfeeding and antibiotic-resistant *Enterobacteriaceae* carriage in young Peruvian children. Specifically, we used geospatially tagged human fecal metagenomes and household survey datasets from 26 countries to determine that increased access to

both improved water and sanitation infrastructure and improved sanitation alone – but not improved water on its own – was associated with lower ARG abundance in human guts (Fuhrmeister, E. R., et al., manuscript submitted to the Lancet family of journals). In peri-urban Peru, we leveraged monthly stool samples from children aged 1-16 months and 52,816 daily household survey visits to reveal that that daily breastfeeding reduced children’s risk of acquiring ESBL-producing *E. coli* and ESBL-producing *Klebsiella*, *Enterobacter*, or *Citrobacter* spp. (Nadimpalli, M. L., et al., manuscript under review at the American Journal of Clinical Nutrition).

7.3 Environmental Surveillance for Monitoring Infectious Diseases

There has been increasing emphasis in the international research community, particularly in the wake of the COVID-19 pandemic, on the importance of One Health surveillance efforts to enhance pandemic preparedness.³³⁷ During a COVID-19 outbreak in Somerville, Massachusetts in 2020, we collected longitudinal high-touch surface samples in public locations and essential business to 1) document surfaces contaminated with SARS-CoV-2 RNA; 2) estimate human risk of infection from touching contaminated surfaces; and 3) determine whether environmental SARS-CoV-2 RNA contamination levels could predict COVID-19 cases in the community.³³⁸ We found that while crosswalk buttons, trash can handles, and door handles were contaminated with SARS-CoV-2 RNA, the estimated risk of infection from touching a contaminated surface was low. In addition, the weekly percentage of positive samples predicted variation in community COVID-19 cases with a 7-day lead time.

In addition to providing an early warning for emerging outbreaks, environmental surveillance can be a useful tool for monitoring infectious disease intervention progress. For example, molecular-based assessment methods to detect and quantify STH material in soil and water might be a cost-effective strategy for measuring STH infection prevalence at low levels to determine when interventions can be stopped. We piloted these methods in Kenya, India, and Benin to compare the prevalence of STH-positive households by qPCR of soil samples and qPCR of human stool samples (Manuel, M. and Pilotte, N., et al., manuscript in preparation).

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Appendices

8.1 Appendix A – Chapter 2

Supporting Information A-1: Generic search string and search strings adapted for six databases

Generic

(animal OR livestock OR cattle OR cow OR bovine OR ruminant OR swine OR pig OR dog OR cat OR goat OR sheep OR poultry OR chicken OR fowl OR duck OR goose OR geese OR turkey OR rabbit OR horse OR donkey OR buffalo OR camel OR yak OR llama OR alpaca) AND (zoonotic OR zoonosis OR ((exposure OR exposed OR contact OR proximity) and (child OR children OR infant OR human))) OR household OR domestic OR community) AND (((antibiotic OR antimicrobial OR drug) AND (resistance OR resistant)) OR resistome)

CAB Abstracts

((animal or livestock or cattle or cow or bovine or ruminant or swine or pig or dog or cat or goat or sheep or poultry or chicken or fowl or duck or goose or geese or turkey or rabbit or horse or donkey or buffalo or camel or yak or llama or alpaca) and (zoonotic or zoonosis or ((exposure or exposed or contact or proximity) and (child or children or infant or human))) or household or domestic or community) and (((antibiotic or antimicrobial or drug) and (resistance or resistant)) or resistome)).ab.

Cochrane Library

(animal OR livestock OR cattle OR cow OR bovine OR ruminant OR swine OR pig OR dog OR cat OR goat OR sheep OR poultry OR chicken OR fowl OR duck OR goose OR geese OR turkey OR rabbit OR horse OR donkey OR buffalo OR camel OR yak OR

llama OR alpaca) in Title, Abstract, Keywords and (zoonotic OR zoonosis OR ((exposure OR exposed OR contact OR proximity) AND (child OR children OR infant OR human)) OR household OR domestic OR community) in Title, Abstract, Keywords and (((antibiotic OR antimicrobial OR drug) AND (resistance OR resistant)) OR resistome) in Title, Abstract, Keywords

EMBASE

(animal:ab,ti OR livestock:ab,ti OR cattle:ab,ti OR cow:ab,ti OR bovine:ab,ti OR ruminant:ab,ti OR swine:ab,ti OR pig:ab,ti OR dog:ab,ti OR cat:ab,ti OR goat:ab,ti OR sheep:ab,ti OR poultry:ab,ti OR chicken:ab,ti OR fowl:ab,ti OR duck:ab,ti OR goose:ab,ti OR geese:ab,ti OR turkey:ab,ti OR rabbit:ab,ti OR horse:ab,ti OR donkey:ab,ti OR buffalo:ab,ti OR camel:ab,ti OR yak:ab,ti OR llama:ab,ti OR alpaca:ab,ti) AND (zoonotic:ab,ti OR zoonosis:ab,ti OR ((exposure:ab,ti OR exposed:ab,ti OR contact:ab,ti OR proximity:ab,ti) AND (child:ab,ti OR children:ab,ti OR infant:ab,ti OR human:ab,ti)) OR household:ab,ti OR domestic:ab,ti OR community:ab,ti) AND (resistome:ab,ti OR ((antibiotic:ab,ti OR antimicrobial:ab,ti OR drug:ab,ti) AND (resistance:ab,ti OR resistant:ab,ti)))

Global Health

((animal or livestock or cattle or cow or bovine or ruminant or swine or pig or dog or cat or goat or sheep or poultry or chicken or fowl or duck or goose or geese or turkey or rabbit or horse or donkey or buffalo or camel or yak or llama or alpaca) and (zoonotic or zoonosis or ((exposure or exposed or contact or proximity) and (child or children or infant or human)) or household or domestic or community) and (((antibiotic or antimicrobial or drug) and (resistance or resistant)) or resistome)).ab.

PubMed

((((animal[Title/Abstract] OR livestock[Title/Abstract] OR cattle[Title/Abstract] OR cow[Title/Abstract] OR bovine[Title/Abstract] OR ruminant[Title/Abstract] OR swine[Title/Abstract] OR pig[Title/Abstract] OR dog[Title/Abstract] OR cat[Title/Abstract] OR goat[Title/Abstract] OR sheep[Title/Abstract] OR poultry[Title/Abstract] OR chicken[Title/Abstract] OR fowl[Title/Abstract] OR duck[Title/Abstract] OR goose[Title/Abstract] OR geese[Title/Abstract] OR turkey[Title/Abstract] OR rabbit[Title/Abstract] OR horse[Title/Abstract] OR donkey[Title/Abstract] OR buffalo[Title/Abstract] OR camel[Title/Abstract] OR yak[Title/Abstract] OR llama[Title/Abstract] OR alpaca[Title/Abstract])) AND (zoonotic[Title/Abstract] OR zoonosis[Title/Abstract] OR ((exposure[Title/Abstract] OR exposed[Title/Abstract] OR contact[Title/Abstract] OR proximity[Title/Abstract])) AND (child[Title/Abstract] OR children[Title/Abstract] OR infant[Title/Abstract] OR human[Title/Abstract])) OR household[Title/Abstract] OR domestic[Title/Abstract] OR community[Title/Abstract])) AND (((antibiotic[Title/Abstract] OR antimicrobial[Title/Abstract] OR drug[Title/Abstract]) AND (resistance[Title/Abstract] OR resistant[Title/Abstract])) OR resistome[Title/Abstract])

Web of Science Core Collection

TS=(animal OR livestock OR cattle OR cow OR bovine OR ruminant OR swine OR pig OR dog OR cat OR goat OR sheep OR poultry OR chicken OR fowl OR duck OR goose OR geese OR turkey OR rabbit OR horse OR donkey OR buffalo OR camel OR yak OR llama OR alpaca) AND TS=(zoonotic OR zoonosis OR ((exposure OR exposed OR contact OR proximity) AND (child OR children OR infant OR human)) OR household OR

domestic OR community) AND TS=((antibiotic OR antimicrobial OR drug) AND (resistance OR resistant)) OR resistome)

Supporting Information A-2: Methods for categorizing full text publications

We screened the full text of all included publications and categorized them based on evidence of household-level zoonotic exposures and reported antibiotic resistance data in human and animal domains. Specifically, publications in Category 1 included (a) evidence of direct human contact or exposure via environmental routes within the household setting to domestic animals, animal feces, or animal products and (b) measurement of ARB or ARGs in human and domestic animal (including animal feces and products) samples matched spatially (i.e., collected in the same administrative division within a country) and temporally (i.e., collected in the same month) (Figure A-1). Publications were classified as Category 2 if they did not meet the criteria for Category 1, but included (a) evidence of direct human contact or exposure via environmental routes within the household setting to domestic animals, animal feces, or animal products and measurement of ARB or ARGs in human or domestic animal (including animal feces and products) samples or (b) measurement of ARB or ARGs in human and domestic animal samples matched spatially. The exclusion criteria applied for the title and abstract screening were also applicable to the full-text screening. We excluded studies for which the full text was not available in English. Category 2 papers were not considered high enough quality to assess zoonotic exchange, and only Category 1 publications were synthesized in this review and are hereinafter referred to as the included studies.

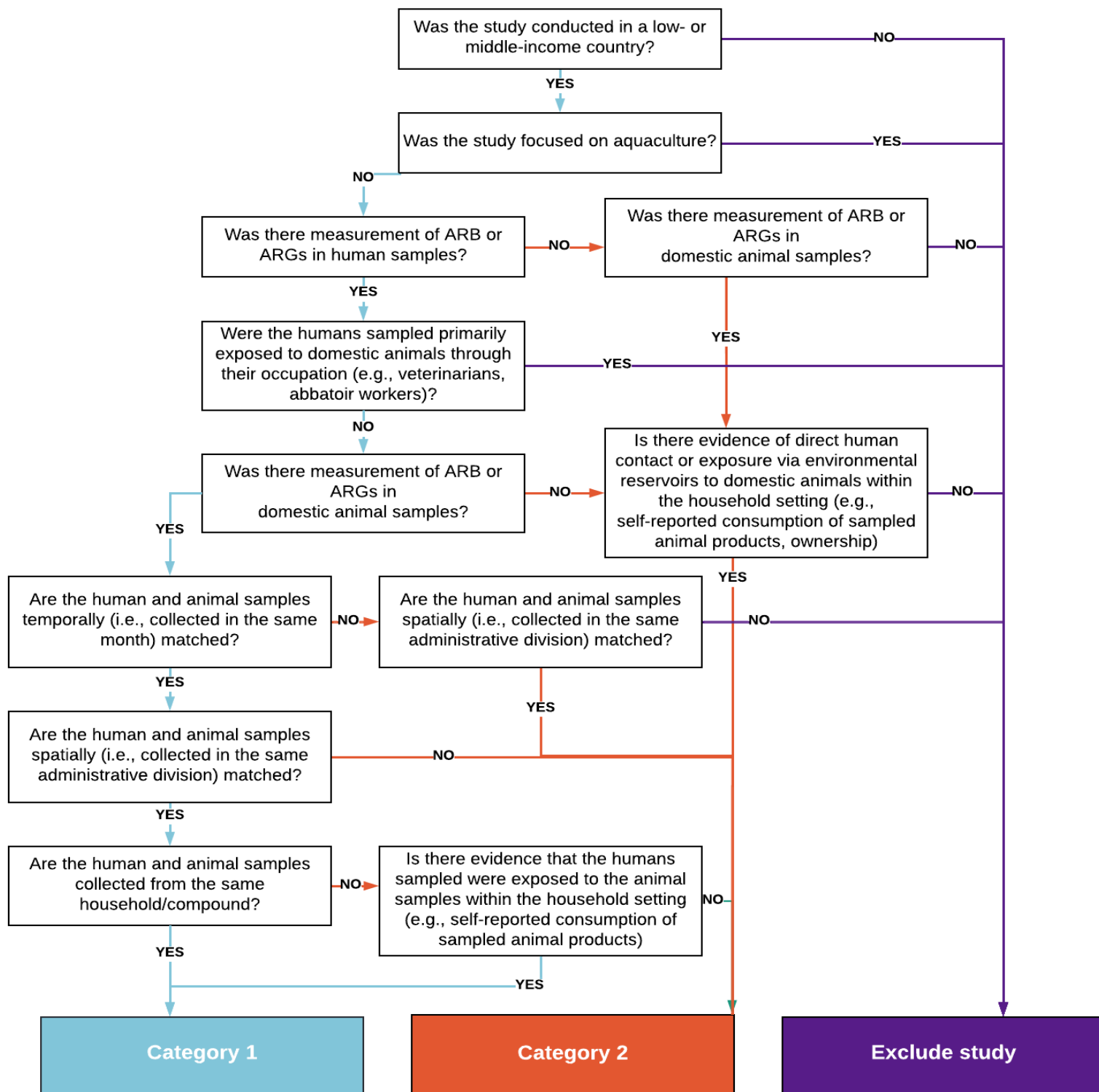


Figure A-1: Full-text screening and categorization flow chart. ARB is an abbreviation for “antibiotic-resistant bacteria” and ARG for “antibiotic resistance gene”. The categories are categorized according to Table 2 of the Checklist for One Health Epidemiological Reporting of Evidence (COHERE).

Table A-1: Data extraction codebook

Variable	Code	Label	Notes
Country	N/A	N/A	
Urbanicity	1	Rural	
	2	Urban	
	3	Peri-urban	
Setting	1	Household	
	2	Compound	
	3	Market	
	4	Park	
	5	School	
	6	Hospital	
	7	Veterinary clinic	
	8	Farm	
	77	Other (specify)	
Farm Size	1	Household/Backyard/Small-scale	
	2	Commercial	
	99	Unknown/Not specified	
Main Findings	N/A	N/A	
Months and Years of Study	N/A	N/A	
Primary Objectives	N/A	N/A	
Study Design	1	Randomized controlled trial	
	2	Case-control study	
	3	Cluster randomized controlled trial	
	4	Controlled interrupted time series	
	5	Historically controlled trial	
	6	Prospective cohort study	
	7	Retrospective cohort study	
	8	Panel study	
	9	Other cross-sectional design	
	10	Modeling	
Sample Domains	1	Human	
	2	Animal	
	3	Environmental	
Human Sample Type	1	Feces	
	2	Rectal swab	
	3	Blood	
	4	Oral/Saliva	
	5	Nasal	
	6	Vaginal	
	7	Skin	
	8	Clinical isolates	

Variable	Code	Label	Notes
	9	Ear	
	10	Wound	
	77	Other (specify)	
Human Sample Size	N/A	N/A	
Human Population	1	Children (0-2 yr)	
	2	Children (2-5 yr)	
	3	School-aged children (5-18 yr)	
	4	Adults (≥ 18 yr)	
Human Sex	1	Male	
	2	Female	
Animal Sample Type	1	Feces	
	2	Rectal swab	
	3	Blood	
	4	Oral/Saliva	
	5	Nasal	
	6	Vaginal	
	7	Skin	
	8	Clinical isolates	
	9	Ear	
	10	Wound	
	11	Cloacal swabs	
	12	Carcass/Slaughtered animal	
	13	Meat	
	14	Eggs	
	15	Dairy (e.g. milk, cheese)	
	77	Other (specify)	
Animal Sample Size	N/A	N/A	
Animal Species	1	Dogs	
	2	Cats	
	3	Cattle	
	4	Goats	
	5	Sheep	
	6	Pigs	
	7	Chickens	
	8	Ducks	
	9	Turkeys	
	10	Geese	
	11	Pigeons	
	12	Buffalo	
	13	Camels	
	14	Rabbits	
	15	Horses	

Variable	Code	Label	Notes
	16	Donkeys	
	17	Guinea pigs	
	18	Yaks/Oxen	
	19	Llamas	
	20	Alpacas	
	21	Unspecified poultry	
	22	Unspecified ruminants	
	77	Other (specify)	
Environmental Sample Type	1	Stored water	
	2	Source water	
	3	Wastewater	
	4	Soil	
	5	Air	
	6	Surfaces	
	7	Unspecified water	
	77	Other (specify)	
Environmental Sample Size	N/A	N/A	
Exposure Assessment Methods	1	Self-report/Survey	
	2	Structured observation	
	3	Household-level proximity to human population	e.g., pets, backyard chickens
	4	Geographic relatedness of sample population	e.g., market and hospital sampling from same village
	77	Other (specify)	
Exposure Routes Assessed	1	Direct contact	e.g., ownership, cohabitation
	2	Handling of manure	e.g., garden fertilizer, fuel source
	3	Food consumption	
	4	Water consumption	
	5	Contaminated fomites	e.g., toys, utensils, surfaces
	6	Hand contamination	
	7	Slaughtering practices	
	77	Other (specify)	
Bacteria Genera Identified/Analyzed	1	Escherichia	
	2	Campylobacter	
	3	Salmonella	
	4	Shigella	
	5	Staphylococcus	
	6	Acinetobacter	
	7	Enterococcus	
	8	Streptococcus	
	9	Clostridium/Clostridioides	
	10	Pseudomonas	

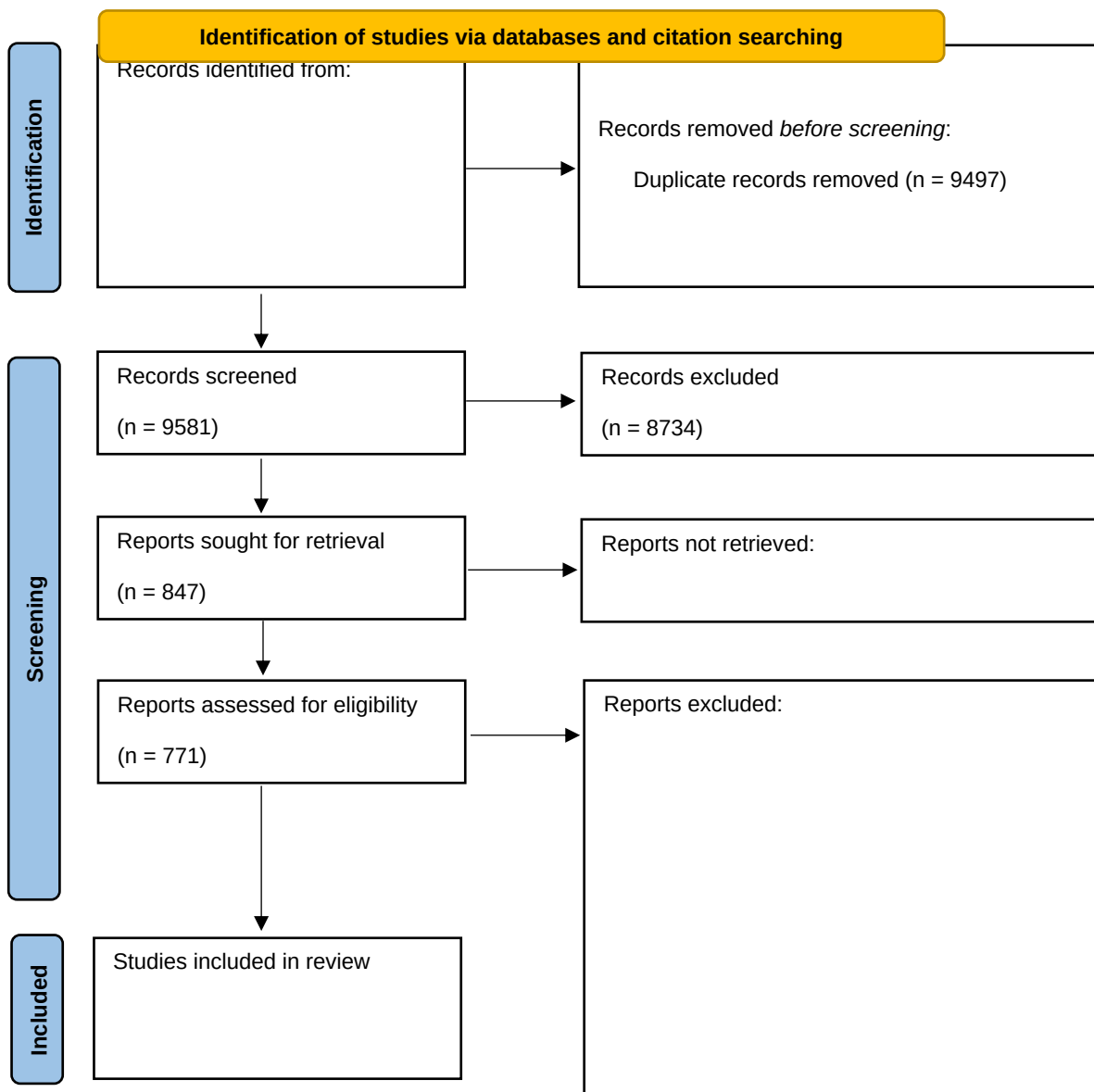
Variable	Code	Label	Notes
	11	Listeria	
	12	Arcobacter	
	13	Yersinia	
	14	Klebsiella	
	77	Other (specify)	
Bacterial Selection Methods	1	Culture-based	e.g., growth media (agar, broth)
	2	Culture-independent/Molecular	e.g., metagenomic sequencing
Bacterial Identification Methods	1	Culture-based	e.g., selective/differential media
	2	Molecular	e.g., PCR, 16s sequencing, metagenomic sequencing
Antibiotic Classes Analyzed	1	Aminoglycosides	Classes defined according to Wikipedia (https://en.wikipedia.org/wiki/List_of_antibiotics)
	2	Carbapenems	
	3	Cephalosporins	
	4	Glycopeptides	
	5	Lincosamides	
	6	Macrolides	
	7	Nitrofurans	
	8	Penicillins	
	9	Polypeptides	
	10	Quinolones/Fluoroquinolones	
	11	Sulfonamides	
	12	Tetracyclines	
	13	Chloramphenicol	
	14	Drugs Against Mycobacteria	
	15	Trimethoprim	
	77	Other (specify)	
Culture-Based Antibiotic Resistance Detection Methods	1	Agar dilution assays	e.g., Kirby-Bauer test/disk diffusion, Etest
	2	Broth dilution assays	
	77	Other (specify)	
Molecular-Based Antibiotic Resistance Detection Methods	1	Whole genome sequencing methods	
	2	Metagenomic sequencing methods	
	3	PCR methods	e.g., qPCR, TaqMan arrays
	77	Other (specify)	

Table A-2: PRISMA 2020 checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Title
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Abstract
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Introduction
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Introduction
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Methods
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Methods
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Supporting Information 1
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Methods
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Methods
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Methods; Table A-1
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Methods; Table A-1
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Not applicable
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Not applicable
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Not applicable
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Not applicable
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Methods
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Methods
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Not applicable
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Not applicable
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Not applicable
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Not applicable
RESULTS			

Section and Topic	Item #	Checklist item	Location where item is reported
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Results; Figure A-1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Not applicable
Study characteristics	17	Cite each included study and present its characteristics.	Table A-3
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Not applicable
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Not applicable
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Results
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Results
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Results
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Not applicable
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Not applicable
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Results
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Discussion
	23b	Discuss any limitations of the evidence included in the review.	Limitations
	23c	Discuss any limitations of the review processes used.	Limitations
	23d	Discuss implications of the results for practice, policy, and future research.	Discussion
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Methods
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Methods
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Methods
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Acknowledgment
Competing interests	26	Declare any competing interests of review authors.	Not applicable
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Not applicable

Adapted From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: [1](#)



Adapted From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: <http://www.prisma-statement.org/>

Figure A-2: PRISMA 2020 flow chart. The categories are categorized according to Table 2 of the Checklist for One Health Epidemiological Reporting of Evidence (COHERE).

