Antimicrobial Resistance in Humans and Animals in the Somali region of Ethiopia: A One-Health Approach

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AHRI	Armauer Hansen Research Institute
AMR	Antimicrobial Resistance
AMS	Antimicrobial Stewardship
AMU	Antimicrobial Use
ARG	Antimicrobial Resistance Genes
BEE	Bidirectional Emic-Etic tool
CAWS	Community Animal Health Workers
CBEF	Community-Based Emergency Fund
CC	Climate Change
CHW	Community Health Workers
CIPARS	Canadian Integrated Program for Antimicrobial Resistance
CLSI	Clinical and Laboratory Standards Institute
CLTS	Community-Led Total Sanitation
CPE	Carbapenemase-Producing *Enterobacteriaceae*
CRE	Carbapenem Resistant *Enterobacteriaceae*
DAEC	Diffusely Adherent *Escherichia coli*
DDD	defined daily dose
DEC	Diarrheagenic *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended Spectrum β-lactamase
ESBL-E	Extended Spectrum β-lactamase *Enterobacteriaceae*
E. coli	*Escherichia coli*
ESKAS	Swiss Government Excellence Scholarships
ETEC	Enterotoxigenic *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
FGD	Focus Group Discussion
GAP	Global Action Plan
GEMS	Global Enteric Multicenter Study
HGT	horizontal gene transfer
HIV	Human Immunodeficiency Virus
IAP	Intrapartum Antimicrobial Prophylaxis
JJU	Jigjiga University One health Initiative
JJUSHYCH	Jigjiga University Sheik Hassen Yabere Comprehensive Hospital

K. pneumoniae	*Klebsiella pneumoniae*
KAP	Knowledge, Attitude, and Practice
LMICs	Low- and Middle Income Countries
mCCD	Modified Charcoal Cefoperazone Deoxycholate Agar
MDR	Multidrug resistant
MLST	Multi-Locus Sequency Typing
MUAC	Mid-Upper Arm Circumference
NAP	National Action Plan
NGS	Next generation sequencing
NICU	Neonatal Intensive Care Unit
NRERC	National Research Ethics Review Committee
OHSES	One Health in Social-Ecological Systems
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses.
S. aureus	*Staphylococcus aureus*
SES	Socio-Ecological System
SNP	Single Nucleotide Polymorphisms
SRS	Somali Regional State
ST	sequence types
STEC	Shiga-Toxin producing *Escherichia coli*
Swiss TPH	Swiss Tropical and Public Health Institute
TCBS	Thiosulfate Citrate Bile Salts Sucrose Agar
UNEP	United Nations Environment Programme
UTI	Urinary Tract Infection
V. cholera	*Vibrio Cholera*
WAAW	World Antimicrobial Resistance Awareness Week
WASH	Water, Sanitation, and Hygiene
WGS	Whole Genome Sequencing
WHO	World Health Organization
XLD	Xylose Lysine Desoxycholate Agar

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Summary

The rampant spread of antimicrobial resistance (AMR) in humans, food-producing animals, agriculture and the environment presents an alarming and significant global crisis. Unless action is taken, AMR is projected to claim 10 million lives annually by 2050, disproportionally impacting low- and middle income countries (LMICs). Due to the severity, vastness, intricacy and multifaceted nature of AMR, a holist One Health approach is widely considered the strategy to address and combat AMR. However, prior evidence to this thesis, demonstrated the dearth of studies that truly applied a One Health approach implementing genomic analysis in LMICs. The broad goal of this research was to provide evidence on AMR genes in children and animals, as well as the knowledge and attitude of rural communities regarding AMR.

The first part of the research provides an overview of the existing evidence of molecular AMR studies utilizing a One Health approach in Africa, aiming to determine the prevalence of resistance genes in humans, animals and the environment. The findings from the systematic review and meta-analysis showed very limited studies truly implementing this approach in Africa. However, within the limited studies, *sul1*, *sul2*, *tetA*, *strB*, and *blaTEM* were the most prevalent genes identified in humans and animals.

Thus, due the scarcity of studies utilizing unified approach in Africa, we examined the genetic characterization of fecal carriage of ESBL-producing *E. coli* in rural children and livestock, studied diarrheagenic pathogens and their resistance genes in urban children, as well as assessed the communities' knowledge and attitude regarding AMR and climate change in the Somali region of Ethiopia. The results showed a high prevalence of ESBL-producing *E.coli* in rural and urban children, and a low prevalence in livestock, predominantly harboring $bla_{\text{CTX-M-15}}$. Conversely, the carbapenemase-producing diarrheagenic *E. coli, Salmonella spp.* and *Shigella spp.* were exclusively detected in urban children. Moreover, the first whole genome sequencing analysis employed in rural communities in Ethiopia, revealed a high diversity of sequence types (ST), with ST2353 being among the most prevalent, harboring multiple resistance genes. Notably, this is the first study to report ST2353 in Ethiopia. The findings also shed light on *Shigella spp.* harboring bla_{NDM} and $bla_{\text{OXA-48}}$, which has been rarely described to date, indicating the widespread of AMR genes in both rural and urban children aged less than five years. These findings are concerning as the identified genes confer resistance to most commonly used antibiotics, potentially hampering treatment of infections caused by these bacteria.

In addition to the spread of AMR genes, a significant association between ESBL carriage in rural children and being both wasted and stunted, water treatment (with chlorine), and chicken ownership were found. Furthermore, the findings highlighted the limited knowledge of AMR

and climate change, with noticeable gender disparity, where females showed lower levels of knowledge compared to males. Further, the limited knowledge of mothers was seen to significantly increase the odds of ESBL-producing *E. coli* carriage in children.

The overall findings indicated the wide spread of AMR resistance genes in urban and rural children, showcasing diverse sequence types such as ST2353, a first report in Ethiopia. Additionally, this research identified *Shigella spp.* harboring carbapenemase genes, rarely reported globally. This is alarming, as these strains might complicate treatment options, possibly prolong hospital stay and increase costs. Moreover, the limited knowledge of AMR and climate change, mainly among females, adds to the burden. Therefore, with the emergence and spread of AMR in the country, it is imperative to implement an integrated AMR surveillance system using a One Health approach that involves both hospital and community settings in order to mitigate and control AMR. Addressing other relevant issues such as malnutrition and water, sanitation and hygiene (WASH) is also of paramount importance. Further, we recommend comprehensive community-based educational programs tailored for women to promote responsible antimicrobial use and environmental conservation.

In addition, the research of this thesis provided the foundation for future research work on various aspects of AMR. Furthermore, the research showed the need for robust study designs suitable for determining the acquisition and dynamics of AMR.

Chapter 1 Introduction

1.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is a growing phenomenon globally and represents a serious threat to human health and the economy (Pulingam et al., 2022). In 2019, it was projected that 4.95 million deaths were attributable to bacterial AMR and its consequences, and 1.27 million of these were caused directly by bacterial AMR (Murray et al., 2022). These fatalities occurred predominantly in sub-Saharan Africa and Southern Asia (Murray et al., 2022). By 2050, it is estimated that AMR will cause the loss of both lives and capital, affecting around 10 million people and causing a loss of $300 billion to $1 trillion annually (Burki, 2018; O'Neill, 2016). However, these forecasts have been denounced by the World Health Organization (WHO) and several researchers (de Kraker, Stewardson, & Harbarth, 2016).

AMR occurs when bacteria, viruses, fungi and parasites develop the ability to survive or multiply despite administration of antimicrobial drugs used to combat infections (Dadgostar, 2019). Multiple different mechanisms of resistance have been described, including for example mutations of the drug target, decreased import of the drug due to decreased membrane permeability, and deactivation of the drug by an enzyme. A good example of the latter is the production of beta-lactamase in bacteria, which allows them to hydrolyze beta-lactam antibiotics and thus become resistant to them (Karen Bush, 1989). When pathogens are overexposed to antimicrobials, the selective growth of resistant strains is fostered (Holmes et al., 2016). Indeed, under drug pressure, susceptible strains are replaced by resistant ones, if fitness cost allow, in a phenomenon called “selective sweep” (Messer & Petrov, 2013). The over- and misuse of antimicrobials in humans, but also in animals and for agricultural purposes, increases drug pressure and thus plays a major role in the spread of AMR (Prestinaci, Pezzotti, & Pantosti, 2015). For instance, in 2018, the global antibiotic consumption was estimated at 40.1 billion defined daily dose (DDD), corresponding to a rate of 14.1 DDD per 1000 population per day (Browne et al., 2021). Additionally, the use of antimicrobials in food-producing animals accounted 99,502 tons, and is expected to increase to 107,472 tons (8%) by 2030 (Mulchandani, Wang, Gilbert, & Van Boeckel, 2023). The increase in antibiotic use is a consequence of the increasing demand for meat products and for the over-the-counter sale of antibiotics, especially in low- and middle income countries (LMICs), where population and economic growth are imminent (Allel et al., 2023). The increased consumption will inevitably lead to an increase in AMR and, possibly, to antimicrobial treatment failures, which could have major consequences in terms of human and animal lives loss.

The complex and multifaceted nature of AMR entails several interlinked risk factors, including socioeconomic and environmental factors that contribute to the emergence and spread of antimicrobial resistance genes, particularly in LMICs. The unnecessary use of antibiotics for the treatment of viral infections, non-adherence to antimicrobial treatment regimens, and patient-related conditions such as obesity, smoking, and alcohol consumption, may also render individuals susceptible to infections or diminish the efficacy of antimicrobial drugs (Chatterjee et al., 2018; Sun, Yao, Zhou, Harbarth, & Lin, 2022). Insufficient access to safe water, inadequate sanitation facilities and hygiene practices stemming from water scarcity or poor sanitation infrastructure increase the risk of infections and transmission of resistant pathogens (O. O. Ikhimiukor, E. E. Odih, P. Donado-Godoy, & I. N. Okeke, 2022). Additionally, poor healthcare systems exacerbate the problem. This encompasses the lack of advanced diagnostic facilities, limited availability of appropriate medications, insufficient implementation of infection prevention control and stewardship programs, and a lack of surveillance and monitoring systems (Sulis, Sayood, & Gandra, 2022). Moreover, the widespread use of antibiotics in livestock farming, agriculture, and aquaculture in LMICs might contribute to the emergence and dissemination of AMR (Mulchandani et al., 2023; Tang et al., 2017).

To overcome the issue of AMR, new strategies encompassing combination therapies and, most importantly, the development of new drugs, are needed. The WHO has defined a prioritized list of pathogens necessitating focused research and development (R&D) for new antibiotics. Categorized as critical are *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant), and *Enterobacteriaceae* (carbapenem-resistant, ESBL-producing). In the high-priority stratum, *Enterococcus faecium* (vancomycin-resistant), *Staphylococcus aureus* (methicillin-resistant, vancomycin-intermediate, and resistant), *Helicobacter pylori* (clarithromycin-resistant), *Campylobacter spp.* (fluoroquinolone-resistant), *Salmonellae* (fluoroquinolone-resistant), and *Neisseria gonorrhoeae* (cephalosporin-resistant, fluoroquinolone-resistant) are spotlighted. Lastly, the medium-priority section contains *Streptococcus pneumoniae* (penicillin-non-susceptible), *Haemophilus influenzae* (ampicillin-resistant), and *Shigella spp.* (fluoroquinolone-resistant) (E. Tacconelli et al., 2018). Six of these pathogens (*E. coli* followed by S*taphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) were identified as the leading causes of death associated with drug resistance (Murray et al., 2022).

1.2 Antimicrobial resistance within the One Health approach

AMR is a complex, multidimensional issue with interlinked drivers in humans, animals, and the environment. For example, animal antibiotic consumption has been positively correlated

with resistance in critical priority human pathogens, and human antibiotic consumption has been linked to animal AMR (Allel et al., 2023). Similarly, antibiotic use in agriculture has also been associated with human AMR (Chang, Wang, Regev-Yochay, Lipsitch, & Hanage, 2015).

The main driver is the misuse and abuse of antimicrobials across these sectors, highlighting AMR as a One Health problem. One Health is defined as an added value of human and animal lives saved, reduced cost and sustained social and environmental services that can be achieved by closer cooperation of human and animal health and other disciplines, not possible when sectors work separately (Zinsstag, Waltner-Toews, & Tanner, 2015).

The World Organization for Animal Health (OIE), the WHO, and the Food and Agriculture Organization of the United Nations (FAO) have joined forces to develop a Global Action Plan on Antimicrobial Resistance (GAP) which prioritizes research and integrated surveillance to unravel the dynamics of AMR spread and use of antimicrobials across human, animal, and environmental settings (Mendelson & Matsoso, 2015). Recognizing the importance of the holistic approach, the tripartite organization were later joined by the United Nations Environment Programme (UNEP) to enhance the collaboration in halting AMR through a One Health approach (WHO, 2023).

Several countries in the Global North including Canada, Denmark, Sweden, Switzerland, and the US have implemented an integrated surveillance system for AMR (Guardabassi, Butaye, Dockrell, Fitzgerald, & Kuijper, 2020; IFIK, 2023; Karp et al., 2017). These systems monitor antimicrobial consumption, trends of AMR, research on identification of new resistance mechanisms and genetic characterizations in humans, animals, and food (Parmley et al., 2012). These systems play a significant role in containing AMR by providing valuable data to inform public health interventions and guide research efforts. The Canadian Integrated Program for Antimicrobial Resistance (CIPARS) exemplifies as a successful approach (Parmley et al., 2012; Zinsstag, Schelling, Waltner-Toews, & Tanner, 2011). CIPARS benefits from a diverse range of professionals, including researchers, diagnostic microbiologists, pharmacologists, and clinicians, by facilitating international comparisons of resistance patterns and supporting AMR research (Deckert et al., 2010; Guardabassi et al., 2020).

LMICs bear the highest global burden of AMR, and also struggle with limited access to safe water, sanitation, and hygiene practices – major drivers of AMR transmission (Odion O Ikhimiukor, Erkison Ewomazino Odih, Pilar Donado-Godoy, & Iruka N Okeke, 2022). Yet, only limited integrated surveillance systems for AMR have been established due to resource constrains, including human capacity, laboratories, essential drugs, robust policies, and formal programs to combat AMR (Cox et al., 2017). Implementing such systems could offer advantages like early detection of AMR pathogens and outbreaks, data-driven decision

making, optimizing resource allocation, ability to monitor AMR trends and manage outbreaks, capacity building, and applying a coordinated response across borders (Karp et al., 2017).

1.3 Antimicrobial resistance in Ethiopia

In Ethiopia, a troubling trend of high AMR levels has been observed across humans, animals and the environment, presenting a significant public health concern nationally and internationally (A. W. Fujita et al., 2022). The main resistant pathogens include *E. coli, K. pneumoniae*, *S. aureus* as well as *Salmonella spp.* (A. W. Fujita et al., 2022; B. A. Gemeda, Assefa, Jaleta, Amenu, & Wieland, 2021). These pathogens exhibit high resistance rates to frequently used antibiotics such as penicillines, trimethoprim/sulfamethoxazole, nalidixic acid, and third generation cephalosporins (Beyene et al., 2017; Garedew, Hagos, Zegeye, & Addis, 2016).

Misuse of these very antibiotics is a main contributor to AMR in the country. Indeed, these antibiotics are commonly used in both humans and animal-producing food, and they are often purchased without a prescription (Biruk Alemu Gemeda et al., 2020; Geta & Kibret, 2021; Gutema, Ali, & Suleman, 2021). Additionally, limited resources such as advanced laboratories, force both human and animal health professionals to rely on empirical treatment, which further contributes to a rise in AMR (Berhe et al., 2021).

Several studies conducted in Ethiopia have assessed AMR in different sectors separately such as humans (Addis et al., 2011; Biadglegne & Abera, 2009), milk (Tadesse et al., 2018), raw meat (Beyi et al., 2017; Messele et al., 2017) and the environment (Ergie, Leng, & Wang, 2019), but only five studies have applied a One Health approach (A. W. Fujita et al., 2022). All of the studies reported very high AMR rates, up to a prevalence of above 50%.

Following the recommendation of the national action plan (NAP), Ethiopia developed a strategy for the prevention and containment of antimicrobial resistance in 2015 and launched the Ethiopian Antimicrobial Resistance Surveillance System in 2017. However, this system currently prioritizes monitoring AMR in humans, leaving a significant gap in understanding how resistance trends develop across animals and the environment. The importance of a One Health approach considering all these interconnected aspects has been highlighted by independent studies (Zinsstag et al., 2015). Recognizing this need, Ethiopia revised its strategic plan in December 2020, placing emphasis on a One Health approach to combat AMR (EFDA, 2021).

1.4 Etiology of diarrheal diseases in LMICs

Sub-Saharan Africa (SSA) faces critical child health challenges, with the highest child mortality rate globally at 75.8 death per 1000 live births (Sharrow et al., 2022). This disproportionately impacts children under five, with half of all global deaths occurring in just five countries, including Ethiopia (WHO, 2022a). In Ethiopia, diarrhea stands out as the second leading cause of child mortality, after neonatal disorders, and contributes to disability-adjusted life years (DALYs) (Misganaw et al., 2022). In fact, over half (57%) of the children under-five experience untreated diarrhea (Alemu et al., 2023).

Diarrhea is primarily caused by different pathogens such as viruses (*Rotavirus),* bacteria (diarrheagenic *E. coli, Campylobacter spp., Shigella spp. and Salmonella spp.*) and protozoa (*Giardia intestinalis*, *Cryptosporidium*, and *Entamoeba histolytica*) (M. Balew & M. Kibret, 2023; Belina et al., 2023; Damtie, Melku, Tessema, & Vlasova, 2020). These pathogens are easily transmitted in resource-limited countries, where due to inadequate infrastructures and poor hygienic practices, individuals can contract infections through contaminated food or drinking water or direct contact between humans (person-to-person) or with animals (Fenta & Nigussie, 2021).

In Ethiopia, studies have revealed different prevalence of enteric pathogens in children with diarrhea. For instance, in one study, diarrheagenic *E. coli* (DEC) was the most prevalent pathogen accounting for 40.7% (Zelelie et al., 2023) of the cases. *Salmonella spp.* and *Shigella spp.* also contribute substantially, with a combined prevalence of around 11% (Dessale, Mengistu, & Mengist, 2023) and *Campylobacter spp.* adds to the burden with another 10% (Diriba, Awulachew, & Anja, 2021). Alarmingly, the majority of these enteric pathogens showed a high resistance level (70-95%) to commonly used antibiotics such as ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, nalidixic acid, trimethoprim-sulfamethoxazole and tetracycline (Dessale et al., 2023; Lengerh, Moges, Unakal, & Anagaw, 2013; Mulu, Belete, Demlie, Tassew, & Sisay Tessema, 2024; Zelelie et al., 2023). This rise in AMR is particularly concerning because it makes common infections caused by these enteric pathogens difficult to treat, potentially leading to longer illness times, increased healthcare costs, and even death.

1.5 Extended spectrum β-lactamase- and carbapenemase-producing *Enterobacteriaceae* in LMICs

Enterobacteriaceae, including *Klebsiella* spp, *E. coli,* and *Salmonella spp*., cause preventable infectious diseases which affect a high proportion of children in SSA (Kowalski et al., 2024).

Due to the increase of AMR in these pathogens, combating these infections has become exceedingly challenging.

First-line treatment of *Enterobacteriaceae* infections is based on β-lactam antibiotics. The emergence and spread of extended spectrum β-lactamase *Enterobacteriaceae* (ESBL-E)- and carbapenemase-producing *Enterobacteriaceae* (CPE), which hydrolyze β-lactam antibiotics, is very alarming (Tängdén & Giske, 2015; Wilson & Török, 2018). ESBLs confer resistance to a broad spectrum of β-lactam antibiotics, including penicillines, third- and fourth generation cephalosporins, and monobactams, but not to carbapenems (K. Bush & Bradford, 2016). Therefore, maintaining the efficacy of carbapenems is imperative, as they represent the last antimicrobial resort for the treatment of resistant infections. However, the spread of carbapenem resistant *Enterobacteriaceae* (CRE) has also been recently reported (Jiayue Ma et al., 2023), representing an additional challenge in the fight against AMR.

LMICs are experiencing a rapid growth of ESBL-E and CPE (Tornimbene et al., 2022). A systematic review conducted in LMICs revealed a high prevalence of both ESBL-E and CPE in humans (72%), animals (75%), and the environment (79%) (O. Ouchar Mahamat et al., 2021). In Ethiopia, high rates of both have been previously reported. Indeed, a review showed high prevalence of ESBLs in *K. pneumoniae* (67%), *E. coli* (62.1%), and *Salmonella spp.* (48.5%) (Tafese B Tufa et al., 2020; Worku, Getie, Moges, & Mehari, 2022) and 25% prevalence of carbapenem-resistant *E. coli* (Worku et al., 2022) in the country.

Furthermore, in hospital settings, higher proportions of ESBL-producing bacteria were observed in the Neonatal Intensive Care Unit (NICU) (82%), orthopedic departments (92%), and waste disposal areas (57%), whereas a lower prevalence was found in flies within the hospital compound (2%) (Tafese Beyene Tufa et al., 2020). Regarding the food chain, 20% of *E. coli* isolates obtained from minced meat, butcher hands, chopping boards and protective clothing were identified to be ESBL-producing, suggesting potential contamination in the food chain (Abayneh, Tesfaw, Woldemichael, Yohannis, & Abdissa, 2019).

Within ESBL-E and CPE, some genes and bacterial lineages are known to be associated with higher levels of AMR. For example, CTX-M-15 is encoded by a gene which is notably found in the globally disseminated *E. coli* clonal and resistant lineage sequence type (ST) 131 (Coque et al., 2008). Similarly, the carbapenemase-producing enzyme KPC has been associated with *Klebsiella pneumoniae* ST258 (van Duin & Doi, 2017). The NDM-1 carbapenemase enzyme presents a distinct challenge (Kumarasamy et al., 2010). Unlike the previous examples, NDM-1 encoding genes are not restricted to a single bacterial clone but can be found in different isolates of *E. coli* and *K. pneumonia* (Kumarasamy et al., 2010; Nordmann, Poirel, Toleman, & Walsh, 2011). Plasmids carrying NDM-1 encoding genes often

carry a multitude of resistance genes, not just for carbapenems, but also for β-lactams, aminoglycosides, and macrolides, making the bacteria truly multidrug-resistant (Kumarasamy et al., 2010; Nordmann et al., 2011). The gene encoding for NDM-1 has also been identified in *E. coli* ST131, a ST known to carry the CTX-M-15 encoding gene, and a significant source of community-acquired infections (Coque et al., 2008). CTX-M-15, NDM, OXA-48, and OXA-181 are all among the most concerning ESBLs and carbapenemase encoding genes prevalent in this region (O. Ouchar Mahamat et al., 2021). A recent study has observed ST131 and ST410 as the most prevalent sequence types of ESBL *E. coli* in Ethiopia (Negeri et al., 2023). This alarming surge of ESBL and CPE in humans, animals, and the environment underscores the potential for difficult-to-treat simple infections, specifically, due to the emergence of multidrug-resistant bacteria like those expressing NDM-1 and CTM-X-15.

1.6 Extended spectrum β-lactamase and carbapenemase-producing commensal *E. coli* (Microbiome)

The extended spectrum β-lactamase and carbapenemase-producing *Enterobacteriaceae* were traditionally linked to hospital settings (Oumar Ouchar Mahamat et al., 2019). However, community carriage of these bacteria has become gradually common, with an eightfold increase over the last two decades (Y. M. Bezabih et al., 2021; Pitout, Nordmann, Laupland, & Poirel, 2005). This trend is alarming because it signifies that the healthy communities are becoming a growing reservoir, thereby contributing to the worldwide spread and emergence of resistant bacteria (Salyers, Gupta, & Wang, 2004). For instance, these resistant bacteria can potentially spread via contaminated water, food chain or contact with contaminated objects. Further, the exchange of resistance genes can happen among the gastrointestinal microbiota, including potential pathogens, especially when host immunity levels are low (Stecher et al., 2012).

In the early life, the microbiome remains dynamic for several years until it achieves a composition that likely sustains into adulthood (Eggesbø et al., 2011; Yatsunenko et al., 2012). During this period, the microbiome may be permanently altered in its phylogenetic composition and associated resistome. Exposure to antibiotics is a common factor that disrupts the microbiome, with children in LMICs particularly at risk, as they are estimated to receive an average of eleven rounds of antibiotics within their first two years of life (Fink, D'Acremont, Leslie, & Cohen, 2020).

Studies have shown the asymptomatic carriage of ESBLs (21.7%) and CPE (2.4%) in children (A. Amare, Eshetie, Kasew, & Moges, 2022). This indicates the ability of commensal *E. coli,* a member of the gut microbiota residing harmlessly in the gut lumen and hardly causing disease, to acquire resistance genes and virulent traits from pathogenic bacteria. This can

occur via horizontal gene transfer (HGT) involving plasmids, transposons and pathogenicity islands and bacteriophages (Puvača & de Llanos Frutos, 2021; Christian JH Von Wintersdorff et al., 2016). *E. coli* can acquire and pass resistance genes, such as those encoding for ESBLs and carbapenemase production, from and to other bacteria via HGT, thus exacerbating the challenge in treating routine infections (Christian JH Von Wintersdorff et al., 2016). This may depend on the density of the strains, for example, in dysbiotic diseases, an increase of ESBL-producing *E. coli* has been observed. This can significantly influence the likelihood of HGT among ESBL-producing *E. coli* with resistance genes (Lerner, Matthias, & Aminov, 2017).

Therefore, profiling the diversity and mobility of commensal resistome is crucial to comprehend the spread of multidrug resistance in hospital and communities. Previous studies in Ethiopia have mainly focused on hospital settings (Negeri et al., 2023; Negeri et al., 2021), largely overlooking the community. To fill this gap, we conducted a study on the genetic characterization of commensal *E. coli* in children and animals. Such study should enlighten the potential reservoir of resistance in the community and inform strategy to halt its dissemination.

1.7 The link between antimicrobial resistance and climate change

AMR and climate change represent important challenges for global health and the ecosystem. Both issues are significantly fueled by human actions. AMR is mainly driven by the misuse and overuse of antimicrobials in humans, animals and the environment. Climate change, on the other hand, is primarily attributed to our continued reliance on fossil fuels, such as coal, oil and natural gas, which contribute to the highest gas emissions.