Table A-3: Individual study characteristics for included studies (N = 79)

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Abbas 2019	Pakistan; urban	A: feces (45); H: feces (60)	dogs, cats	<i>Escherichia</i>	Amin., Carb., Ceph., Nitr., Peni., Quin., Chlo., Glyc.	P: agar dilution assays; G: PCR	DC
Abdel-moein 2012	Egypt; urban	A: oral/saliva (114), nasal (55), ear (10), wound (5); H: oral/saliva, nasal (50)	dogs, cats	<i>Staphylococcus</i>	Amin., Ceph., Glyc., Linc., Macr., Peni., Quin./Fluo., Sulf., Tetr., Myco.	P: agar dilution assays; G: PCR	DC
Abunna 2016	Ethiopia; urban	A: feces (75), cloacal swabs (100); H: skin (4); E: poultry drinking water (8), poultry litter (10), chicken feed (8)	chickens	<i>Salmonella</i>	Amin., Ceph., Peni., Quin./Fluo., Tetr., Chlo., Trim.	P: agar dilution assays	DC
Ahmed 2020	Egypt; rural	A: dairy (80), skin (20); H: feces (20), skin (20); E: stored water (20); cattle bedding (20)	cattle	<i>Escherichia</i>	Ceph.	G: PCR	DC, FC, WC, CF, HC
Akano 2009	Nigeria; urban	A: groin (4); H: nasal (7), clinical isolates (9), ear (1)	dogs	<i>Staphylococcus</i>	Amin., Macr., Peni., Sulf., Tetr., Chlo.	P: agar dilution assays	DC
Atterby 2019	Cambodia; rural	A: feces (285); H: feces (307)	cattle, pigs, chickens, ducks, geese	<i>Escherichia, Klebsiella</i>	Amin., Carb., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays; G: PCR	DC, HM, FC, WC, HC, SP
Bich 2019	Vietnam; rural	A: feces (45), cooked food (75); H: feces (93); E: water (91)	dogs, cats, pigs, chickens, ducks, geese	NA	Carb., Quin./Fluo.	G: PCR	DC, FC, WC
Carvalho 2016	Brazil; urban	A: feces (134); H: feces (178)	dogs	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Mono.	P: agar dilution assays; G: PCR, pulsed-field gel electrophoresis	DC
Caudell 2018	Tanzania; mixed	A: dairy (181); H: feces (226); E: surfaces (191)	NA	<i>Escherichia</i>	Amin., Ceph., Peni., Quin., Sulf., Tetr., Chlo., Trim.	P: agar dilution assays	DC, FC, WC, CF
Dang 2018	Vietnam; rural	A: feces (1500); H: feces (100)	pigs	<i>Escherichia</i>	Ceph.	P: agar dilution assays; G: PCR	DC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Derakhshandeh 2018	Iran; urban	A: feces (31); H: feces (44)	dogs	<i>Escherichia</i>	Ceph., Sulf. Chlo., Phenicol	G: PCR	DC
Diab 2021	Egypt; rural	A: rectal swabs (180), dairy (720); H: feces (180)	camels	<i>Escherichia</i>	Amin., Linc., Peni., Quin./Fluo., Tetr.	P: CLSI breakpoint	DC
Dione 2011	Gambia; rural	A: feces (210); H: feces (990)	goats, sheep, chickens	<i>Salmonella</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Trim.	P: agar dilution assays	DC
Eger 2021	Rwanda; NA	A: rectal swab (120); H: rectal swab (480)	NA	<i>Escherichia</i>	Poly.	P: broth dilution assays; G: whole genome sequencing methods	DC
Fabri 2021	Brazil; NA	A: nasal (241); H: nasal (208)	dogs	<i>Staphylococcus</i>	Amin., Ceph., Peni.,	P: agar dilution assays; G: PCR	DC
Fernandes 2018	Brazil; NA	A: rectal swab, oral/saliva (4); H: feces (2); E: surfaces (12)	dogs	<i>Pseudomonas</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	G: whole genome sequencing methods, multilocus sequence typing	DC, CF
Findik 2018	Turkey; NA	A: rectal swab, nasal swab (33); H: nasal (33)	dogs	<i>Staphylococcus</i>	Ceph., Glyc., Peni., Quin./Fluo., Sulf., Tetr.	P: agar dilution assays	DC
Ghoneim 2020	Egypt; urban	A: cloacal swabs (36); H: feces (50); E: stored water (15)	chickens	<i>Campylobacter</i>	Amin., Macr., Peni., Quin./Fluo., Tetr., Chlo.	P: agar dilution assays; G: PCR	DC, FC, WC
Gwida 2020	Egypt; rural	A: feces (82); H: feces (50)	cattle, buffalo	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Poly., Quin./Fluo., Sulf., Trim., Monobactams, Tigecycline, Fosfomycin	P: broth dilution assays; G: microarray	DC
Gwida 2021	Egypt; urban	A: feces (90), dairy (90); H: feces (80)	cattle	<i>Escherichia</i>	Amin., Ceph., Macr., Peni., Quin./Fluo., Sulf., Tetr.	P: agar dilution assays	DC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Hartertinger 2021	Peru; rural	A: rectal swab (<80), cloacal swabs (<80); H: feces (40); E: stored water (40), source water (40), soil (83), surface water (26)	dogs, cats, cattle, pigs, unspecified poultry	<i>Escherichia</i>	Amin., Carb., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Trim., Monobactams	P: agar dilution assays; G: PCR	DC, WC, HC
Hassell 2019	Kenya; urban	A: feces (2782); H: feces (333); E: water (<277), garbage (<277)	unspecified poultry/ruminants, wild bats, rodents, birds, carnivores, primates	<i>Staphylococcus</i>	Ceph., Glyc., Peni., Quin./Fluo., Sulf., Tetr.	P: agar dilution assays	DC, FC, WC
Hedman 2019	Ecuador; rural	A: feces (349); H: feces (220)	chickens	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays	DC
Hedman 2020	Ecuador; rural	A: cloacal swabs (439); H: feces (375)	chickens	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays	DC
Hickman 2021	Thailand; NA	A: feces (143); H: feces (144)	pigs	<i>Escherichia</i>	Amin., Carb., Peni., Poly., Quin./Fluo., Chlo., Trim., Fosfomycin, Tigecycline	P: broth dilution assays; G: whole genome sequencing methods	DC
Huber 2021	Thailand; NA	A: rectal swab (NA); H: feces (NA)	pigs	<i>Escherichia</i>	Amin., Carb., Ceph., Quin./Fluo., Sulf., Tetr., Chlo., Trim.	P: agar dilution assays	DC
Iramiot 2020a	Uganda; rural	A: rectal swab (148); H: rectal swab (152)	cattle	<i>Escherichia</i> , <i>Klebsiella</i> , <i>Serratia</i> , <i>Kluyvera</i> , <i>Enterobacter</i>	Amin., Carb., Ceph., Nitr., Peni., Quin./Fluo., Tetr.	P: BD Phoenix automated identification and susceptibility testing system	DC
Iramiot 2020b	Uganda; rural	A: feces (12); H: feces (30)	cattle	<i>Escherichia</i>	Amin., Carb., Ceph., Nitr., Peni., Quin./Fluo., Sulf., Tetr.	P: BD Phoenix automated identification and susceptibility testing system; G: whole genome sequencing methods	DC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Kalter 2010	Peru; mixed	A: rectal swab (NA), carcass/slaughtered animal (NA); H: rectal swab (636), skin (636)	chickens	<i>Escherichia</i>	Ceph., Peni., Quin./Fluo., Sulf.	P: agar dilution assays	DC, FC, HC
Kariuki 1997	Kenya; rural	A: feces (246); H: feces (168)	chickens	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Myco.	P: agar dilution assays; G: iso-electric focusing on ployacrylamide gels	DC, FC, WC
Kariuki 1999	Kenya; rural	A: rectal swab (62); H: feces (42); E: tap water (12)	chickens	<i>Escherichia</i>	Amin., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Myco., Trim.	P: agar dilution assays; G: pulsed-field gel electrophoresis	DC, WC
Kariuki 2002	Kenya; rural	A: feces (560), carcass/slaughtered animal (220); H: feces (<402), blood (<402); E: wastewater (47)	cattle, goats, pigs, chickens, camels	<i>Salmonella</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Myco.	P: agar dilution assays; G: pulsed-field gel electrophoresis	DC, FC, CF
Kariuki 2006	Kenya; urban	A: rectal swab (NA), carcass/slaughtered animal (NA), unspecified food; H: feces (467), blood (2246); E: surface water (NA)	cattle, goats, pigs, chickens,	<i>Salmonella</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays; G: pulsed-field gel electrophoresis	DC
Lalhruaipuii 2021	India; NA	A: rectal swab (102); H: feces (55), rectal swab (34); E: stored water (55)	cattle, sheep	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Myco., Monobactams	P: agar dilution assays; G: multilocus sequence typing	DC, WC
Leangapichart 2021	Thailand; NA	A: feces (839); H: feces (226)	pigs	<i>Klebsiella</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays; G: whole genome sequencing methods	DC
Li 2019	China; rural	A: feces (1153); H: feces (747); E: flies (180)	dogs, cats, cattle, goats, pigs, chickens, ducks, donkeys	<i>Escherichia</i>	Amin., Carb., Ceph., Nitr., Peni., Poly., Quin./Fluo., Sulf., Tet., Tigecycline, Fosfomycin	P: agar dilution assays; G: whole genome sequencing methods, PCR	DC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Loayza-Villa 2020	Ecuador; urban	A: feces (32), rectal swab (6), cloacal swabs (4); H: peritoneal fluid (1)	dogs, chickens, ducks, turkeys, pigeons, rabbits, guinea pigs	<i>Escherichia</i>	Poly.	P: broth dilution assays; G: whole genome sequencing methods, PCR	DC
Lunha 2020	Thailand; rural	A: rectal swab (839); H: feces (272)	pigs	<i>Escherichia</i>	Amin., Carb., Ceph., Quin./Fluo., Sulf., Tetra., Chlo.	P: agar dilution assays	DC
Lupindu 2014	Tanzania; urban	A: feces (446); H: feces (200); E: surface water (200), soil (400)	cattle	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Myco.	P: agar dilution assays; G: PCR	DC, HM, FC, WC
Lupindu 2015	Tanzania; urban	A: feces (446); H: feces (200); E: surface water (200), soil (400)	cattle	<i>Escherichia</i>	Peni., Tetr.	P: agar dilution assays	DC, HM, FC, WC
Madupuru 2017	India; urban	A: nasal (40); H: nasal (80)	dogs	<i>Staphylococcus</i>	Ceph., Peni.	P: agar dilution assays; G: PCR	DC
Momoh 2018	Nigeria; rural	A: nasal (300); H: nasal (101)	pigs	<i>Staphylococcus</i>	Amin., Ceph., Glyc., Linc., Macr., Peni., Quin./Fluo., Sulf., Tetr., Myco., Lipopeptide, Fusidic acid, Mupirocin, Oxazolidinones, Tigecycline	P: broth dilution assays	DC, SP
Montealegre 2018	Bangladesh; rural	A: feces (102); H: feces (50); E: soil (52)	cattle, chickens	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Monobactams	P: agar dilution assays; G: PCR	DC
Montealegre 2020	Bangladesh; rural	A: feces (27); H: feces (14); E: soil (19)	cattle, chickens	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetra., Chlo., Monobactams	P: agar dilution assays	DC
Moretto 2021	Brazil; rural	A: feces (NA); H: feces (50); E: source water (2), surface water (10)	cattle, pigs, horses	<i>Escherichia</i>	Carb., Ceph., Peni., Quin./Fluo., Monobactams	P: agar dilution assays; G: PCR methods	DC, WC
Moser 2018	Ecuador; rural	A: cloacal swabs (376); H: feces (799)	chickens	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluor., Sulf., Tetr., Chlo.	P: agar dilution assays; G: dot-blot hybridization on library-on-a-slide array platform	DC, FC, WC, AIR

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Mourabit 2020	Morocco; urban	A: nasal (421); H: nasal (50)	cattle, goats, sheep	<i>Staphylococcus</i>	Amin., Ceph., Linc., Macr., Quin./Fluoro., Sulf., Chlo., Myco., Streptogramins, Fusidic acid	P: agar dilution assays; G: PCR	DC
Muloi 2019	Kenya; urban	A: rectal swab (633); H: feces (321)	cattle, goats, sheep, pigs, chickens, ducks, turkeys, geese, pigeons, rabbits, guinea fowl, wildlife	<i>Escherichia`</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Trim.	P: agar dilution assays	DC
Muloi 2021, preprint	Kenya; urban	A: feces (2081)	cattle, goats, sheep, pigs, chickens, ducks, turkeys, geese, pigeons, rabbits, guinea fowl, wildlife	<i>Escherichia</i>	NA	G: whole genome sequencing methods	DC
Ogundipe 2020	Nigeria; urban	A: meat (148), oropharyngeal swabs (142); H: nasal (311); E: surfaces (133)	chickens	<i>Staphylococcus</i>	Amin., Glyc., Linc., Macr., Quin./Fluo., Sulf., Tetr., Amphenicols, Oxazolidinones, Quinupristin/Dalfopristin, Pleuromutilin	P: broth dilution assays; G: whole genome sequencing methods	DC, FC, CF
Padungtod 2006	Thailand; NA	A: rectal swab (210), cloacal swabs (212), carcass/slaughtered animal (191), meat (45); H: feces (28)	cattle, pigs, chickens	<i>Campylobacter</i>	Amin., Macr., Peni., Quin./Fluo., Tetr., Chlo.	P: agar dilution assays, broth dilution assays	DC, FC,
Pehrsson 2016	El Salvador; rural, Peru; urban	A: feces (14); H: feces (263); E: stored water (<22), source water (<22), wastewater (43), soil (34), latrine samples (36)	dogs, cattle, chickens, guinea pigs	NA	Ceph., Linc., Mac., Peni., Quin./Fluo., Sulf., Chlo., Trim., Monobactams, Tigecycline	G: metagenomic sequencing, mass spectrometry	DC, WC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Phumthanakorn 2018	Thailand; urban	A: nasal (18), groin (19), pyoderma (6); H: nasal (18); E: surfaces (32)	cattle, goats, sheep, pigs, rabbits, unspecified poultry	<i>Staphylococcus</i>	Peni.	P: agar dilution assays; G: multilocus sequence typing, pulsed-field gel electrophoresis	DC
Poulsen 2012	Vietnam; urban	A: feces (NA), cloacal swabs (NA); H: urine (31)	chickens	<i>Enterococcus</i>	Amin., Glyc., Macr., Peni., Quin./Fluor., Tet., Chlo. Orthosomycin, Lipopeptide, Oxazolidinones, Salinomycin, Quinupristin-dalfopristin, Tigecycline	P: broth dilution assays	DC
Puii 2019	India; NA	A: rectal swab (164); H: feces (55); E: stored water (<34), source water (<34)	pigs	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Monobactams	P: agar dilution assays, broth dilution assays; G: PCR	DC, FC, WC
Purohit 2017	India; rural	A: feces (4); H: feces (22); E: stored water (22), source water (2), wastewater (2)	dogs, goats, chickens, horses	<i>Escherichia</i>	Amin., Carb., Ceph., Nitr., Peni., Quin./Fluo., Polymyxin	P: agar dilution assays; G: PCR	DC, WC
Riccobono 2012	Bolivia; urban	A: rectal swab (21); H: rectal swab (21)	chickens	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluor., Sulf., Tetra., Chlo	P: agar dilution assays; G: PCR methods	DC
Rousham 2021	Bangladesh; mixed	A: feces (NA), carcass/slaughtered animal (NA); H: feces (200); E: source water (120), wastewater (120)	unspecified poultry	<i>Escherichia</i>	Amin., Carb., Ceph., Macr., Nitr., Peni., Quin./Fluor., Sulf., Tetr., Trim.	P: agar dilution assays, broth dilution assays; G: PCR	DC, FC, WC, SP
Rwego 2008	Uganda; rural	A: feces (114); H: feces (65)	cattle, goats, sheep, gorillas	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tet., Chlor.	P: agar dilution assays	DC
Sahoo 2012	India; rural	A: feces (417); H: feces (417); E: stored water (417)	cattle	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluor., Sulf., Tetr.	P: agar dilution assays	DC, WC
Saleha 2006	Malaysia; urban	A: skin (60), ear (60); H: nasal (60), skin (60)	dogs, cats	<i>Staphylococcus</i>	Amin., Mac., Peni., Tet.	P: agar dilution assays	DC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Salinas 2019	Ecuador; rural	A: feces (203); H: feces (64)	dogs, cats, cattle, sheep, pigs, chickens, ducks, geese, rabbits, horses, guinea pigs, quail	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluoro., Sulf., Tetr., Chlo.	P: agar dilution assays, broth dilution assays; G: whole genome sequencing methods	DC, FC,
Salinas 2021	Ecuador; rural	A: feces (39); H: feces (29)	dogs, chickens	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tet., Chlo., Trim.,	P: agar dilution assays; G: whole genome sequencing methods	DC
Shittu 2007	Nigeria; urban	A: rectal swab (100); H: rectal swab (30)	dogs, cattle, goats, sheep, chickens	<i>Escherichia</i>	Amin., Nitr., Peni., Quin./Fluor., Sulf., Tetr., Trim.,	P: agar dilution assays, broth dilution assays	DC
Sonola 2021a	Tanzania; mixed	A: cloacal swabs (236), carcass/slaughtered animal (101); H: feces (243); E: soil (290)	chickens, rodents	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Tetr., Chlo.	P: agar dilution assays	DC
Sonola 2021b	Tanzania; mixed	A: cloacal swabs (286), carcass/slaughtered animal (101); H: nasal (284); E: soil (285)	chickens, rodents	<i>Staphylococcus</i>	Amin., Linc., Macr., Peni., Quin./Fluo., Tetr.	P: agar dilution assays	DC
Subbiah 2020	Tanzania; mixed	A: feces (NA); H: feces (NA); E: water (NA)	dogs, cattle, goats, sheep, chickens	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Trim.	P: agar dilution assays	DC, FC, WC
Subramanya 2021	Nepal; rural	A: rectal swab (158), cloacal swabs (86); H: rectal swab (NA); E: drainage/sewage swabs (NA)	cattle, goats, chicken, buffalo	<i>Escherichia</i>	Amin., Carb., Ceph., Nitr., Peni., Quin./Fluo., Tetr., Tigecycline	P: agar dilution assays, broth dilution assays; G: whole genome sequencing methods, PCR	DC
Tansawai 2019	Thailand; NA	A: feces (467); H: rectal swab (32)	chickens, ducks	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Myco., Trim., Monobactams	P: agar dilution assays; G: PCR, sequencing PCR products	DC
Trung 2016	Vietnam; mixed	A: feces (204); H: rectal swab (510); E: soil (54), water (34)	chickens	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays	DC

Study Identifier	Country; Urbanicity	Sample Types & Sizes ^a	Animal Species	Bacteria Genera	Antibiotic Classes ^b	Antibiotic Resistance Detection Methods ^c	Exposure Routes ^d
Trung 2017a	Vietnam; mixed	A: feces (204); H: rectal swab (510)	chickens	<i>Escherichia</i>	Poly.	P: agar dilution assays; G: whole genome sequencing methods, PCR	DC
Trung 2017b	Vietnam; mixed	A: feces (204); H: feces (510)	chickens	<i>Salmonella</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays, broth dilution assays	DC
Trung 2019	Vietnam; mixed	A: feces (204); H: rectal swab (510)	chickens	<i>Escherichia</i>	Ceph.	P: agar dilution assays; G: whole genome sequencing methods	DC
Ueda 2015	Vietnam; rural	A: rectal swab (47); H: feces (199)	chickens	<i>Escherichia</i>	Amin., Carb., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Fosfomycin	P: agar dilution assays; G: PCR	DC
Venkatvasan 2020	India; urban	A: nasal (110); H: nasal (44)	goats	<i>Staphylococcus</i>	Peni.	P: agar dilution assays; G: PCR	DC
Weil 2020	Uganda; rural	A: feces (NA); H: feces (11)	chickens	NA	Amin., Glyc., Linc., Macr., Quin./Fluo., Tetr.,	G: PCR	DC
Weiss 2018	Uganda; rural	A: feces (259); H: feces (204)	dogs, cattle, goats, sheep, mountain gorilla, black and white colobus red colobus red-tailed guenon chimpanzee	<i>Escherichia</i>	Amin., Ceph., Peni., Quin./Fluo., Sulf., Tetr., Chlo.	P: agar dilution assays; G: PCR, sequencing polymerase chain reaction products	DC
Wu 2020	Thailand; rural	A: rectal swab (140); H: feces (140)	dogs	<i>Salmonella</i>	Amin., Peni., Quin./Fluo., Sulf., Tetr., Chlo., Trim.	P: agar dilution assays	DC
Zou 2019	China; rural	A: feces (36); H: feces (6); E: stored water (43), surface water (25), wastewater (36), soil (23), sediment (39)	pigs, unspecified bird	<i>Escherichia</i>	Amin., Carb., Ceph., Nitr., Peni., Quin./Fluo., Sulf., Tetr., Fosfomycin, Florfenicol, Polymyxin	P: agar dilution assays; G: PCR methods	DC, FC, WC

^a Codes for Sample Sizes and Types: A: Animal; H: Human; E: Environment.

^b Codes for Antibiotic Classes: Amin.: Aminoglycosides; Carb: Carbapenems; Ceph.: Cephalosporins; Glyc.: Glycopeptides; Linc.: Lincosamides; Macr.: Macrolides; Nitr.: Nitrofurans; Peni.: Penicillins; Poly.: Polypeptides; Quin./Fluo.: Quinolones/Fluoroquinolones; Sulf.: Sulfonamides; Tetr.: Tetracyclines; Chlo.: Chloramphenicol; Myco.: Drugs against Mycobacteria; Trim: Trimethoprim

^c Codes for Antibiotic Molecular Resistance: P: Phenotypic; G: Genotypic

^d Codes for Exposure Routes: DC: direct contact; HM: handling of manure; FC: food consumption; WC: water consumption; CF: contaminated fomites; HC: hand contamination; SP: slaughtering practices

8.2 Appendix B – Chapter 3

Supporting Information B-1: Additional methods details

Study Design

The intervention arms included water treatment, improved sanitation, handwashing with soap, combined WSH, nutritional interventions, and combined WSH and nutritional interventions (WSHN). The WSH interventions were designed to improve the environmental conditions of the children in the first two years of life to reduce early-life exposure to fecal pathogens. The water treatment intervention included installation of chlorine dispensers at community water locations plus bottled chlorine provided to enrolled households. The sanitation intervention included pit latrine upgrades with a reinforced slab and drop hole cover, as well as child potties and scoops to remove human or animal feces from households and compounds. The handwashing intervention included dual dispenser tippy-tap devices operated with independent pedals, each providing one container for soapy water (soap delivered by community promoters) and one container for rinse water, installed near the latrine and kitchen area.¹ The nutrition intervention included lipid-based nutrient supplements (LNS) and age-appropriate recommendations on maternal nutrition and infant feeding practices. Local promoters visited study compounds at least every other month to deliver relevant behavior change messages for the treatment group assigned.

Clusters were defined as one or two, and in rare cases three, adjoining villages with at least six eligible pregnant women. Nine clusters were used to form a geographic block;

¹ Amin, N., et al. 2014. Microbiological evaluation of the efficacy of soapy water to clean hands: A randomized, non-inferiority field trial. *Am J Trop Med Hyg*, 91(2): 415-423.

clusters were randomized into the eight study arms (six intervention arms, active control, passive control) within each block (geographically pair-matched randomization increases the efficiency of effect estimates as study outcomes are expected to be correlated with geographical characteristics). The EED subset of the trial for which the one- and two-year environmental assessments were conducted consisted of 54 clusters per arm in four study arms (control, nutrition, WSH, WSHN), with seven households per cluster. This resulted in a total sample size of 1,500 households for the environmental assessment (375 households per arm), where 750 households (WSH and WSHN arms) received the combined WSH intervention, and 750 households (control and nutrition arms) served as comparisons. We assumed an intra-class correlation coefficient of 0.2 based on a previous large-scale evaluation in rural Bangladesh and a design effect of 2.2.² With this sample size of 750 households per comparison group, we expected the following minimum detectable effect sizes in intervention (WSH and WSHN pooled) vs. comparison (control and nutrition pooled) groups: (1) 0.2 log₁₀ reduction in *E. coli* concentration in stored water, child hand rinse, and toy rinse samples and (2) 7.6 flies difference from 11.5 flies in control in the latrine and food preparation areas.

Sample and Data Collection

We collected samples of stored drinking water, child hand rinses, and sentinel toy object rinses, as well as enumerated and speciated flies caught in the food preparation and latrine areas of the compound. We also recorded the presence of visible dirt on

² Huda, T. M. N., et al. 2012. Interim evaluation of a large scale sanitation, hygiene and water improvement programme on childhood diarrhea and respiratory disease in rural Bangladesh. *Soc Sci Med*, 75(4): 604-611.

children's and caregivers' hands, including fingernails, fingerpads, and palms. We collected stored drinking water samples from a subset of households in the water treatment single arm (those geographically matched with EED enrolled clusters) and from a subset of households in the handwashing single arm (those geographically matched with EED enrolled clusters). These subsets were selected based on household enrollment in the same randomization block as the EED cohort. We calculated fly densities at latrines and food preparation areas in a subset of households in the sanitation single arm enrolled into the same blocks as the EED cohort.

To sample drinking water, field staff asked the respondent to provide a glass of water as if giving it to the respondent's under-five children and recorded whether the water had been obtained directly from the source or from a storage container. Field staff asked the respondent to pour the water from the glass into a sterile Whirlpak bag; if the respondent reported treating the water with chlorine, a Whirlpak bag with sodium thiosulfate tablets was used to neutralize the residual chlorine. Stored water field blanks were collected by pouring a bottle of distilled water (prepared in the lab and carried to the field in a cooler) into a Whirlpak collection bag in the field. To collect child hand rinse samples, field staff asked the respondent to place the child's left hand into a sterile Whirlpak bag pre-filled with 250 mL of distilled water. The hand was massaged from the outside of the bag for 15 seconds, followed by 15 seconds of shaking. The procedure was repeated with the right hand in the same bag, and the rinse water was preserved in the Whirlpak bag. Hand rinse field blanks were collected by opening and closing an unused hand rinse Whirlpak bag in the field. Field staff provided a previously sterilized plastic toy ball to the under-five child and encouraged the respondent to prompt the

child to play with the ball. Field staff returned to the households 24 hours later to collect a rinse sample from the ball by placing the ball into a sterile Whirlpak bag pre-filled with 300 mL of distilled water and massaging it from the outside of the bag for 15 seconds, followed by 15 seconds of shaking. Toy rinse field blanks were collected by opening and closing an unused toy rinse Whirlpak bag in the field. Field staff identified a suitable location in the food preparation area (away from smoke) and latrine area (under a roof or protected from rain). At each location, they placed one scudder fly grill on the ground and took two 90 second measurements within 30 seconds of each other to record the number of flies landing on the grill and categorize the captured flies as house flies (*Musca domestica*, *Fannia canicularis*, *Stomoxys*), bottle flies (*Lucilia*, *Chrysomya*, *Calliphora*), flesh flies (*Sarcophaga*), or other flies using a visual identification chart.

All water, hand rinse, and toy rinse samples were preserved on ice in coolers and transported to the field laboratory to be processed on the same day, typically within eight hours of collection. 100 mL was filtered per stored water sample and stored water field blank sample, 50 mL filtered per hand rinse sample and hand rinse field blank sample, and 100 mL filtered per toy rinse sample and toy rinse field blank sample. Lab technicians targeted processing 5% duplicates for each sample type by filtering the same volume from the same collection bag. Lab blanks were also processed daily by each lab technician by filtering 50 mL of distilled water. Stored water and hand rinse samples were incubated at 35 °C, while toy samples were incubated at 44.5 °C.

Previous work demonstrated fecal coliforms incubated at 44.5°C on toy balls to be an

indicator of environmental contamination associated with sanitation conditions.³ *E. coli* was the primary indicator of microbial contamination (reported in main text); total and fecal coliform counts were secondary indicators (reported in SI only).

Statistical Analyses

Post-incubation on MI media, stored water, hand rinse, and toy rinse samples classified as “too numerous to count” were assigned a value of 500 colonies per plate. Colony forming units (CFU) of *E. coli* were log₁₀-transformed for analysis of discrete variables. Samples below the detection limit were substituted with half the lower detection limit (0.5 colonies per plate) prior to log transformation.

Our primary environmental outcomes for all analyses included *E. coli* prevalence and concentration in water, hand rinse, and toy rinse samples, and the presence and number of flies at food preparation areas and latrines (intervention impact analyses only). Secondary outcomes for the intervention impact analyses included fecal coliform prevalence and concentration in water, hand rinse, and toy rinse samples and the percentage of children and caregivers with visible dirt on hands. We report subgroup analyses by year of data collection (year one versus year two) in the main text and by season (wet versus dry season) in the SI only.

We pre-screened covariates to assess whether they were associated with each outcome prior to including them in adjusted statistical models.⁴ We used the likelihood

³ Vujcic, J., et al. 2014. Toys and toilets: cross-sectional study using children’s toys to evaluate environmental faecal contamination in rural Bangladeshi households with different sanitation facilities and practices. *Trop Med Int Health*, 19(5): 528-536.

⁴ Pocock, S. J., et al. 2002. Subgroup analysis, covariate adjustment and baseline comparisons in clinical trial reporting: current practice and problems. *Stat Med*, 21(19): 2917-2930.

ratio test to assess the association between each outcome and each covariate and included covariates with a p -value < 0.2 in the adjusted analysis. We also excluded covariates that had little variation in the study population (prevalence $< 5\%$).

The following baseline covariates were tested for inclusion in the adjusted models:

- Month of measurement, to account for seasonal variation
- Child age (days)
- Child sex
- Maternal age (years)
- Maternal education level (no education/incomplete primary, complete primary, incomplete secondary, complete secondary)
- Number of children <18 years in the household
- Number of individuals living in the compound
- Distance (in minutes) to the household's primary drinking water source
- Housing materials (floor, walls, roof)
- Household assets assessed individually
 - Electricity, radio, television, mobile phone, clock, bicycle, motorcycle, stove, gas cooker, car
 - Number of cows, goats, dogs, and chickens (intervention impacts analyses only)
- Intervention arm (animal ownership analyses only)

Additionally, we considered the following covariates for all environmental contamination outcomes (*E. coli* in stored water, child hand rinses, sentinel toy rinses and fly counts):

- Staff member ID of staff member who collected measurement
- Most recent time it rained (now, today, yesterday, day before yesterday, last week, or before last week, as reported by respondent)

We also tested the following covariates that would be expected to affect fecal indicator bacteria levels for these specific types of samples. While these covariates were measured at the time of the environmental assessments (rather than at baseline), we did not expect them to be impacted by the study interventions.

Sentinel Toys

- Location of toy ball at time of sample collection (indoors, outdoors)
- How much children played with ball (several times (4+), few times (2-3), once, never)
- Whether children from other compounds played with ball
- Where children played with ball (indoors, outdoors)
- Whether respondent cleaned ball prior to sample collection
- Condition of ball at time of sample collection (intact/bulging, deflated/burst/torn)

Fly Counts

- Location of food preparation area (indoors vs. outdoors)

The following covariates were also considered for child health outcomes (diarrhea, stunting, length-for-age Z-scores):

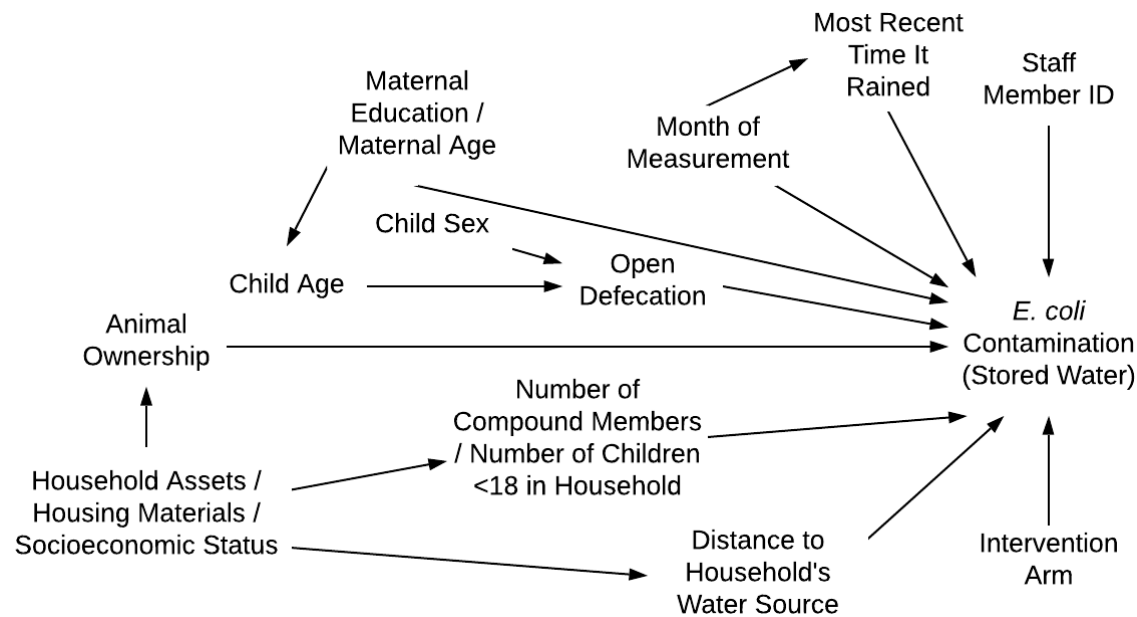
- Maternal height (cm)

- Food insecurity of household measured using the Household Hunger Scale (HHS)⁵ (little-to-no hunger, moderate-to-severe hunger)

Supporting Information B-2: Additional results

Blanks and Duplicates

E. coli was detected in 0.1% (1/832) of lab blanks, and the log-transformed *E. coli* concentration in the positive blank was 3.0 CFU/100 mL. Across sample types and sampling rounds, *E. coli* was detected in 2.1% (22/1035) of field blanks, and the log-transformed geometric mean *E. coli* concentration among positive blanks was 1.0 CFU/100 mL. Agreement between *E. coli* presence in duplicates was 91% (kappa statistic = 0.71). The Pearson correlation coefficient between *E. coli* concentrations in duplicates was 0.97.



⁵ FANTA. 2011. Household hunger scale (HHS): Indicator definition and measurement guide. <https://www.fantaproject.org/monitoring-and-evaluation/household-hunger-scale-hhs>