The link of AMR and climate change is multifaceted and dynamic. Climate conditions and climate change related extreme events exacerbate the threat of AMR. Rising temperatures create a favorable condition for bacterial growth and increase the spread of resistance genes in microorganisms, which can happen through HGT. Additionally, increased temperature have been associated with increased antibiotic resistance in common pathogens such as *E. coli, K. pneumonia* and *S. aureus* (MacFadden, McGough, Fisman, Santillana, & Brownstein, 2018). For instance, *Campylobacter, E. coli, Salmonella* and *V. cholera* thrive in warmer climates, and diarrheal infections caused by these pathogens are increasing (Omazic et al., 2019; Semenza & Menne, 2009). Moreover, some strains of these pathogens might produce heat shock proteins or biofilm to withstand the increased temperature (Yin, Wang, Liu, & He, 2019).

On the other hand, extreme weather events, such as drought and flooding have direct implications on human and animal health, as well as the environment. These events disrupt the ecosystem and displace people, resulting in overcrowding in particular areas, which leads

to increased interaction between humans and animals (Zinsstag et al., 2018). This closer contact facilitates the transmission of zoonotic diseases, potentially harboring novel antibiotic resistance genes (Zinsstag et al., 2018). The overflowing of contaminated water from sewage lines may amplify AMR transmission in areas with poor sanitation infrastructure through sewage, a known reservoir for AMR genes (Karkman, Do, Walsh, & Virta, 2018). Furthermore, droughts can compromise sanitation infrastructure and force people to share limited water sources, facilitating waterborne disease outbreaks like diarrhea (G. Cissé, 2019). The food insecurity related to these extreme events adds to the burden of infection to malnutrition, which further increases vulnerability to infections caused by resistant pathogens (Roberta Magnano San Lio, Giuliana Favara, Andrea Maugeri, Martina Barchitta, & Antonella Agodi, 2023; Springmann et al., 2016).

1.7.1 Knowledge and attitude of antimicrobial use and resistance in LMICs

Between 2016 and 2020, Ethiopia witnessed a 16% increase in defined daily dose (DDD) consumption rates of antibiotics (Gutema et al., 2021). This extensive antibiotic use, spanning from healthcare facilities to communities, agriculture, and animal husbandry, resulted in usage rates ranging from 63% to 97% (Fentie et al., 2022; Geta & Kibret, 2021). Additionally, self-medication with antibiotics increased, exceeding 40% in both human and animal sectors, with most antibiotics obtained without a prescription (72%) (Geta & Kibret, 2021; Muhie, 2019).

Some populations, such as pastoralists, whose livelihood relies on livestock and livestock products, especially, are exposed to antibiotics, both through their livestock as well as personal consumption. These communities heavily depend on animals for their subsistence and income. Antimicrobials are often used for livestock production to maintain good health and productivity of the animals (Robinson et al., 2016). Most of this antimicrobials used for food animals are obtained without prescription or consultation of veterinarians in LMICs (Alhaji & Isola, 2018).

According to recent studies, despite the widespread use of antimicrobials in both the human and animal sectors, approximately 80% of pastoralists were unable to describe antimicrobials and their use, and 77% of farmers reportedly store them under suboptimal conditions (Biruk Alemu Gemeda et al., 2020; Tufa et al., 2018). Furthermore, 98% of pastoralists consume milk or meat from animals treated with antimicrobials, unaware of the necessity of withdrawal periods (Biruk Alemu Gemeda et al., 2020). In animal husbandry, misuse of antimicrobials is concerning, with animals frequently given incorrect doses (either higher or lower than recommended) at a frequency of 2-5 times per month (Geta & Kibret, 2021). Astonishingly, 80% of the pastoralists interviewed in (Biruk Alemu Gemeda et al., 2020) had never heard of AMR, and none of the 10 public health officials interviewed had implemented stewardship

programs to enhance antimicrobial use in healthcare facilities (Fentie et al., 2022). This could be due to the nomadic lifestyle of pastoralist populations that hinders access to education on this marginalized group, which increases the misuse of antimicrobial in humans and agriculture.

In response to the crisis of AMR in the context of the One Health approach, both the Global Action Plan (GAP) and Ethiopian National Action Plan (NAP) emphasized, as one of their five objectives, the necessity to improve the awareness and understanding of AMR through effective communication, education, and training targeted at the public, farmer, healthcare and veterinary professionals (WHO, 2015). Additionally, the GAP highlighted the importance of incorporating antimicrobial use (AMU) and resistance into school curricula (WHO, 2015).

The misuse and overuse of antimicrobials across sectors is influenced by multiple factors. These include knowledge, expectations and social pressure, nature of practices, interaction and communication of patients and prescribers, economic status, characteristics of health care systems – such as access to diagnostic facilities and alternative medicine – and the regulatory environment (E. Gebeyehu, Bantie, & Azage, 2015; Mallah, Orsini, Figueiras, & Takkouche, 2022; Tufa, Regassa, Amenu, Stegeman, & Hogeveen, 2023).

The most significant gap is in implementing education and awareness interventions on the judicious use of antimicrobials across different target audiences (Dejene, Birhanu, & Tarekegn, 2022). Countries have primarily confined their education and awareness efforts on global commemorations, particularly during the World Antimicrobial Resistance Awareness Week (WAAW), characterized by small-scale antimicrobial resistance campaigns scattered across different sectors (WHO, 2022b). Yet, considering the complexity, multifaceted, cross-sectoral dimensions of AMR and its crosscutting nature, one-off campaigns are inadequate to instigate sustainable behavioral change or have a lasting impact.

1.7.2 Knowledge and attitude related to climate change in LMICs

Globally, climate change and its related extreme events pose a significant public health threat, compelling urgent interventions due to its increasing and disproportionate impact on human health and the ecosystem (Guéladio Cissé et al., 2022). Due to the multitude of climate-related effects on global health**,** such as heat waves-related illnesses, respiratory illnesses, zoonotic and food borne diseases, water and vector-borne diseases, impaired mental health, storms and floods related deaths, it is estimated that an additional 250,000 lives will be lost annually between 2030 and 2050 (WHO, 2014).

Climate change has natural causes, however, current trends are largely attributed to anthropogenic activities, mostly the burning of fossil fuels, industrial pollution, deforestation

and land use changes (Barker et al., 2007). The continued reliance on these practices, and engagement in activities that generate greenhouse gases, are projected to exacerbate the intensity of climate change, posing an existential threat to public health, food security, economic development and the environment (Rayner & Jordan, 2016). Specifically, LMICs will bear the heaviest burden, despite being the least contributors to greenhouse gas emissions (Easterling et al., 2007; Sanson & Burke, 2020). These countries are often more vulnerable due to their resource-constrained infrastructure and lack of capacity for preparedness and adaptation to the impact of climate change (Morton, 2007). Successfully transitioning to a low-carbon future requires major changes in individual behavior patterns, along with intervention from political and economic stakeholders, as a crucial strategy in addressing this pressing issue (Whitmarsh, Seyfang, & O'Neill, 2011).

Knowledge is a key driver of global action on climate and its impact in all sectors (Shi, Visschers, Siegrist, & Arvai, 2016). A strong understanding of the climate crisis, as established by science, appears to be essential for the public's willingness to change behavior and support climate change policies (Hundera, Mpandeli, & Bantider, 2019; Shi et al., 2016). Attitude towards it is another important factor, as communities change the information about climate change into psychological awareness (Chowdhury, Ahmed, Ahmed, & Haq, 2021). Both knowledge and attitude influence the transformations needed to mitigate the negative impact of climate change, and to adapt climate change mitigation strategies (Busch, Henderson, & Stevenson, 2019; Trott, 2020). However, there is a tremendously limited knowledge and percepton of climate change in Africa, with a significant proportion of people having never heard of it (T. M. Lee, Markowitz, Howe, Ko, & Leiserowitz, 2015). Low levels of knowledge, negative attitude and poor practice have been reported in Ethiopia and Nigeria (Ebuehi & Olusanya, 2013; Melore & Nel, 2020). Despite this, communities retain some forms of indigenous knowledge, which could be valuable for adaptation strategy (Melore & Nel, 2020).

The public concern about the adaptation and mitigation of climate change is shaped by their level of knowledge and attitude (Corner, 2012; Shi et al., 2016). For instance, farmers hold different perspectives on climate change than the scientific community (Nguyen, Seddaiu, & Roggero, 2019). In essence, scientists approach climate change through specific frameworks, while farmers depend on their social values, interactions within their local societies, and their own constructs of climate change understanding to adapt to it (Nguyen et al., 2019). Therefore, the scientific adaptive strategies may not be favorable to local communities, as they often lack local and traditional human and ecological knowledge. Pastoralists' knowledge gained through extensive experience, observation and traditional practices, is well-suited to the temporal, spatial, and cultural diversity of their communities (Abate, 2016). Subsequently, it enables them to better grasp the implications of climate change on health and strategically respond to

its adverse effects (Mamba, Salam, & Peter, 2015; Rao, Ndegwa, Kizito, & Oyoo, 2011). Thus, to integrate the traditional and scientific knowledge, it is inevitable to comprehend the communities' knowledge and attitude in the vicious cycle of climate change. Such a two-way exchange promotes mutual respect and facilitates a more inclusive approach to addressing climate change.

1.8 Jigjiga University One Health Initiative (JOHI) project

This thesis is part of a bigger project called Jigjiga University One health Initiative (JOHI), established through a partnership between Jigjiga University (JJU), the Armauer Hansen Research Institute (AHRI) and the Swiss Tropical and Public Health Institute (Swiss TPH) in the Somali regional state. This project aims to develop and implement innovative solutions that promote health, resilience, and sustainability in the pastoralist and agro-pastoralist communities in the Somali region, utilizing a transdisciplinary approach.

The Somali region of Ethiopia, formed in 85% by pastoralist populations, is one of the regions with the highest child mortality rates and lowest access to education in the country (Ebrahim & Atteraya, 2023; Gezahegn, 2006; Oh, 2019). Along this burden, the region is highly vulnerable to the consequences of the climate crisis and AMR, due its unique characteristics. For instance, the region's livelihood depends on livestock and livestock products, which are influenced by climatic conditions (Bogale & Erena, 2022). Furthermore, in these instances, human-animal proximity may facilitate microbial exchange between species (Zinsstag et al., 2011). In recent years, the region has also been exposed to climate crises like droughts and flooding, which have triggered outbreaks of diarrheal disease, especially in children (G. Cissé, 2019; Guéladio Cissé et al., 2022; Zinsstag et al., 2018). These outbreaks escalate antimicrobial use, always administered empirically due to the limited diagnostic capacity available in the region. This exercise enhances alarm about the inappropriate use of antimicrobials and the emergence of treatment-resistant pathogens. Moreover, the region's wide border with Djibouti, Kenya and Somalia, facilitates the import of unregulated medication, without country quality checks, increasing the concern about the use of poor quality antibiotics. Because of political reasons and the lack of capacity in the area, the Somali region has been excluded from the national AMR surveillance system, and its consequences have been felt by the population.

The paucity of research on AMR truly applying the One Health approach in the country and the exclusion from the national AMR surveillance system in the region created a blind spot hindering the understanding of AMR prevalence in rural communities and animals at the regional level. Therefore, this thesis, under the umbrella of the JOHI project, aimed to implement a holistic One Health approach to understand the prevalence of AMR genes in rural

and urban children and livestock, as well as community's knowledge and attitude on AMR and climate change.

In addition to this thesis, the project enabled me to be involved in other project initiatives in the last years, such as "Gut microbiomes and their unique dietary habits" and "Prevalence and associated risk factors of intestinal parasitic infections" among children in pastoralist and agro-pastoralist communities in the Adadle woreda of the Somali Regional State of Ethiopia. The outcomes of these projects are provided in the appendix of this thesis.

Chapter 2 Aim and objectives

2.1 Aim

The main goal of this thesis was to use a holistic One Health approach to determine the prevalence of antimicrobial resistance genes between humans and animals, the resistance profile of diarrheagenic pathogens, as well as the community knowledge regarding AMR and climate change in the Somali region.

2.2 Objectives

Given the above aim, the specific objectives were:

- To conduct a systematic review on molecular studies on AMR genes utilizing One Health in Africa;
- To assess the genetic characterization of fecal carriage of ESBL-producing *E. coli* in healthy children and livestock;
- To assess the genetic characterization of diarrhea pathogens and their resistance genes;
- To evaluate the knowledge, attitude and practices of rural communities regarding AMR and climate change.

Each of the specific objectives are presented in separate chapters of this thesis (chapters 3 to 6). An overall discussion and conclusions are presented in chapter 7 and 8.

Chapter 3 Systematic review and meta-analysis of integrated studies on antimicrobial resistance gene in Africa - A One Health perspective.

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3.1 Abstract

Background: Increasing antimicrobial resistance (AMR) raises serious health and financial concerns. However, the main drivers of the emergence, spread and subsequent colonization of resistant bacterial strains between humans, animals and the environment are still poorly understood.

Objective: The aim of this review was to identify molecular studies on AMR in One Health settings in Africa and to determine the prevalence of antimicrobial resistance genes in humans, animals and the environment. Due to the very low number of studies including environmental samples, the meta-analysis only includes data obtained from animals and humans.

Methods: The PubMed, Web of Science and Scopus databases were searched, identifying 10'464 publications on AMR in Africa from January 1st, 2000 until June 1st, 2020. Inclusion criteria were: 1) Integrated studies assessing AMR simultaneously in an animal-human, animal-environment, human-environment or animal-human-environment context, 2) Genotypic characterization of AMR and 3) temporal and spatial relationship between samples from humans and animals. Statistical random effects model meta-analysis was performed.

Results: Overall, 18 studies met our eligibility criteria and were included in this review. The majority of studies (N = 6) investigated *E. coli* and Salmonella spp. (N = 6). The most prevalent AMR genes in animals included *sul1* (36.2%), *sul2* (32.0%), *tetA* (31.5%), *strB* (30.8%) and *blaTEM* (30.0%), whereas *sul2* (42.4%), *tetA* (42.0%), *strB* (34.9%), *blaTEM* (28.8%) and *sul1* (27.8%) were most prevalent in humans. We observed no clear pattern for a higher prevalence in either the animal or the human reservoir.

Conclusion: To date, there is only limited data for AMR in a One Health perspective in Africa. Prospective and longitudinal studies using an integrated One Health approach assessing the environment, animals and humans at the same time are needed to better understand the main drivers of AMR sharing in Africa.

3.2 Introduction

By 2050, 10 million lives could be lost each year to antimicrobial resistant bacterial strains (AMR) (O'Neill, 2016). High selection pressure due to antibiotic overuse, inadequate prescribing (Kakkar, Walia, Vong, Chatterjee, & Sharma, 2017), massive use to promote growth in livestock production and agricultural use (Evelina Tacconelli & Pezzani, 2019) are regarded as the most important drivers. Following acquisition of resistance, AMR is disseminated by clonal spread of bacteria and horizontal gene transfer (HGT), i.e. by plasmids

or integrons (Rozwandowicz et al., 2018), resulting in accumulation of antimicrobial resistance genes (ARGs) in bacteria. The collection of all ARGs in both pathogenic and non-pathogenic bacteria within an individual organism or a given environment (i.e. surface water, animal or human gastrointestinal tract etc.) is referred to as the resistome (Wright, 2007).

Recent resistome studies demonstrated that environmental reservoirs such as ground and surface water (Aga et al., 2018; O'Neill, 2016) and animal hosts represent an important pool of mobile ARGs (Hu et al., 2016; Pal, Bengtsson-Palme, Kristiansson, & Larsson, 2016). Further, the exchange of ARGs between bacteria from different reservoirs, such as farm animals, farm soil and clinical pathogens, was found to occur via HGT (Hu et al., 2013; B. Li et al., 2015). As an example, extended-spectrum beta-lactamase (ESBL) transmission from livestock to humans has been reported from China (H. Zheng et al., 2012). Feces from livestock, primarily poultry and pig, have been shown to contaminate soil and water mostly with ESBL-*Escherichia coli* (Junying Ma et al., 2012). Human fecal excrements have likewise been shown to transfer AMR to the environment (e. g. quinolones (qnr genes)) (Woolhouse, Ward, Van Bunnik, & Farrar, 2015). Multidrug resistant (MDR) soil bacteria have been found to contain resistance cassettes with the same nucleotide sequence as resistance genes found in diverse human pathogens (Forsberg et al., 2012). As humans, animals and the environment are in close contact and interconnected in a complex way, AMR is a quintessential One Health issue. AMR research in low-income countries is highly underrepresented and most resistome studies have focused on industrialized settings (Hu et al., 2013; B. Li et al., 2015; Smillie et al., 2011).

In low- and middle-income countries (LMIC), few studies have assessed AMR simultaneously in humans, animals, food and the environment, and those available often suffer from poor design and bias (Dhaka et al., 2016) or assess only selected sections of the socio-ecological system (SES). Only one study used a comprehensive SES design combined with a metagenomic analysis but sampled relatively few animals in a cross-sectional study with no clear conclusion on attribution or spread of AMR (Pehrsson et al., 2016) .

A One Health perspective is increasingly needed, especially in Africa, where lack of access to safe drinking water and close contact between animals and humans might lead to a changed landscape compared to more industrialized settings (Collignon & McEwen, 2019). Strategies targeted at reducing antibiotic misuse in humans and animals in LMICs have been proposed (Nadimpalli et al., 2018) . However, it might be that clonal dissemination plays an even more critical role on AMR spread than antimicrobial selection pressure (Davis, Hancock, & Besser, 2002).

This report presents a systematic review of scientific literature published between January 2000 and June 2020 on AMR resistant bacterial strains in Africa. The objective of this review was to identify studies targeting genotypic characterization of antimicrobial resistance genes simultaneously in animals, humans and the environment, examine the prevalence of shared resistance genes between these different sources and summarize evidence on the phylogenetic relationship of the assessed bacterial strains.

3.3 Methods

Search strategy

The literature search was performed in PubMed, Scopus and Web of Science between May and June 2020. The search was performed by two independent reviewers and compared. The following search-terms, with a publication limit of 1st January 2000 – 1st June 2020, were used to retrieve relevant articles published: *("antimicrobial resistance" OR "antibiotic resistance" OR "antimicrobial susceptibility" OR "Resistome") AND (Africa OR "Horn of Africa" OR Ethiopia OR Eritrea OR Somalia OR Djibouti OR Kenya OR Sudan OR Nigeria OR Egypt OR Congo OR "South Africa" OR Tanzania OR Algeria OR Morocco OR Uganda OR Mozambique OR Ghana OR Angola OR "Ivory coast" OR Madagascar OR Cameroon OR Niger OR "Burkina Faso" OR Mali OR Malawi OR Zambia OR Senegal OR Chad OR Zimbabwe OR Rwanda OR Tunisia OR Guinea OR Benin OR Burundi OR Togo OR "Sierra Leone" OR Libya OR "Central African Republic" OR Liberia OR Mauritania OR Namibia OR Botswana OR Lesotho OR Gambia OR Gabon OR Mauritius OR Eswatini OR Comoros OR "Cape Verde" OR Seychelles)*. Only publications in English were included. EndNote X9 was used to manage citations. Duplicate entries were identified by considering the title of the article, the author and the year of publication.

Selection criteria

Articles were reviewed separately by two independent reviewers. After removing duplicates, the remaining 6,754 articles were screened based on title and abstract. The following inclusion criteria were defined:

Integrated: Studies assessing AMR simultaneously in an animal-human, human-environment, animal-environment or human-animal-environment context.

Genotypic characterization of AMR: Studies applying genotypic methods to target specific ARGs either by polymerase chain reaction (PCR) or whole genome sequencing (WGS).

In total, 6,677 studies were excluded for the following reasons: did not use genetic methods for the detection of ARGs, studied AMR in an isolated manner for human, animal or

environmental samples only, ineligible geographic location or resistance in other microorganisms (viruses, protozoa or helminths). From the 77 studies remaining after title/abstract screening, 5 were not accessible online and could not be retrieved by contacting the authors. The remaining 72 studies were included for full text analysis. Subsequently, articles were filtered for studies where samples from different environments were temporally and spatially related and phylogenetic relationship was assessed. This led to the exclusion of another 45 articles. From the remaining 32 articles, another fourteen articles were excluded, due to methodological reasons, such as missing information on the origin of the samples, genotypic assessment of only a single bacterial strain, or inconsistency in results (results mentioned in text were different from results shown in figure/table). Eighteen articles were finally included in the systematic review. Figure 1 summarizes the flow-chart of selection steps and articles retained.

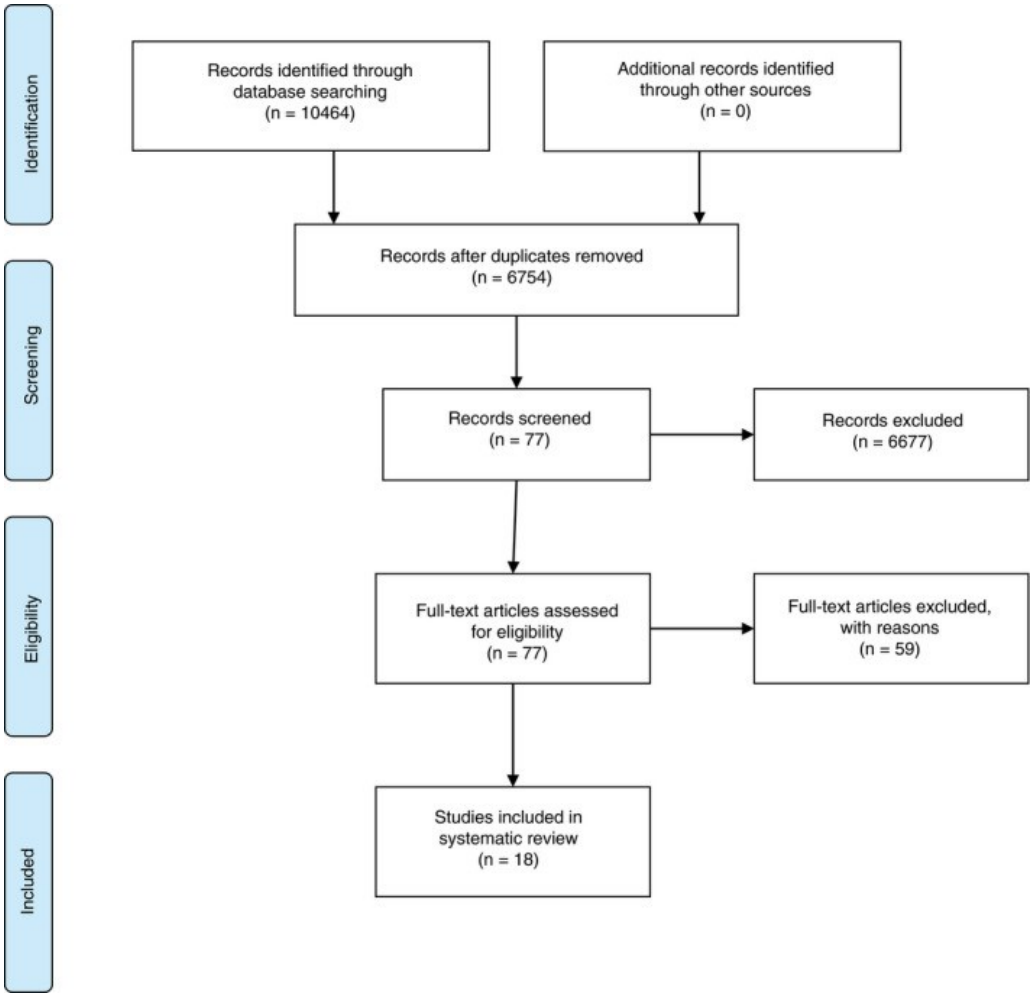

Figure 1. Search Strategy and PRISMA flow diagram

Data extraction

Data from selected studies were extracted under the following parameters: (a) Study identifier: first author, year of publication, country, sampling population (specific animal, human or environmental source/host), reservoirs studied (animal-human, animal-environment, human-environment); (b) methodology: antimicrobial susceptibility testing, genotyping method for detection of ARGs; (c) results: bacterial species isolated, number of bacterial isolates from each source (animal, human, environment) and number of each ARG found for each strain. Data extraction was performed by two independent researchers and compared.

Selection of resistance genes studied

For the analysis, only genes that were studied in at least two different studies were considered. The following genes occurred only in a single study and were therefore not included in the final analysis: *vanB, vanA, tet(L), blaACT, blaNDM, blaMOX-CMY, aac(6')-aph(2''), aac(3)-IId, aac(3')-IIa, aac(3')-Iva, aph(6)-Id, aph(3')-Via, aph(3'')-Ib, aph(3')-Ic, aph(3')-Ia, dfrC, drfA18, drfA10, mph(c), dhfr1, dhfr5, dhfr12, dhfr13, dfrG, norA, rpoB(H481N9), aadD, spc, ant(4')-Ib, ampC, mrx, ere(B), tet(X), ant2, int1, int2, arsB-mob, qacEdelta1, qacL, mrs(A), mrs(E), mph(E), parE(D434N9), inu(F), ere(A), oqxA, oqxB, blaEC.*

Statistical analysis

We included in this meta-analysis studies reporting the number of samples and the number of AMR positive samples to estimate the relative risk. Studies were grouped on the basis of bacterial species. A pooled risk ratio (RR) was then calculated if for the given bacterial species, if the gene was tested in at least two different studies. Heterogeneity was assessed by the I^2 and τ^2 statistics. We exclusively used random-effects models, irrespective of whether heterogeneity was present or not. For all statistical analyses we used the R software environment version 4.0.3 and the “meta” package version 4.14-0. We used the function “metabin” using the Mantel-Haenszel method with inverse variance weighting for pooling (Mantel & Haenszel, 1959).

3.4 Results

Overview of the selected studies

Based on the eligibility criteria, a total of 18 original studies with a count of 1,988 isolated bacterial strains (981 Escherichia sp., 316 Campylobacter spp., 278 Staphylococcus spp., 413 Salmonella spp.) were included for this systematic review and meta-analysis. An overview of the selected studies is given in Figure 2. Selected studies and study identifiers are listed in Table 1. Although the time interval searched was between 2000 and 2020, the earliest study

that met the eligibility criteria was from 2014, and the number of studies per year increased in recent years (Figure 2C). Studies were available from ten different countries: Tunisia, Algeria, Egypt, Nigeria, Ghana, Ethiopia, Uganda, Zambia, Botswana and South Africa (Figure 2A), and included data from four bacterial genera: Escherichia sp., Salmonella spp., Staphylococcus spp. and Campylobacter spp., with six, six, four and two studies respectively (Figure 2B). The majority of the studies (15/18) detected ARGs by PCR, however, five studies applied WGS, with some studies applying both methods (Figure 2E). In 15/18 studies, samples were collected from human and animal sources (Figure 2D). Only three studies included sampling from environmental sources, with one study examining animal and environmental samples and two studies assessing human and environmental samples. There was not a single study meeting our inclusion criteria that covered all three domains.