Figure B-1: Directed acyclic graph for *E. coli* contamination in stored water

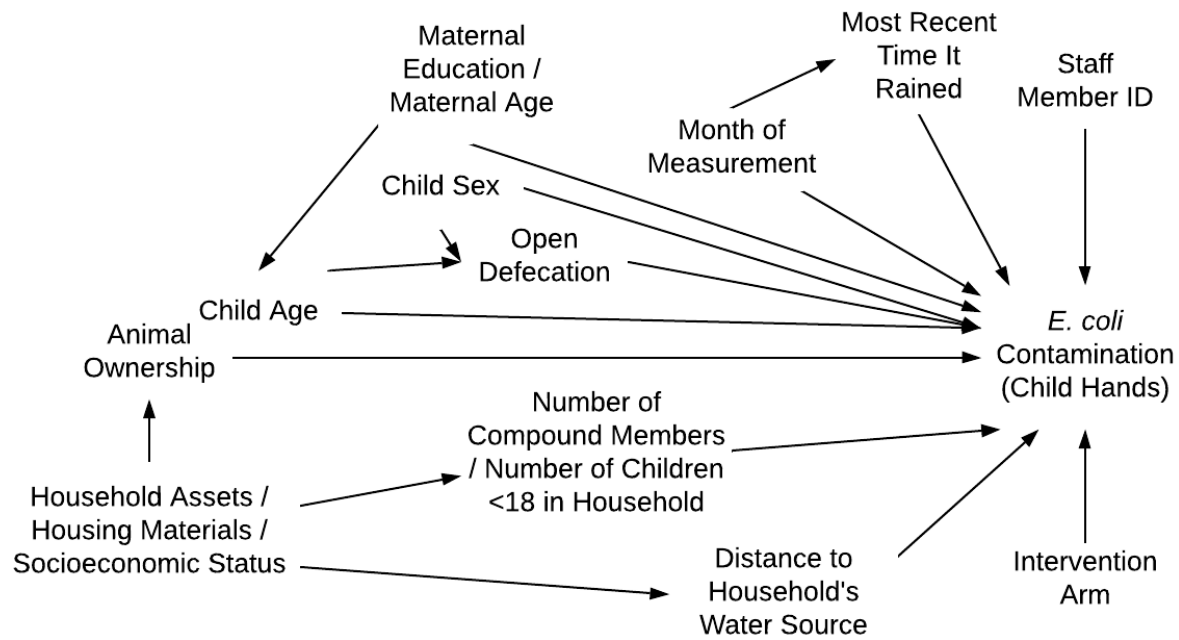


Figure B-2: Directed acyclic graph for *E. coli* contamination on child hands

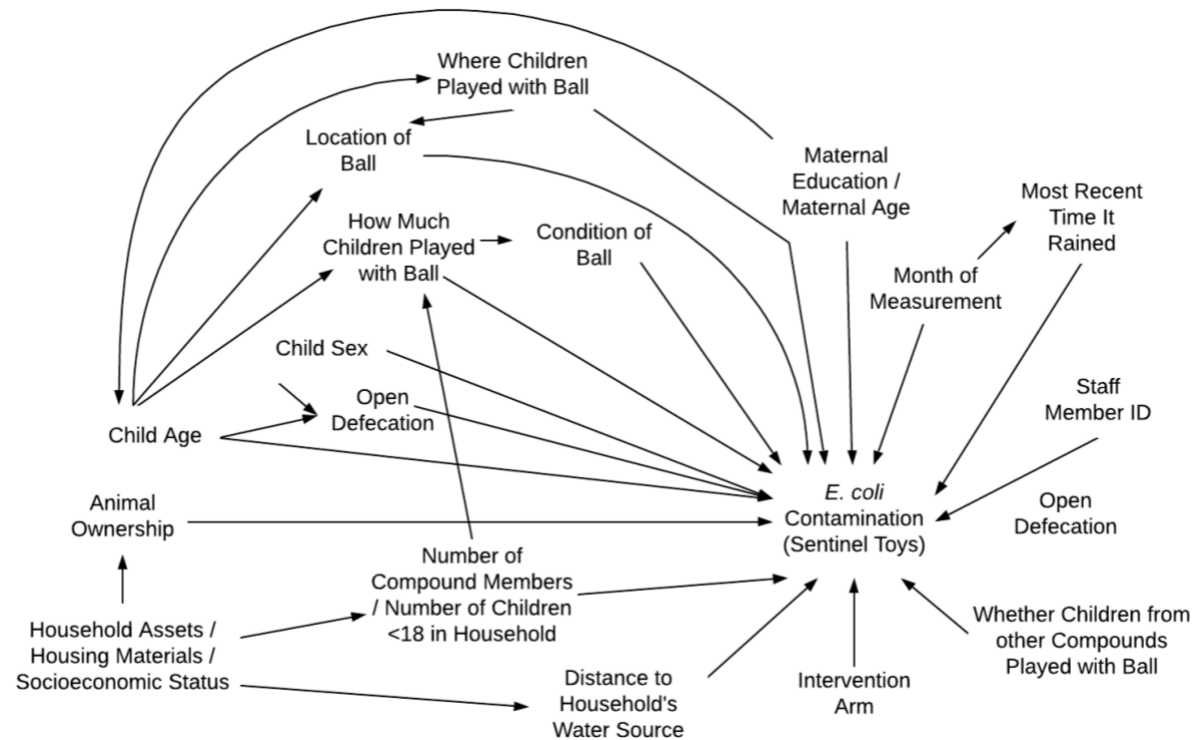


Figure B-3: Directed acyclic graph for *E. coli* contamination on sentinel toys

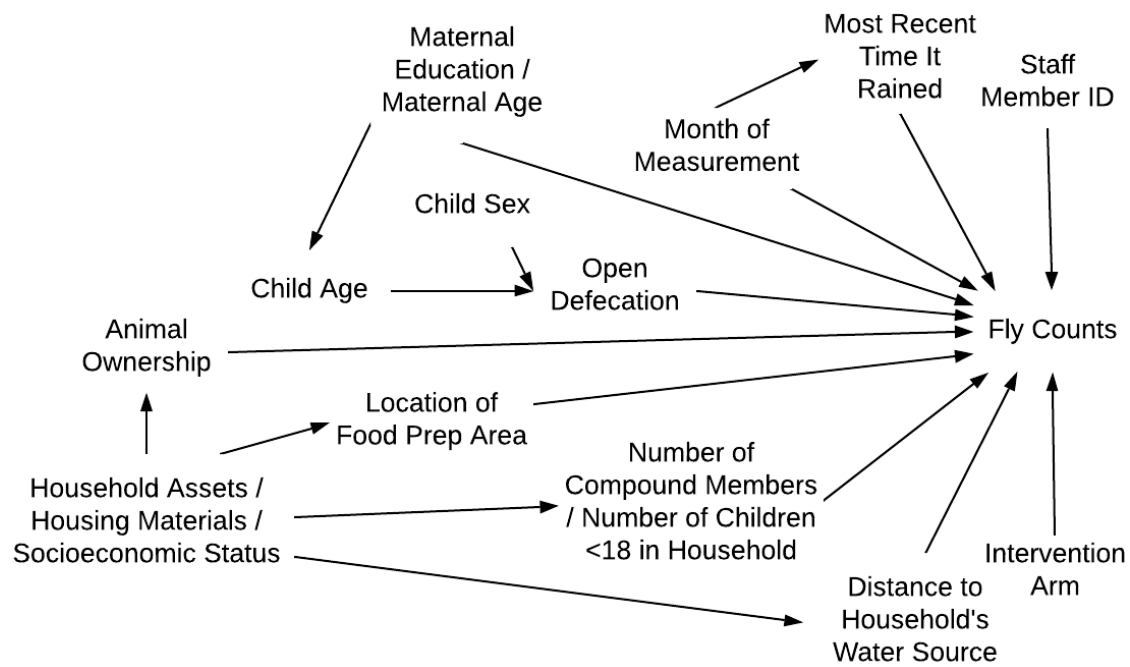


Figure B-4: Directed acyclic graph for fly counts

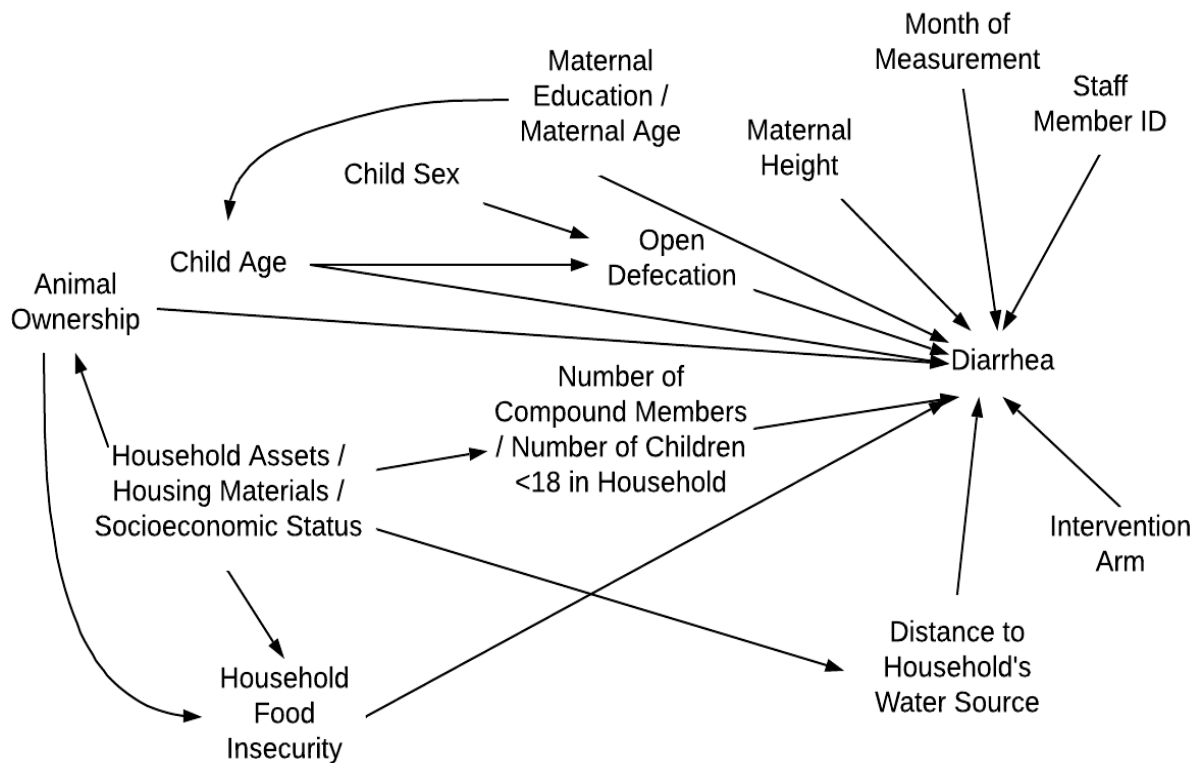


Figure B-5: Directed acyclic graph for diarrhea

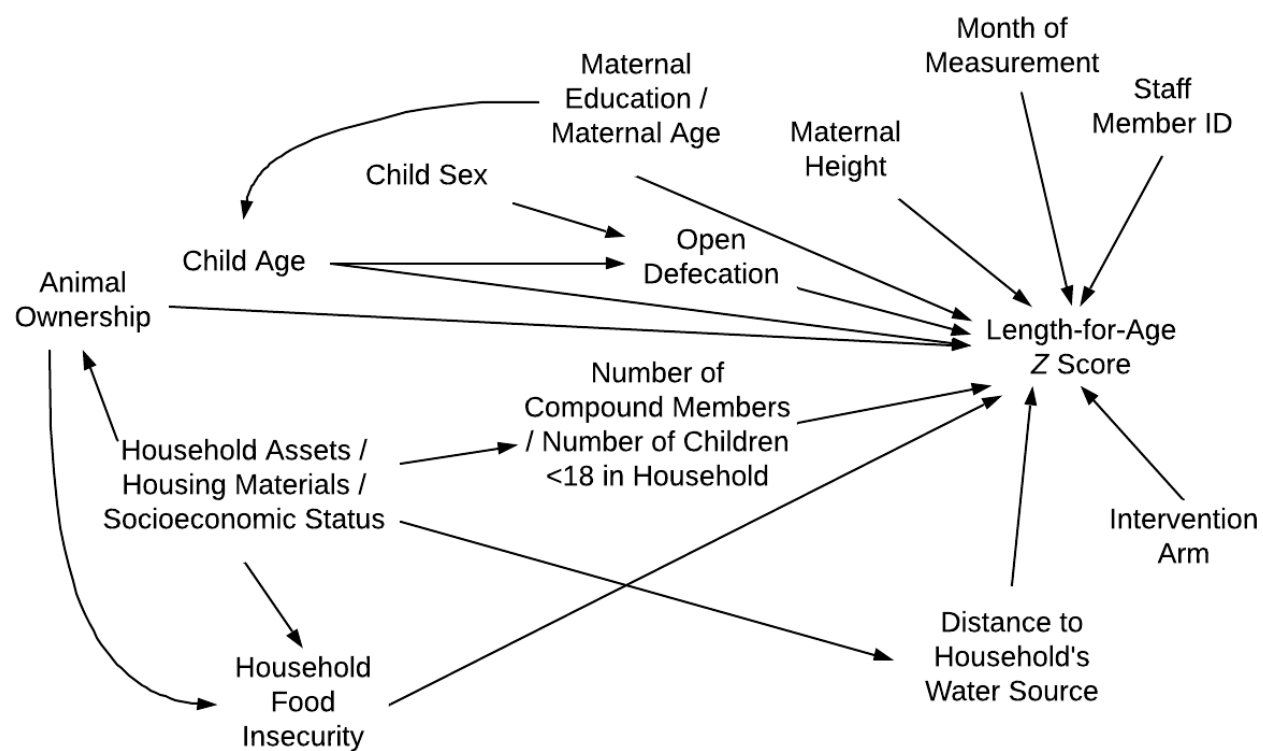


Figure B-6: Directed acyclic graph for length-for-age Z-scores

Table B-1: *E. coli* measured at one- and two-year assessments, combined and separately, interventions vs. control.

One- and Two-Year Assessments Combined															
Arms	N	Prev.	Log10 Mean (CFU/ 100 mL)	Prevalence Ratio						Log10 Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals	
Stored Water															
Control/Nutrition	1376	0.94	1.48	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1231	0.67	0.96	0.70	0.67	0.74	0.70	0.66	0.74	-0.53	-0.63	-0.43	-0.53	-0.63	-0.43
Water	635	0.68	0.97	0.73	0.68	0.78	0.72	0.68	0.78	-0.51	-0.62	-0.40	-0.49	-0.61	-0.38
Handwashing	667	0.93	1.44	0.98	0.95	1.00	0.98	0.95	1.00	-0.06	-0.16	0.03	-0.10	-0.20	-0.01
Child Hands															
Control/Nutrition	1378	0.90	1.74	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1313	0.91	1.80	1.00	0.98	1.03	1.00	0.98	1.03	0.04	-0.04	0.13	0.08	-0.01	0.17
Sentinel Toys															
Control/Nutrition	1276	0.73	0.58	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1146	0.72	0.62	0.97	0.93	1.02	0.97	0.91	1.03	0.02	-0.07	0.10	-0.02	-0.11	0.07
One-Year Assessment															
Arms	N	Prev.	Log10 Mean (CFU/ 100 mL)	Prevalence Ratio						Log10 Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals	
Stored Water															
Control/Nutrition	550	0.94	1.49	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	504	0.52	0.63	0.55	0.48	0.61	0.54	0.48	0.61	-0.90	-1.07	-0.74	-0.87	-1.03	-0.70
Water	251	0.62	0.83	0.66	0.58	0.76	0.65	0.57	0.75	-0.64	-0.86	-0.43	-0.66	-0.89	-0.42
Handwashing	275	0.95	1.45	1.01	0.97	1.05	1.02	0.98	1.05	-0.05	-0.22	0.12	-0.08	-0.26	0.09
Child Hands															
Control/Nutrition	518	0.91	1.78	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	520	0.91	1.79	1.00	0.95	1.04	1.00	0.95	1.04	0.00	-0.16	0.17	0.07	-0.10	0.23
Sentinel Toys															
Control/Nutrition	687	0.79	0.66	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	628	0.79	0.74	0.98	0.93	1.04	0.98	0.89	1.07	0.06	-0.08	0.19	0.02	-0.13	0.16
Two-Year Assessment															
Arms	N	Prev.	Log10 Mean (CFU/ 100 mL)	Prevalence Ratio						Log10 Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals	
Stored Water															
Control/Nutrition	826	0.94	1.48	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	727	0.76	1.19	0.81	0.76	0.85	0.81	0.76	0.85	-0.29	-0.40	-0.18	-0.30	-0.41	-0.19
Water	384	0.72	1.07	0.76	0.72	0.81	0.77	0.72	0.82	-0.43	-0.54	-0.32	-0.37	-0.49	-0.25
Handwashing	392	0.91	1.42	0.95	0.92	0.99	0.95	0.92	0.98	-0.07	-0.19	0.05	-0.10	-0.22	0.03
Child Hands															
Control/Nutrition	860	0.90	1.71	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	793	0.92	1.80	1.01	0.98	1.04	1.01	0.98	1.04	0.07	-0.05	0.20	0.13	0.00	0.26
Sentinel Toys															
Control/Nutrition	589	0.66	0.49	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	518	0.64	0.48	0.96	0.88	1.04	0.97	0.89	1.07	-0.04	-0.15	0.07	-0.04	-0.15	0.06

Table B-2: Total coliforms (water and hand samples) or fecal coliforms (toy samples) measured at one- and two-year assessments, combined and separately, interventions vs. control.

One- and Two-Year Assessments Combined															
Arms	N	Prev.	Log10 Mean (CFU/100 mL)	Prevalence Ratio						Log10 Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals	
Stored Water															
Control/Nutrition	1376	0.99	2.56	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1231	0.81	1.80	0.82	0.79	0.85	0.82	0.79	0.85	-0.76	-0.85	-0.67	-0.75	-0.83	-0.66
Water	635	0.84	1.85	0.85	0.82	0.89	0.85	0.82	0.89	-0.69	-0.83	-0.56	-0.66	-0.79	-0.54
Handwashing	664	0.98	2.52	0.99	0.98	1.00	0.99	0.98	1.01	-0.03	-0.09	0.03	-0.03	-0.09	0.02
Child Hands															
Control/Nutrition	1376	0.99	2.71	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1312	0.99	2.72	1.00	0.99	1.01	1.00	0.99	1.01	-0.01	-0.05	0.04	-0.01	-0.06	0.04
Sentinel Toys															
Control/Nutrition	1276	0.90	1.23	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1146	0.90	1.25	0.98	0.96	1.01	0.98	0.94	1.02	-0.01	-0.09	0.07	-0.02	-0.11	0.07
One-Year Assessment															
Arms	N	Prev.	Log10 Mean (CFU/100 mL)	Prevalence Ratio						Log10 Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals	
Stored Water															
Control/Nutrition	550	0.99	2.54	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	504	0.71	1.34	0.71	0.67	0.77	0.71	0.67	0.77	-1.22	-1.36	-1.08	-1.17	-1.31	-1.03
Water	251	0.82	1.66	0.82	0.77	0.87	0.82	0.76	0.87	-0.87	-1.09	-0.65	-0.86	-1.10	-0.63
Handwashing	272	0.98	2.50	0.99	0.97	1.01	0.99	0.97	1.01	-0.03	-0.10	0.04	-0.01	-0.09	0.07
Child Hands															
Control/Nutrition	516	0.99	2.63	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	520	0.99	2.62	1.00	0.98	1.01	1.00	0.98	1.01	-0.02	-0.10	0.06	-0.01	-0.08	0.07
Sentinel Toys															
Control/Nutrition	687	0.93	1.36	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	628	0.93	1.38	0.98	0.94	1.02	0.97	0.92	1.03	-0.01	-0.14	0.12	0.03	-0.12	0.18
Two-Year Assessment															
Arms	N	Prev.	Log10 Mean (CFU/100 mL)	Prevalence Ratio						Log10 Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals		Log10 Diff.	95% Confidence Intervals	
Stored Water															
Control/Nutrition	826	0.99	2.57	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	727	0.88	2.11	0.89	0.86	0.92	0.89	0.86	0.92	-0.46	-0.56	-0.36	-0.46	-0.57	-0.36
Water	384	0.86	1.97	0.87	0.83	0.92	0.87	0.83	0.92	-0.59	-0.74	-0.43	-0.55	-0.71	-0.39
Handwashing	392	0.99	2.54	1.00	0.98	1.01	1.00	0.98	1.01	-0.04	-0.13	0.04	-0.04	-0.12	0.04
Child Hands															
Control/Nutrition	860	0.99	2.75	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	792	1.00	2.78	1.00	1.00	1.01	1.00	1.00	1.01	0.00	-0.05	0.05	0.01	-0.05	0.06
Sentinel Toys															
Control/Nutrition	589	0.86	1.07	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	518	0.86	1.10	0.99	0.94	1.04	0.98	0.93	1.05	-0.02	-0.14	0.10	-0.06	-0.18	0.06

Table B-3: Fly counts and dirt on hands/fingernails measured at one- and two-year assessments, combined and separately, interventions vs. control

One- and Two-Year Assessments Combined															
Arms	N	Prev.	Mean	Prevalence Ratio						Fly Count Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Fly Diff.	95% Confidence Intervals		Fly Diff.	95% Confidence Intervals	
Flies Caught in Food Preparation Area															
Control/Nutrition	1882	0.62	3.37	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1386	0.62	3.26	1.01	0.94	1.08	1.00	0.94	1.07	-0.12	-0.60	0.36	-0.22	-0.70	0.26
Sanitation	1001	0.641	3.25	0.99	0.93	1.06	0.98	0.92	1.05	-0.30	-0.88	0.27	-0.23	-0.78	0.31
Flies Caught in Latrine Area															
Control/Nutrition	1793	0.67	3.72	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1354	0.64	3.19	0.96	0.91	1.01	0.96	0.91	1.01	-0.49	-0.87	-0.11	-0.52	-0.89	-0.15
Sanitation	971	0.70	3.52	1.02	0.96	1.08	1.01	0.96	1.07	-0.49	-1.00	0.01	-0.42	-0.95	0.10
Visible Dirt on Caregiver Hands															
Control/Nutrition	3605	0.26	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	2442	0.23	-	0.86	0.78	0.95	0.86	0.75	0.98	-	-	-	-	-	-
Visible Dirt under Caregiver Fingernails															
Control/Nutrition	3605	0.54	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	2442	0.48	-	0.90	0.85	0.96	0.91	0.84	0.98	-	-	-	-	-	-
Visible Dirt on Child Hands															
Control/Nutrition	3390	0.36	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	2288	0.34	-	0.92	0.85	1.01	0.87	0.78	0.97	-	-	-	-	-	-
Visible Dirt under Child Fingernails															
Control/Nutrition	3394	0.67	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	2290	0.66	-	0.99	0.94	1.03	0.95	0.89	1.02	-	-	-	-	-	-
One-Year Assessment															
Arms	N	Prev.	Mean	Prevalence Ratio						Fly Count Difference					
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Fly Diff.	95% Confidence Intervals		Fly Diff.	95% Confidence Intervals	
Flies Caught in Food Preparation Area															
Control/Nutrition	562	0.65	3.80	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	590	0.65	3.68	0.97	0.89	1.06	0.98	0.89	1.07	-0.28	-1.18	0.63	-0.24	-1.17	0.69
Sanitation	395	0.64	3.30	0.94	0.81	1.10	0.93	0.79	1.09	-0.42	-1.50	0.66	-0.69	-1.87	0.49
Flies Caught in Latrine Area															
Control/Nutrition	535	0.66	3.53	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	578	0.59	2.84	0.88	0.79	0.97	0.86	0.77	0.95	-0.59	-1.16	-0.02	-0.66	-1.24	-0.08
Sanitation	384	0.69	3.25	0.97	0.84	1.12	0.94	0.81	1.10	-0.18	-0.92	0.57	-0.55	-1.43	0.33
Visible Dirt on Caregiver Hands															
Control/Nutrition	1494	0.26	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1027	0.21	-	0.81	0.70	0.93	0.91	0.75	1.11	-	-	-	-	-	-
Visible Dirt under Caregiver Fingernails															
Control/Nutrition	1494	0.47	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1027	0.40	-	0.87	0.79	0.96	0.91	0.80	1.05	-	-	-	-	-	-
Visible Dirt on Child Hands															
Control/Nutrition	1285	0.31	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	882	0.28	-	0.87	0.74	1.03	0.79	0.61	1.02	-	-	-	-	-	-
Visible Dirt under Child Fingernails															
Control/Nutrition	1289	0.57	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	884	0.56	-	1.00	0.91	1.09	1.01	0.89	1.15	-	-	-	-	-	-

Arms	N	Prev.	Mean	Two-Year Assessment						Fly Count Difference					
				Prevalence Ratio											
				Unadjusted			Adjusted			Unadjusted			Adjusted		
				Prev. Ratio	95% Confidence Intervals		Prev. Ratio	95% Confidence Intervals		Fly Diff.	95% Confidence Intervals		Fly Diff.	95% Confidence Intervals	
Flies Caught in Food Preparation Area															
Control/Nutrition	1320	0.60	3.18	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	796	0.60	2.95	1.01	0.93	1.10	1.01	0.93	1.10	-0.20	-0.72	0.33	-0.16	-0.67	0.35
Sanitation	606	0.64	3.21	1.01	0.93	1.09	1.01	0.93	1.09	-0.24	-0.95	0.47	-0.02	-0.64	0.60
Flies Caught in Latrine Area															
Control/Nutrition	1258	0.67	3.80	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	776	0.67	3.45	1.02	0.96	1.08	1.01	0.95	1.08	-0.34	-0.78	0.10	-0.48	-0.91	-0.06
Sanitation	587	0.71	3.69	1.04	0.96	1.13	1.03	0.95	1.11	-0.46	-1.09	0.16	-0.56	-1.17	0.05
Visible Dirt on Caregiver Hands															
Control/Nutrition	2111	0.26	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1415	0.24	-	0.90	0.79	1.02	0.84	0.69	1.01	-	-	-	-	-	-
Visible Dirt under Caregiver Fingernails															
Control/Nutrition	2111	0.58	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1415	0.54	-	0.92	0.87	0.99	0.91	0.83	1.00	-	-	-	-	-	-
Visible Dirt on Child Hands															
Control/Nutrition	2105	0.39	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1406	0.37	-	0.95	0.86	1.04	0.91	0.79	1.05	-	-	-	-	-	-
Visible Dirt under Child Fingernails															
Control/Nutrition	2105	0.74	-	-	-	-	-	-	-	-	-	-	-	-	-
WSH/WSH+Nutrition	1406	0.72	-	0.98	0.93	1.03	0.94	0.88	1.01	-	-	-	-	-	-

Table B-4: *E. coli* and fly prevalence and concentration measured at one- and two-year assessments, combined and separately, interventions vs. control, subgroup analysis by season.

One- and Two-Year Assessments Combined																						
Arms	Prevalence										Inter-action P-Value	Concentration										
	Wet Season					Dry Season						Wet Season					Dry Season					
	N	Prev.	Prev. Ratio	95% Confidence Intervals		N	Prev.	Prev. Ratio	95% Confidence Intervals			N	Log10 Mean (CFU/ 100 mL)/ Mean (Flies)	Log Diff./ Fly Count	95% Confidence Intervals		N	Log10 Mean (CFU/ 100 mL)/ Mean (Flies)	Log Diff./ Fly Count	95% Confidence Intervals		
Stored Water																						
Control/Nutrition	737	0.94	-	-	-	637	0.94	-	-	-	-	731	1.51	-	-	-	635	1.44	-	-	-	-
WSH/ WSH+Nutrition	665	0.65	0.69	0.64	0.75	565	0.68	0.72	0.67	0.77	0.544	664	0.95	-0.57	-0.70	-0.44	564	0.966	-0.47	-0.61	-0.34	0.282
Water	361	0.65	0.69	0.62	0.76	274	0.72	0.77	0.71	0.84	0.091	361	0.95	-0.57	-0.75	-0.39	274	1.003	-0.44	-0.56	-0.32	0.262
Handwashing	363	0.93	0.98	0.94	1.02	304	0.92	0.97	0.93	1.01	0.834	362	1.47	-0.05	-0.18	0.08	304	1.395	-0.07	-0.21	0.07	0.812
Child Hands																						
Control/Nutrition	743	0.89	-	-	-	632	0.92	-	-	-	-	724	1.68	-	-	-	623	1.81	-	-	-	-
WSH/ WSH+Nutrition	714	0.89	0.99	0.96	1.03	597	0.93	1.02	0.99	1.04	0.370	705	1.71	-0.02	-0.13	0.10	593	1.905	0.13	-0.01	0.26	0.127
Sentinel Toys																						
Control/Nutrition	627	0.72	-	-	-	649	0.74	-	-	-	-	616	0.59	-	-	-	644	0.57	-	-	-	-
WSH/ WSH+Nutrition	591	0.73	0.99	0.93	1.04	555	0.71	0.96	0.89	1.03	0.534	587	0.67	0.02	-0.07	0.11	552	0.57	0.01	-0.13	0.15	0.873
Flies Caught in Food Preparation Area																						
Control/Nutrition	946	0.65	-	-	-	936	0.59	-	-	-	-	946	3.78	-	-	-	936	2.95	-	-	-	-
WSH/ WSH+Nutrition	745	0.62	0.98	0.91	1.05	641	0.62	1.03	0.93	1.14	0.396	745	3.35	-0.54	-1.26	0.18	641	3.15	0.24	-0.28	0.76	0.075
Sanitation	527	0.68	1.00	0.92	1.09	474	0.60	0.97	0.87	1.09	0.724	527	3.63	-0.48	-1.35	0.38	474	2.82	-0.11	-0.72	0.49	0.462
Flies Caught in Latrine Area																						
Control/Nutrition	912	0.70	-	-	-	881	0.63	-	-	-	-	912	4.17	-	-	-	881	3.25	-	-	-	-
WSH/ WSH+Nutrition	731	0.64	0.93	0.87	1.00	623	0.63	0.97	0.89	1.07	0.488	731	3.20	-0.82	-1.29	-0.34	623	3.17	-0.22	-0.78	0.34	0.111
Sanitation	515	0.70	0.96	0.89	1.04	456	0.69	1.09	1.00	1.18	0.041	515	3.74	-0.84	-1.58	-0.09	456	3.27	-0.12	-0.70	0.46	0.110

One-Year Assessment																						
Arms	Prevalence											Concentration										
	Wet Season					Dry Season					Inter-action P-Value	Wet Season					Dry Season					Inter-action P-Value
	N	Prev.	Prev. Ratio	95% Confidence Intervals	N	Prev.	Prev. Ratio	95% Confidence Intervals	Log10 Mean (CFU/ 100 mL)/ Mean (Flies)	Log Diff./ Fly Count		95% Confidence Intervals	N	Log10 Mean (CFU/ 100 mL)/ Mean (Flies)	Log Diff./ Fly Count	95% Confidence Intervals						
Stored Water																						
Control/Nutrition	313	0.95	-	-	-	235	0.93	-	-	-	-	311	1.59	-	-	-	233	1.35	-	-	-	-
WSH/ WSH+Nutrition	301	0.56	0.59	0.51	0.69	86	0.64	0.49	0.41	0.58	0.086	300	0.73	-0.86	-1.09	-0.63	202	0.478	-0.93	-1.16	-0.71	0.658
Water	165	0.61	0.64	0.54	0.77	120	0.93	0.70	0.57	0.87	0.519	165	0.85	-0.72	-1.02	-0.43	86	0.795	-0.51	-0.78	-0.24	0.301
Handwashing	155	0.96	1.01	0.96	1.07	202	0.47	1.01	0.94	1.09	0.998	154	1.56	-0.06	-0.26	0.15	120	1.316	-0.03	-0.30	0.24	0.859
Child Hands																						
Control/Nutrition	295	0.93	-	-	-	220	0.89	-	-	-	-	285	1.88	-	-	-	218	1.64	-	-	-	-
WSH/ WSH+Nutrition	321	0.93	1.00	0.95	1.05	197	0.87	0.99	0.91	1.08	0.872	317	1.93	0.05	-0.13	0.22	196	1.562	-0.04	-0.34	0.27	0.639
Sentinel Toys																						
Control/Nutrition	327	0.77	-	-	-	360	0.81	-	-	-	-	321	0.72	-	-	-	357	0.60	-	-	-	-
WSH/ WSH+Nutrition	322	0.79	1.01	0.94	1.08	306	0.78	0.95	0.88	1.04	0.347	319	0.79	0.05	-0.10	0.19	303	0.69	0.07	-0.15	0.29	0.868
Flies Caught in Food Preparation Area																						
Control/Nutrition	304	0.69	-	-	-	231	0.63	-	-	-	-	314	4.43	-	-	-	248	3.00	-	-	-	-
WSH/ WSH+Nutrition	345	0.59	0.95	0.85	1.06	233	0.60	1.03	0.89	1.19	0.364	356	3.86	-0.83	-2.18	0.52	234	3.39	0.46	-0.72	1.64	0.169
Sanitation	203	0.68	0.96	0.79	1.18	181	0.69	0.91	0.71	1.18	0.759	207	3.58	-1.08	-2.51	0.35	188	2.99	0.52	-1.13	2.18	0.162
Flies Caught in Latrine Area																						
Control/Nutrition	304	0.69	-	-	-	231	0.63	-	-	-	-	304	3.90	-	-	-	231	3.04	-	-	-	-
WSH/ WSH+Nutrition	345	0.59	0.84	0.74	0.95	233	0.60	0.90	0.75	1.07	0.538	345	2.92	-0.96	-1.78	-0.15	233	2.74	-0.16	-1.10	0.78	0.250
Sanitation	203	0.68	0.94	0.78	1.12	181	0.69	1.02	0.82	1.28	0.542	203	3.62	-0.55	-1.59	0.49	181	2.83	0.36	-0.90	1.63	0.304

Two-Year Assessment																						
Arms	Prevalence											Concentration										
	Wet Season					Dry Season					Inter-action P-Value	Wet Season					Dry Season					Inter-action P-Value
	N	Prev.	Prev. Ratio	95% Confidence Intervals		N	Prev.	Prev. Ratio	95% Confidence Intervals			N	Log10 Mean (CFU/ 100 mL)/ Mean (Flies)	Log Diff./ Fly Count	95% Confidence Intervals		N	Log10 Mean (CFU/ 100 mL)/ Mean (Flies)	Log Diff./ Fly Count	95% Confidence Intervals		
Stored Water																						
Control/Nutrition	424	0.94	-	-	-	402	0.95	-	-	-	-	420	1.46	-	-	-	402	1.50	-	-	-	-
WSH/ WSH+Nutrition	364	0.73	0.78	0.71	0.85	188	0.76	0.84	0.79	0.89	0.199	364	1.14	-0.32	-0.47	-0.17	362	1.238	-0.26	-0.39	-0.12	0.515
Water	196	0.68	0.72	0.65	0.80	184	0.91	0.81	0.74	0.88	0.099	196	1.04	-0.46	-0.62	-0.31	188	1.10	-0.39	-0.55	-0.23	0.530
Handwashing	208	0.90	0.95	0.91	1.00	363	0.80	0.95	0.91	0.99	0.913	208	1.40	-0.07	-0.25	0.112	184	1.446	-0.08	-0.23	0.08	0.968
Child Hands																						
Control/Nutrition	448	0.86	-	-	-	412	0.94	-	-	-	-	439	1.54	-	-	-	405	1.90	-	-	-	-
WSH/ WSH+Nutrition	393	0.87	0.99	0.94	1.05	400	0.97	1.02	0.99	1.05	0.356	388	1.53	-0.05	-0.21	0.122	397	2.075	0.19	0.02	0.37	0.059
Sentinel Toys																						
Control/Nutrition	300	0.66	-	-	-	289	0.66	-	-	-	-	295	0.46	-	-	-	287	0.53	-	-	-	-
WSH/ WSH+Nutrition	269	0.66	0.97	0.87	1.08	249	0.62	0.95	0.84	1.08	0.874	268	0.52	0.006	-0.14	0.149	249	0.43	-0.09	-0.25	0.07	0.381
Flies Caught in Food Preparation Area																						
Control/Nutrition	632	0.63	-	-	-	688	0.57	-	-	-	-	632	3.45	-	-	-	688	2.93	-	-	-	-
WSH/ WSH+Nutrition	389	0.60	0.98	0.88	1.09	407	0.61	1.04	0.93	1.17	0.405	389	2.89	-0.60	-1.49	0.30	407	3.01	0.17	-0.41	0.74	0.155
Sanitation	320	0.68	1.04	0.94	1.14	286	0.59	0.97	0.85	1.11	0.462	320	3.66	-0.26	-1.39	0.88	286	2.71	-0.22	-0.82	0.39	0.946
Flies Caught in Latrine Area																						
Control/Nutrition	608	0.71	-	-	-	650	0.63	-	-	-	-	608	4.30	-	-	-	650	3.33	-	-	-	-
WSH/ WSH+Nutrition	386	0.69	1.00	0.93	1.09	390	0.65	1.03	0.93	1.14	0.696	386	3.46	-0.61	-1.23	0.00	390	3.44	-0.10	-0.73	0.53	0.256
Sanitation	312	0.72	1.01	0.91	1.13	275	0.69	1.08	0.95	1.23	0.435	312	3.82	-0.83	-1.84	0.17	275	3.55	-0.08	-0.72	0.57	0.201

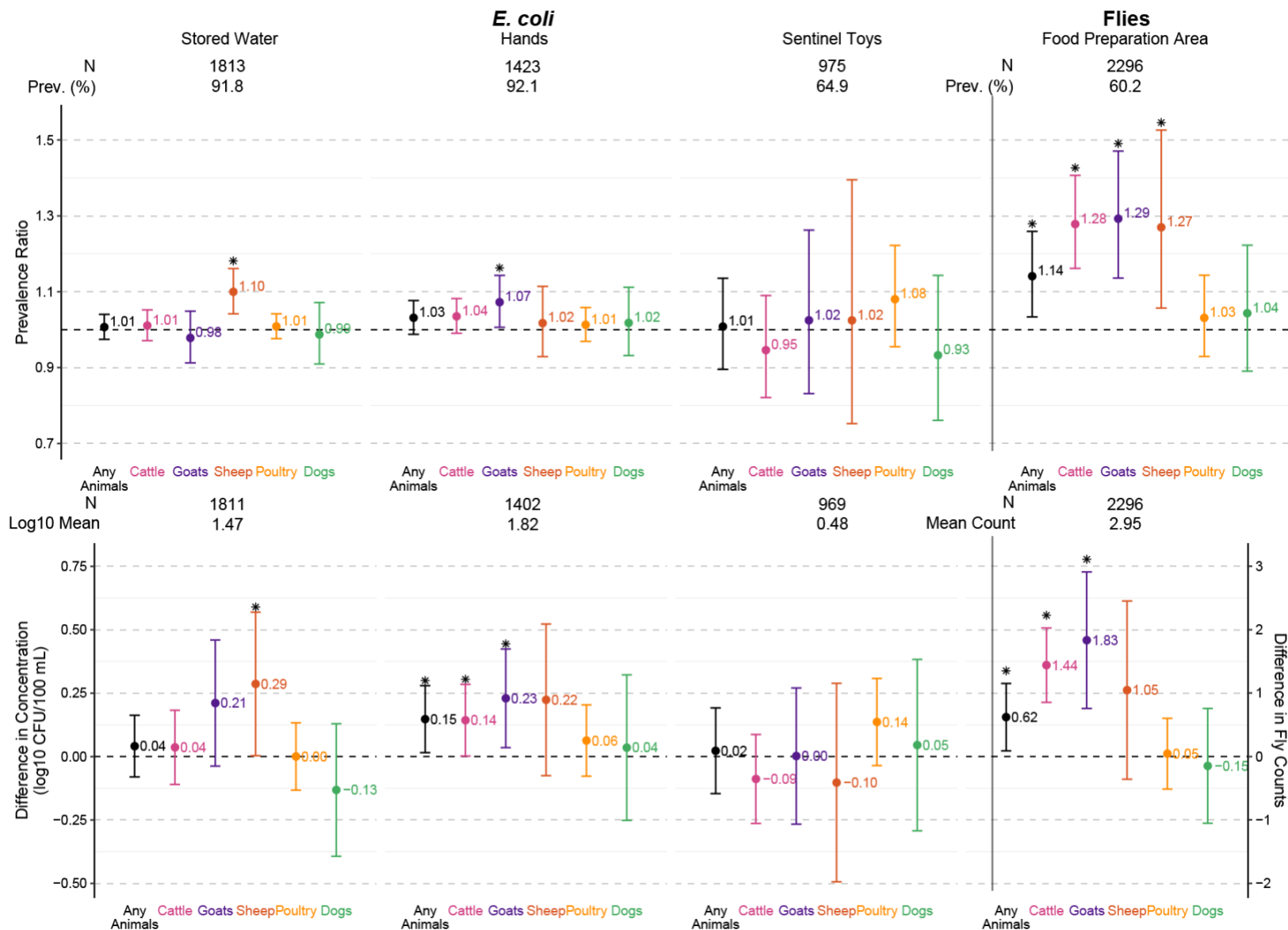


Figure B-7: Associations between $\log_{10}$ number of animals (capped at five per species) owned by households and environmental contamination. Prevalence ratios (top) and differences in $\log_{10}$ concentrations (bottom) for *E. coli* in stored water, on child hands, and on toys for each additional $\log_{10}$ animal owned (left); prevalence ratios (top) and differences in fly counts for flies at food preparation areas for each additional $\log_{10}$ animal owned (right). Asterisks indicate significance at a significance level of 0.05.

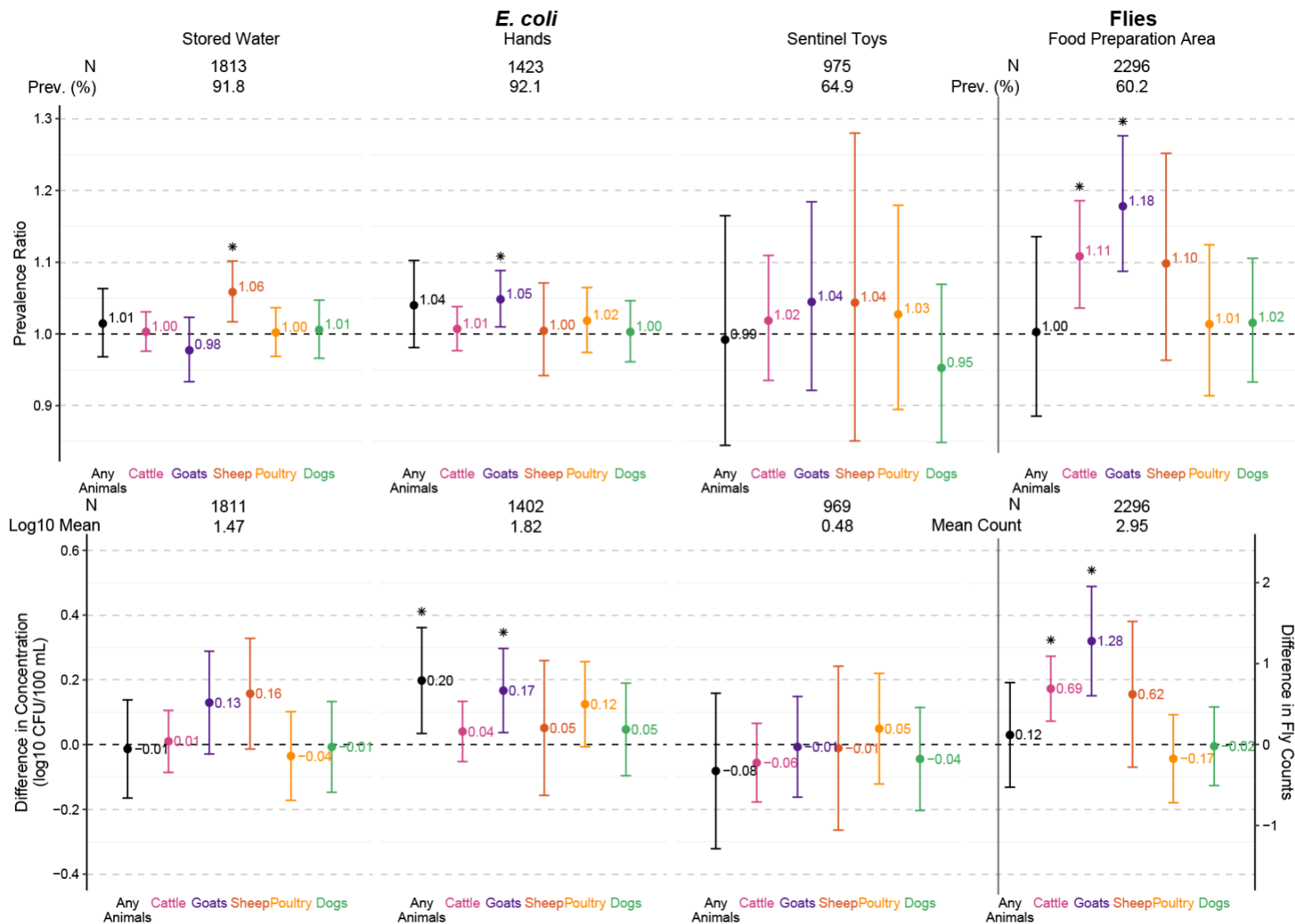


Figure B-8: Associations between binary household animal ownership and environmental contamination. Prevalence ratios (top) and differences in $\log_{10}$ concentrations (bottom) for *E. coli* in stored water, on child hands, and on toys in animal-owning compared to non-animal-owning households (left); prevalence ratios (top) and differences in fly counts for flies at food preparation in animal-owning compared to non-animal-owning households (right). Asterisks indicate significance at a significance level of 0.05.