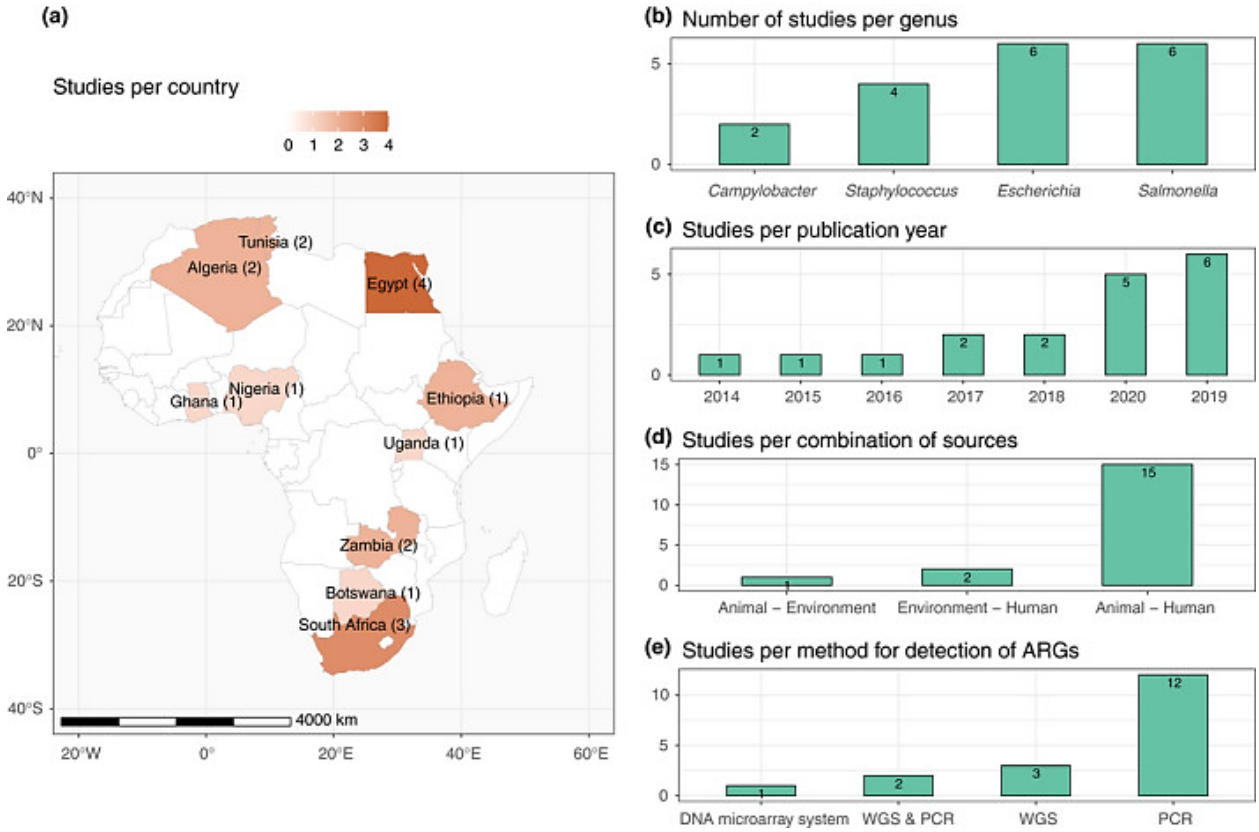

Figure 2. Summary of the selected studies showing number of studies (a) per country, (b) per bacterial genus, (c) per combination of sources and (e) applied methods for detection of ARGs

Table 1. Overview of the selected studies

ID	Reference	Country	Pathogen	Source	Detection ARGs	ARGs tested	Phylogeny	*n*
1	Agabou, A.; Clonal relationship between human and avian ciprofloxacin-resistant Escherichia coli isolates in North-Eastern Algeria (2015)	Algeria	*Escherichia*	Human, chicken	PCR	*blaKPC*, *blaOXA-48-like*, *blaVIM*, *blaIMP*, and *blaNDM*, *blaTEM*, *blaSHV*, *blaCTX*, *qnrA*, *qnrB*, *qnrS*, *qepA*, *aac(6')-Ib-cr*, *oqxAB*	MLST	94
2	Ahmed, H.A.; Characterizationof Virulence-Associated Genes, Antimicrobial Resistance Genes, and Class 1 integrons in Salmonella enterica serovar Typhimurium Isolates from Chicken meat and Humans in Egypt (2016)	Egypt	*Salmonella*	Chicken meat, humans	PCR	*blaTEM*, *aadB*, *aadC*, *aadA*, *floR*, *tetA(A)*, *tetA(B)*, *sul1*,*invA*, *avrA*, *mgtC*, *stn*, *bcfC*	gyrA gene sequencing	78
3	Ajayi, A.; Molecular diversity and antibiotic resistance gene profile of Salmonella enterica serovars isolated from humans and food animals in Lagos, Nigeria (2019)	Nigeria	*Salmonella*	Humans, food animals (cattle, sheep, chicken)	PCR	*qnrA*, *qnrB*, *qnrS*, *gyrA*, *blaSHV*, *blaCTX*, *blaTEM*, *tet(B)*, *tet(A)*	gyrA gene sequencing	71
4	Amoako, D. G.; Genomic analysis of methicillin-resistant Staphylococcus aureus isolated from poultry and occupational farm workers in Umgungundlovu District, South Africa (2019)	South Africa	*Staphylococcus*	Poultry, occupational farm workers	Whole genome Sequencing, PCR	*mecA*, *blaZ*, *aac(6')-aph(2")*, *aadD*, *spc*, *ant(4')-Ib*, *erm(A)*, *erm(C)*, *msr(A) and mph(C)*, *tet(M)*, *tet(K)*, *dfr(C)*, *gyrA*	MLST, spa typing, clonal complex prediction	145
5	Chukwu, M. O.; Antibiotic resistance profile and clonality of E. coli isolated from water and paediatric stool samples in the north-west, province South Africa (2019)	South Africa	*Escherichia*	Pediatric stool samples, water	PCR	*blaCTX*, *blaSHV*, *blaCMY*, *blaDHA*	ERIC-PCR	240

6	Chukwu, M. O.; Characterization and Phylogenetic Analysis of Campylobacter Species Isolated from Paediatric Stool and Water Samples in the Northwest Province, South Africa (2019)	South Africa	*Campylobacter*	Pediatric stool samples, water	PCR	*gyrA*, *tetO*	ERIC-PCR	257
7	De vries, S. P. W.; Phylogenetic analyses and antimicrobial resistance profiles of Campylobacter spp. from diarrhoeal patients and chickens in Botswana (2018)	Botswana	*Campylobacter*	Humans with diarrhea, chickens	whole genome sequencing	*tetO*, *gyrA*, *blaOXA*	Core genome alignments, SNP based tree	90
8	Dhaouadi, S.; Prevalence of meticillin-resistant and -susceptible coagulase-negative staphylococci with the first detection of the mecC gene among cows, humans and manure in Tunisia (2020)	Tunisia	*Staphylococcus*	Cows with mastitis, humans, manure	PCR	*mecA*, *mecC*, *blaZ*, *tet(K)*, *erm(A)*, *erm(B)*	PFGE	49
9	Djeffal, S.; Prevalence and clonal relationship of ESBL-producing Salmonella strains from humans and poultry in northeastern Algeria (2017)	Algeria	*Salmonella*	Humans, poultry	PCR	*blaCTX*, *blaTEM*	MLST	83
10	Eguale, T.; Genetic markers associated with resistance to beta-lactam and quinolone antimicrobials in non-typhoidal Salmonella isolates from humans and animals in central Ethiopia (2017)	Ethiopia	*Salmonella*	Cattle, poultry, swine, human	PCR, sequencing	*blaTEM*, *blaSHV*, *blaPER*, *blaPSE*, *blaOXA*, *gyrA*, *gyrB*, *parC*, *parE*, *qnrA*, *qnrB*, *qnrD*, *qnrS*, *qepA*, *aac(6')-Ib-cr*, *blaCMY*, *blaCTX*	MLST	72
11	Egyir, B.; Whole genome sequence profiling of antibiotic resistant Staphylococcus Aureus isolates from livestock and farm attendants in Ghana (2020)	Ghana	*Staphylococcus*	Livestock, farm attendants	Whole genome sequencing, PCR	*mecA*, *mecC*	MLST	25

12	Elhariri, M.; Virulence and Antibiotic Resistance Patterns of Extended-Spectrum Beta-Lactamase-Producing Salmonella enterica serovar Heidelberg Isolated from Broiler Chickens and Poultry Workers: A Potential Hazard (2019)	Egypt	*Salmonella*	Broiler chickens, poultry workers	PCR	*blaCMY*, *blaTEM*, *blaSHV*, *blaOXA*, *blaPSE*, *blaCTX*, *amoC*	invA gene sequencing	33
13	Gwida, M.; Microarray-based detection of resistance and virulence factors in commensal Escherichia coli from livestock and farmers in Egypt (2020)	Egypt	*Escherichia*	Farmers, livestock	DNA microarray system	*aadA*, *strA*, *strB*, *aac(3')-Iva*, *ant2*, *qnrA*, *qnrS*, *tet(A)*, *tet(B)*, *tet(X)*, *sul1*, *sul2*, *sul3*, *dfrA*, *aar*, *mph(A)*, *mrx*, *ereB*, *cmlA*	Split network tree construction, detected by microarray	47
14	Iramiot, J. S.; Whole genome sequences of multi-drug resistant Escherichia coli isolated in a Pastoralist Community of Western Uganda: Phylogenomic changes, virulence and resistant genes (2020)	Uganda	*Escherichia*	Cattle, humans in pastoralis community	Whole genome sequencing	*blaOXA*, *blaTEM*, *catA*, *clmA*, *drfA*, *oqxB*, *qacL*, *qacE*, *qnrS*, *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*	SNP based tree	42
15	Kalai, W.; Antimicrobial susceptibility and MLVA analysis of S. Typhimurium strains isolated from human and poultry samples in Tunisia (2018)	Tunisia	*Salmonella*	Poultry, humans	PCR	*blaTEM*, *blaSHV*, *blaCTX*, *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(G)*, *sul1*, *sul2*, *gyrA*, *parC*, *qnrA*, *qnrB*, *qnrS*, *aac(c')-Ib*, *oqxAB*, *qepA*	MLVA	45
16	Mainda, G.; Whole Genome Sequence Analysis Reveals Lower Diversity and Frequency of Acquired Antimicrobial Resistance (AMR) Genes in E. coli From Dairy Herds Compared With Human Isolates From the Same Region of Central Zambia (2019)	Zambia	*Escherichia*	Human, dairy Herds	Whole genome sequencing	*strB*, *strA*, *sul2*, *tet(A)*, *tet(B)*, *sul2*, *blaTEM*, *aadA*	SNP based tree	296
17	Ramadan, H.; Antimicrobial Resistance, Genetic Diversity and Multilocus Sequence Typing of Escherichia coli from	Egypt	*Escherichia*	Humans, retail chicken, ground beef	PCR	*blaCTX*, *blaTEM*, *blaCMY*, *blaSHV*, *blaOXA*, *catA1*, *catA2*, *floR*, *tetA*, *tetB*, *sul1*,	PFGE, MLST	120

	Humans, Retail Chicken and Ground Beef in Egypt (2020)					*sul2*, *strA*, *strB*, *dhfr1*, *dhfr5*, *dhfr12*, *dhfr13*, *mphA*		
18	Youn, J. H.; Prevalence and characterization of Staphylococcus aureus and Staphylococcus pseudintermedius isolated from companion animals and environment in the veterinary teaching hospital in Zambia, Africa (2014)	Zambia	*Staphylococcus*	Companion animals, environment in veterinary teaching hospital	PCR	*catA*, *ermA*, *ermB*, *ermC*, *aac(6')-Ie-aph(2'')-Ia*, *mecA*, *blaZ*, *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)*, *vanA*, *vanB*	MLST, spa typing	48

Assessment of shared ARGs

We calculated the average prevalence for every single resistance gene from animal and human sources separately (Figure S1). For human isolates, *tetA* was the gene with the highest prevalence, followed by *sul2, floR*, *strB* and *sul1*. For animal samples, *blaZ* was the most prevalent gene (detected uniquely in Staphylococcus spp.), followed by *tetK, sul1, floR* and *sul2*. The average frequency for most of the genes is similar between the isolates from human and animal sources, highlighting the high number and degree of shared resistance genes between the two compartments. For most of the bacterial species, the number of studies and sample size was small. The analysis for Campylobacter spp. included 316 samples from 2 different studies, the analysis for Staphylococcus spp. included a total of 278 isolates across 4 different studies, and for Salmonella spp. 413 samples across 6 different studies were included. For Escherichia sp., the sample size was bigger, with a total of 981 isolates across 6 different studies.

Resistance genes found in *Escherichia sp.*

Although here we summarized studies by genus, the selected studies for Escherichia sp. exclusively examined *E. coli* isolates. Six studies on *E. coli* met our eligibility criteria, in which a total of 981 isolates were examined. From animal sources, the following resistance genes were found most frequently: *sul1* (36.2%), *sul2* (32.0%), *tetA* (31.5%), *strB* (30.8%) and *blaTEM* (30.0%). In human isolates, resistance genes have been detected following a similar frequency pattern: *sul2* (42.4%), *tetA* (42.0%), *strB* (34.9%), *blaTEM* (28.8%), *sul1* (27.8%). Subsequently, we assessed if genes were more frequently detected in human or in animal isolates using random effect models. No clear pattern emerged for the majority of the genes (Figure 3). The range of observed prevalences among studies inter-study differences were substantial, whereas some studies reported higher prevalence in humans and others in animals for the same gene. There were two genes with consistent trends: The aminoglycoside adenylyl transferase gene *aadA1* was detected more frequently in animal isolates across all studies, with a pooled risk ratio (RR) of 2.83 (95% confidence interval (CI): 1.13 to 7.11, Fig. 3A). The opposite was found for the chloramphenicol acetyl transferase *catA1*, which was consistently higher amongst *E. coli* isolated from humans, with a pooled RR of 0.39 (CI: 0.15 to 1.00), and no heterogeneity ($I^2 = 0\%$, $\tau^2 = 0$, Fig. 3C). For the other genes, no clear pattern was detected, with most of the genes having a pooled RR close to 1, suggesting a similar probability of occurrence for in humans and animals (Fig. 3 C-F).

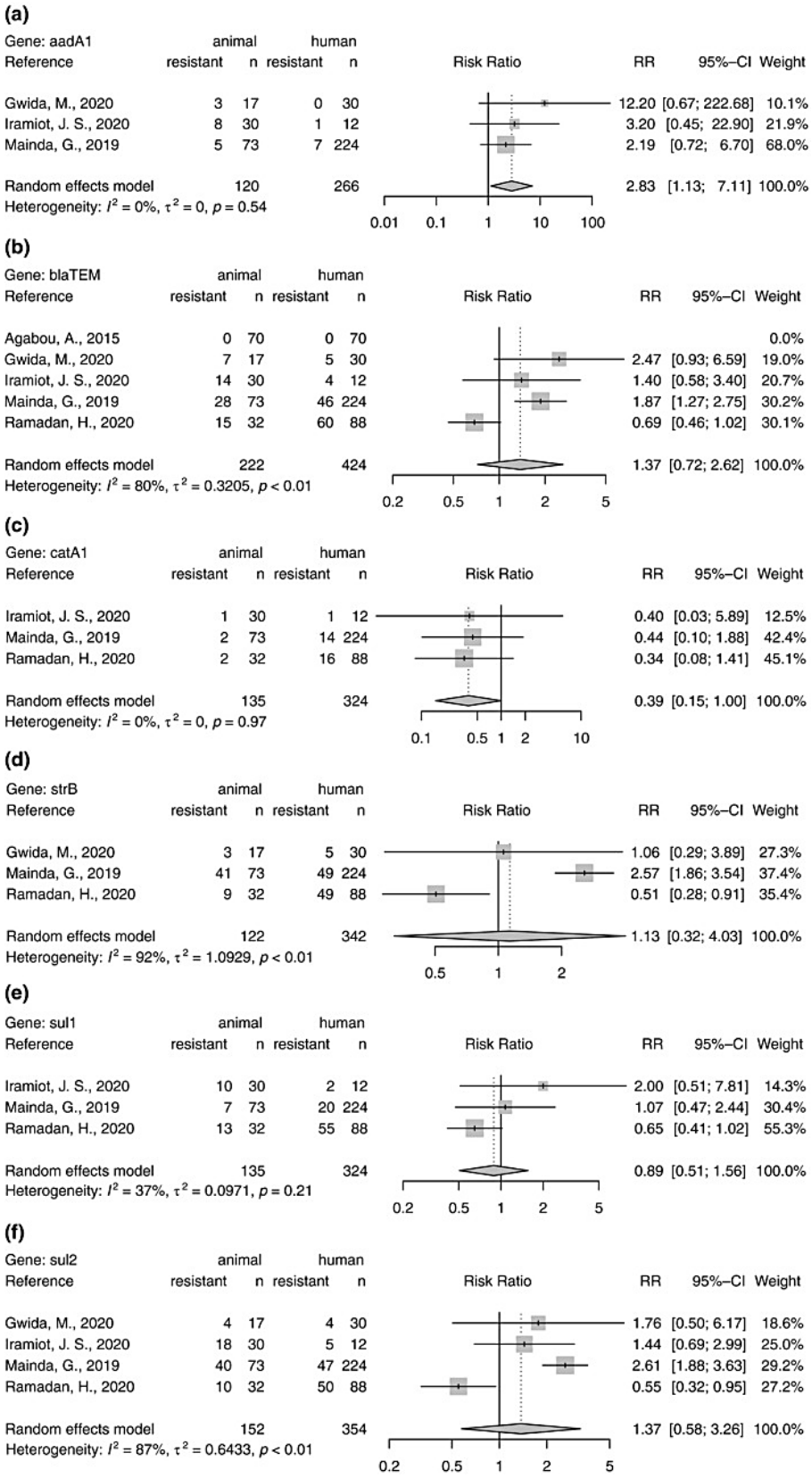

Figure 3. Forest plots for (a) aadA1, (b) blaTEM, (c) cat(A), (d) strB, (e) sul1 and (f) sul2

Resistance genes found in *Salmonella spp.*

6 studies on Salmonella spp. met our eligibility criteria, including 413 isolates. From animal sources, the following resistance genes were found most frequently: *floR* (75%), *aadA2* (70%), *sul1* (50.3%). For human samples, a similar pattern was observed: *floR* (70%), *sul1* (55.6%) and *aadA1* (50%). No clear pattern emerged for the majority of the genes in the random effect models comparing frequencies in humans and animals (Figure S4), suggesting little evidence that there is a difference between humans and animals. However, due to small sample size, the power is low.

Resistance genes found in *Staphylococcus spp.*

Four studies on Staphylococcus spp. were included in this review, compromising a total of 278 isolates. In isolates from animal sources, the most frequent genes were *blaZ* (30.9%), *tetK* (28.4%) and *tetM* (16.2%). For human isolates the most frequently detected gene was *catA* (40%), followed by *tetK* (36.7%) and *blaZ* (34%). There was no evidence for a significant difference in occurrence of the genes between human and animal samples (Figure S5).

Resistance genes found in *Campylobacter spp.*

Two studies on Campylobacter were included in this review, including a total of 316 isolates. In total, only 3 genes (*blaOXA, gyrA, tetA*) were detected with the following frequencies in humans 70%, 49.5% and 16.2%, and in animals 40%, 36.6% and 17.1%, respectively. Since the two studies did not include the same genes that were analyzed, no pooled RR could be produced and no data for the meta-analysis can be shown.

Overall, these results suggest that many resistance genes co-occur in animal and in human reservoirs with prevalences varying between different studies and settings. However, the co-occurrence of resistance genes does not necessarily mean that these genes share the same origin. Therefore, for this review, we included exclusively studies where samples were spatially and temporally related and where some kind of genetic relationship between samples from different sources was examined.

In the studies identified, the following genetic relationships between samples from different sources were found:

Agabou et al. identified seven major clonal groups across human and avian ciprofloxacin resistant *E. coli* from chickens and their farmers in Algeria, of which four were found simultaneously in human and in chicken isolates. Multi-locus sequency typing (MLST) further provided evidence of a genetic linkage of samples belonging to the same clonal group between human and animal isolates, suggesting that these pathogenic resistant strains share

the same origin. (Agabou et al., 2016) Iramiot et al. used Single Nucleotide Polymorphisms (SNPs) to cluster phylogenomic groups of samples from humans and cattle in pastoralist communities in western Uganda and found that 67% of *E. coli* isolated from cattle were closely related to those found in humans. (Iramiot, Kajumbula, Bazira, de Villiers, & Asiimwe, 2020) By using hybridization profiles, Gwida et al. investigated the relationship between multidrug resistant (MDR) isolates from different food producing animals (buffalo, cattle) and in-contact farmers. Due to the high similarity of hybridization patterns between some human and animal isolates, the authors assume a direct transmission between human and animal or vice versa of multi resistant strains. (Gwida et al., 2020) Ramadan et al. determined the existence of sequence types using MLST among *E. coli* isolates from diarrheic patients, retail chicken and beef in Mansoura, Egypt. Across 116 *E. coli* isolates, chicken and beef samples shared six sequence types, and human and animal samples shared two sequence types (one shared between human and chicken and one between human and beef). (Ramadan et al., 2020)

On the contrary, a study by Mainda et al. tested the relationship between resistance genes in *E. coli* from cattle and humans inhabiting the same region of Zambia by WGS and found no clear evidence for a genetic relationship of the isolates. (Mainda et al., 2019) Knowing that many ARGs are carried by mobile genetic elements such as plasmids, phylogenetic relationship alone may not be enough to infer transmission of ARGs, reiterating the difficulty of establishing transmission routes. (Woolhouse et al., 2015)

Estimates from different studies are significantly different, with some studies reporting higher prevalence of a given AMR gene/resistant bacterial strain in animal isolates and others in human isolates. Due to small sample size and high heterogeneity, none of the pooled effect estimates was found significant. Overall, these results suggest that a high number of frequent ARGs are shared between human and animal sources, however, certain strains and genetic elements might occur preferentially in one of the two compartments with a high variability between different settings and studies.

We last also assessed if there are co-occurrence patterns of specific resistance genes (Fig. S6). As the sample sizes of the individual studies differ and it is not always reported if a gene was not found or not reported, the graph shows only weak evidence of co-occurrence of different resistance genes and mainly highlights the need for further molecular studies on integrated AMR studies in Africa.

3.5 Discussion

Our meta-analysis revealed that while there are few studies assessing sharing of AMR genes between animals and humans, there is no single study with a comprehensive One Health approach focusing simultaneously on animals, humans and their wider environment.

A recent study by Chukwu et al. investigated the resistance profiles of *E. coli* pathotypes isolated from pediatric stool samples and drinking water in South Africa and examined the clonality of the isolates. The overall similarity between isolates from water and human sources was estimated between 80-90%, suggesting that domestic water plays an important role in the transmission of *E. coli* within the studied community setting (Chukwu, King Abia, Ubomba-Jaswa, Obi, & Dewar, 2019) and emphasizing the need for a comprehensive analysis of AMR in the wider context.

Figure 4 summarizes the relationship of humans, livestock, food, excreta, water and the environment reported in the 18 studies. For every related compartment (shown by arrows), we assessed the shared bacterial species and the most prominent type of antimicrobial resistance. Our analysis highlights the lack of data for many of the potential transmission routes and emphasizes the need for targeted One Health studies on AMR resistance.

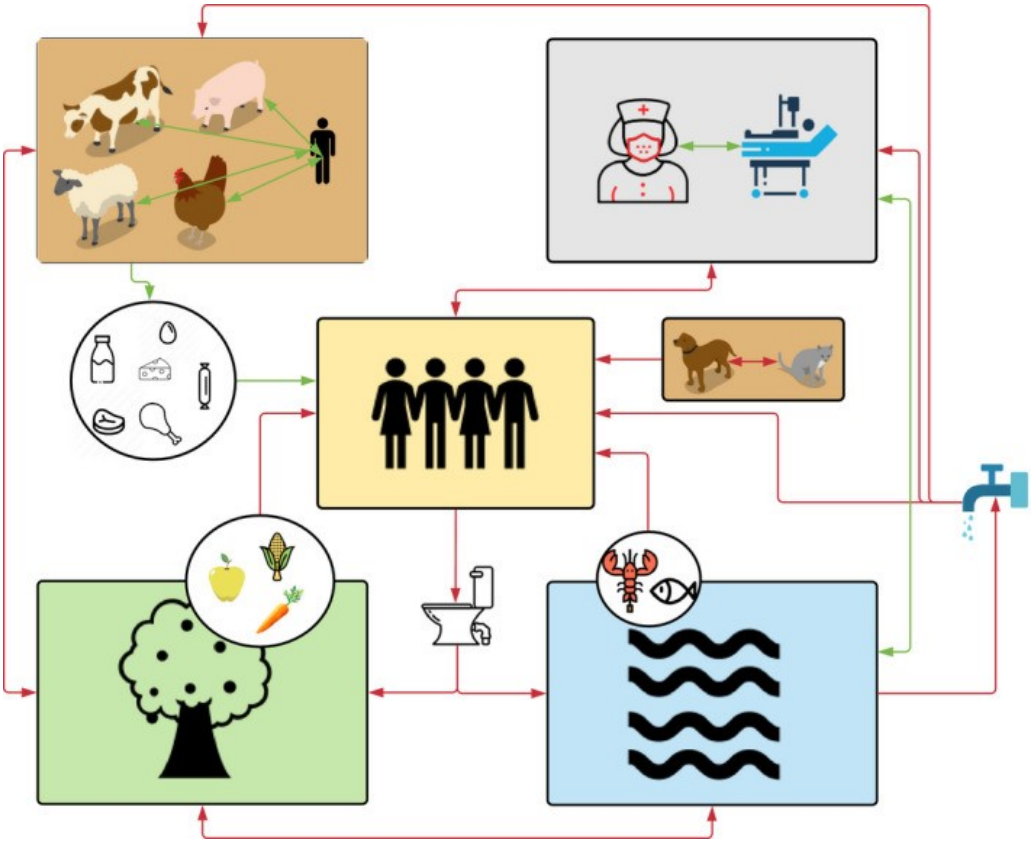

Figure 4. A potential schematic of the complex flow of antimicrobial-resistant bacteria in a human-animal-environment system.

Green arrows indicate that we identified studies assessing the shared occurrence between the connected reservoirs and red arrows indicate that literature is missing.

Our study further highlights that most of the studies performed so far isolated only limited bacterial strains and that strains which are more difficult to isolate (i.e. Campylobacter spp) are underrepresented. In our review, we could show that AMR prevalence varies widely between the studies and there is no clear trend to higher prevalence of any particular resistance gene in either animals or humans. The small sample size in each study could be, at least partially, responsible for the contradicting results when comparing prevalence in human and animal settings. However, different drivers of AMR emergence and spreading could also lead to divergent results. Future studies should not only have an integrated approach but increase also the number and type of strains assessed for AMR resistance, assessing the overall pool of resistance genes in a given environment ("resistome") as well as the phenotypic resistance of isolated strains.

All of the studies analyzed are cross-sectional and there is no temporal dimension. Therefore, while the studies allow for comparison of AMR gene sharing, they do not allow for assessing actual transmission routes. For future research, prospective cohort studies should be considered. For this review, only English publications were considered. Since Africa has many French speaking countries, this clearly represents a limitation to the study. However, a retrospective search of French publications in PubMed found only 48 articles (compared to 2,784 in English) of which none corresponded to the inclusion criteria fixed for this meta-analysis.

This review does not include any studies using culture-free methods such as metagenomics as none of these analyzed samples from more than one domain.

The results or our study do not show a clear predominance of AMR genes in animals (cattle, pigs and poultry) compared to humans. Based on the current knowledge, we can argue that livestock production in Africa *per se* is not a major driver for AMR emergence. This means that continuous attention should be paid to antibiotic use in livestock and humans. Antibiotic use in Africa oscillates between persistent lack of supply and un-controlled sale and use. National authorities must urgently regulate the sale and use of antibiotics for humans and animals in a better way.

AMR is a public health problem that transcends species and national borders. New types of AMR strains can disseminate globally following initial endemic emergence, as exemplified by several resistant clones that spread internationally (J. Hawkey et al., 2019; Johnson & Woodford, 2013). Indeed, emerging AMR in low-income settings has been shown to be an important source of worldwide spread (Karanika, Karantanos, Arvanitis, Grigoras, &

Mylonakis, 2016; Rousham, Unicomb, & Islam, 2018). To date, a single study on AMR in a low-income setting in Latin America used a comprehensive SES design combined with a metagenomic analysis to determine the resistome. However, the study sampled relatively few animals and as a cross-sectional study did not allow for clear conclusion on acquisition or spread of AMR (Pehrsson et al., 2016).

The simultaneous approach to human and animal health combined with robust epidemiological study design has a high potential to elucidate understanding of drivers for emergence and spread of ARGs. Only results from comprehensive One Health approaches, which integrate at the same time humans, animals, and their environment, will allow for inferring the most important transmission routes in Africa and for designing more efficient AMR control policies.

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3.6 Supplementary

Resistance genes isolated across all bacterial species

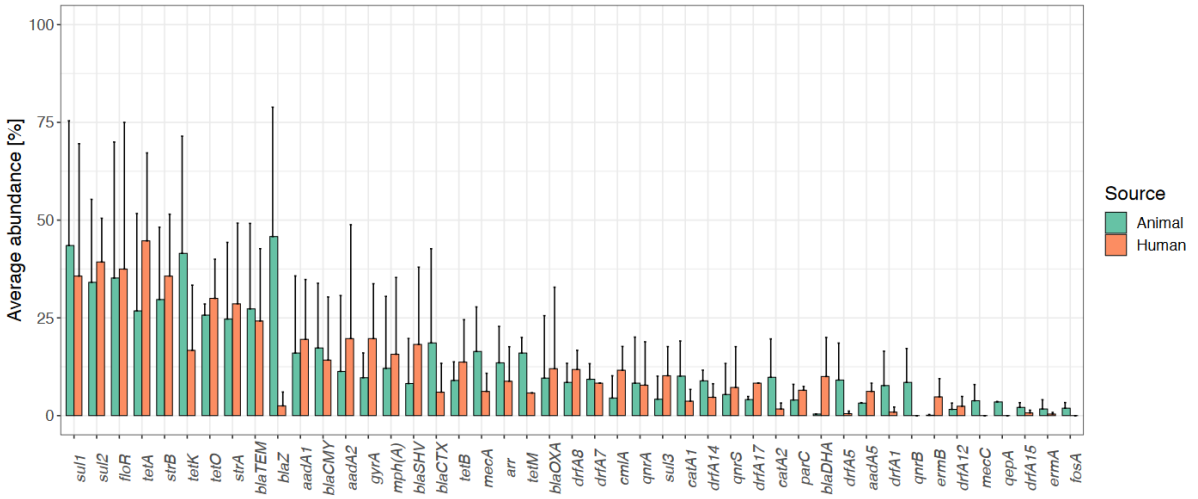

Figure S1. Bar plot showing the prevalence of resistance genes in bacterial isolates from animal and human sources. The study includes data from 15 studies, assessing ARGs in animal and human samples simultaneously

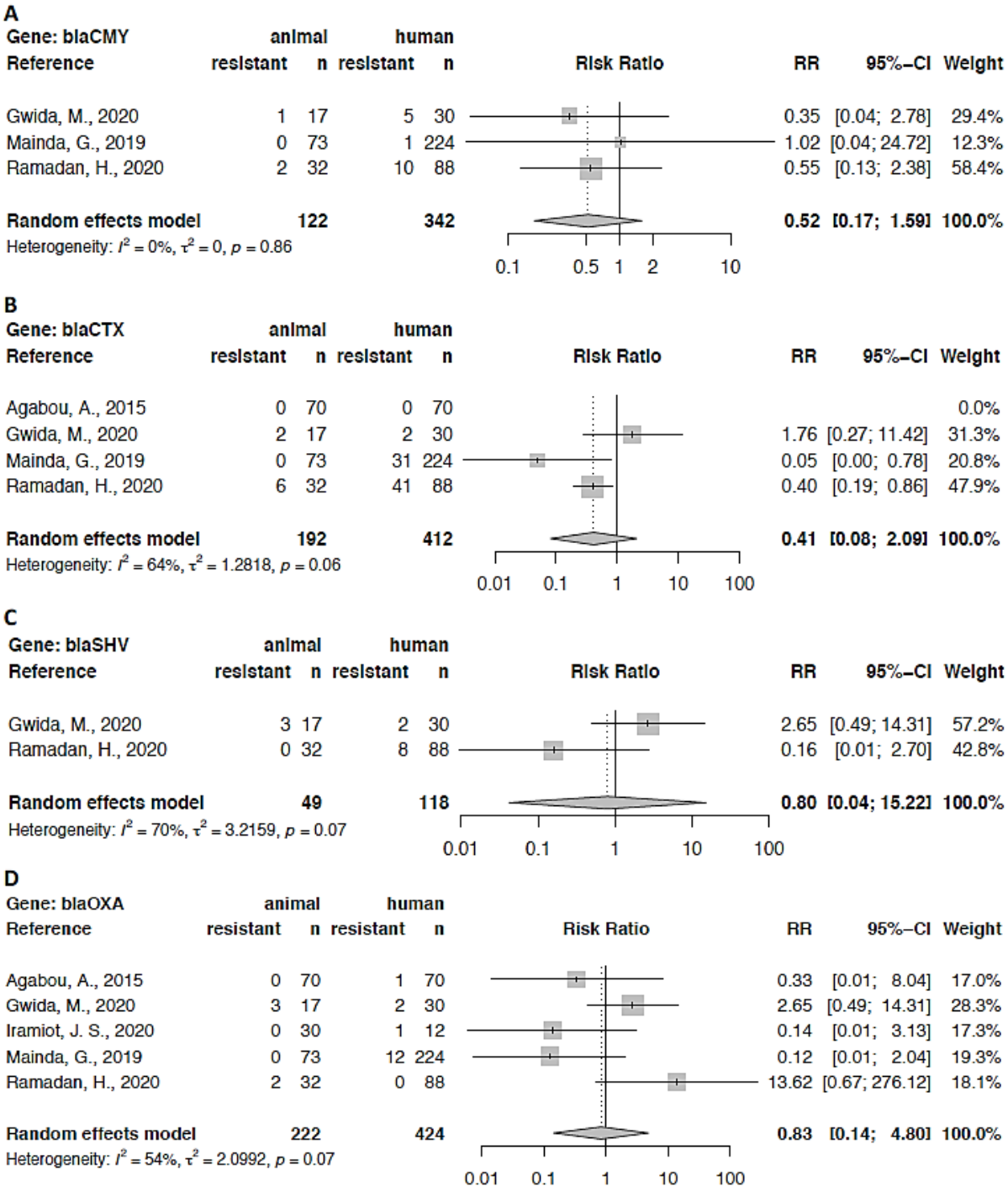

Figure S2. Forest plots for beta-lactamase resistance genes: (A) blaCMY, (B) blaCTX, (C)19 blaSHV, (D) blaOXA

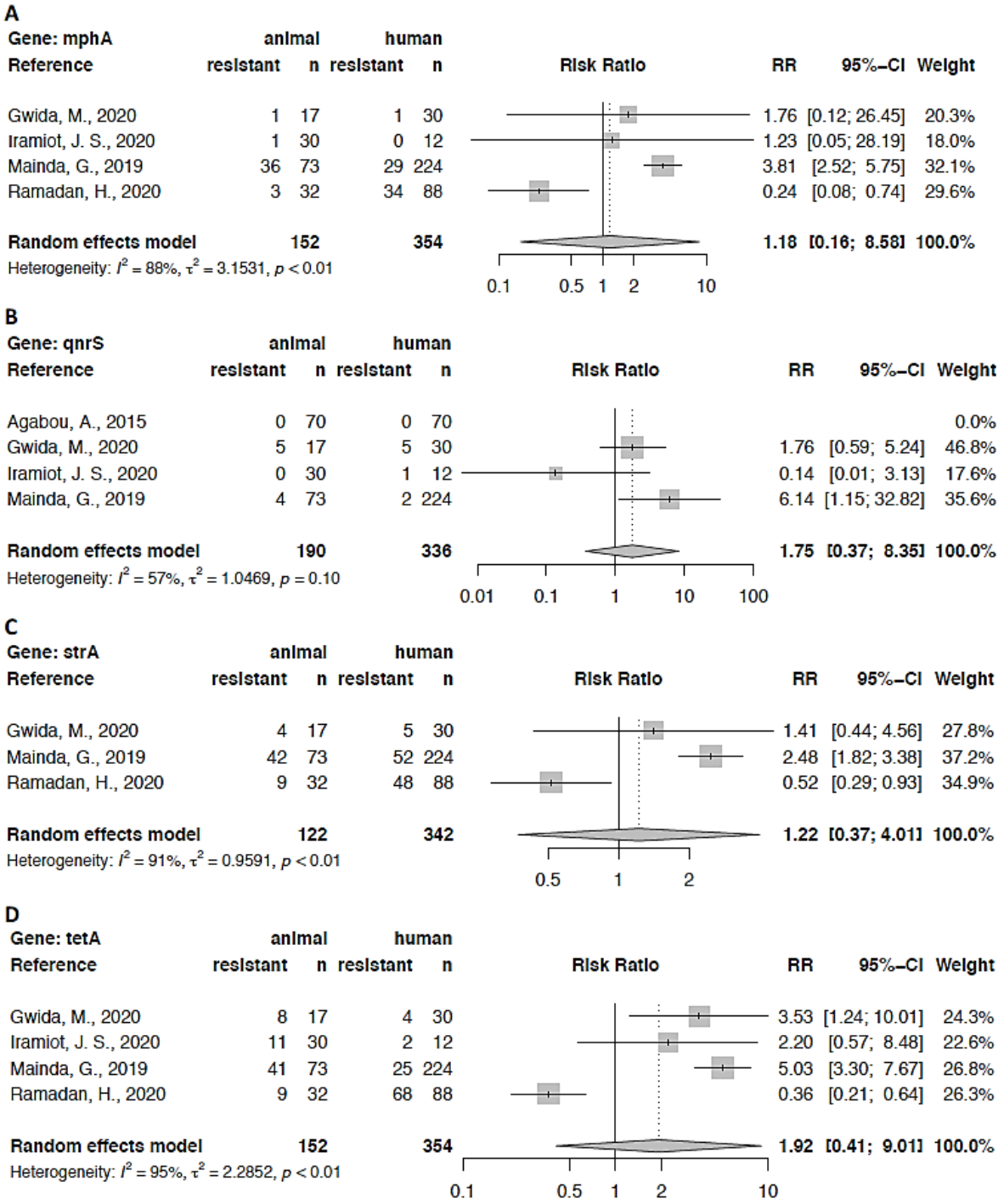

Figure S3. Forest plots for (A) mphA, (B), qnrS (C), strA (D) tetA

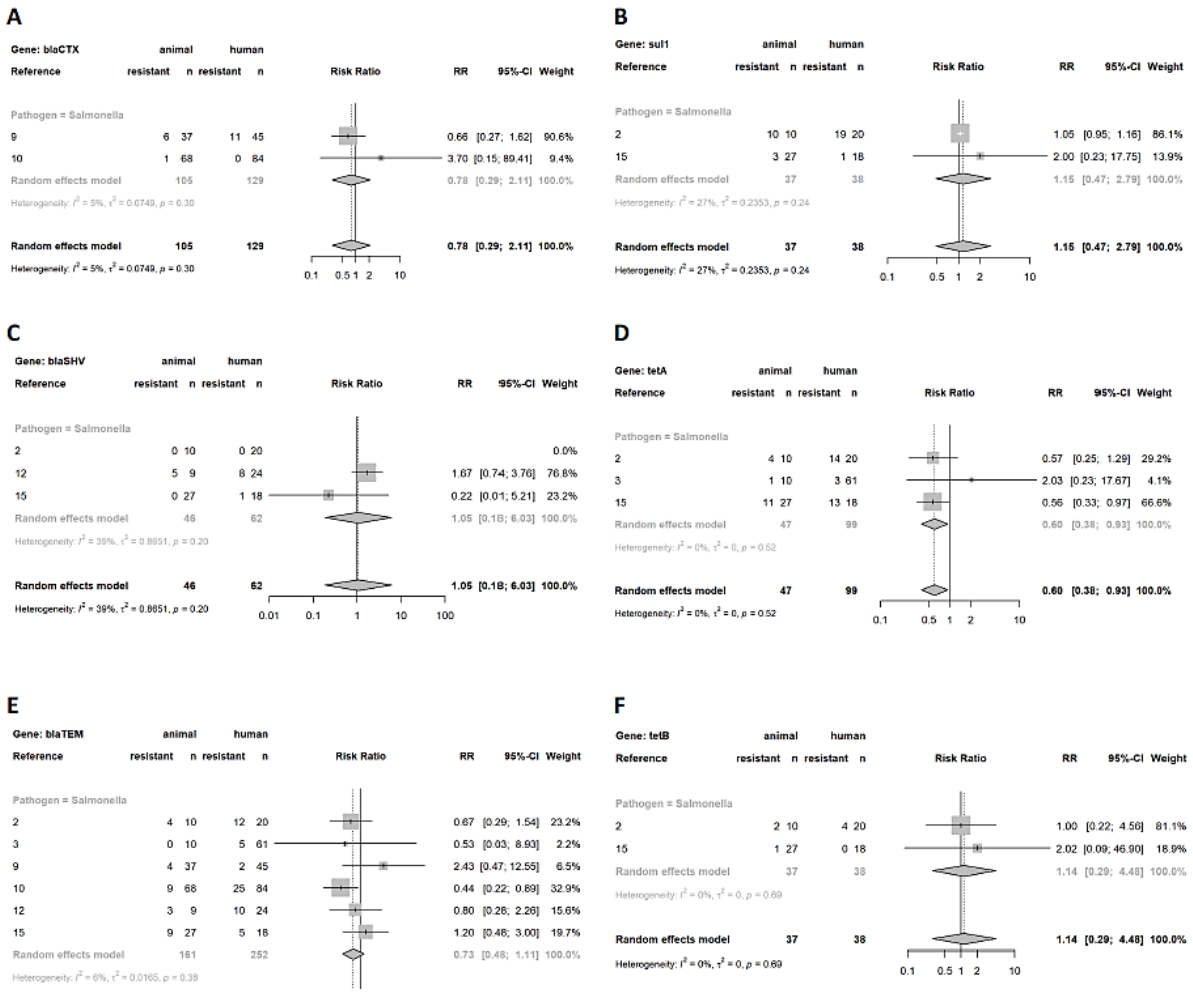

Figure S4. Forest plots for **(A)** blaCTX, **(B)** sul1, **(C)** blaSHV, **(D)** tetA, **(E)** blaTEM and **(F)** tetB detected in Salmonella spp.

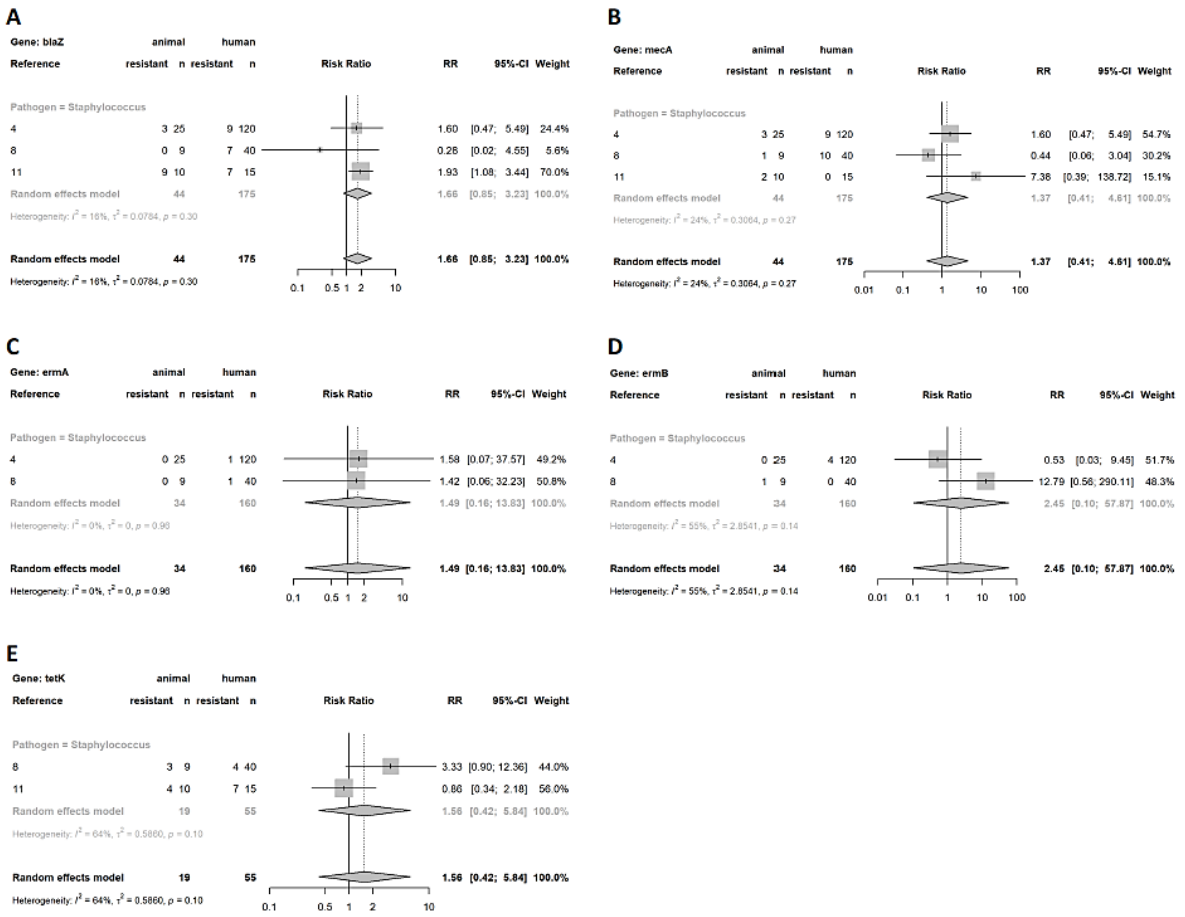

Figure S5. Forest plots for (A) blaZ, (B) mecA, (C) ermA, (D) ermB and (E) tetK detected in Staphylococcus spp.

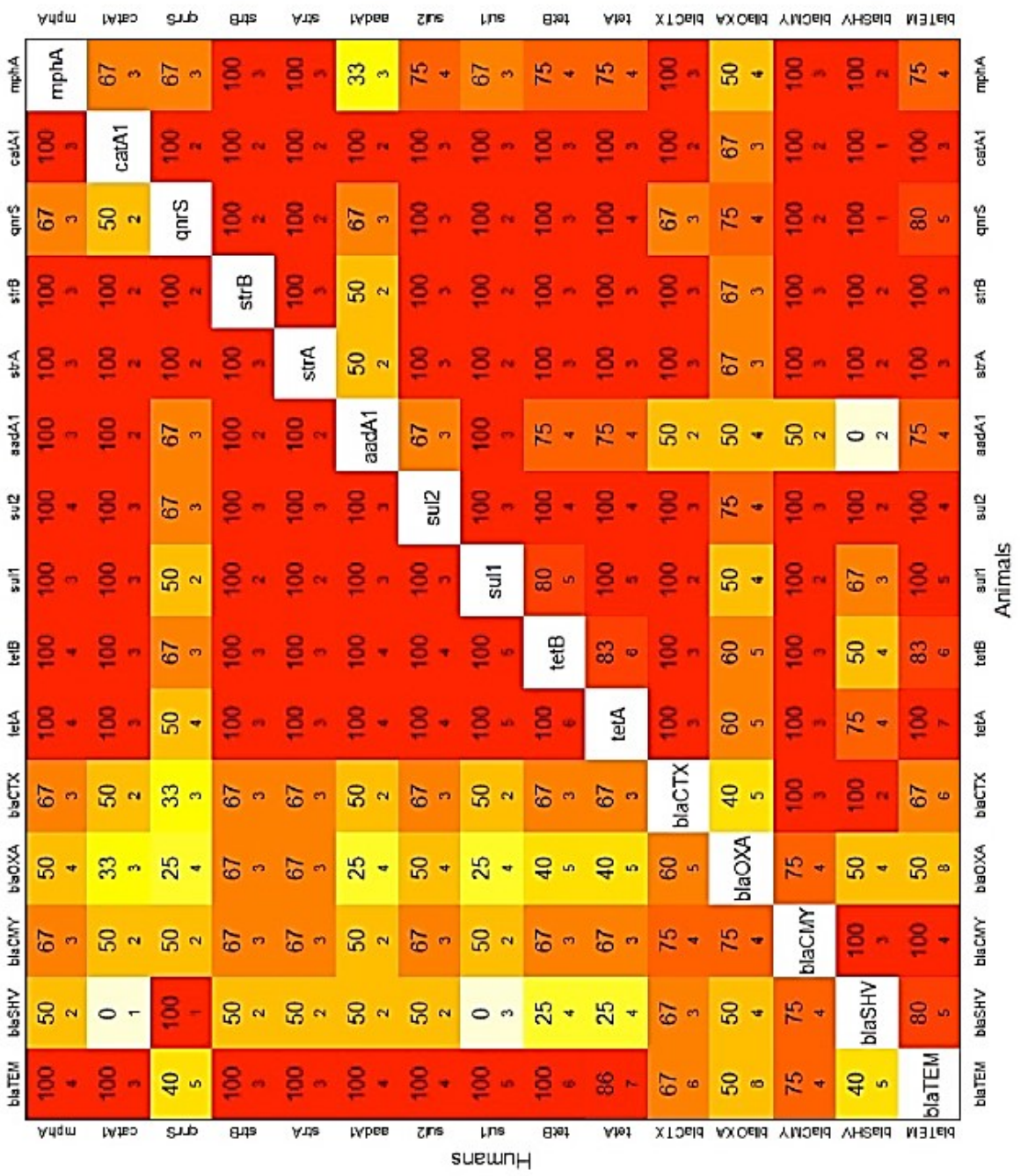

Figure S6. Heatmap showing the frequency of simultaneous detection of a gene across all studies, shown separately for humans (top left triangle) and animals (bottom-right triangle). The number at the top indicates the percentage of studies that detected both genes. The number at the bottom indicates the absolute number of studies which investigated both genes

Chapter 4 Fecal carriage of ESBL-producing *E. coli* and genetic characterization in rural children and livestock in the Somali region, Ethiopia: A One Health Approach.

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4.1 Abstract

Background: The emergence and spread of Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* pose significant challenges for treatment of infections globally. This challenge is exacerbated in sub-Saharan African countries, where the prevalence of ESBL-producing *E. coli* is high. This, combined with the lack of a strong and supportive healthcare system, leads to increased morbidity and mortality due to treatment failures. Notably, studies in Ethiopia have primarily focused on hospital settings, leaving a gap in understanding ESBL prevalence in rural communities, where human-animal proximity may facilitate microbial exchange.

Methods: We conducted a community-based study in the rural Somali region of Ethiopia, simultaneously examining the fecal carriage of ESBL-producing *E. coli* in children aged 2-5 years and their livestock (cattle, camel, goat). Fecal samples from 366 children and 243 animals underwent phenotypic screening for ESBL-producing *E. coli*. Following phenotypic confirmation, ESBL resistance genes were identified via conventional PCR. Whole-genome sequencing (WGS) was performed on a subset of isolates from human feces.

Results: We found that 42.8% of children and 3.7% of livestock harbored ESBL-producing *E. coli*. The ESBL gene $bla_{\text{CTX-M-15}}$ was predominant in human (82.7%) and livestock (100%) isolates. In the 48 human *E. coli* isolates subjected to WGS, a high diversity resulting in 40 sequence types (STs) was observed. Among these, ST-2353 was the most prevalent (5/48), followed by ST-10 and ST-48 (3/48) and ST-38, ST-450, and ST-4750 (2/48). These STs were associated with multiple resistance genes, such as $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-35}}$. In conclusion, we report a high prevalence of ESBL *E. coli* in rural children, which outnumbers its prevalence in livestock. The predominant resistance gene detected is $bla_{\text{CTX-M-15}}$.