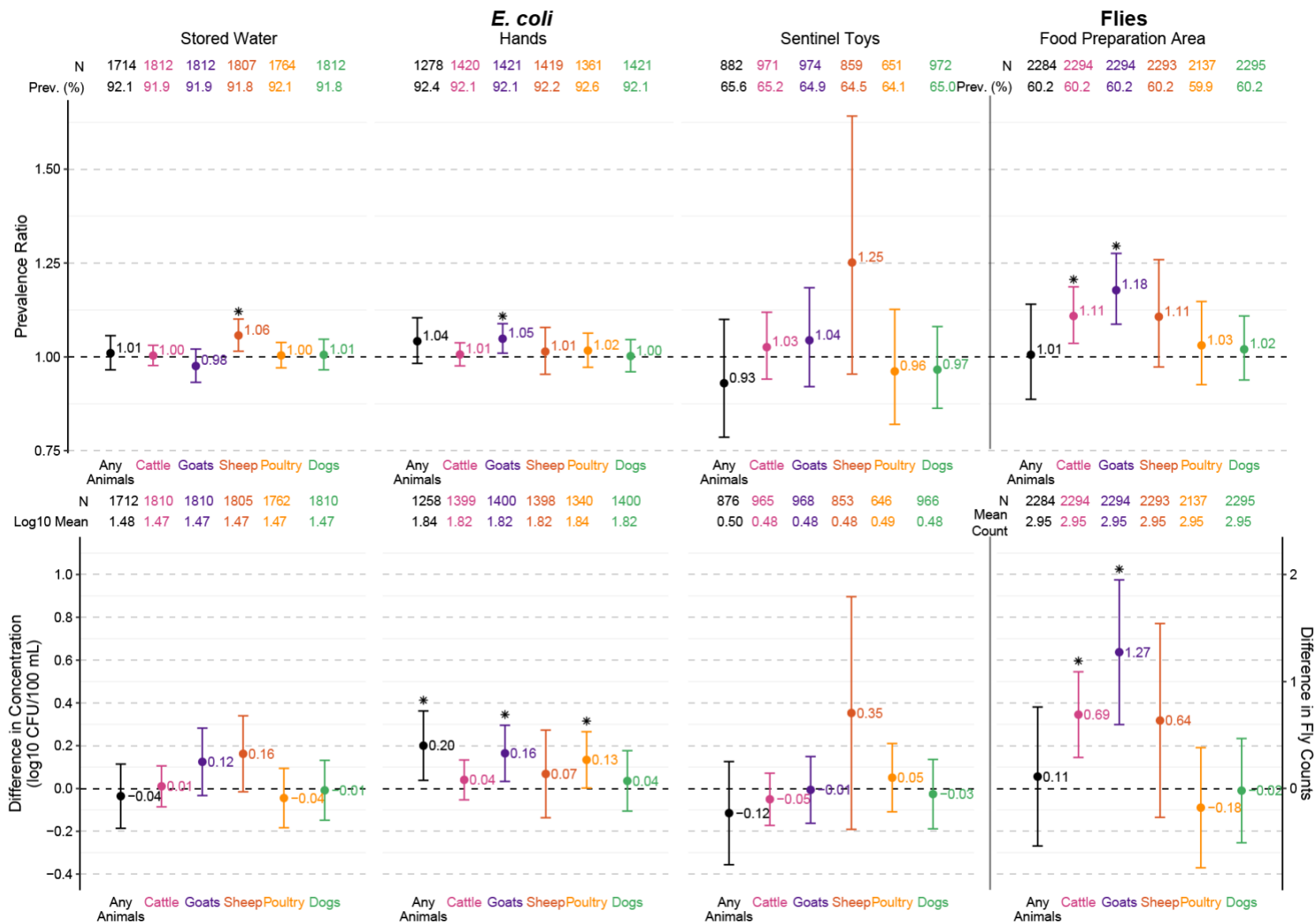


Figure B-9: Associations between binary household animal ownership (households with extreme predicted probabilities excluded) and environmental contamination. Prevalence ratios (top) and differences in $\log_{10}$ concentrations (bottom) for *E. coli* in stored water, on child hands, and on toys in animal-owning compared to non-animal-owning households (left); prevalence ratios (top) and differences in fly counts for flies at food preparation in animal-owning compared to non-animal-owning households (right). Asterisks indicate significance at a significance level of 0.05.



Figure B-10: Associations between $\log_{10}$ median number of animals owned by households in the same village and environmental contamination. Prevalence ratios (top) and differences in $\log_{10}$ concentrations (bottom) for *E. coli* in stored water, on child hands, and on toys for each additional $\log_{10}$ animal owned (left); prevalence ratios (top) and differences in fly counts for flies at food preparation areas for each additional $\log_{10}$ animal owned (right). Asterisks indicate significance at a significance level of 0.05.

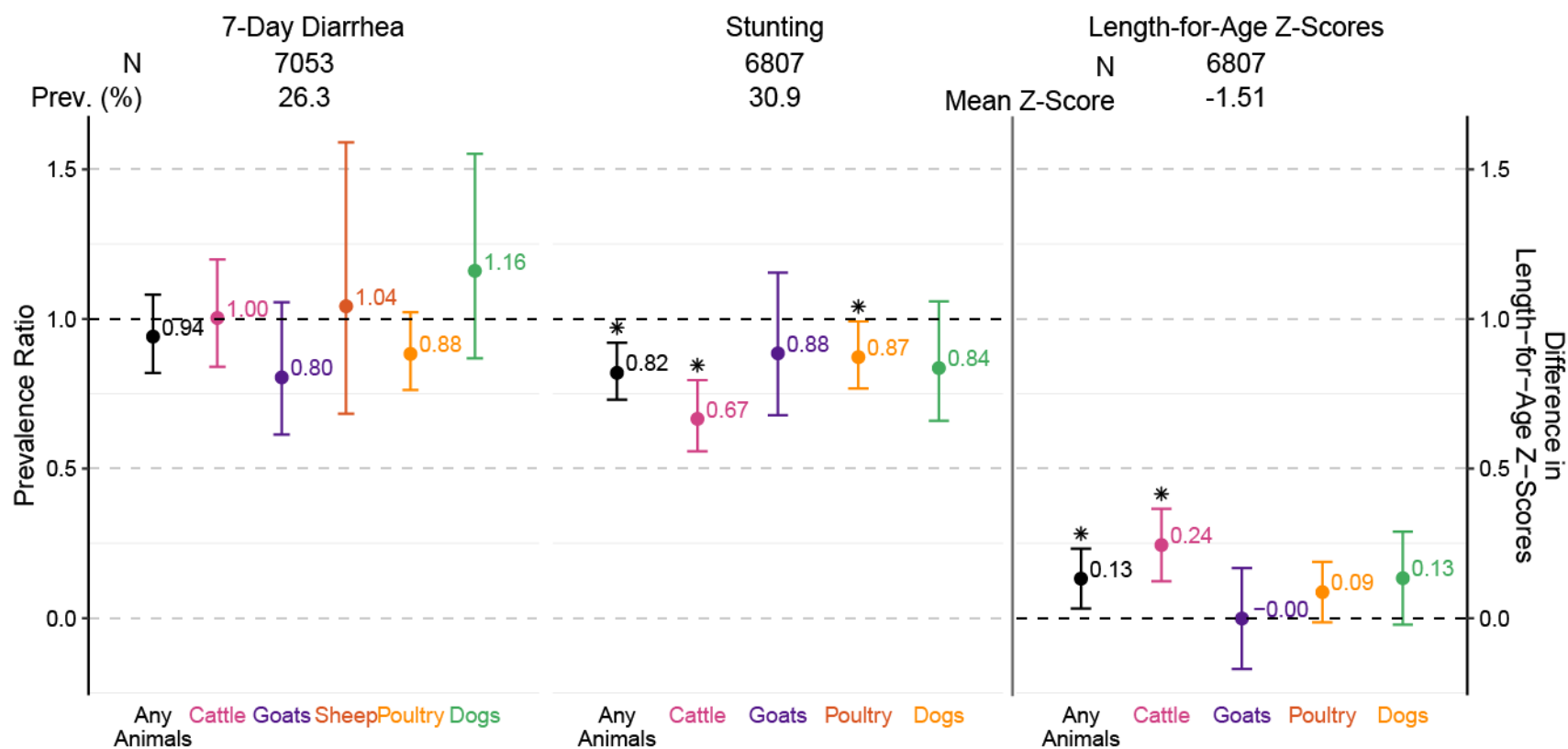


Figure B-11: Associations between $\log_{10}$ number of animals (capped at five per species) owned by households and child health. Prevalence ratios for diarrhea and stunting for each additional $\log_{10}$ animal owned (left); differences in length-for-age Z-scores for each additional $\log_{10}$ animal owned (right). Asterisks indicate significance at a significance level of 0.05.

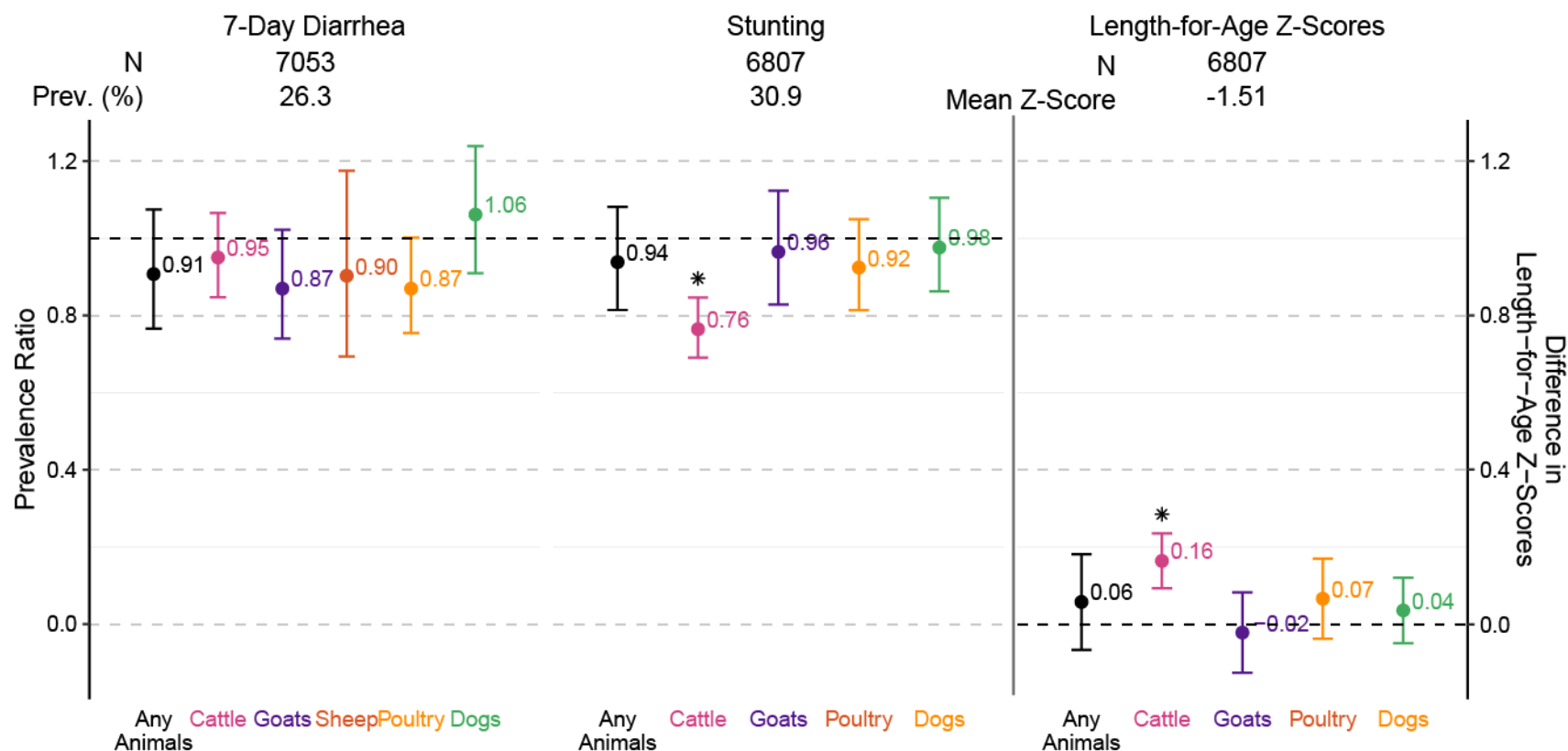


Figure B-12: Associations between binary household animal ownership and child health. Prevalence ratios for diarrhea and stunting in animal-owning compared to non-animal-owning households (left); differences in length-for-age Z-scores in animal-owning compared to non-animal-owning households (right). Asterisks indicate significance at a significance level of 0.05.

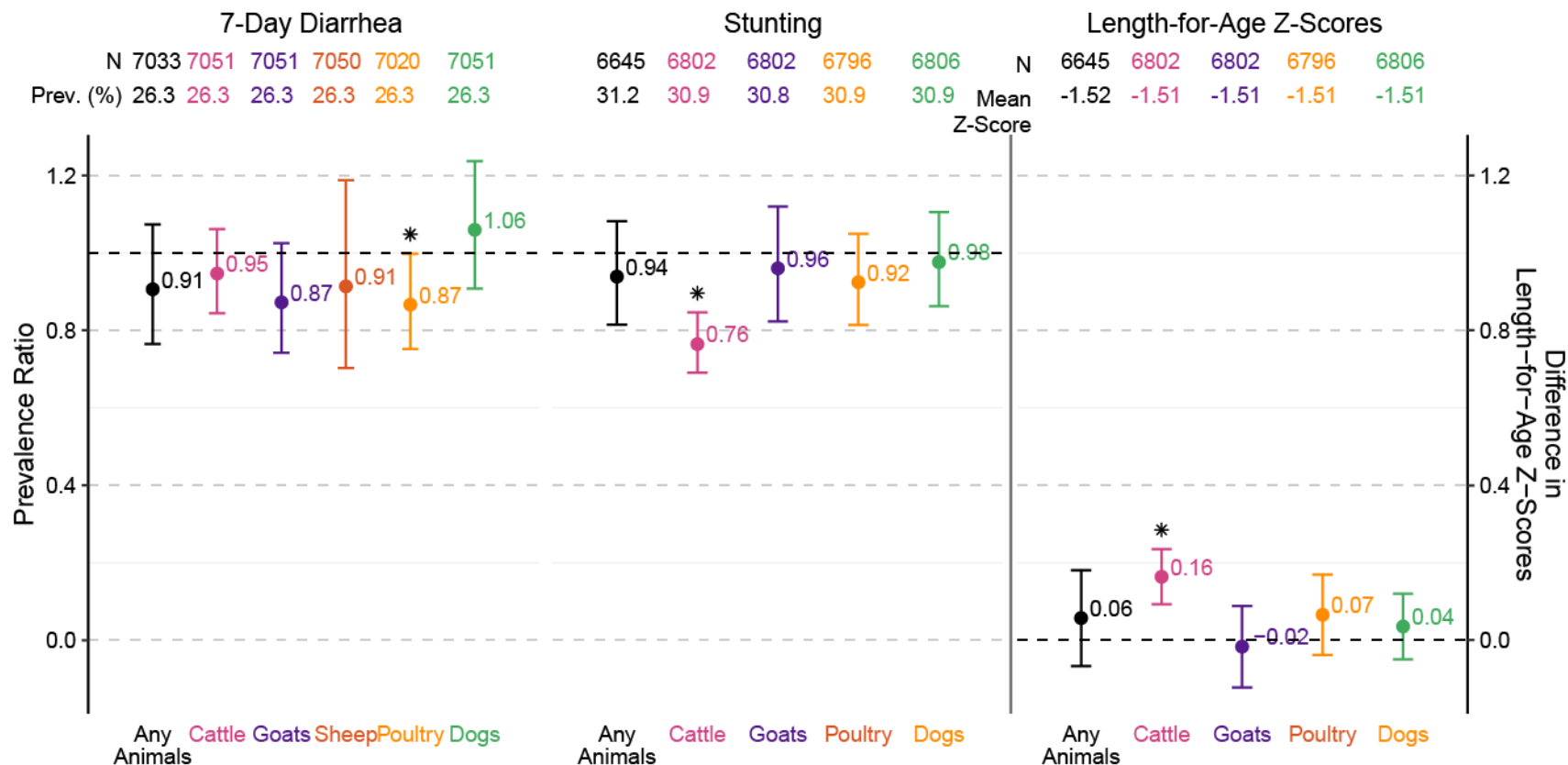


Figure B-13: Associations between binary household animal ownership (households with extreme predicted probabilities excluded) and child health. Prevalence ratios for diarrhea and stunting in animal-owning compared to non-animal-owning households (left); differences in length-for-age Z-scores in animal-owning compared to non-animal-owning households (right). Asterisks indicate significance at a significance level of 0.05.

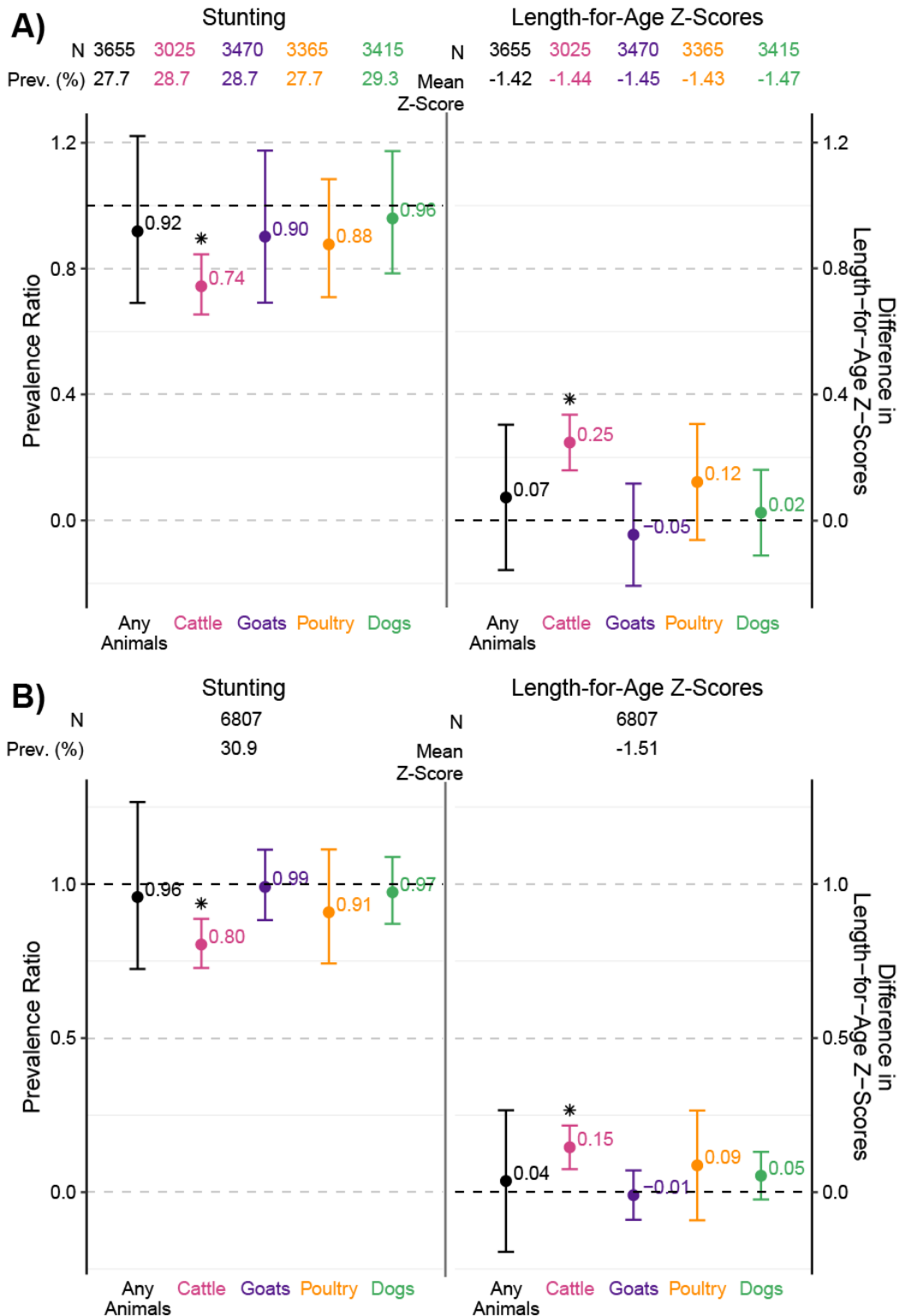


Figure B-14: Associations between binary household animal ownership and stunting outcomes, accounting for households that changed animal ownership status between enrollment and year two. Prevalence ratios for stunting (left) and differences in length-for-age Z-scores (right) in animal-owning compared to non-animal-owning households (left), where (A) excludes households that changed ownership status and (B) reclassifies households that obtained animals during the study as animal-owning households at enrollment. Asterisks indicate significance at a significance level of 0.05.