Conclusion: We found high and low prevalence of ESBL-producing *E. coli* in rural children and livestock, largely $bla_{\text{CTX-M-15}}$. These isolates displayed a high diversity of sequence types (STs) with ST-2353 being the dominant ST. Our study is the first to report the association of ST-2353 with multi-drug resistance genes in Ethiopia. Further research using an integrated approach including other domains such as water and food products is needed to truly understand and combat AMR transmission and acquisition in this region.

Key words: Antimicrobial resistance; Extended spectrum beta lactamase; *E. coli;* One Health

4.2 Introduction

The emergence and spread of multidrug-resistant bacterial strains, particularly Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* (*E. coli*), pose significant challenges for treatment of infections globally (Díaz-Agero Pérez et al., 2019). This challenge is exacerbated in sub-Saharan African countries, including Nigeria, Tanzania and Ghana, where the prevalence of ESBL-producing *E. coli* is high, both in humans (up to 60%) and animals (up to 56%) (Andriatahina et al., 2010; Ayinla & Mateus, 2023; Linda Falgenhauer et al., 2019; Moremi, Claus, Vogel, & Mshana, 2017; Ojo, Schwarz, & Michael, 2016). This, combined with the lack of a strong and supportive healthcare system, leads to increased morbidity and mortality due to treatment failures (Rousham et al., 2018) . Robust laboratory diagnostics and strong surveillance systems for antimicrobial resistance are urgently needed, yet, they are still lacking in many parts of Sub-Saharan Africa (Williams, Isaacs, & Berkley, 2018).

ESBLs, enzymes that hydrolyze a broad range of beta-lactamases, are considered multi-drug resistant (K. Bush & Bradford, 2016). CTX-M, a dominant ESBL type, has become widespread globally after the late 1990s, outpacing TEM and SHV (Cantón, González-Alba, & Galán, 2012). Nowadays, its prevalence is high in infections of humans and food-producing animals, and it is considered one of the main contributing factors to multidrug resistance in *E. coli* in both low- and middle-income (LMICs) and Western countries (P. M. Hawkey, 2008; Hawser et al., 2011; Wickramasinghe et al., 2012).

ESBL genes, commonly found in plasmids, primarily spread through horizontal gene transfer (Bonnet, 2004; Carattoli et al., 2014). Recent findings suggest they can also exist in the chromosome, indicating potential clonal transmission (Hirai et al., 2013; Rodríguez et al., 2014). Transmission of ESBL-producing *E. coli* from animals to humans, often discussed in the context of contaminated animal-origin food, is a concern (Leverstein-van Hall et al., 2011). Surveillance relies on identifying similar clones, plasmids, or sequence types in human and animal populations, to infer transmission (Liebana et al., 2013). The interconnectedness of infections in both species underscores the need for a One Health approach to fully understand the dynamics and spread of resistant clones.

In Ethiopia, recent studies have revealed a high prevalence of *bla*$_{CTX\text{-}M}$, especially the *bla*$_{CTX\text{-}M\text{-}15}$ variant (87.7% - 88.4%), within hospital settings, which also conferred resistance to non-beta-lactam antibiotics, such as fluoroquinolones and aminoglycosides (Negeri et al., 2023; Negeri et al., 2021; Sewunet et al., 2022). Additionally, these studies reported different *E. coli* phylo-groups, with several isolates linked to the ST-131 clone, which is mainly associated with *bla*$_{CTX\text{-}M\text{-}15}$ (Negeri et al., 2023; Negeri et al., 2021; Sewunet et al., 2022). Notably, studies in

Ethiopia have primarily focused on hospital settings, leaving a gap in understanding ESBL prevalence in rural communities, where human-animal proximity may facilitate microbial exchange.

To fill this gap, we conducted a community-based study to investigate the fecal carriage of ESBL-producing *E. coli* in children aged two to five years and livestock of the same households and genetically characterized the isolated clones in the feces of children and livestock. By identifying the prevalence of ESBL-producing *E. coli* and their genetic characteristics, this study yields new insights to guide interventions aimed at curbing the dissemination of antimicrobial resistance and enhancing treatment strategies for infectious diseases caused by these bacteria.

4.3 Methods

Study area

The study was carried out from May 2021 until June 2021 in Adadle district in the Shebele zone, Somali Regional State (SRS), Ethiopia.

Sample size

Based on a previous study conducted in Addis Ababa, we expected the prevalence of ESBL-producing *E. coli* to be 42% (Kassu Desta et al., 2016) and assumed an inter-cluster correlation coefficient of 0.15. We calculated a sample size of 360 eligible children, with 180 children from pastoralist and 180 children from agro-pastoralist communities to be sufficient to estimate the prevalence with a margin of error of 10% at the 95% confidence level.

Selection criteria

Based on the nature of livelihood, eight of the thirteen Kebele in the Adadle district were randomly selected. Among the selected Kebele, four were pastoralist (Malkasalah, Todab, Harsug, Boholhagere) and four were agro-pastoralist (Bursaredo, Dabafyd, Gabal, Higlo).

A pre-enrolment screening was carried out in each Kebele to assess children between 2 and 5 years of age. All children were screened for stunting and wasting based on WHO guidelines. All stunted (height for age score, HAZ < -2), and wasted (weight for height score, WHZ < -2 or mid-upper arm circumference (MUAC) < 11.5 cm) were enrolled in the study. Among the screened children who were non-stunted and/or non-wasted, a random selection process was employed from an Excel file containing information on all pre-screened households/children in the community. The random selection process continued until the desired sample size was achieved. Children outside the age range (below 2 years and above 5 years) and those who

had received antibiotic treatment within the last 14 days were excluded from the study. Informed consent was obtained from all participants' legal guardians.

Anthropometric measurements

Height measurements were taken with children standing against a WHO standard wooden measuring board, ensuring the correct posture and position. Weights were recorded using a WHO standard weight scale, either the child alone or with the mother by adjusting the weight scale (Onis, 2008). MUAC measurements were performed using a WHO standard tape measure. To ensure the accuracy of height, weight and MUAC measurements, each anthropometric measurement was repeated at least twice or until the measurements were within 1 mm, 100 g, or 1 mm of one another, respectively.

Data collection

A comprehensive questionnaire was administered to the mother of the child by trained field workers in the local language using Open Data Kit (ODK). The questionnaire covered different sections, including demographic characteristics, child health status, WASH behavior, breastfeeding and diet, and anthropometric measurements. As birth records were not available in the community, the child's age was estimated based on group discussion involving the child's mother, as well as other family and community members. These discussions considered factors such as seasonal events (floods, drought, and rainy season) and festive occasions (Ramadan, festival, and other religious events) that occurred before and after the child's birth. Additionally, leveraging the close-knit relationships within the community, efforts were made to engage in discussions to collectively recollect and remind each other about significant events and timeframes related to the child's birth.

Stool sample collection

Trained field workers provided mothers with instructions in the local language a day before collecting fecal samples. Mothers used sterile specimen cups, and upon receipt, samples were placed in an ice box and transported within 2-4 hours to a field laboratory. There, samples were aliquoted into six cryotubes, two with glycerol (40% glycerol mixed 1:1 with the stool) and four without, stored at 4°C. Subsequently, samples were sent to Gode within 24 hours, stored at -20°C, and then shipped to the Armauer Hansen Research Institute in Addis Ababa, where they were preserved at -80°C for long-term storage.

Additionally, from the selected households that owned animals, we assessed different types of animals (camels, cattle, and goats). We sampled at least one animal per species, proportionally to their relative numbers. Animal fecal samples were collected by a veterinary professional through either rectal insertion of gloved hands or aseptic collection of fresh stool

(if the animals defecated in the presence of the team). Animal samples underwent a process similar to human samples - immediate shipment to the field laboratory, aliquoting, storage at -20°C, and final storage in Addis Ababa at -80°C for future analysis

Bacterial isolation and identification

Fecal samples were aseptically inoculated onto MacConkey agar plates (Allen, 2005), and subsequently, a cefotaxime disc was placed at the center of the plate. The plates were then incubated for 24 hours at 37ºC. The colony that was closest to the cefotaxime disc was meticulously selected based on its distinctive morphology and pigmentation characteristics indicating it was an *E. coli* for further identification. The selected colonies were re-isolated, and subsequently, the colony was confirmed as *E. coli* through a biochemical test: isolates were classified as *E. coli* if they exhibited a positive indole test, negative citrate, positive lysine decarboxylation, gas and acid production, mannitol fermentation, negative urea hydrolysis, and if they were motile.

Antibiotic susceptibility test

The disc diffusion method, was used to determine the antibiotic susceptibility using Müller-Hinton agar according to the recommendation of the Clinical and Laboratory Standard Institute (CLSI) guidelines (Performance, 2016). Susceptibility tests were performed for 19 antibiotics from 10 different classes, including penicillins (ampicillin (AMP) 10µg and amoxicillin (AML) 10µg, beta-lactamase inhibitor combinations (ampicillin-sulbactum (SAM) 30µg and amoxicillin/clavulanic acid (AMC) 30µg), cephalosporin (cefazolin (KZ) 30µg, cefpodoxime (CPD)) 10µg, cefuroxime (CXM) 30µg, ceftriaxone (CRO) 30µg, cefotaxime (CTX) 30µg, cefipime (FEP) 30µg, ceftazidime (CAZ) 30µg), carbapenem (imipenem (IPM) 10 µg and ertapenem (ETP) 10µg), monobactams (aztreonam (ATM) 30µg), fluoroquinolones (ciprofloxacin (CIP) 5µg), aminoglycosides (gentamycin (CN) 30µg), macrolides (azithromycin (AZM) 15 µg), tetracycline (TE) 30 µg), and cotrimoxazole (SXT) 25 µg (Oxoid, United Kingdom). Prior to spreading the suspension on the Müller-Hinton agar plate, McFarland 0.5 was measured. Based on the inhibition zone diameters, antibiotics were assigned to susceptible, intermediate, and resistant as indicated by CLSI (Performance, 2016). Multidrug resistance was defined as non-susceptible to at least one antibiotic agent in three or more antimicrobial classes (Magiorakos et al., 2011).

ESBL screening and confirmatory test

According to CLSI, any strain showing resistance against cefotaxime (CTX) 30µg, ceftazidime (CAZ) 30µg, or ceftriaxone 30 µg was considered as a potential extended-ESBL producer. Confirmatory tests were performed utilizing a disc of cefotaxime (CTX) 30µg and one of

ceftazidime (CAZ) 30µg alone, and a disc of cefotaxime and one of ceftazidime combined with clavulanic acid 10 µg. Strains were considered ESBL-producers if an increase of inhibition zone diameter of 5 mm or greater was observed in the discs of CTX or CAZ combined with clavulanic acid compared to the inhibition observed in the CTX or CAZ alone discs (Oxoid, United Kingdom). *E. coli* ATCC 25922 and *E. coli* ATCC BAA-2326 were used as negative and positive controls (Humphries et al., 2018).

Molecular testing for β-lactamase genes

The DNA of the confirmed ESBL-producing isolates was extracted using Wizard® HMW DNA Extraction Kit, according to the manufacturer's instructions (Kit). The presence of three ESBL genes (*blaCTX-M, blaTEM, and blaSHV*) was detected using a multiplex PCR approach within a single reaction tube. Details regarding the PCR reaction, cycling, and primers for amplifying the ESBL resistance genes can be found in supplementary materials 1.

Whole genome sequencing

The extracted DNA from human and animal ESBL-producing isolates were shipped to Swiss Tropical Public Health Institute (Swiss TPH) for whole genome sequencing (WGS). DNA was quantified with the Qubit dsDNA HS Assay Kit (Invitrogen, Germany). Isolates were selected for WGS using the MinION platform (Oxford Nanopore Technologies, UK) based on DNA concentrations (>33 ng/µL) and their antibiotic susceptibility results. The sequencing library was prepared according to the manufacturer's instructions using the Native Barcoding Kit 96 (SQK-LSK114.96) and loaded onto the R10.4.1 flow cell and sequenced on the MinION Mk1C using super-accurate base calling.

Bioinformatics Analysis

De novo assembly was conducted using Flye 2.9.1 at the scientific computing core facility of the University of Basel (Kolmogorov, Yuan, Lin, & Pevzner, 2019). The Bacterial and Viral Bioinformatics Resource Center (BV-BRC) was used for annotation and phylogenetic analysis of the assemblies. The assemblies were annotated using the RAST 2.0 toolkit (Brettin et al., 2015). Only assemblies with less than 20 contigs were considered for further analysis. Sequence type of assembled contigs were determined using MLST (Larsen et al., 2012). Phylogenetic trees were generated by aligning protein and nucleotide sequences using MUSCLE, MAFFT and RAxML (Alcock et al., 2022; Katoh, Misawa, Kuma, & Miyata, 2002; Larsen et al., 2012). Resistome analysis was performed using CARD (6.0.0) and ResFinder (4.2.2) (Alcock et al., 2022; Bortolaia et al., 2020).

Statistical analysis

R statistical software version 4.1.3 was used to perform the statistical analysis (R Core Team, 2013). Initial descriptive analysis of variables was performed using the gtsummary package. Multivariate analysis employed logistic regression to assess the association between ESBL-producing *E. coli* and independent variables. The initial model included variables (age, sex, education, sanitation practice, hygiene, source of water, owning livestock, nutritional status) based on literature knowledge, with stepwise removal of those contributing insufficient information ($p>0.2$). Variables with $p<0.2$ were retained. Model fitness was assessed using likelihood ratio test, AIC (Akaike Information Criterion), and adjusted R square. Significance was determined at $p<0.05$. R packages ape, ggplot2, ggtree, and ggtreeExtra were used for analyzing resistance genes and visualizing phylogenetic trees.

4.4 Results

Description of study population

A total of 346 children were included in this study. Pastoralists and agro-pastoralists were evenly represented in terms of age and sex among the enrolled children. Over half of the children showed a normal growth (61%), while a quarter (25%) of them were wasted, and 7.8% were stunted. Further, 5.2% of the children suffered concomitantly from stunting and wasting. Characteristics of the study group are summarized in table 1.

Table 2. Characteristic of pastoralist and agro-pastoralist in the Adadle district, Somali region, Ethiopia.

Variables	**N = 346 (%)**
Settlement area	
Pastoralist	177 (51.2%)
Agro-Pastoralist	169 (48.8%)
Education of the mothers	
Formal education	50 (14.5%)
Non-formal education	36 (10.4%)
Illiterate	260 (75.1%)
Sex of the child	
Female	176 (50.9%)
Male	170 (49.1%)
Age group of the child	
2 years	150 (43.4%)
3 years	98 (28.3%)
4-5 years	98 (28.3%)

Number of children per household	
1-3 children	78 (22.5%)
4-6 children	160 (46.2%)
>= 7 children	108 (31.2%)
Completed all required vaccinations	
No	309 (89.3%)
Yes	37 (10.7%)
Has a vaccination card	
No	328 (94.8%)
Yes	18 (5.2%)
Nutritional status	
Normal growth	212 (61.3%)
Wasted	88 (25.4%)
Stunted	27 (7.8%)
Stunted and wasted	18 (5.2%)
Overweight	1 (0.3%)

As summarized in table S1 in the supplementary file, the two main sources of water for pastoralists and agro-pastoralists were rainwater/birkad (89%) and river water (95%), respectively. Most of pastoralist and agro-pastoralist (96%) used open space for defecation. The 14 households (4%) that had a toilet shared it with 28 households. Dumping waste in the street or open spaces within the compound was the predominant method of waste disposal in both pastoralist and agro-pastoralist societies (84%), while 16% opted to burn the waste. Most mothers reported that they used water to wash the children's hands (93%), while a small subfraction of mothers reported (4%) using water and soap. At the time of the sampling, more than half of the agro-pastoralists and pastoralists had soap, while 29% did not have soap very often and 13% never had soap in their house.

Nearly half of agro-pastoralist households (43%) and 12% of pastoralist households treated the water prior to consumption (mainly chlorination (98.9%)). The majority of agro-pastoralists possessed cattle (94.7%), donkeys (81.1%), goats (62.7%), sheep (53.3%), and both camels and chickens (10% each). Among pastoralists, predominant livestock ownership included goats (87.6%), donkeys (58.8%), sheep (44.1%), camels (36.2%), and cattle (29.9%).

Phenotypic test results for ESBL *E. coli* carriage

A total of 609 fecal samples, comprising 366 from humans and 243 from animals (including 77 goats, 136 cows, and 30 camels) were analysed. For human isolates, 159 (43%) of the *E.*

coli isolates were ESBL-producers (24.5% in pastoralists and 18.8% in agropastoralists). Furthermore, 7.8% of the *E. coli* isolates from goats (6/77) and 2.2% from cows (3/136) were ESBL-producers. Regarding animal ESBL-producing isolates, 7 (77.8%) were identified among animals of agro-pastoralists, while 2 (22.2%) were found among those of pastoralists.

Susceptibility pattern for ESBL-producing *E. coli*

For the human isolates, ESBL-producing *E. coli* strains exhibited complete resistance to several commonly used antibiotics including amoxicillin, cefotaxime, cefuroxime, ceftriaxone, cefazolin, and cefpodoxime. Almost all ESBL-producing *E. coli* isolates (98.7%) were resistant to ampicillin and 51.6% were resistant to tetracycline.

Regarding co-trimoxazole, 57.9% of the isolates were classified as resistant, 2.5% as intermediate, and 37.7% as susceptible. Among ESBL-producing *E. coli*, 47.8%, 42.1%, and 27.7% were non-susceptible, and 32.1%, 54.7%, and 25.2% were intermediate to aztreonam, cefipime, and ceftazidime, respectively. In addition, 15.7% and 12.6% of the isolates were resistant to azithromycin and ciprofloxacin. Ampicillin-sulbactam (8.8%), gentamycin (6.9%), and amoxicillin-clavulanic acid (5.9%) had the lowest rates of resistance. Intermediate susceptibility was observed in 16.5% for amoxicillin-clavulanic acid, 15.1% for ampicillin-salbactum, and 2.5% for gentamycin. All carbapenems (imipenem and ertapenem) were found to be effective against the ESBL-producing *E. coli* isolates, except for 1.3% of ertapenem, which exhibited intermediate susceptibility.

Additonally, nine ESBL isolates obtained from animal fecal samples exhibited complete (100%) resistance to ampicillin, cefotaxime, and amoxicillin. Furthermore, an 88.9% resistance rate was observed for cefepime, followed by cefazolin (55.6%), ceftriaxone (44.4%), cefuroxime (44.4%), and aztreonam and ampicillin-sulbactam, which both exhibited an equal resistance rate of 33.3%. In ESBL-producing *E. coli* isolates from animals, ampicillin-clavulanic acid, ceftazidime, gentamicin, and ciprofloxacin all exhibit an equal resistance rate of 11.1%. The data is summarized in Figure 5.

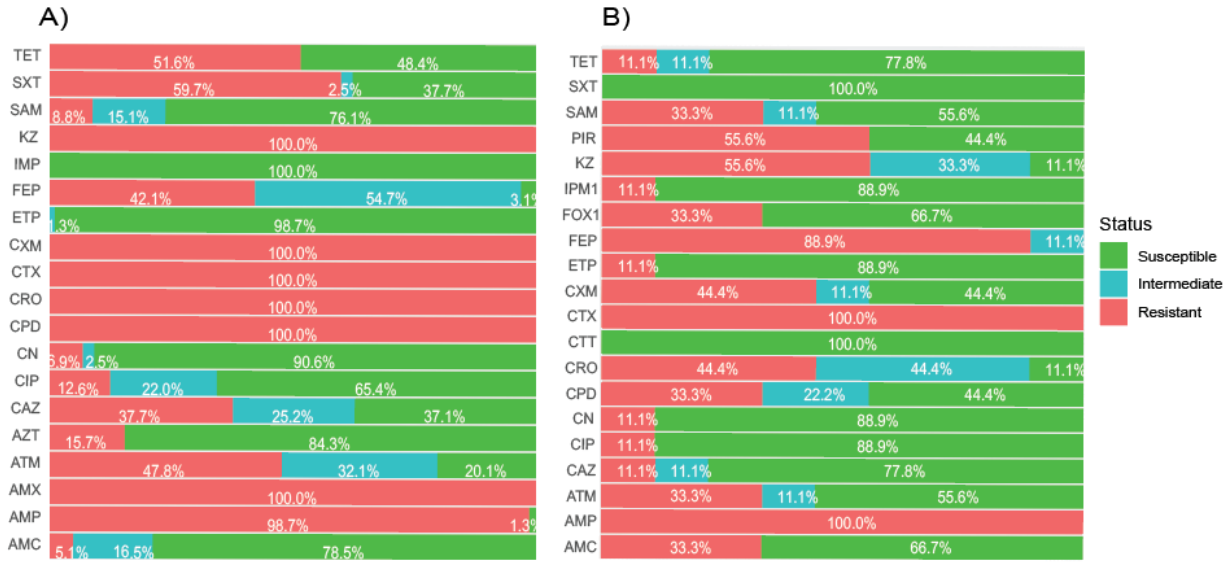

Figure 5. Antimicrobial resistance pattern of ESBL-producing *E. coli* isolated from the feces of children aged 2-5 years (figure A, 159/366) and from livestock (Figure B, 9/243) in the Adadle district, Somali region, Ethiopia.

Antimicrobial agents tested include: AMX (Amoxicillin), AMC (Amoxicillin-clavulanic acid), AMP (Ampicillin), ATM (Aztreonam), AZT (Azithromycin), CAZ (Ceftazidme), CIP (Ciprofloxacin), CN (Gentamycin), CPD (Cefpodoxime), CRO (Ceftriaxone), CTX (Cefotaxime), CCT (Cefotetan), ETP (Ertapenem), FEP (Cefepime), FOX (Cefoxitin), IMP (Imipenem), KZ (Cefazolin), SAM (*Ampicillin-sulbactam)*, SXT (Co-trimoxazole), PIR (Piperacillin), TET (Tetracycline).

All ESBL-producing *E. coli* strains exhibited multi-drug resistance (MDR), rendering them non-susceptible to three or more antibiotic drug classes (Figure S1 in Supplementary File 1). Thus, ESBL-carriage is very prevalent, especially in children, in a community setting and is much lower in the feces of livestock animals.

Predictors of ESBL-producing *E. coli* carriage

In the multivariable analysis, education was significantly associated with ESBL-producing *E. coli*. Children whose mothers or household heads were illiterate had twice the odds of carrying ESBL-producing *E. coli* compared to children whose mothers were formally educated (aOR =2.65, 95%CI = 1.27-5.48). The odds of children who were both stunted and wasted were three time higher to harbor ESBL-producing *E. coli* compared to children with normal growth (aOR = 3.14, 95%CI = 1.02-9.07). Moreover, pastoralist children had 2.65 times higher odds of being colonized with ESBL producing *E. coli* compared to agro-pastoralist children (aOR =2.65, 95%CI = 1.30-5.41). Counterintuitively, children who drank water treated with chlorine showed a positive association with ESBL-producing *E. coli* (aOR =2.09, 95%CI = 1.10-3.98). Additionally, the possession of chicken increased the odds of infection with ESBL-producing *E. coli* five times (aOR =5.13, 95%CI = 1.66-15.68). The sex and age of the child were not

found to be significantly associated with infection with ESBL-producing *E. coli.* Similarly, although owing soap or washing ands with water and soap showed atrend to decrease the risk of contracting ESBL-producing *E. coli,* this association was not statistically significant (Figure 6).

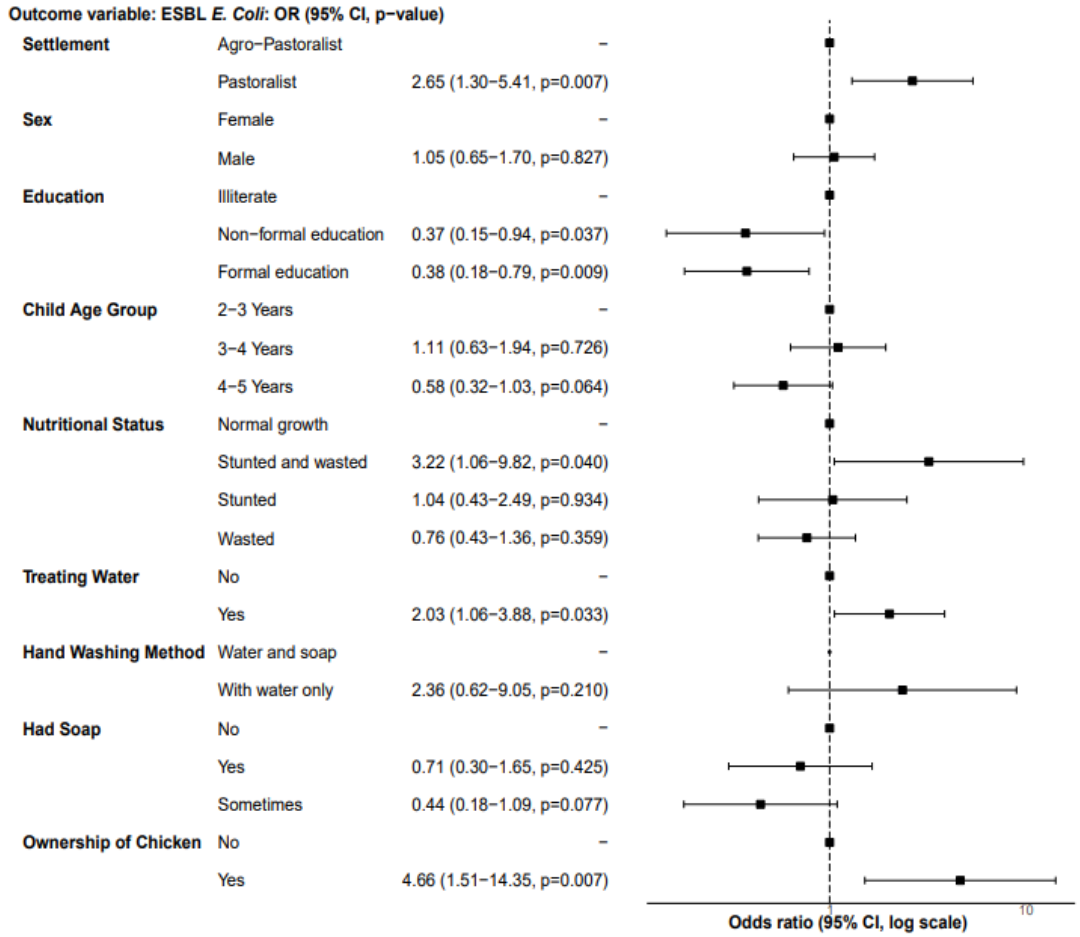

Figure 6. Risk factors (multivariable model) associated with fecal carriage of ESBL-producing *E. coli* among children living in the Adadle district, Somali region, Ethiopia.

Genetic characterization of ESBL strains (conventional PCR)

During PCR screening, we found that the *bla*$_{\text{CTX-M-15}}$ gene was the most prevalent resistance gene in both human (82.8%) and animal (100%) isolates. The prevalence of the *bla*$_{\textit{CTX-M-15}}$ resistance gene was similar in isolates from the feces of pastoralists and agro-pastoralists, both almost at 80% (Figure S2 of the Supplementary Materials 1). Comparing the nine isolates from animals alongside those from children residing in the same household, we observed that only two households within the pastoralist group demonstrated simultaneous presence of *bla*$_{\text{CTX-M-15}}$ in both their children and livestock (Figure S3).

Multi locus sequence types (MLST), phylogenetic groups and plasmid MLST

Out of the 48 human *E. coli* isolates subjected to WGS analysis, a sequence type (ST) by MLST using the Achtman scheme could be assigned to 44 isolates (91.7%), with five isolates having a single nucleotide polymorphism (SNP) in one gene (2 in *adk*, 2 in *fumC*, 1 in *mdh*). The most common ST was ST2353 with five *E. coli* isolates, followed by ST10 and ST48 with three *E. coli* isolates each and ST38, ST450 and ST4750 with two *E. coli* isolates each. All other 27 *E. coli* isolates had singular ST. A total of nine *E. coli* isolates were assigned to clonal complex ST10 (3 ST-10, 3 ST48, 1 ST-227, 1 ST378 and 1 ST617) and two isolates to clonal complex ST38 (2 ST38).

The predominant beta-lactam resistance gene among the 48 *E. coli* isolates was $bla_{\text{CTX-M-15}}$, identified in 72.9% (35/48) of the whole-genome sequenced isolates, followed by $bla_{\text{TEM-1B}}$ identified in 47.9% (23/48), ampC beta-lactamase in 14.6% (7/48), $bla_{\text{OXA-1}}$in 8.3% (4/48) and $bla_{\text{OXA-1}}$ in 4.2% of the isolates. The beta-lactam resistance genes $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-35}}$ were the least prevalent, each detected in a single isolate.

Four *E. coli* isolates (8.3%) did not carry any known beta-lactam resistance genes, 20 *E. coli* isolates (41.7%) carried one, 19 *E. coli* isolates (39.6%) carried two and five *E. coli* isolates (10.4%) carried three beta-lactam resistance genes. Seventy-five percent (15/20) of *E. coli* isolates with one beta-lactam resistance gene carried only $bla_{\text{CTX-M-15}}$, while 68.4% of *E. coli* isolates with two beta-lactam resistance genes carried both $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$.

Quinolone resistance conferred by mutations in the *gyrA* gene was detected in 33.3% (16/48) of *E. coli* isolates. Serine at position 83 was mutated to either Leucine (S83**L**, 10/16), Alanine (S83**A**, 3/16) or Valine (S83**V**, 3/16). In addition to S83**L**, three *E. coli* isolates had *gyrA* mutation D87**N**. Four *E. coli* isolates (8.3%) had *parC* mutation S57**T** and four *E. coli* isolates (8.3%) had *parC* mutation S80**I** combined with *parE* mutation S458**A**. The plasmid-encoded *qnrS1* and *qnrS13* genes were detected in 39.6% (19/48) and 2.1% (1/48) of the sequenced isolates, respectively.

Aminoglycoside resistance genes were present in 60.4% (29/48) of *E. coli* isolates. Four different aminoglycoside (3") (9) adenylyltransferase (*aadA*) genes were detected in 15 *E. coli* isolates (31.3%), with *aadA1* accounting for 66.7% (10/15), *aadA2* and *aadA24* for 13.3% (2/15) each and *aadA5* for 6.7% (1/15) of the aadA genes found. Eighteen *E. coli* isolates (37.5%) had the plasmid-encoded aph(3")-Ib and aph(6)-Id aminoglycoside resistance genes, one had only aph(3")-Ib and one only aph(6)-Id. The aminoglycoside resistance genes aac(3)-IId, aac(6')-Ib-cr, ant(2")-Ia and aph(3')-Ia were detected in one *E. coli* isolate each.

Fosfomycin resistance mutation E448**K** in *glpT* was detected in 62.5% of *E. coli* isolates (30/48), with additional mutation E350**Q** in *uhpT* in four *E. coli* isolates. Phenicol resistance gene *catA1* was detected in 10.4% (5/48) of the isolates, one *E. coli* isolate containing

additionally *catB3*. Phenicol resistance mutation S3**N** in *marR*. Polymyxin resistance mutations E123**D** and Y358**N** in *pmrB* were detected in 2.1% (1/48) and 18.8% (9/48) of the isolates, respectively.

Sulfonamide resistance genes were detected in 62.5% of *E. coli* isolates (30/48), with *sul1* in 30.0% (9/30), *sul2* in 63.3% (19/30) and both genes in 6.7% (2/30) of the isolates. Trimethroprim resistance genes *dfrA* were detected in 58.3% of *E. coli* isolates (28/48), most commonly *dfrA14* (9), followed by *dfrA1* (7), *dfrA5* (3), *dfrA17* (3), *dfrA7* (2), *dfrA15* (2), *dfrA8* (1) and *dfrA19* (1). Tetracycline resistance genes *tetA*, *tetB* and *tetD* were detected in 35.4% (17/48), 16.7% (8/48) and 2.1% (1/48) of *E. coli* isolates, respectively. Macrolide resistance gene *mphA* was detected in 18.8% (9/48) of the isolates, with one isolate simultaneously carrying *ermB*. The results are summarized in Figure 7.

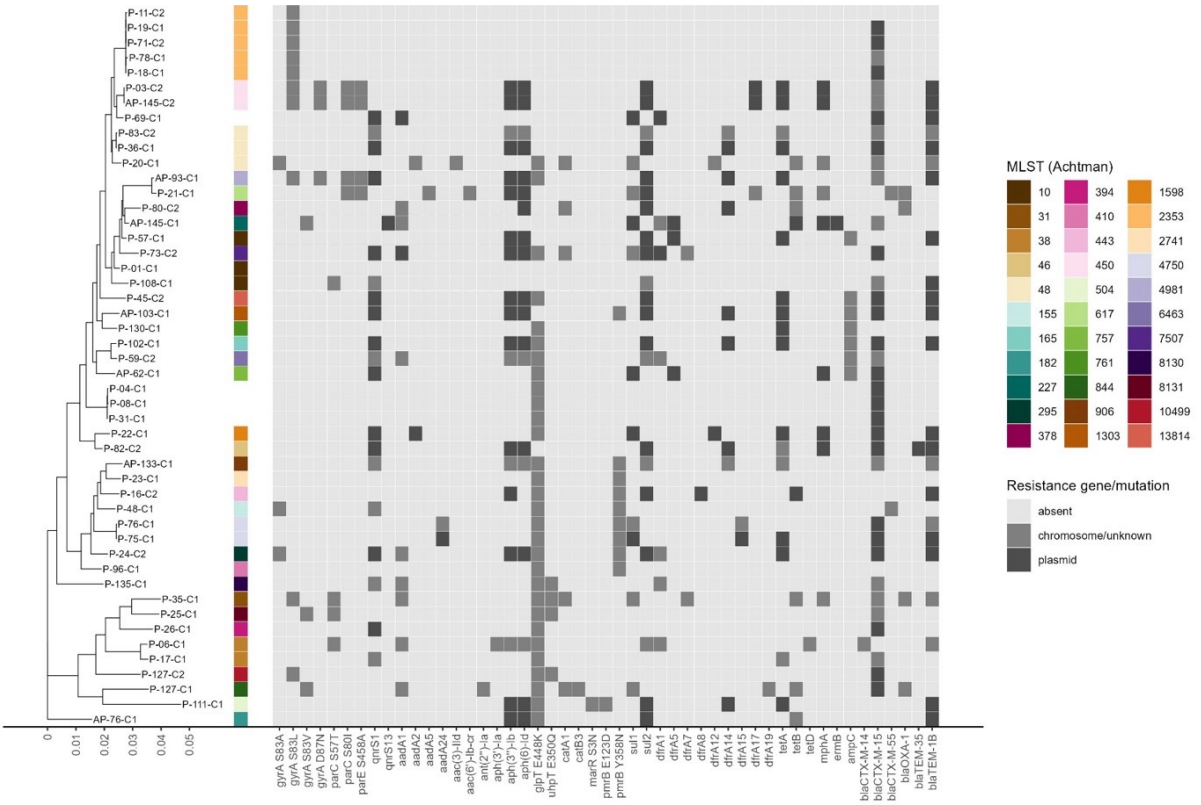

Figure 7. Phylogenetic analysis of AMR markers and MLST based sequence types in 48 ESBL-producing *E. coli* isolated from 2-5 year old children in the Adadle woreda, Somali Region, Ethiopia.

The presence of AMR markers is shown in light gray if located in the genome or an unidentified plasmid and dark gray if located on a plasmid. MLST are indicated with the color code indicated on the right of the figure. P: Isolate from pastoralist children, AP: isolate from agropastorliast children.

Comparison of genotypes and phenotypes

Resistance to different antibiotics was inferred from WGS data. The presence of specific genes (Figure 8, in blue) indicated resistance to specific antibiotics (Figure 4, in red). For

example, the presence of at least one of the following genes, CTX-M-type, OXA-type, TEM-type, or ampC, indicated resistance to amoxicillin, ampicillin, aztreonam, cefotaxime, ceftriaxone, cefazolin, cefuroxime, and cefepodoxime. The same procedure was used for all other antibiotics, as indicated in Figure 8.

The resistance results inferred from WGS data were then compared with the results of the phenotypic assay. We found high concordance rate (>90%) between genotype and phenotype for azithromycin (93%), amoxicillin (91%), and ampicillin (91%). Conversely, chloramphenicol (87%), as well as several cephalosporins including cefotaxime, ceftriaxone, cefazolin, cefuroxime, and cefepodoxime, each showed a low concordance rate of 84%. Tetracycline (81%) and trimethoprim/sulfamethoxazole (72%) showed even lower concordance rates, with ciprofloxacin (63%) registering the lowest concordance rate among them. The results are summarized in Figure 8.

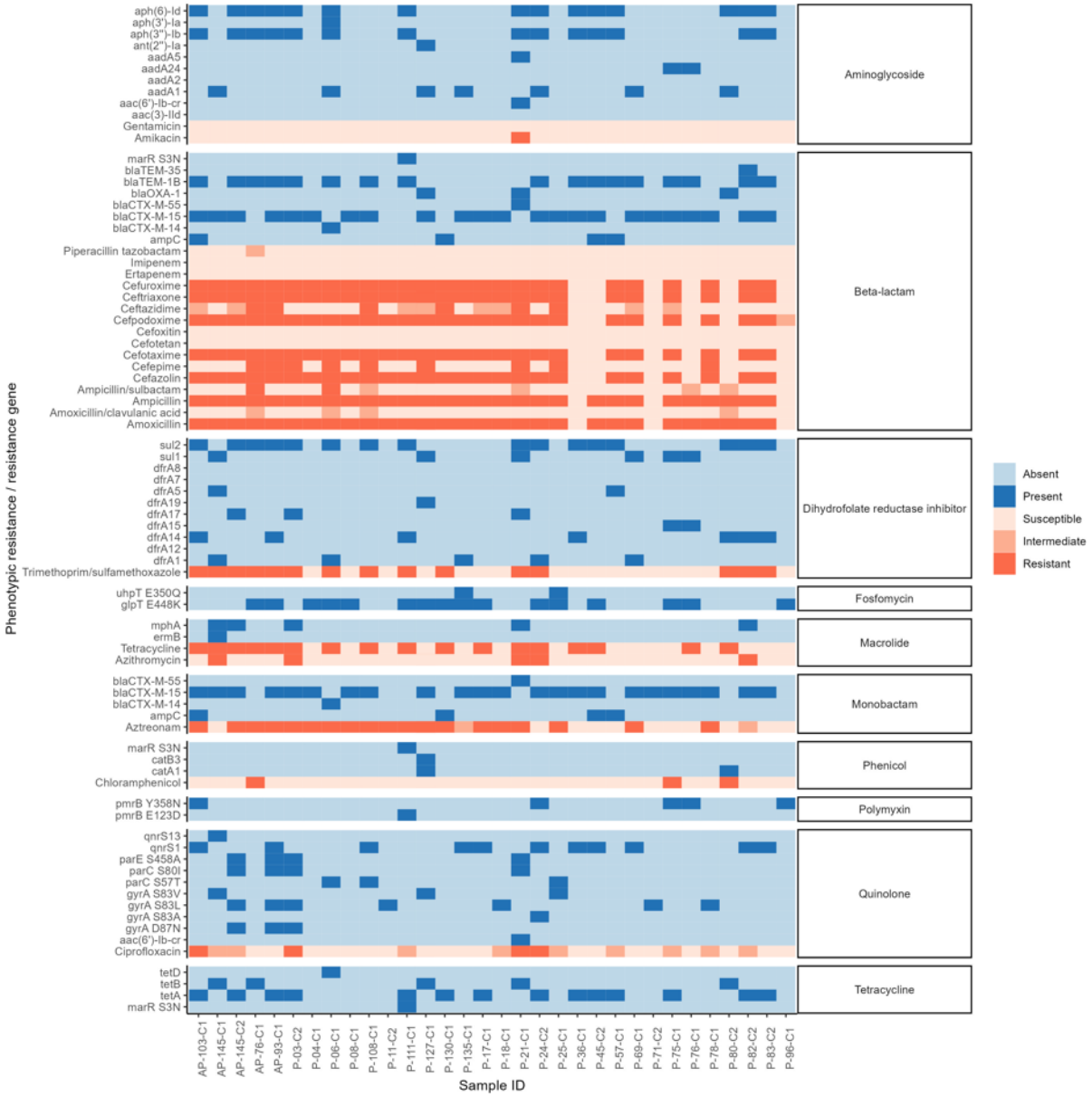

Figure 8. Concordance and discordance between genotype and phenotype for selected ESBL-producing *E. coli* isolates from Adadle district, Somali region, Ethiopia

4.5 Discussion

Over the past two decades, there has been a significant increase in the global prevalence of communities carrying ESBL-producing *E. coli* (Y. M. Bezabih et al., 2021). In LMICs, the colonization of ESBL-producing *E. coli* has risen steadily in both community and healthcare settings, with the community carriage rate approaching that of healthcare settings (Yihienew M. Bezabih et al., 2022). To our knowledge, this is the first study to employ WGS for profiling of the phylogenomic of ESBL-producing *E. coli* in children under the age of five in rural communities in Ethiopia.

The results show a high carriage (42.9%) of ESBL-producing *E. coli* in children under the age of five living in these communities via phenotypic analysis. This finding aligns with a study conducted among hospitalized children in Addis Ababa (K. Desta et al., 2016) and rural children in Ghana (Akenten et al., 2023). The elevated prevalence observed could be attributed to the widespread availability of antimicrobials and misuse of antibiotics (Ayukekbong, Ntemgwa, & Atabe, 2017a). Additionally, in rural communities, limited access to clean water and sanitation may contribute the spread of ESBL-producing *E. coli* resistant clones and genes (Berendes, Kirby, Brown, & Wester, 2020). On the other hand, low prevalence of ESBL-producing *E. coli* carriage was found in livestock, which aligns with study performed in Kenya (Nüesch-Inderbinen et al., 2020). In rural communities, limited access to veterinary care may result in reduced antibiotic usage in livestock, thereby decreasing the selection pressure for antibiotic-resistant bacteria (Muhummed et al., 2024). This, in turn, could contribute to the lower prevalence of ESBL-producing *E. coli* strains in the livestock population.

In the PCR analysis, the resistance gene $bla_{\text{CTX-M-15}}$ emerged as the most frequently observed gene among *E. coli* isolates that exhibited an ESBL phenotype in our study, both in the children (120/159 isolates) and in livestock (9/9 isolates). Importantly, $bla_{\text{CTX-M-15}}$ is known as the primary ESBL resistance gene responsible for human infections worldwide (Alonso et al., 2017). Moreover, recent research conducted in Africa corroborates this finding, highlighting $bla_{\text{CTX-M-15}}$ as the dominant ESBL-producing *E. coli* gene in livestock populations (Valentin et al., 2014). This geographical distribution strongly suggests a significant spread of resistance genes among both humans, animals and other domains such as water and food products, highlighting the importance of understanding and addressing this issue from a One Health perspective.

In the MLST analysis, a high diversity of 40 different sequence types (STs) was observed, with ST2353, ST10, ST38, ST48, and ST450 being the most prevalent. Studies conducted in

Ethiopia and Ghana also demonstrated a substantial diversity of STs, accounting for over 30 STs (Linda Falgenhauer et al., 2019; Sewunet et al., 2022). ST10 and ST48 exhibited multi-drug resistance genes, primarily *bla*$_{\text{CTX-M-15}}$ and *bla*-$_{\text{TEM-1B}}$, along with other non-beta-lactamase resistance genes. Globally, ST10 and ST48 are recognized as clonal genetic entities known to harbor multi-drug resistance genes, primarily in humans, with a notable prevalence of *bla*$_{\text{CTX-M-15}}$ (Aibinu, Odugbemi, Koenig, & Ghebremedhin, 2012; Day et al., 2019). Additionally, ST38 showed a lineage associated with the carriage of *bla*-$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$, which is consistent with prior research findings in England, Scotland, Wales, and China (Day et al., 2019; J. Zhang et al., 2014)

Notably, ST2353, typically associated with highly pathogenic diarrheagenic *E. coli* strains (Joffré, von Mentzer, Svennerholm, & Sjöling, 2016; Kwon et al., 2017) and historically less reported for its resistance gene carriage, emerged as one of the predominant sequence types in our study. Remarkably, ST2353 was found to harbor multiple resistance genes, encompassing *bla*$_{\text{CTX-M-15}}$, *bla*-$_{\text{TEM-1b}}$, *gyrA*, and *tetA*. This observation underscores the potential for gene evolution over time, signifying the spread of resistance mechanisms across diverse clonal sequence types. This gene can impact the efficacy of antibiotics treatment on a population level. Hence, our findings underscore the imperative need for further comprehensive investigations aimed at elucidating the mechanisms underlying genetic mutations and the emergence of allelic variants associated with antibiotic resistance.

We report a high concordance rate (>90%) between genotypic and phenotypic resistance for certain antibiotics, as similarly reported by others (Moura et al., 2024; Zankari et al., 2013). However, lower concordance rates (<90%) were observed for certain antibiotics classes, with fluoroquinolones showing the lowest agreement. Most cases were so-called “major errors”, i.e. we observed genotypic resistance but phenotypic susceptibility (Yee, Dien Bard, & Simner, 2021). This phenomenon may be attributed to the suppression of gene expression through transcriptional regulation, other gene silencing mechanisms or compensatory mutations (Enne, Delsol, Roe, & Bennett, 2006; Zhong, Guo, Seow, Ming, & Schlundt, 2021). Similar results were noted in other studies conducted in Singaopore and France (Moura et al., 2024; Zhong et al., 2021). Further research is needed to fully explain this phenomenon.

In the multivariable analysis, we found that children whose mother were illiterate had higher odds of carrying ESBL-producing *E. coli* (Tola, Abera, Gebeyehu, Dinku, & Tullu, 2021). Furthermore, age and sex of the child were not significantly associated with ESBL-producing *E. coli* carriage. These results are consistent with prior studies conducted in Ethiopia, Guinea-Bissau, and Madagascar (Herindrainy et al., 2011; Isendahl et al., 2012; Tola et al., 2021).

The observed link between maternal illiteracy and ESBL-producing *E. coli* colonization suggests the importance of community education in implementing effective strategies to combat this public health concern.

Children who were both stunted and wasted were significantly more likely to be colonized by ESBL-producing *E. coli*, with a three times higher odds compared with those without malnutrition (de Lauzanne et al., 2022; Tellevik et al., 2016; Woerther et al., 2011). This finding can be explained by the fact that malnutrition weakens the immunity system, making children more susceptible to infections, and more likely to be treated with antibiotics (Rytter, Kolte, Briend, Friis, & Christensen, 2014). This connection may also involve the influence of microbiota and the creation of niches for colonization. Given the well-established link between antibiotic use and the emergence and spread of AMR, addressing malnutrition through public health interventions could contribute to the reduction of infections and colonization by AMR carrying bacteria.

Furthermore, the study revealed that there was a significant correlation between ESBL carriage and settlement type, water treatment, and chicken ownership. Pastoralists were found to have higher odds of ESBL-producing *E. coli* colonization compared to agro-pastoralists. The nomadic nature of pastoralists, involving frequent travels in search of water and food, may foster shared water sources and inadequate sanitation and hygiene practices. Consequently, this situation could escalate the risk of waterborne diseases like diarrhea and facilitate the transmission of ESBL-producing *E. coli* ("<Ayele_NCCR_Dialogue_11.pdf>," ; G. Cissé, 2019). As infectious diseases become more prevalent in such contexts, there is an associated rise in antibiotic usage, a well-established precursor to AMR (R. Magnano San Lio, G. Favara, A. Maugeri, M. Barchitta, & A. Agodi, 2023).

Despite chlorine being the most commonly used method for water treatment in LMICs (Nielsen et al., 2022), this study found a positive association between children who consumed chlorine-treated water and ESBL-producing *E. coli*. This finding aligns with a randomized control trial conducted in Bangladesh, which revealed that water chlorination did not significantly decrease the fecal carriage of ESBL-producing *E. coli* in children (Montealegre et al., 2022). Other studies conducted in China and South Africa also demonstrated the tolerance of chlorine in relation to AMR (Krige, 2009; Xiao et al., 2021). Furthermore, a study conducted in China highlighted that chlorination promotes horizontal gene transfer through natural transformation, thus facilitating the spread and emergence of AMR (Jin et al., 2020). Therefore, we hypothesize that the widespread use of chlorine in LMICs for water treatment may not be as effective as previously thought in reducing the prevalence and transmission of antimicrobial-resistant *E. coli* strains.

In this study, possessing chickens showed a significant relationship with ESBL-producing *E. coli*, with the odds of colonization being five times higher. Previous studies conducted in Ethiopia, Kenya, Nigeria, Ghana, Pakistan, and the Netherlands have suggested that chickens are the primary source of ESBL-producing *E. coli* transmission to humans (Aworh et al., 2020; Badr et al., 2022; L. Falgenhauer et al., 2018; Langata, Maingi, Musonye, Kiiru, & Nyamache, 2019; Liaqat et al., 2022; Overdevest et al., 2011; M. M. Rahman et al., 2020). This suggests that there is a need of effective measures to control the spread of antimicrobial resistance in animal husbandry, with a focus on poultry as the main carriers.

We attempted to collect fecal samples from children and livestock within the same households to investigate the circulation of resistance genes between the two populations. However, we encountered a limitation as we were unable to perform Whole Genome Sequencing (WGS) for the animal isolates due to issues related to the quality of DNA samples that were shipped to Switzerland. This limitation restricted our ability to explore the genetic aspects of resistance gene transmission between human and animal populations comprehensively.

Conclusion

Our study represents the first study of molecular epidemiology of ESBL-producing *E. coli* isolated from rural children and livestock in Ethiopia. We found high and low prevalence of ESBL-producing *E. coli* in rural children and livestock, respectively, largely mediated by the gene $bla_{\text{CTX-M-15}}$ encoded on a plasmid. The isolates displayed a high diversity of STs, with the predominant types being ST-2353, ST-10, ST-48, ST-38, and ST-450. Our study is the first to report that ST-2353 is associated with multi-drug resistance genes in Ethiopia. Further research including other domains such water and food products are needed to comprehensively study this diversity and the spread of antimicrobial resistance genes to better understand their acquisition. We suggest implementing an integrated One Health surveillance system, which would be able to monitor transmission events and detect resistant bacteria in a timely manner from both humans and animals.

Declaration

Ethics approval and consent to participate

Ethical approval was obtained from the Swiss Ethics Committee of Northwest and Central Switzerland (Ethikkommision Nordwest- und Zentralschweiz; REQ-2020-00608), the Review Committee of Armauer Hansen Research Institute in Addis Ababa, Ethiopia (AF-10-015), the Review Committee of the University of Jigjiga in Ethiopia (JJU-RERC030/2020), and National Research Ethics Review Committee (NRERC) (D2/152/533/4). A written consent was obtained from the parent or legal guardian of all participating children before study enrolment

(signed or finger print). Data was recorded using the Open Data Kit and securely stored on a server at the Swiss TPH in Basel. All personally identifiable information was maintained by the local study team in Ethiopia and securely stored in a closed cupboard.

Consent for Publication: Not applicable

Availability of data and materials: On reasonable request, the corresponding author will provide the datasets used or analyzed during the current study.

Competing interest: **The authors declare no competing interests.**

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Authors' contributions: AM, RT, JZ, GC and PV developed the research questions. AM, AA, YO, and SH conducted laboratory work, supervised by EI and PV. SH and TS performed the bioinformatics analysis. JH contributed to data analysis. AM wrote the first draft of the manuscript and all authors (AA, SH, YO, RT, SY, TS, JH, JZ, GC, and PV) revised and approved the final text.