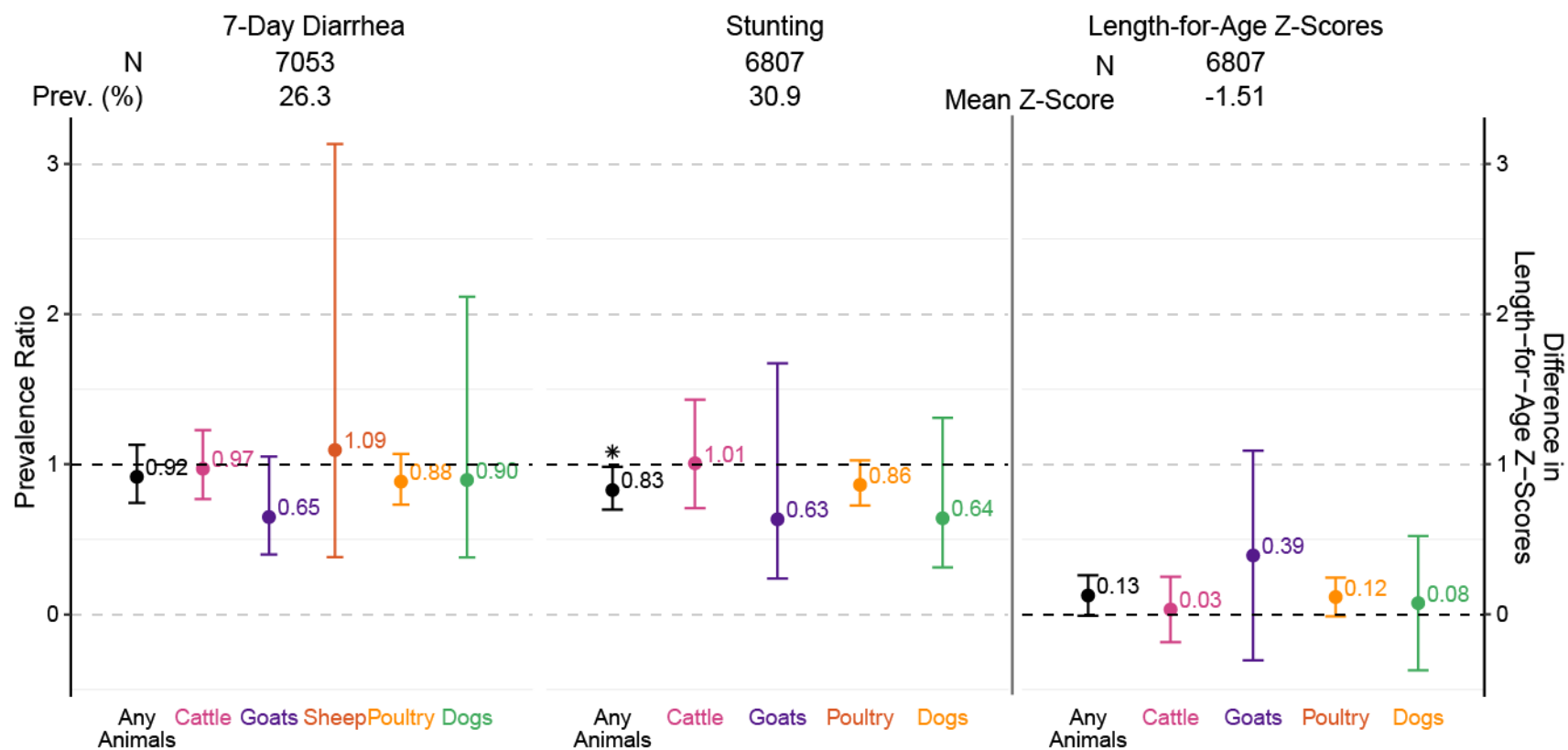


Figure B-15: Associations between $\log_{10}$ median number of animals owned by households in the same village and child health. Prevalence ratios for diarrhea and stunting for each additional $\log_{10}$ animal owned (left); differences in length-for-age Z-scores for each additional $\log_{10}$ animal owned (right). Asterisks indicate significance at a significance level of 0.05.

8.3 Appendix C – Chapter 4

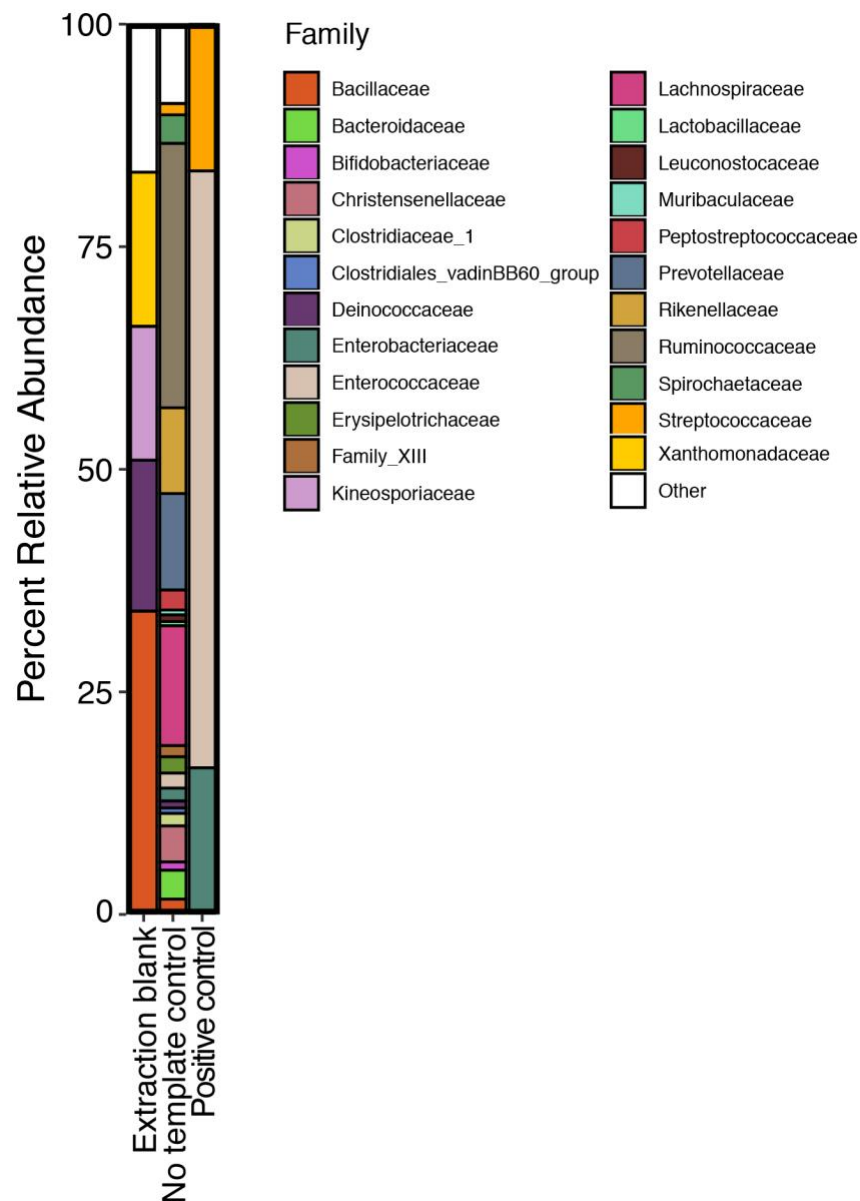


Figure C-1: Relative abundance of the 30 most abundant families in all controls from 16S rRNA gene analysis. All other taxa are grouped into “Other”.

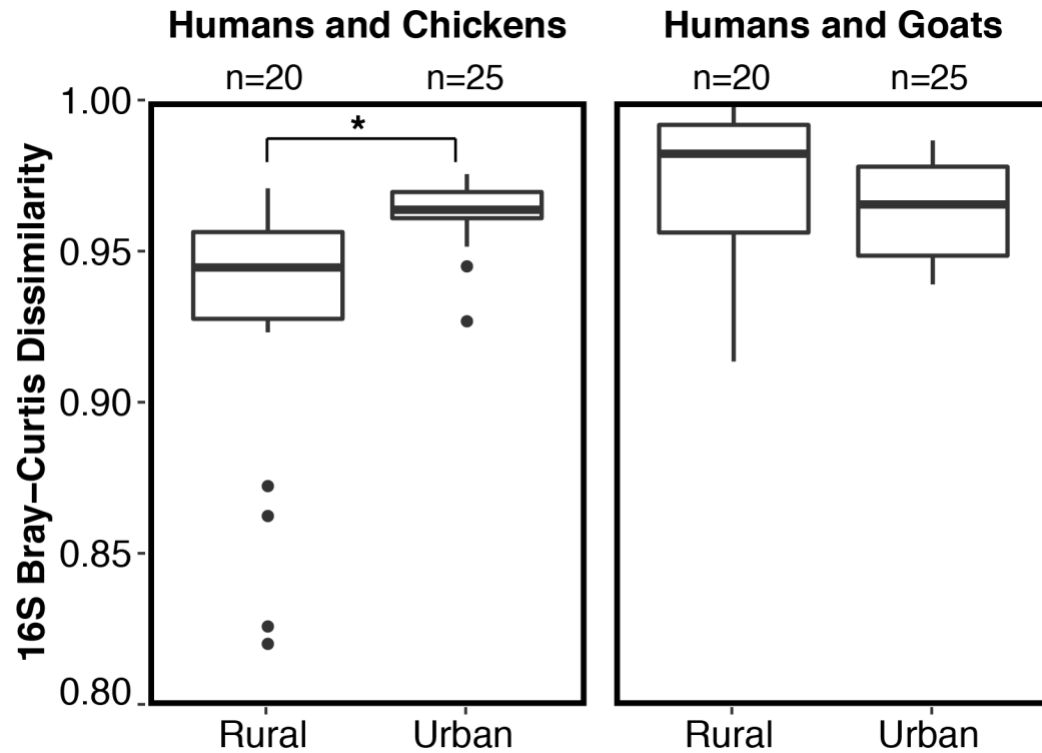


Figure C-2: Bray-Curtis dissimilarity between bacterial community compositions in humans and chickens (left) and in humans and goats (right) with taxa present in no template control removed

Table C-1: Descriptive statistics for long-read sequencing and assembly

Fecal Host	Raw Reads				Assembled Contigs		
	Number Reads (millions)	Mean Length [SD] (bp)	Total Data (Gbp)	Bacterial Data (Gbp)	Number Contigs (thousands)	N50 (kbp)	Median Length (kbp)
Rural Chicken	6.415	668.8 [1013]	4.291	2.747	1.636	34.33	21.79
Urban Chicken	9.834	809.2 [1151]	7.958	5.129	4.598	44.95	21.54
Rural Goat	11.19	2096 [2699]	23.44	14.98	38.60	44.73	24.15
Urban Goat	8.821	2391 [3367]	21.09	14.77	24.52	60.33	31.62
Rural Human	6.610	2864 [2799]	18.93	14.22	17.73	60.71	22.82
Urban Human	9.964	2327 [2406]	23.18	18.05	27.00	53.86	22.03

Table C-2: Comparisons of normalized abundance of antibiotic resistance genes in humans, chickens, and goats using Wilcoxon rank sum tests

Comparison	Location	Chicken		Goat		Human		Z-score	p-Value
		N	Rank Sum	N	Rank Sum	N	Rank Sum		
Chicken vs. Human	Rural	4	15	NA	NA	4	21	-0.866	0.387
	Urban	4	18	NA	NA	4	18	0.000	1.000
Goat vs. Human	Rural	NA	NA	3	6	4	22	-2.121	0.034
	Urban	NA	NA	4	10	4	26	-2.309	0.021
Chicken vs. Goat	Rural	4	22	3	6	NA	NA	2.121	0.034
	Urban	4	26	4	10	NA	NA	2.309	0.021

Table C-3: Comparisons of normalized abundance of antibiotic resistance genes, paired by drug class, in rural versus urban communities using Wilcoxon signed-rank tests

Fecal Host	N	Z-score	p-Value
Chicken	13	-2.411	0.016
Goat	13	-2.385	0.017
Human	13	-0.841	0.400

Table C-4: Comparisons of Bray-Curtis dissimilarity indices between resistomes within humans, chickens, and goats using Wilcoxon rank sum tests

Host	Rural		Urban		p-Value
	Median	IQR	Median	IQR	
Chicken	0.655	0.169	0.794	0.221	0.064
Goat	0.534	0.046	0.324	0.046	<0.001
Human	0.395	0.231	0.289	0.068	0.005

Table C-5: BLASTN confirmation of potential pathogens classified to the strain or subspecies level and carrying antibiotic resistance genes

Fecal Host	Potential Pathogen	NCBI Tax ID	Accession	Accession Length	Maximum Score	Total Score	Query Length	Query Coverage	E-Value	Percent Identity
Rural Human	<i>Escherichia coli</i> PCN033	1001989	CP006632.1	4987957	44128	2.972E+05	190736	83%	0	98.13%
Urban Human	<i>Clostridioides difficile</i> M120	699035	FN665653.1	4047729	5547	9364	33932	23%	0	99.46%
Urban Goat	<i>Clostridioides difficile</i> M120	699035	FN665653.1	4047729	3086	5568	34911	22%	0	98.61%

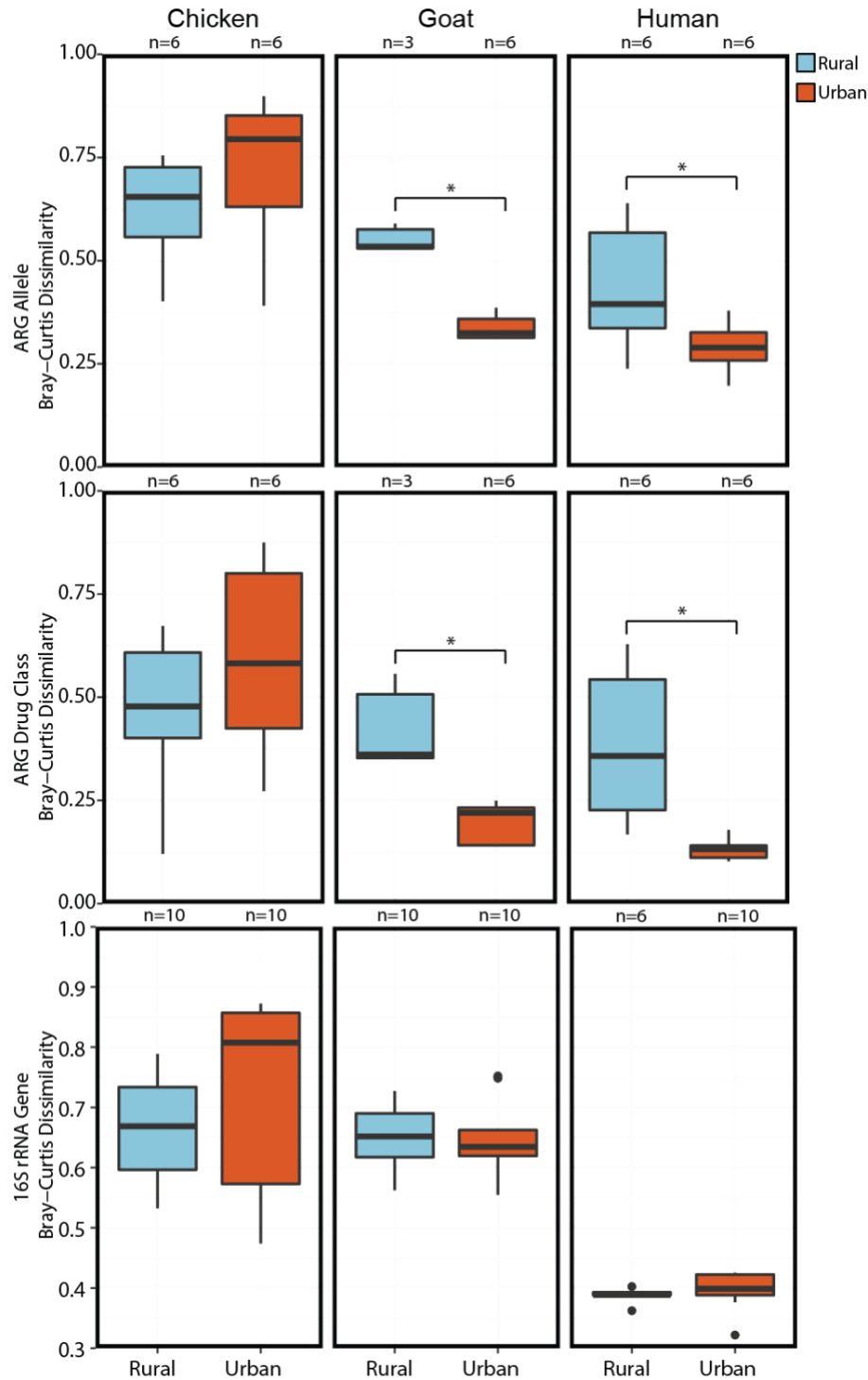


Figure C-3: Bray-Curtis dissimilarity between resistomes (top and middle; long-read sequencing) and bacterial community compositions (bottom; 16S rRNA gene analysis) within chickens, goats, and humans. The line through each box denotes the median Bray-Curtis dissimilarity index, the lower and upper box boundaries denote the first and third quartiles, respectively, and the whiskers extend to the minimum and maximum indices up to 1.5 times the interquartile range, with outliers plotted as dots. A Bray-Curtis dissimilarity index equal to 0.00 indicates identical community compositions and closer to 1.00 indicates more dissimilarity between community compositions.

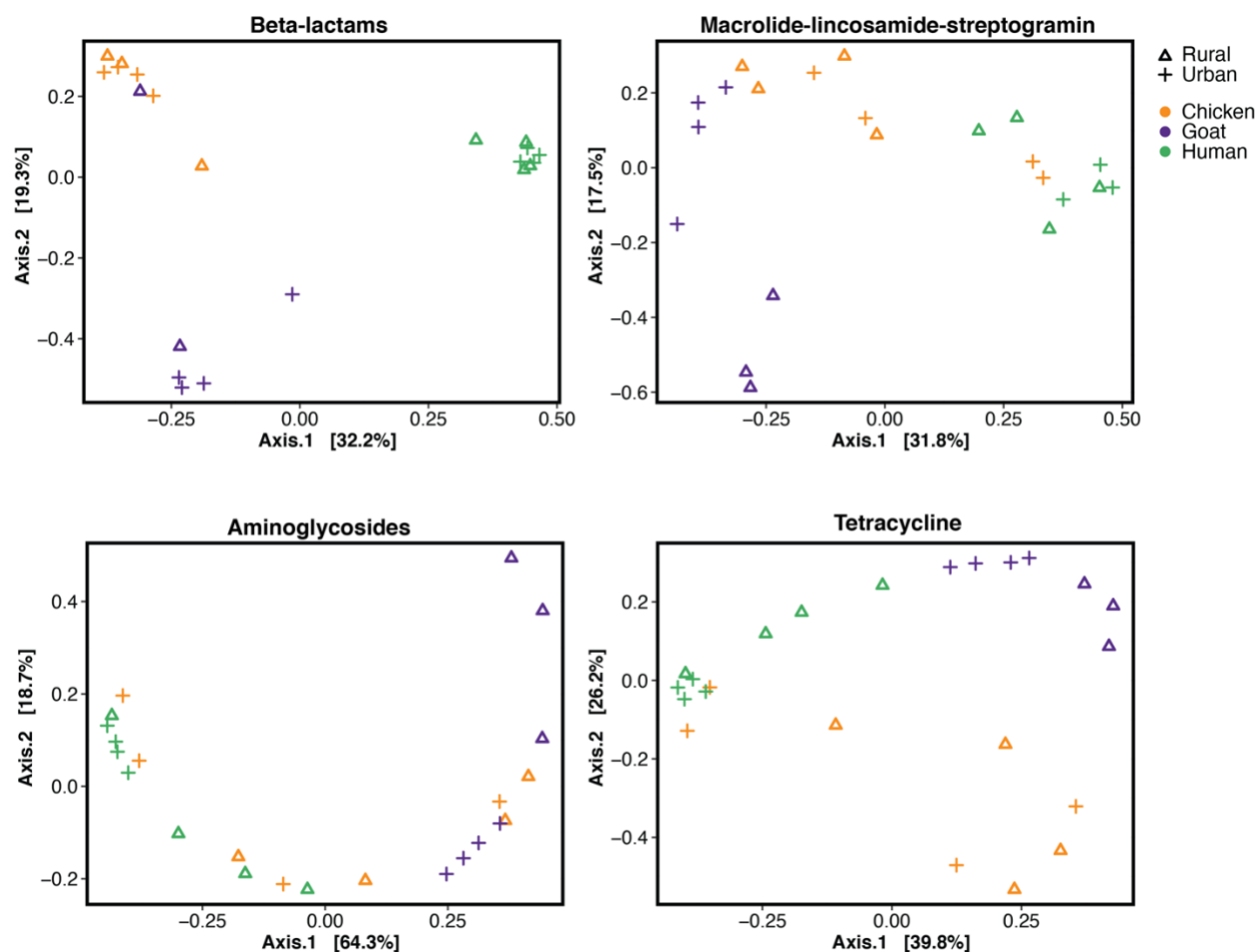


Figure C-4: Principal coordinate analyses (PCoAs) of Bray-Curtis dissimilarity between antibiotic resistance gene alleles in humans, chickens, and goats for four most abundant drug classes. Color indicates fecal host and symbol indicates rural, urban, or control.