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4.6 Supplementary

Table S1: Household WASH Characteristics and Livestock Ownership among Pastoralist and Agro-pastoralist Communities in Adadle District, Somali Region, Ethiopia

Variables	Agro-Pastoralist, N = 169	Pastoralist, N = 177	N = 346
Water Source			
Birkad /Borehole	3 (1.8%)	157 (88.7%)	160 (46.2%)
River water	161 (95.3%)	5 (2.8%)	166 (48%)
Tank truck	5 (3.0%)	15 (8.5%)	20 (5.8%)
Treat water			

Variables	Agro-Pastoralist, N = 169	Pastoralist, N = 177	N = 346
No	97 (57.4%)	155 (87.6%)	252 (72.8%)
Yes	72 (42.6%)	22 (12.4%)	94 (27.2%)
Toilet type			
Outdoor	168 (99%)	164 (93%)	332 (96%)
Pit latrine	1 (0.6%)	13 (7.3%)	14 (4.0%)
Shared toilet			
No	151 (89.3%)	167 (94.4%)	318 (91.9%)
Yes	18 (10.7%)	10 (5.6%)	28 (8.1%)
Waste disposal			
Burned	25 (14.8%)	29 (16.4%)	54 (15.6%)
Dumped in the street/open space	144 (85.2%)	148 (83.6%)	292 (84.4%)
Hand washing method			
Water and soap	15 (8.9%)	5 (2.8%)	20 (5.8%)
With water only	154 (91.1%)	172 (97.2%)	326 (94.2%)
Household had soap			
No	31 (18.3%)	15 (8.5%)	46 (13.3%)
Yes	95 (56.2%)	103 (58.2%)	198 (57.2%)
Sometimes	43 (25.4%)	59 (33.3%)	102 (29.5%)
Cattle			
No	9 (5.3%)	124 (70.1%)	133 (38.4%)
Yes	160 (94.7%)	53 (29.9%)	213 (61.6%)
Camel			
No	152 (89.9%)	113 (63.8%)	265 (76.6%)
Yes	17 (10.1%)	64 (36.2%)	81 (23.4%)
Chicken			
No	151 (89.3%)	177 (100.0%)	328 (94.8%)
Yes	18 (10.7%)	0 (0.0%)	18 (5.2%)
Goat			
No	63 (37.3%)	22 (12.4%)	85 (24.6%)
Yes	106 (62.7%)	155 (87.6%)	261 (75.4%)
Sheep			
No	79 (46.7%)	99 (55.9%)	178 (51.4%)
Yes	90 (53.3%)	78 (44.1%)	168 (48.6%)
Donkey			
No	32 (18.9%)	73 (41.2%)	105 (30.3%)
Yes	137 (81.1%)	104 (58.8%)	241 (69.7%)

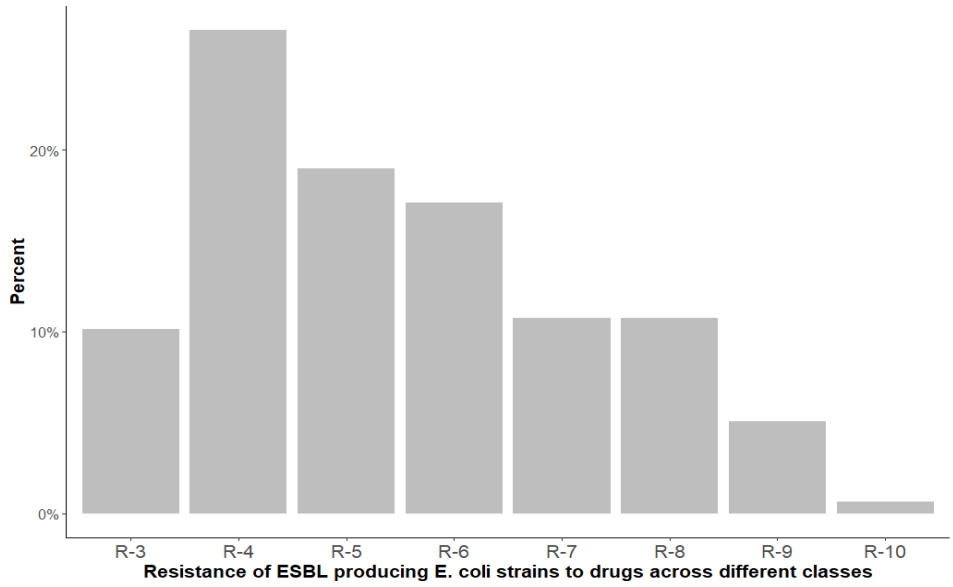

Figure S1: Multidrug resistance pattern in ESBL-producing *E. coli* among children in Adadle district Somali region, Ethiopia. R-3: Resistance to three drugs from different classes; R-4: Resistance to four drugs from different classes; R-5: Resistance to five drugs from different classes; R-6: Resistance to six drugs from different classes; R-7: Resistance to seven drugs from different classes; R-8: Resistance to eight drugs from different classes; R-9: Resistance to nine drugs from different classes; R-10: Resistance to ten drugs from different classes.

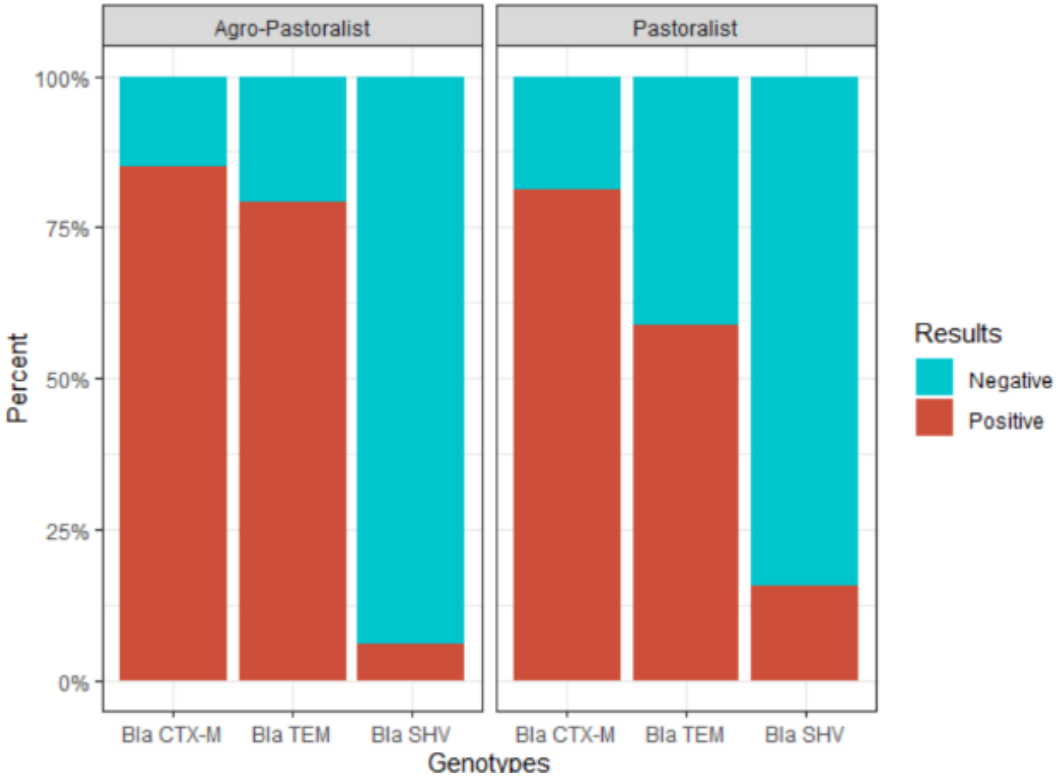

Figure S2: Characterization of the resistance genes in ESBL-producing *E. coli* among children in Adadle district, Somali region, Ethiopia

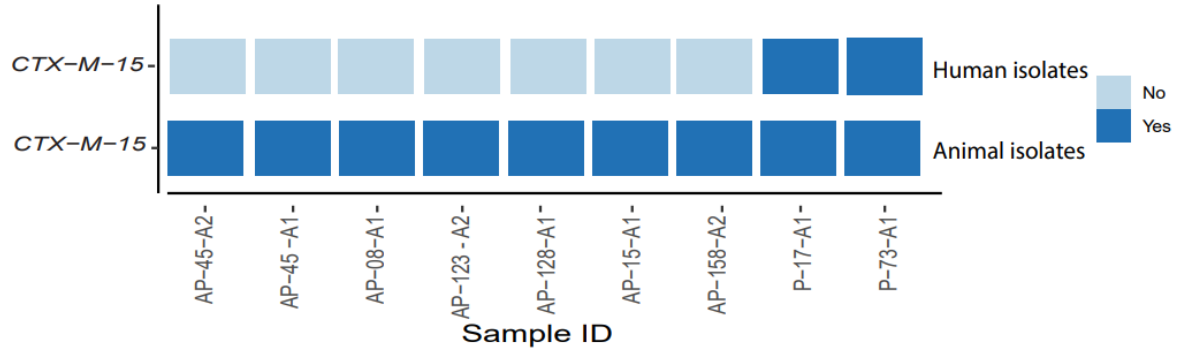

Figure S3: Comparing resistance gene carried by human and animal isolates from Adadle district, Somali region, Ethiopia

Methods and Materials

Molecular testing for β-lactamase genes (conventional PCR)

Table S2: Primer Sequences for PCR amplifications for ESBL genes

Target gene	Oligo name	Oligo Sequence 5' and 3'	Size (bp)	Reference
*bla*CTX-M	CTX-M-F	CGCTGTTGTTAGGAAGTGT	754	(Ramachandran, Shanthi, & Sekar, 2017)
	CTX-M-R	GGCTGGGTGAAGTAAGTGA		
*bla*TEM	TEM-F	TTTCGTGTCGCCCTTATTC	404	(Mohammed, Gadzama, Zailani, & Aboderin, 2016)
	TEM-R	ATCGTTGTCAGAAGTAAGTTG		
*bla*SHV	SHV-F	CGCCTGTGTATTATCTCCC	294	(Mohammed et al., 2016)
	SHV-R	CGAGTAGTCCACCAGATCC		

PCR conditions

The reaction comprised 12.5 µL of 2X HotStartTaq multiplex PCR Master Mix (QIAGEN), 1.5 µL of each primer (forward and reverse, 0.2 µM), 1.5 µL of template DNA (300 ng), and 9.5 µL of nuclease-free water.

PCR cycling conditions comprised an initial denaturation step at 95°C for 15 minutes, followed by 35 cycles of denaturation for 30 seconds, annealing at 58°C for 90 seconds, and extension at 72°C for 90 seconds. A final elongation step was carried out at 72°C for 10 minutes. The results ere visualized through gel electrophoresis S3.

Chapter 5 Molecular characterization of diarrheal pathogens and their ESBL and Carbapenem resistance genes in the Somali region of Ethiopia: A matched case-control study

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5.1 Abstract

Background: The increase of ESBL- and carbapenemase-producing pathogens causing diarrhea, a leading cause of child mortality in sub-Saharan Africa, has exacerbated treatment challenges. In the Somali region of Ethiopia, empirical treatment is common due to the scarcity of diagnostic facilities, and there is a dearth of studies on diarrheal pathogens and their resistance profile, thus, addressing this issue is crucial. Therefore, to fill this gap and support treatment strategies, we conducted a case-control study on the diarrheal pathogens and associated resistance genes in Jigjiga, Somali region of Ethiopia, using molecular techniques.

Methods: Cases were children under five hospitalized for diarrhea. Controls were matched to cases by age, sex and neighborhood of residence. Demographic and anthropometric data were recorded from both cases and controls. A total of 176 stool samples were collected from each group to detect enteropathogens including bacteria (*Camplyobacter, diarrheagenic E. coli, Shigella spp. Salmonella spp.* and *Vibrio cholerae*), protozoa (*Entamoeba spp. and Giardia intestinalis*) and viruses (rotavirus). Bacterial pathogens were cultured and screened by PCR. Enteropathogens isolates were then tested for ESBL and carbapenem resistance using disc diffusion test and PCR.

Results: The overall detection rate of at least one potential pathogen was significantly higher in cases than controls, with an OR of 3 (95% CI: 1.8-5.2). Diarrheagenic *E. coli* (DEC) were more prevalent in cases: EPEC (34% vs. 17%), ETEC (25% vs. 7%) EAEC (22% vs. 15%), and STEC (21% vs. 3%). *Salmonella spp.* (12% vs. 3%), *Shigella spp.* (10% vs. 2%), and *Campylobacter jejuni* (8% of vs. 2%) were also more detected in cases. *Rotavirus* (4%) was exclusively identified in cases. *Campylobacter jejuni, Salmonella spp.,* STEC, and ETEC significantly associated with diarrhea. The ESBL resistance gene $bla_{\text{CTX-M-15}}$ was found in *Shigella spp*. (64.3% vs. 21.4%), DEC (48.5% vs. 18.2%), and *Salmonella spp*. (42.9% vs. 14.3%). Regarding carbapenem resistance genes, the $bla_{\text{NDM-5}}$ were detected in *Salmonella spp*. (16% vs. 7%), *Shigella spp*. (14% vs. 5%) and DEC (14%) only in cases. The $bla_{\text{OXA-48}}$ gene was exclusively detected in cases, with 16% in *Salmonella spp*., and 14% each in DEC and *Shigella spp..*

Conclusion: A high prevalence of $bla_{\text{CTX-M-15,}}$ $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-48}}$ was found in DEC *Salmonella spp.,* and *Shigella spp..* Interestingly, we found *Shigella spp.* harboring bla_{NDM} and $bla_{\text{OXA-48}}$, which has been rarely described to date, making this a first reported instance in Ethiopia. Further research employing whole genome sequencing is needed to elucidate the spread of *Enterobacteriaceae* and the prevalence of resistance genes in specific pathogens.

Key words: Extended spectrum β-lactamase; Carbapenem; Antimicrobial resistance

5.2 Introduction

Diarrheal diseases persist as a significant global health challenge, remaining the second leading cause of mortality among children under five, with approximately half a million reported deaths annually (J. Liu et al., 2016). Most of these fatalities occur in sub-Saharan Africa and Southern Asia (L. Liu et al., 2012). The etiology of diarrheal diseases is multifactorial, primarily attributed to various pathogens including viruses, bacteria, protozoa, helminths, and fungi, with transmission being notably high in populations lacking access to safe water, sanitation facilities, and adequate hygiene practices [2].

Though the specific attribution of enteric pathogens to diarrheal diseases varies by region, rotavirus, adenovirus, diarrheagenic *Escherichia coli* (DEC), *Campylobacter*, *Salmonella spp.*, and *Shigella spp.* consistently emerge as primary contributors to diarrhea worldwide (Breurec et al., 2016; Hugho et al., 2023; Troeger et al., 2018). Low- and middle-income countries (LMICs) are particularly affected, with rotavirus standing out as the main cause of gastroenteritis in children (Sadiq & Khan, 2023). Infections caused by DEC strains such as enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), shiga-toxin producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC), and by other bacterial pathogens like *Campylobacter*, *Salmonella*, and *Shigella spp.,* have also been associated with diarrheal diseases in these regions (R. Das, Haque, Chisti, Faruque, & Ahmed, 2021; Kiiru, Maina, Mwaniki, Songoro, & Kariuki, 2024; Kotloff et al., 2013; Moharana et al., 2019; Sadiq & Khan, 2023). Because of the wide spectrum of possible hosts and the environmental adaptability of these enteric bacteria, co-infections with other bacteria, viruses or parasites can occur and lead to severe disease. For instance, DEC has been shown to cause hybrid infections, as seen in the case of the fatal EAEC/EHEC infections in Europe (Rasko et al., 2011).

Besides co-infection, the emergence of extended-spectrum beta-lactamase (ESBL) and carbapenem resistance genes among enteric pathogens has become a global concern, compromising the effectiveness of first line antibiotics and complicating treatment strategies (Abera et al., 2023). In ESBL-producing bacteria, the prevalence of the $bla_{\text{CTX-M}}$ gene has surged worldwide, particularly in sub-Saharan African regions, where pathogens like DEC, *Salmonella spp.*, and *Shigella spp* have started harboring this gene, causing antibiotic resistant infections which are challenging to treat (Fakorede, Amisu, Saki, & Akinyemi, 2023; Simbarashe Karambwe, Afsatou Ndama Traoré, & Natasha Potgieter, 2024; Kowalski et al., 2024; Park et al., 2021). The last treatment option for these infections is carbapenem, which, however, is also threatened by the emergence of resistance genes such as $bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$,

bla$_{OXA-181}$ and *bla*$_{OXA-48}$. These genes have also been increasingly detected in DEC and *Salmonella spp.* (Dembélé et al., 2021; Kowalski et al., 2024; Prah et al., 2021). To date, to the best of our knowledge, no carbapenem resistance has been described in *Shigella* spp.

In Ethiopia, several studies have identified rotavirus, DEC, *Campylobacter*, *Shigella spp.* and *Salmonella spp.* in children with diarrhea, and the majority of the bacteria displayed phenotypic resistance to the most commonly used antibiotics such as ampicillin, amoxicillin, tetracycline, and cephalosporin (Damtie et al., 2020; Dessale et al., 2023; Diriba et al., 2021; Zelelie et al., 2023). *bla*$_{CTX-M}$ emerged as the predominant gene identified in *E. coli* strains in the country (Kowalski et al., 2024). However, there remains a scarcity in the genetic characterization of etiology of diarrhea and distribution of ESBL or carbapenem resistance in DEC, *Shigella spp*, and *Salmonella spp.*. Moreover, there is a significant gap in research within the Somali region, which has experienced outbreaks of diarrheal diseases in the past years. To address this gap and support treatment strategies on both regional and national level, we conducted a comprehensive molecular characterization study of diarrheal pathogens and resistance genes in Jigjiga, the capital city of the Somali region of Ethiopia.

5.3 Methods

Study setting and design

This matched case-control study was conducted from June 2022 to March 2023 in Jigjiga, Somali Region, Ethiopia. Participants were recruited from Jigjiga University Sheik Hassen Yabere Comprehensive Hospital (JJUSHYCH) and Karamardha Hospital, the only hospitals serving the Jigjiga area, along with communities residing in the same area. Cases included children under 60 months hospitalized for diarrhea, who were able to provide a stool sample, and who tested negative for human immunodeficiency virus (HIV). Controls, selected from the community were matched to cases based on age (±2 months for infants aged 0-11 months, ±3 for toddlers aged 12-23 months, ±3 months for children aged 24-59 months), sex (male, female) and residing neighborhood (details in the method section in the supplementary). Only children who were in good health with no history of diarrhea and antibiotic use during the seven days before sampling were included as control. The controls were not tested for HIV, but if the parents disclosed that the child was seropositive, the child was not included in the study. In cases where no matching control was found in the neighborhood, we moved to the next zone within the Kebele. Cases and controls were not included more than once.

Sample size

Based on the hospital annual report, we assumed that Jigjiga hospitals see approximately 200 cases of diarrhea in children per year. We aimed to recruit 50% of these cases, with an

assumed detection rate of *E. coli* of 45% and a prevalence of antimicrobial resistance (AMR) of 80%. For controls, we assumed a detection rate of 33% for *E. coli* and an AMR prevalence of 65%. The calculated sample size was 135 children in each group, assuming an AMR prevalence of 80% and a precision of 5% points.

Stool sample collection

Pediatricians in both hospitals examined all cases and made the decisions regarding the need of hospitalization. Trained nurses collected fresh stool samples aseptically from cases and immediately transported them to the microbiology laboratory at JJUSHYCH within 30 minutes of collection. For controls, trained field workers provided mothers with instructions in the local language a day before collecting fecal samples. Fresh stool samples were then collected in the morning, placed in an icebox, and transported to the microbiology laboratory at JJUSHYCH within 30-60 minutes. Upon receipt, samples were aliquoted into four cryotubes, two with glycerol (40% glycerol mixed 1:1 with the stool) and two without, all of which were stored at -20°C. Additionally, one aliquot was placed into Cary-Blair culture medium, and stored at 4°C. Within 24 hours of collection, all samples were shipped to Armauer Hansen Research Institute (AHRI) in Addis Ababa. Upon arrival, samples in the Cary-Blair culture medium were immediately processed within 2-4 hours, while the remaining cryotubes were stored at -80°C for long-term preservation.

Data collection

The caretakers of both cases and controls responded to the same standardized questionnaire, which included demographic characteristics, child health status, WASH behavior, breastfeeding and diet, and anthropometric measurements. The anthropometric measurements were done using the World Health Organization (WHO) standard wooden measuring board for height, weight scale for weight, and tape measure for mid-upper arm circumference (MUAC). Additional details are summarized in the supplementary methods section.

Bacterial isolation and identification

The swabs from the Cary-Blair tube were inoculated onto specific agar media for the detection of different pathogens. These included MacConkey agar No:3 for the detection of *E. coli*, xylose lysine deoxycholate agar (XLD) and *Salmonella-Shigella spp.* agar for *Salmonella spp.* and *Shigella spp*., modified charcoal cefoperazone deoxycholate agar (mCCD) for *Campylobacter*, and thiosulfate citrate bile salts sucrose agar (TCBS) (Oxoid, UK) for *Vibrio cholerae*. Subsequently, all plates were incubated overnight at 37°C, except for the *Campylobacter* media, which were incubated for 48-72 hours at 42°C to facilitate growth.

After 24-hour incubation on MacConkey agar, pink colonies were identified as *E. coli* and subsequently inoculated onto Sorbitol MacConkey agar. On XLD agar, *Salmonella* was identified by colonies displaying a red or pink color with a black center, while *Shigella spp.* colonies appeared red or pink but transparent. On *Salmonella-Shigella spp.* agar, *Salmonella* colonies were colorless with a black center, and *Shigella spp.* colonies were colorless and transparent. Furthermore, the identification of *E. coli, Salmonella, and Shigella spp.* was carried out through standard biochemical tests, encompassing oxidase, catalase, sulfide indole motility, citrate, lysine decarboxylase, triple-sugar iron, and urea media (Oxoid, UK). Subsequently, DNA was extracted from these isolates for confirmation using conventional PCR for *E. coli* and real-time PCR for *Salmonella spp. and Shigella spp..* Additional details are summarized in the supplementary methods section.

On the mCCD agar, *Campylobacter jejuni* ATCC 33291 and *E. coli* ATCC *25922* were used as positive and negative controls, respectively. After 48-72 hours, colonies were selected based on their morphology (grey, moist spreading, dry with or without metallic sheen) and microscope appearance (slender, curved, “gull wing” shaped). Selected colonies were then tested using standard biochemical tests, including catalase, oxidase, urea utilization, Triple Sugar Iron (TSI), hippurate hydrolysis, resistance to nalidixic acid and cephalothin, and hydrogen sulfide production. Subsequently, DNA was extracted from these isolates for confirmation using real-time PCR. Additional details are summarized in the supplementary methods section.

For *Vibrio cholerae*, large yellow and green colonies identified on TCBS were sub-cultured onto Trypicase Soy Agar (TSA) and incubated at 37°C overnight. In instances where no colonies resembling *Vibrio spp.* were observed following overnight incubation on the TCBS plates, sub-cultures from TSA were subjected to oxidase testing. If oxidase test yielded a negative result, no additional testing for *Vibrio spp.* was conducted. Given that all our samples tested negative for oxidase, further analysis was deemed unnecessary.

Parasitology and virology analysis

Upon arrival of the stool samples at the microbiology laboratory of JJUSHCH, parasitology and virology analyses were conducted, and the results were immediately sent to the attending physicians for use in treatment. In parasitology analysis, the thick-smear Kato-Katz detection method was utilized to detect helminth ova, along with the direct smear method conducted in duplicate to identify protozoan cysts and trophozoites (Lanker et al., 2023). Subsequently, the samples were checked for protozoa including *Giardia intestinalis* and *Entamoeba spp*. (Lanker et al., 2023). The presence of *Rotavirus* antigens was tested using Xpect *rotavirus* assay

(Oxoid, Thermo Fisher Scientific, Basingstoke, UK), according to the manufacturer's guidelines (Thermofisher, 2024).

Antibiotic susceptibility test

The disc diffusion method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Performance, 2016), was used to determine antibiotic susceptibility using Müller-Hinton agar. Susceptibility tests were performed for 26 antibiotics from 12 different classes, including: penicillins 10µg (ampicillin (AMP) 10µg and amoxicillin (AML) 10µg); beta-lactamase inhibitor combinations (ampicillin-sulbactum (SAM) 30µg and amoxicillin/clavulanic acid (AMC) 30µg); penicillin and beta-lactamase (piperacillin/tazobactam (PIR) 40µg); amphenicols (chloramphenicol (CAF) 10 µg); cephalosporin (cefazolin (KZ) 30µg, cefpodoxime (CPD)), cefuroxime (CXM) 30µg, ceftriaxone (CRO) 30µg, cefotaxime (CTX) 30µg, cefotetan (CTT) 30µg, cefoxitin (FOX) 10µg, cefipime (FEP) 30µg, ceftazidime (CAZ) 30µg); carbapenem (ertapenem (ETP) 10µg, imipenem (IPM) 10µg and meropenem(MEM) 10µg); monobactams (aztreonam (ATM) 30µg); fluoroquinolones (ciprofloxacin (CIP) 5µg and nalidixic acid (NA) 30µg); aminoglycosides (Amikacin (AK) 30µg and gentamycin (CN) 30µg); macrolides (azithromycin (AZM) 15 µg); tetracycline (TET) 30 µg), and cotrimoxazole (SXT) 25 µg (Oxoid, United Kingdom). McFarland 0.5 was measured prior to spreading the suspension on the Müller-Hinton agar plate. Pathogens were classified as susceptible, intermediate, or resistant based on the diameters of inhibition zones according to CLSI (Performance, 2016). Multidrug resistance was defined as non-susceptibility to at least one antibiotic agent in three or more antimicrobial classes (Magiorakos et al., 2011). The details of ESBL and carbapenem confirmatory tests can be found in the methods section in the supplementary.

Molecular detection for Diarrheagenic *E.coli, Campylobacter jejuni, Shigella spp.* and *Salmonella spp.*

Furthermore, intestinal pathotypes of *E. coli,* such as enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterotoxogenic *E. coli* (ETEC), entero-invasive *E. coli* (EIEC) and entero-aggregative *E. coli* (EAEC) were identified using a multiplexed assay on conventional PCR. Specific target genes were amplified for each pathotype: *eae* and *bfpA* for EPEC, *stx1* and *stx2* for STEC, *LT* for ETEC, *CVD432* for EAEC, and *ipaH* for EIEC. PCR cycling conditions comprised an initial denaturation step at 96^{o}C for 4 minutes, followed by 35 cycles of denaturation for at 95^{o}C for 20 seconds, annealing at 57°C for 20 seconds, and extension at 72°C for 1 minute. A final elongation step was carried out at 72°C for 7 minutes (Panchalingam et al., 2012). Then, amplification was visualized through gel electrophoresis. Primers sequences can be found in supplementary table S4.

Moreover, a real-time PCR assay was used for the identification of *Salmonella ssp.* (invA), *Shigella spp*. (ipaH), and *Campylobacter jejuni* (cadF). For the multiplex real-time PCR assay, cycling conditions were as follows: Initial denaturation step at 95°C for 30 seconds, followed by 34 cycles of annealing and extension at 60°C for 45 seconds. Primers sequences can be found in supplementary table S4.

Molecular characterization for ESBL and Carbapenem resistance genes.