Table C-6: Quality control parameters for DNA extraction for long-read sequencing. Fragment size distributions were not determined for extraction blanks and are marked “NA”. For samples with fragment size distributions marked “NA”, peaks could not be detected in the sample’s electropherogram by the Agilent Bioanalyzer.

Fecal Host	Concentration (ng/uL)	Purity		Fragment Size Distribution		
		OD 260/280	OD 260/230	From (bp)	To (bp)	Average (bp)
Rural Chicken 1	2.172	1.65	0.10	NA	NA	NA
Rural Chicken 2	2.227	1.61	0.59	683	27808	5440
Rural Chicken 3	1.480	1.91	0.47	NA	NA	NA
Rural Chicken 4	1.146	2.08	0.30	NA	NA	NA
Urban Chicken 1	39.12	1.66	1.45	562	28274	5521
Urban Chicken 2	16.58	1.12	0.73	606	28157	4454
Urban Chicken 3	3.014	1.28	0.02	NA	NA	NA
Urban Chicken 4	4.250	1.73	0.53	521	26442	4932
Rural Goat 1	53.71	1.67	0.82	656	29090	6103
Rural Goat 2	85.33	1.76	1.22	689	37014	5921
Rural Goat 3	121.3	1.82	1.84	491	32469	5978
Rural Goat 4	67.46	1.71	1.07	337	42375	6059
Urban Goat 1	102.5	1.83	1.81	556	35499	7202
Urban Goat 2	96.72	1.79	1.34	NA	NA	NA
Urban Goat 3	220.7	1.81	1.49	30	29556	5670
Urban Goat 4	147.5	1.76	1.24	405	30975	7748
Rural Human 1	120.1	1.85	1.84	620	26875	5994
Rural Human 2	144.7	1.87	1.92	332	37713	5150
Rural Human 3	114.3	1.87	1.99	667	31653	7051
Rural Human 4	55.23	1.87	1.74	665	45836	7777
Urban Human 1	121.6	1.87	0.82	504	31770	6400
Urban Human 2	37.58	1.87	1.55	416	35499	5727
Urban Human 3	40.15	1.89	1.41	570	30605	5138
Urban Human 4	38.32	1.86	1.62	NA	NA	NA
Extraction Blank 1	<0.100	1.97	0.08	NA	NA	NA
Extraction Blank 2	<0.100	1.36	0.31	NA	NA	NA
Extraction Blank 3	<0.100	4.97	0.07	NA	NA	NA

Table C-7: Illumina primers

Illumina 5' Adapter	Golay Barcode	Primer Pad	Primer Linker	Primer
CAAGCAGAAGACGGCATA CGAGAT ^a	-	AGTCAGCCAG	CC	GGACTACNVGGGTWTCTAAT
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AATGATACGGCGACCACCG AGATCTACACGCT	TCCATACCGGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
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AATGATACGGCGACCACCG AGATCTACACGCT	CCTAACGGTCCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
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AATGATACGGCGACCACCG AGATCTACACGCT	TACAATATCTGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
AATGATACGGCGACCACCG AGATCTACACGCT	AATTTAGGTAGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
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AATGATACGGCGACCACCG AGATCTACACGCT	GCCTCTACGTCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
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AATGATACGGCGACCACCG AGATCTACACGCT	ACTCTTACTTAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA

^a This is the reverse primer used. The Illumina adapter is the reverse complement, the pad is the reverse pad, the linker is the reverse linker, and the primer is the 806rB primer. All others listed are the barcoded forward primers with the 515f primer.

8.4 Appendix D – Chapter 5

Supporting Information D-1: Additional methods details

Data from Urban Kenya

Study Design

We previously leveraged sample collection from a Bill and Melinda Gates Foundation investment awarded to our research team to field test an environmental surveillance platform for monitoring infectious disease in two urban Kenyan communities, Kibera and Dagoretti South (Figure D-1). The environmental surveillance project visited 120 households (half of which owned poultry) to collect matched sample sets, including: poultry cloacal swabs (up to 3 per poultry-owning household), dog feces (1 per poultry- and dog-owning household), child under 5 years stool (up to 2 per household), child 5-15 years stool (up to 2 per household), adult over 15 years stool (1 per household), soil (1 per household) and stored drinking water (1 per household). Here, we used pooled *E. coli* samples from 8 poultry-owning households ($n = 48$ samples total) in Kibera to obtain short- and long-read sequencing data.

Sample Collection and *E. coli* Isolation

Samples were transported without preservatives (with the exception of cloacal swabs in 2 mL of Cary-Blair media) from the field to the Kenya Medical Research Institute (KEMRI) laboratory on ice and processed within 6 hours of collection. Viable *E. coli* were cultured from each sample type onto Tryptone Bile X-glucuronide (TBX) agar plates incubated at 44.5 °C for 18 hours. Specifically, 100 mL of undiluted water was membrane filtered and incubated on TBX plates. Bacteria were eluted from 5.0 g of each soil sample using 50 mL distilled water and vortexing vigorously for 2 minutes; a

10⁻² dilution was prepared for each eluted sample, and 10 mL of the eluant was filtered and incubated on TBX agar. Fecal samples (cloacal swabs; dog feces; human stool) were directly streaked onto TBX plates. Following incubation, up to five presumptive *E. coli* colonies (soil and water: 4.25 (1.36) average (standard deviation) colonies, cloacal swabs and stool: 4.81 (0.67)) from each sample type were transferred to 1 mL of Luria-Bertani (LB) broth and incubated at 37 °C for 18 hours. Pooled *E. coli* samples (100 µL) were stored in a solution of 30% glycerol and 70% Tryptic Soy Broth (TSB) at -80 °C before being shipped to Tufts University on dry ice.

DNA Extraction and Genomic Sequencing

Genomic DNA was extracted from pooled *E. coli* samples (incubated in 5 mL inoculated TSB at 37 °C for 18 hours) using the Qiagen DNeasy PowerSoil Pro kit. DNA extracts were sequenced using Illumina HiSeq (short reads) to obtain 150 bp, paired-end reads (1.5 Gbp per sample), and Nextera library preparation, at the Broad Institute.

Sequencing of DNA extracts using the ONT MinION (long reads), and ONT library preparation (SQK-LSK109 ligation kit), was conducted at Tufts University; twelve samples were multiplexed on each ONT flow cell to target 2 Gbp per sample ($n = 48$ total samples).

E. coli Strain Characterization

Illumina short reads were processed using FastQC to inspect quality, and the BBTools suite of bioinformatics tools (sourceforge.net/projects/bbmap) to remove PhiX (Illumina control library), adapter sequences, low-quality read ends (Phred quality score < 20),

and short trimmed reads (length < 50 bp).¹ The Strain Genome Search tool (StrainGST) was run on the trimmed Illumina reads to determine the presence and relative abundance of *E. coli* strains; all *E. coli* genomes were downloaded from the National Center for Biotechnology Information (NCBI) on September 23, 2021 for creating the StrainGST reference database.² We assigned Clermont phylotypes to the identified strains using the ClermonTyping tool.³ ARGs were identified by aligning trimmed reads, using DIAMOND, to the Comprehensive Antibiotic Resistance Database (CARD), using a minimum match length of 25 amino acids and identity of 95%.^{4,5}

Data from the Broad Institute for Simulation

For each isolate, genomic DNA extracts were input into Nextera XT library preparation and sequenced on a NovaSeq S4 flow cell run for 300 cycles to obtain 150 bp, paired-end reads (112 Gbp, on average, per sample). DNA extracts were also sequenced using ONT's GridION and 1D ligation library preparation; eight samples were multiplexed on each MinION flow cell.

Trim Galore (<https://github.com/FelixKrueger/TrimGalore>) was used to trim adapters from Illumina reads, which were subsampled to approximately 100x depth of genome coverage, when available. *E. coli* strains were identified from the trimmed Illumina reads

¹ Leggett, R. M., et al. 2013. Sequencing quality assessment tools to enable data-driven informatics for high throughput genomics. *Front Genet*, 4: 288.

² Van Dijk, L. R., et al. 2022. StrainGE: a toolkit to track and characterize low-abundance strains in complex microbial communities. *Genome Biol*, 23(1): 74.

³ Beghain, J., et al. 2018. ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping. *Microb Genom*, 4(7): e000192.

⁴ Buchfink, B., et al. 2021. Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat Methods*, 18(4): 366-368.

⁵ Alcock, B. P., et al. 2020. CARD 2020: antibiotic resistomes surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res*, 48(D1): D517-D525.

using StrainGST (reference database curated from NCBI in September 2021).² ONT reads were demultiplexed with Deepbiner, trimmed of remaining adapters with Porechop (<https://github.com/rrwick/Porechop>), and subsampled to approximately 50x depth of genome coverage.⁶ For each sample, two hybrid assemblies were generated using Unicycler: one combining Illumina 100x data with the 50x subsampled ONT data and another combining Illumina 100x data with the full ONT data set (if >50x).⁷ Illumina reads (using bwa mem) and ONT reads (using minimap2) were aligned to Unicycler contigs; the resulting alignments were input to Pilon for assembly polishing.^{8,9,10} ARGs were identified by aligning assembled genomes, using DIAMOND, to the CARD, using a minimum match length of 25 amino acids and identity of 95%, and plasmid groups were identified using Mobsuite.^{4,5,11}

⁶ Wick, R. R., et al. 2018. Deepbiner: Demultiplexing barcoded Oxford Nanopore reads with deep convolutional neural networks. *PLoS Comput Biol*, 14(11): e1006583.

⁷ Wick, R. R., et al. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol*, 13(6): e1005595.

⁸ Li, H. and Durbin, R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25(14): 1754-1760.

⁹ Li, H. 2018. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics*, 34(18): 3094-3100.

¹⁰ Walker, B. J., et al. 2014. Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One*, 9(11): e112963.

¹¹ Robertson, J. and Nash, J. H. E. 2018. MOB-suite: software tools for clustering, reconstruction and typing of plasmids from draft assemblies. *Microb Genom*, 4(8): e000206.

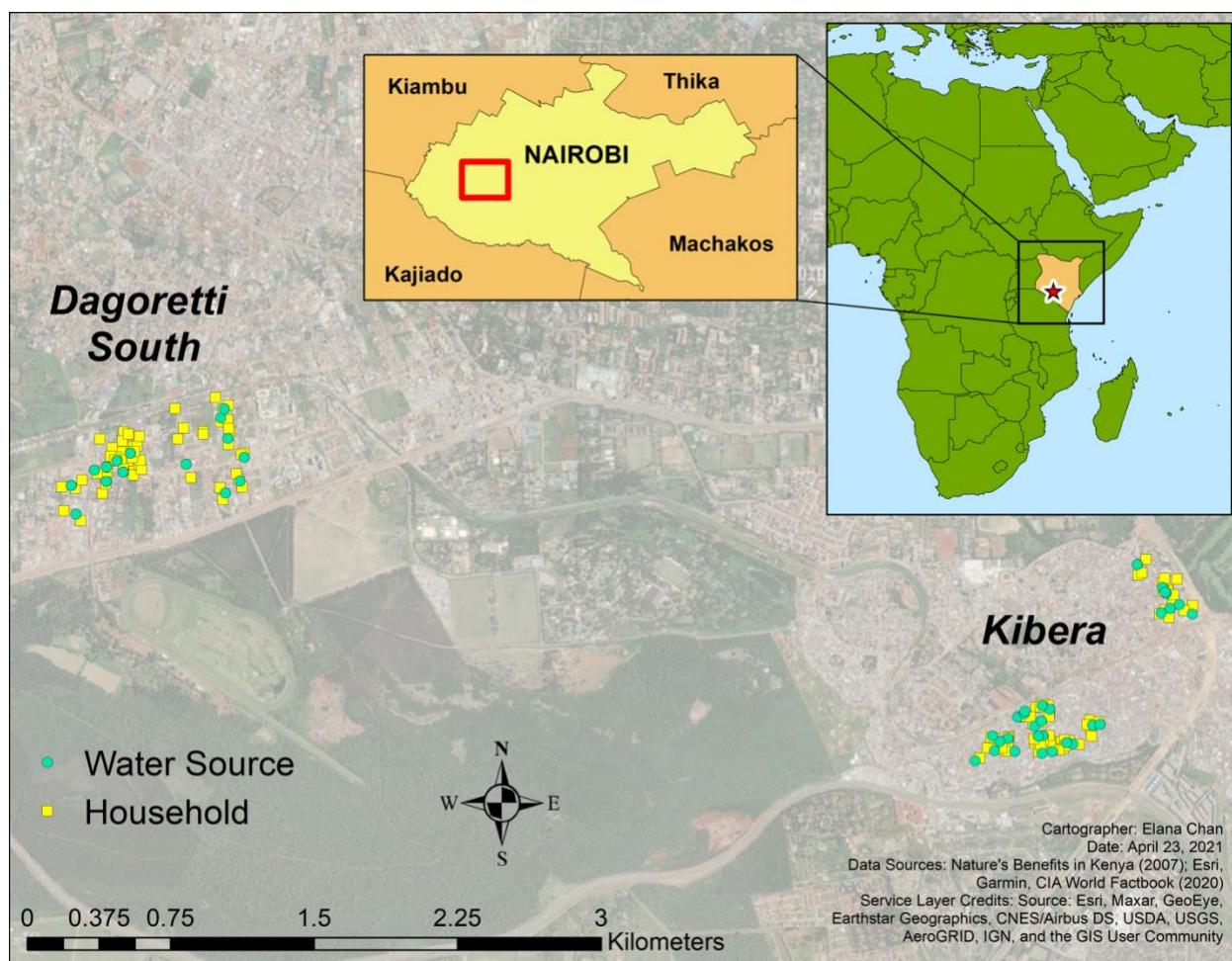


Figure D-1: Locations of households and primary drinking water sources in the two study communities (Dagoretti South; Kibera) in Nairobi County, Kenya

Supporting Information D-2: Additional results

E. coli Strain Characterization of Real Data

The Illumina short-read sequencing and trimming of the real data yielded, on average, 20.2 million paired-end reads (including forward and reverse reads; Table D-1); the maximum number of short reads was 36.2 million, obtained from a young child sample in HH024. The ONT long-read sequencing and trimming yielded an average sequencing length of 1.55 Gbp, with the maximum sequencing length (2.43 Gbp) obtained from a young child sample in HH055.

On average, 81.2% of the short read data was attributable to the *E. coli* genomes comprising the StrainGST reference database. StrainGST identified between 1 and 5 strains from the short reads in each of the real samples (Figure D-2). The identified strains primarily belonged to Clermont phylotypes A (49.1%, 57/116) and B1 (30.2%, 35/116); patterns were similar among the 78 unique strains (A: 48.7%, 38/78; B1: 26.9%, 21/78). In addition, 22 *E. coli* strains were detected in two or more of the real samples (Figure D-1). There were 12 cases of matching strains across two or more samples within the same household, and 11 cases of matching strains within the same village.

Table D-1: Summary of sequencing statistics for real data from 8 poultry-owning households in Kenya. Statistics are presented for Illumina and ONT reads that have been demultiplexed and/or trimmed of adapter sequences. Illumina statistics include forward and reverse reads.

Identifier	Village	Number Samples	Avg. Number Illumina Reads (millions)	Avg. Illumina Sequencing Length (Gbp)	Avg. Number ONT Reads (thousands)	Avg. ONT Sequencing Length (Gbp)	ONT N50 (kbp)
HH004	Mamokinda	7	27.46	4.128	260.1	2.161	10.15
HH009	Ka Wuon Opuk	7	11.66	1.754	231.9	2.013	10.50
HH020	Gatwekera	5	12.90	1.941	206.7	1.785	10.49
HH021	Gatwekera	6	29.23	4.394	93.47	0.929	12.69
HH024	Gatwekera	6	32.07	4.821	90.78	0.774	11.24
HH031	Gatwekera	7	13.01	1.959	169.0	1.395	10.14
HH055	Kichinjio	5	23.80	3.580	257.8	2.002	9.650
HH110	Kichinjio	5	10.79	1.625	143.9	1.233	10.41
Overall	-	48	20.21	3.040	182.8	1.548	10.41

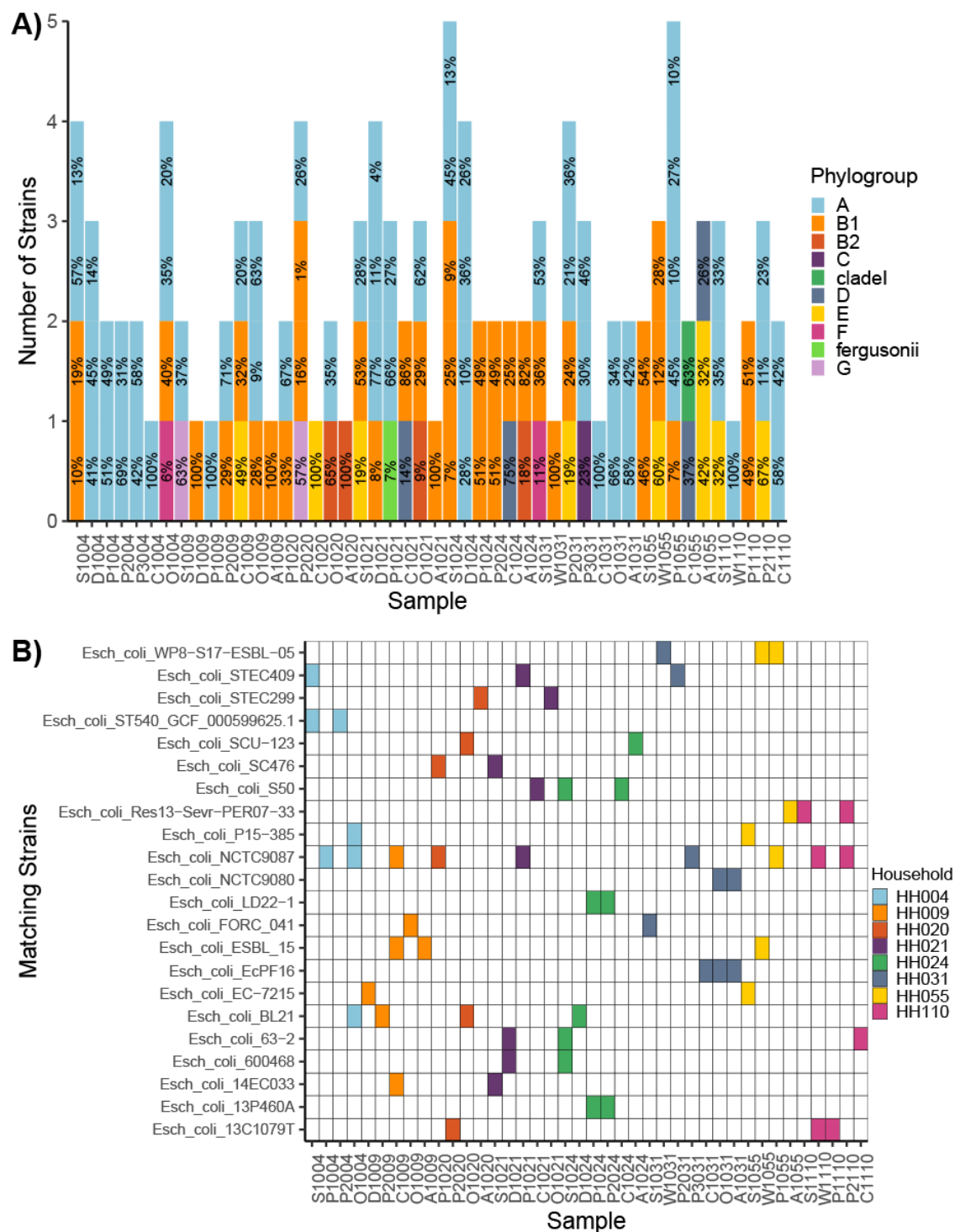


Figure D-2: *E. coli* strain A) multiplicity and B) matching across real samples, as identified from raw Illumina reads by StrainGST. For sample names, S=soil; W=water; D=dog feces; P=poultry cloacal swab; C=young child stool; O=older child stool; and A=adult stool. In plot A), percentages indicate the relative abundance of that strain in the total strain composition for that sample. Phylogroups were assigned using the ClermonTyping tool. In plot B), rectangles are filled if the *E. coli* strains on the y-axis were detected in each sample. Some samples ($n=8$) are not listed, as they did not contain any strains matching those from other samples.