A conventional PCR assay was used for the identification of resistance genes. The ESBL (*bla*$_{CTX\text{-}M\text{-}15}$) and carbapenem (*bla*$_{KPC}$*, bla*$_{OXA48}$*, and bla*$_{NDM\text{-}5}$) resistance genes were identified using singleplex and multiplex PCR, respectively. The PCR cycling conditions were set as follows: 40 cycles of denaturation at 95°C for 15 seconds, annealing at 52.8°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. Then, amplification was visualized through gel electrophoresis. Details of the primers can be found in supplementary table S4.

Statistical analysis

Statistical analysis was conducted using R statistical software version 4.3.2 (R Core Team, 2013). Initial descriptive analysis of variables was performed using the gtsummary package. Continuous variables were shown as mean (±SD) and discrete variable as percentage. The nutritional status was calculated using the zscorer package. Stunting, wasting, and underweight were defined as HAZ, WHZ, WAZ < −2. Conditional logistic regression was used to assess the association between pathotype genes against cases and controls. The initial model included all the pathotypes (*Campylobacter* jejuni, EPEC, ETEC, STEC, EAEC, EIEC, *Salmonella* spp., *Shigella spp*., *Giardia intestinalis* and *Entamoeba spp*. and rotavirus). Rotavirus was subsequently excluded from the model as it was exclusively detected in five children (all cases). Significance was determined at $p<0.05$. Results are presented as adjusted and unadjusted OR (aOR) with 95% CI.

5.4 Results

Between June 2022 and February 2023, 287 children were recruited for the study. Three children tested positive for HIV, and we were not able to recruit controls for six cases. Subsequently, 138 cases (50%) and 138 controls (50%) were analyzed. However, two samples lacked the respective completed questionnaires, resulting in 136 cases and 136 controls included in the final analysis. The male-to-female ratio for both cases and controls was roughly 1:1, with a mean age of 13 months. The distribution of children's ages between

0-11 months, 12-24 months, and 25-59 months was 137 (50.4%), 90 (33.1%), and 45 (16.5%), respectively.

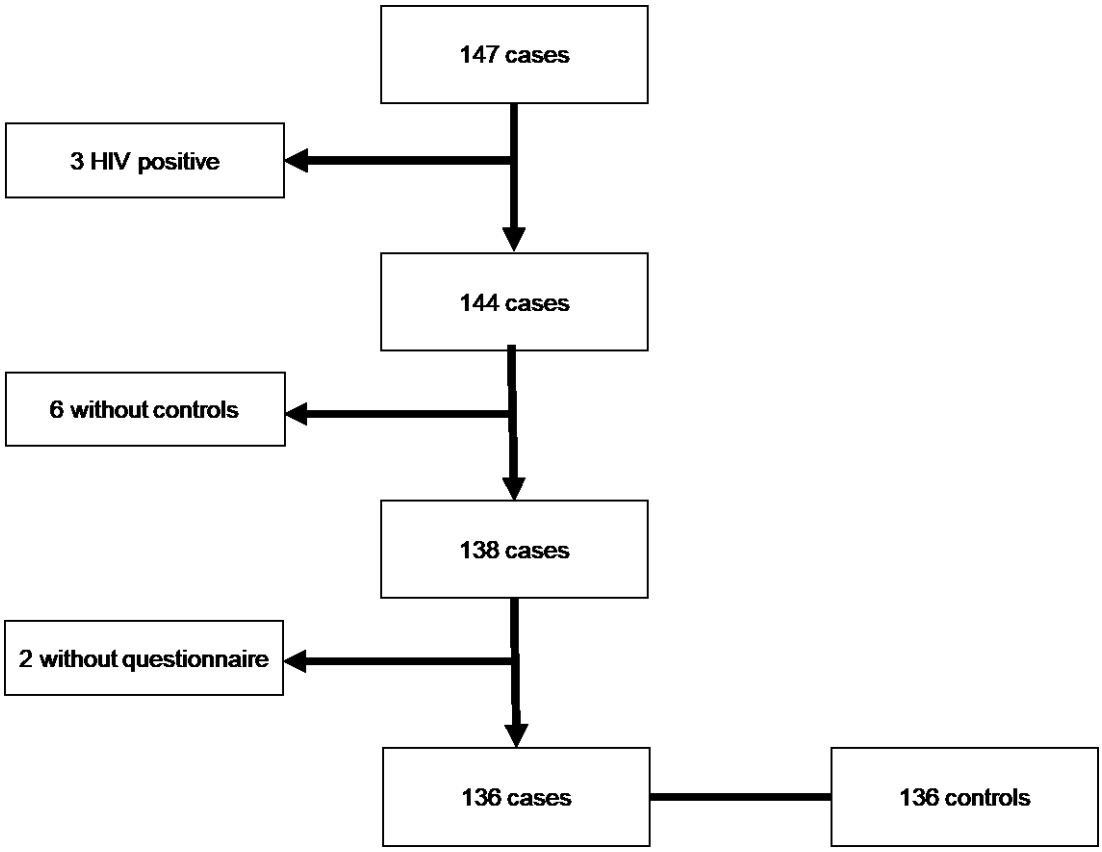

Figure 9. Flow chart of selection.

Table 3. Characteristics of children with and without diarrhea in Jigjiga, Somali region, Ethiopia

Variable	Case, N = 136[1]	Control, N = 136[1]	Overall, N = 272[1]
Sex			
Male	73 (53.7%)	73 (53.7%)	146 (53.7%)
Female	63 (46.3%)	63 (46.3%)	126 (46.3%)
Child Age (Mean (SD))	13.3 (9.7)	13.2 (9.4)	13.3 (9.5)
Currently breastfeeding			
No	39 (28.7%)	5 (3.7%)	44 (16.2%)
Yes	97 (71.3%)	131 (96.3%)	228 (83.8%)
Exclusive breastfeeding (6 months)			
No	76 (55.9%)	96 (70.6%)	172 (63.2%)
Yes	60 (44.1%)	40 (29.4%)	100 (36.8%)
Other milk than breast milk (6 months)			

Variable	Case, N = 136[1]	Control, N = 136[1]	Overall, N = 272[1]
No	5 (6.6%)	1 (1.0%)	6 (3.5%)
Yes	71 (93.4%)	95 (99.0%)	166 (96.5%)
Boiling milk before giving to the child			
No	11 (15.5%)	63 (66.3%)	74 (44.6%)
Yes	60 (84.5%)	32 (33.7%)	92 (55.4%)
Giving water (general)			
No	13 (9.6%)	13 (9.6%)	26 (9.6%)
Yes	123 (90.4%)	123 (90.4%)	246 (90.4%)
Routine vaccination			
No	21 (15.4%)	32 (23.5%)	53 (19.5%)
Yes	115 (84.6%)	104 (76.5%)	219 (80.5%)
Finished the routine vaccination dose			
No	75 (65.2%)	68 (65.4%)	143 (65.3%)
Yes	40 (34.8%)	36 (34.6%)	76 (34.7%)
Stunting (HAZ < −2 SD)			
No	65 (58.0%)	84 (62.7%)	149 (60.6%)
Yes	47 (42.0%)	50 (37.3%)	97 (39.4%)
Underweight (WAZ < −2 SD)			
No	76 (57.6%)	103 (77.4%)	179 (67.5%)
Yes	56 (42.4%)	30 (22.6%)	86 (32.5%)
Wasting (WHZ < −2 SD)			
No	81 (70.4%)	115 (87.1%)	196 (79.4%)
Yes	34 (29.6%)	17 (12.9%)	51 (20.6%)

[1]n (%); Mean (SD)

The cases presented clinical symptoms of vomiting (81.6%), fever (73.5%), cough (50.7%), and edema (5.1%). General dehydration conditions observed during the data collection included restlessness (23.5%), inability to drink (23.5%), dry mouth (20.2%), dry eyes (16.2%), thirstiness (14.3%), sunken eyes (13.9%), and lethargy (12.7%) (Table S1).

Pathogens

The overall detection rate of at least one potential pathogen was significantly higher in cases than controls, with an OR of three (95% CI: 1.8-5.2). Of these isolates, at least one of the bacteria in analysis was found in 32% of cases and 18% of controls. Diarrheagenic *E. coli*

(DEC) was substantially higher in cases compared to controls (OR: 2.4, 95% CI 1.4-4.0). Specifically, EPEC was found in 34% of cases and 17% of controls, ETEC in 25% of cases and 7% of controls, EAEC in 22% of cases and 15% of controls, STEC in 21% of cases and 3% of controls, and EIEC in 4% of cases and 1% of controls. Additionally, *Salmonella spp.* was present in 12% of cases and 3% of controls, *Shigella spp.* in 10% of cases and 2% of controls, and *Campylobacter jejuni* in 8% of cases and 2% of controls. *Rotavirus* (4%) was exclusively detected in cases. Among the 94 samples demonstrating parasite ova, *Entamoeba spp.* was found in 16% of cases and 5% of controls. Conversely, *Giardia intestinalis* was more prevalent in controls (24%) than in cases (17%). The results are summarized in Figure 10.

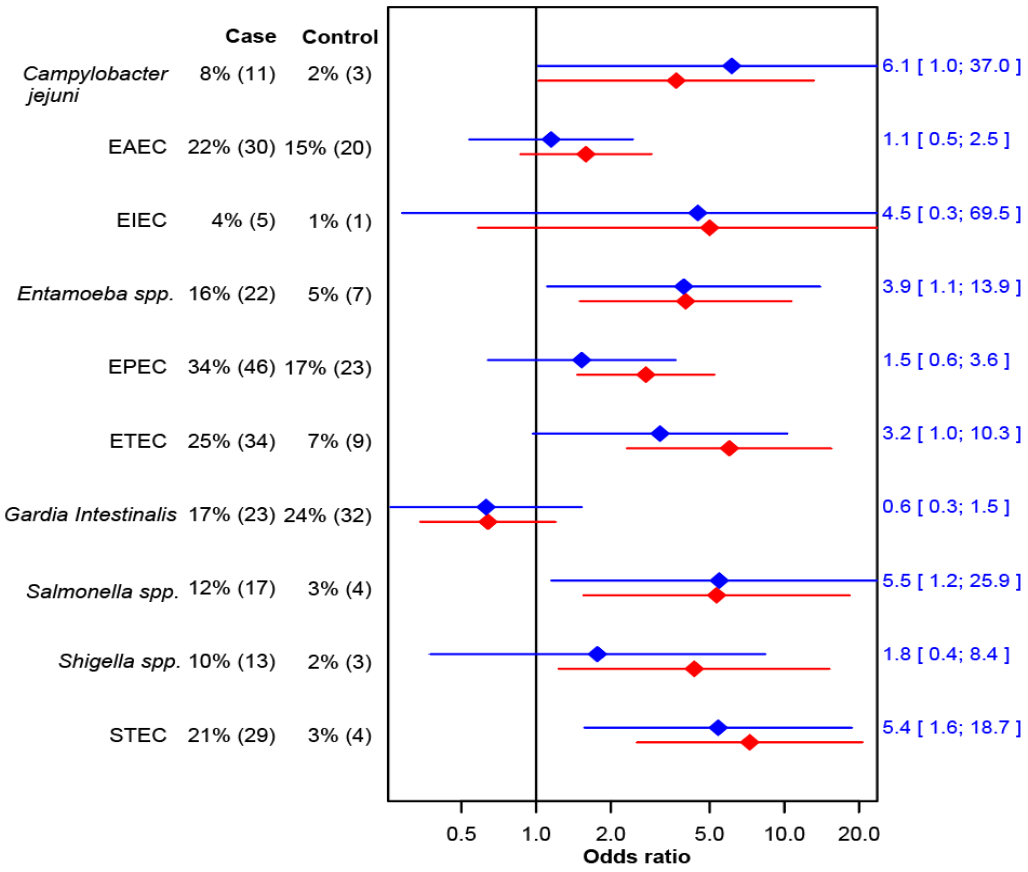

Figure 10. Multivariable (blue) and univariable (red) conditional logistic regression analysis of enteric pathogens associated with diarrhea cases in Jigjiga, Somali region, Ethiopia.

In conditional logistic regression analysis, significant associations were observed between children with diarrhea and several pathogens. *Campylobacter jejuni, Salmonella spp.,* STEC, and ETEC showed significant associations, with odds ratios (ORs) of 6.1 (95% CI: 1.0-37.0), 5.5 (95% CI: 1.2-25.9), 5.4 (95% CI: 1.6-18.7), and 3.2 (95% CI: 1.0-10.3), respectively. Additionally, *Entamoeba spp.* emerged as a significantly associated enteric parasite with diarrhea (OR: 3.9, 95% CI: 1.1-13.9). However, *Shigella spp.,* and EPEC only demonstrated significant associations with diarrhea in the univariable analysis.

The co-infection of enteric pathogens was higher in cases than in controls (OR: 3.1, CI: 1.79 – 5.20). In diarrhea cases, the most common co-infection was between diarrheagenic *E. coli* (DEC) and *Giardia intestinalis* 27% (12/43), followed by DEC-*Entameoba spp.* 20.9% (9/43), and DEC-*Shigella spp*. 18.6% (8/43). Co-infection of DEC with either *Campylobacter jejuni* or *Salmonella spp.* were present in 14% (6/43) of cases. DEC's lowest co-infection was with *Rotavirus* 4.6% (2/43). Results are summarized in Figure 11.

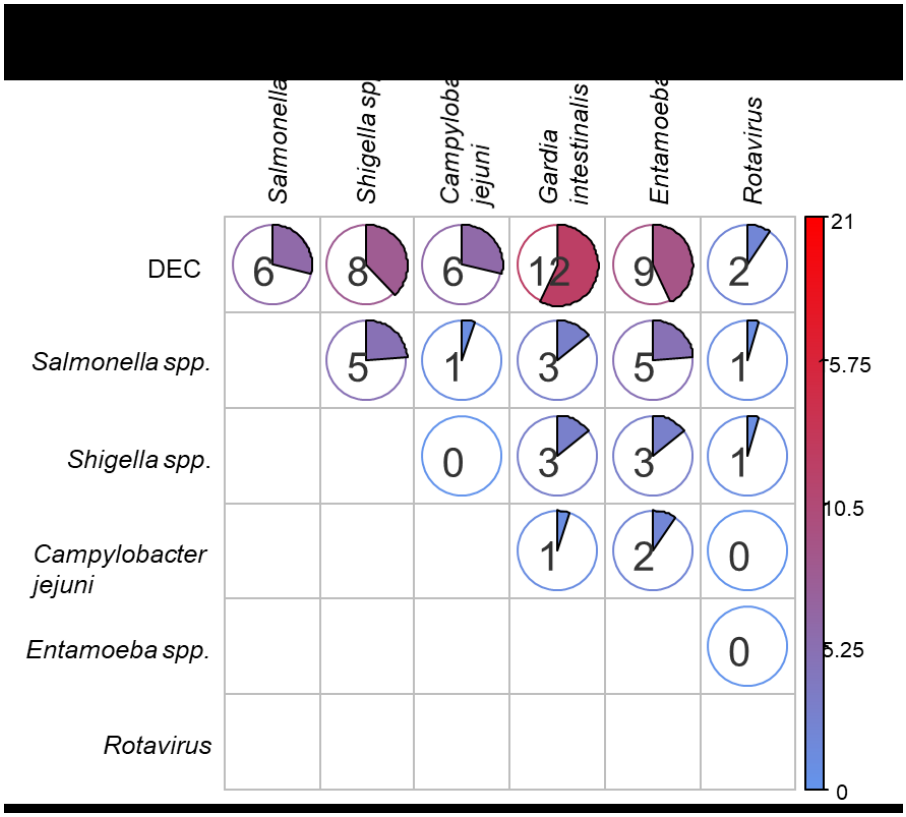

Figure 11. Co-infection of enteric pathogen detected in cases in Jigjiga, Somali region, Ethiopia.

The overall virulent co-infection in DEC hybrid was 19%. Among these, the highest co-infection of two virulent pathogens was found in EPEC/ETEC at 23% (12/52), followed by EPEC/EAEC at 13% (7/52), and EPEC/STEC at 7.7% (4/52). The combination of three virulent DEC strains was recorded as follows: EPEC/STEC/ETEC at 13% (7/52), EPEC/STEC/EAEC at 7.7% (4/52), and EPEC/ETEC/EAEC at 5.7% (3/52). Six isolates showed a combination of four pathogens (EPEC/STEC/ETEC/EAEC). The details are summarized in table S2.

Prevalence of enteric pathogens in diarrhea cases and controls across different age groups

The enteric pathogens differed between cases and controls across distinct age groups. Among the cases, EPEC was detected in 32% (21/65) of infants, while EAEC, ETEC, STEC, and *Campylobacter jejuni* were present in 25% (16/65), 22% (14/65), 18% (12/65), and 14% (9/65) of infants, respectively. *Salmonella spp*. and *Shigella spp.* were each found in 7.7% (5/65) of infants, whereas rotavirus was found in 4.6% (3/65) and EIEC in 3.1% (2/65). In toddlers, EPEC was the most prevalent pathogen, accounting for 40% (19/48) of the cases, followed by ETEC in 29% (14/48), STEC in 25% (12/48), EAEC in 19% (9/48), *Salmonella spp*. in 19% (9/48), and *Shigella spp.* in 15% (7/48). *Campylobacter jejuni* was identified in 4.2% (2/48) of cases, as were EIEC at 4.2% (2/48), and rotavirus at 2.1% (1/48), constituting the lowest

observed pathogens among toddlers. The lowest enteropathogen was detected in children (23-59 months) as shown in Figure 12.

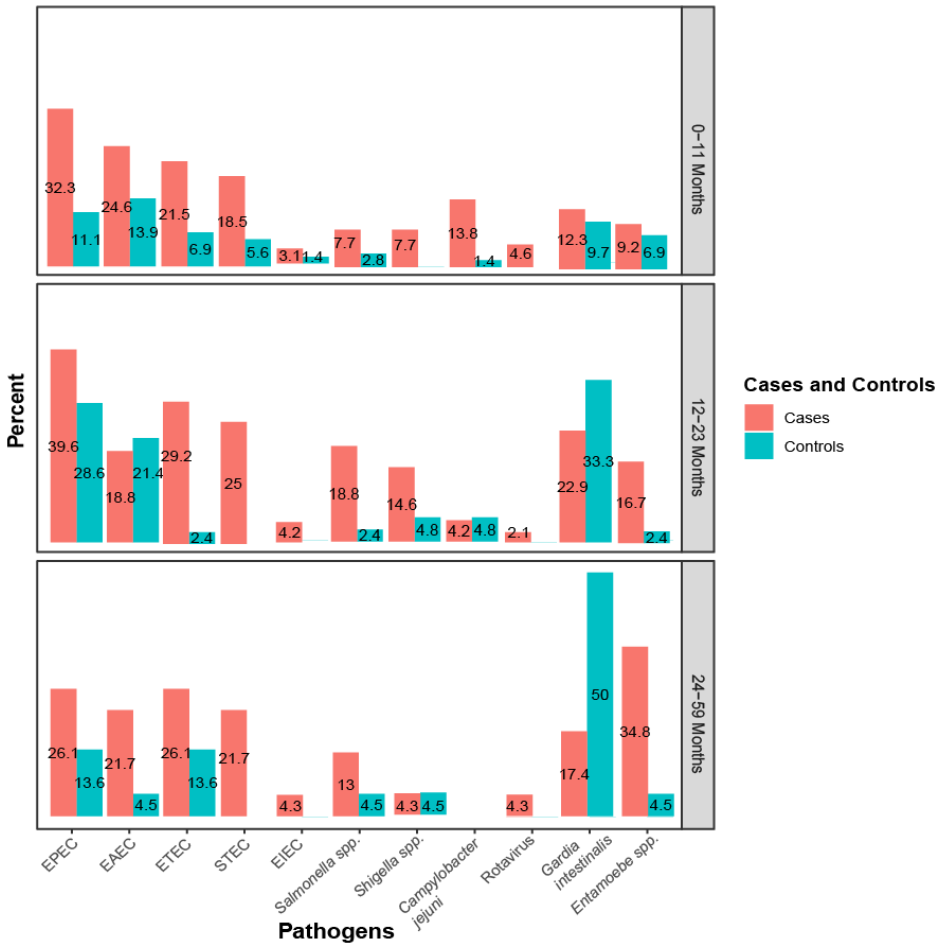

Figure 12. Pathogens distribution across age category among cases and controls in Jigjiga, Somali region, Ethiopia

Regarding enteropathogen distribution based on children's nutritional status among cases, EPEC was the most prevalent diarrheagenic *E. coli* identified in 36% (15/42) of children categorized as normal. Among the stunted, wasted and underweight children the EPEC gene was found in 33% (11/33), 33% (13/39) and 32% (7/22) of cases, respectively. Moreover, ETEC (33%) and STEC (27%) was mainly found in stunted children, while EAEC (32%) showed high prevalence among underweight children and EIEC was most frequently detected in wasted children. *Campylobacter jejuni* was present in 10% (4/39) of wasted children, and in 9.1% of stunted and underweight children each, while the lowest occurrence was registered in normal children at 4.8% (2/42). *Salmonella spp.* was found in 18% (4/22) of underweight cases, 15% (5/33) in stunted cases, 10% (4/39) in wasted cases and 9.5% (4/42) in children classified as normal. Stunted, normal, wasted and underweight children were found to harbor *Shigella spp.* in 15% (5/33), 9.5% (4/42), 7.7% (3/39), and 4.5% (1/41) of cases, respectively.

In contrast, most of the enteropathogens were detected in low prevalence rate or were not detected at all in the control group. Further details of the results are summarized in table S3.

Susceptibility pattern of Diarrheagenic *E. coli, Salmonella spp., and Shigella spp.*

The diarrheagenic *E. coli* isolates obtained from cases and controls demonstrated nearly similar resistance patterns, showing high resistance rates to amoxicillin (93% vs. 91.3%), ampicillin (93% vs. 91.3%), tetracycline (77.2% vs. 73.9%), and trimethoprim-sulfamethoxazole (77.2% vs. 69.6%). In cases, 70.9%, 69.6%, 69.6%, 68.4% and 67.1% of isolates were non-susceptible to cefpodoxime, ceftriaxone, cefazolin, cefuroxime, and cefotaxime, respectively, while susceptibility rates were recorded at 29.1%, 27.8%, 30.4%, 29.1% and 27.8% for the same antibiotics. Additionally, isolates showed intermediated resistance against cefotaxime (5.1%), ceftriaxone (2.5%), and cefuroxime (2.5%). Conversely, in controls, nearly over half (54.3%) of isolates showed resistance to cefpodoxime, and less than half of the isolates exhibited resistance to cefotaxime, cefazolin, an cefuroxime, all recording similar rates (47.8%), with ceftriaxone registering the least resistance (39.1%).

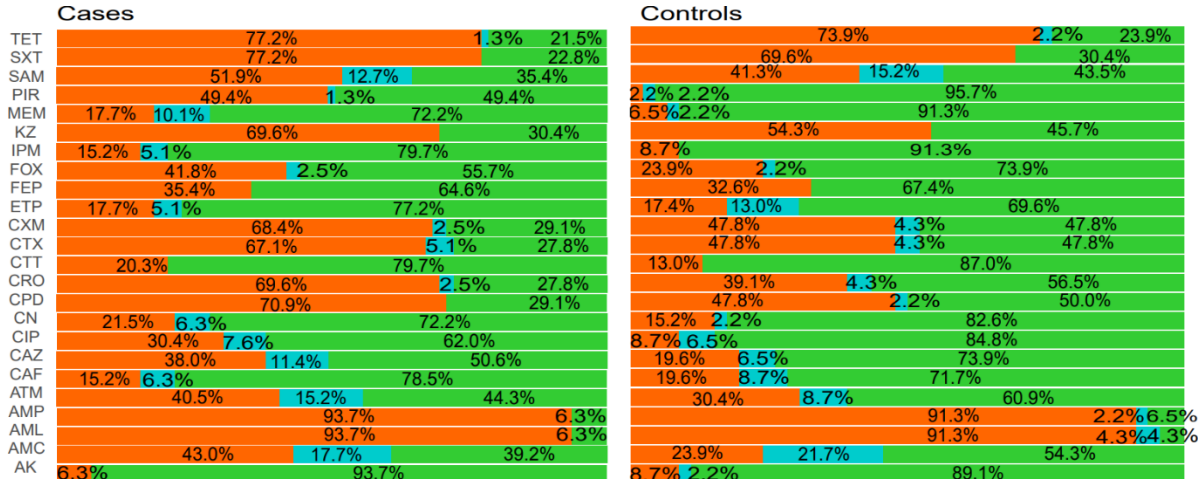

Figure 13. Antimicrobial resistance pattern for diarrheagenic *E. coli* isolates from children with and without diarrhea in Jigjiga, Somali region, Ethiopia.

Antimicrobial agents tested include: TET (Tetracycline), SXT (trimethoprim-sulfamethoxazole), SAM (*Ampicillin-sulbactam)*, PIR (Piperacillin/tazobactam), MEM (Meropenem), KZ (Cefazolin), IMP (Imipenem), FOX (Cefoxitin), FEP (Cefepime), ETP (Ertapenem), CXM (Cefuroxime), CTX (Cefotaxime), CTT (Cefotetan), CRO (Ceftriaxone), CPD (Cefpodoxime), CN (Gentamycin), CIP (Ciprofloxacin), CAZ (Ceftazidme), CAF (Chloramphenicol), ATM (Aztreonam), AMP (Ampicillin), AML (Amoxicillin), AMC (Amoxicillin-clavulanic acid), AK (Amikacin).

Regarding *Salmonella spp.* from both groups, high resistance rates were found for amoxicillin (78.6% vs. 100), ampicillin (78.6% vs. 60%), and cefazolin (78.6% vs. 60%), and tetracycline (64.3% vs. 60%). Cefuroxime showed a resistance rate of 64.3% in cases; however, in the

controls, 40% showed intermediate resistance, while 60% were susceptible. Among cases, 50% of isolates were non-susceptible to amoxicillin-clavulanic acid, cefpodoxime, cefotaxime, nalidixic acid ampicillin-sulbactam and 42.9% to trimethoprim-sulfamethoxazole, cefoxitin, and ceftriaxone. Carbapenems showed the least resistance rate in 7.1% of the isolates. In contrast, most of the antibiotics showed less resistance rate in controls than cases, as illustrated in Figure S1.

In regards to *Shigella spp.* isolates (13 cases vs. 3 controls), conferred non-susceptibility to amoxicillin, ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and cefazolin showed high at rates of 92.9%, 92.9%, 78.6%, 71.4%, and 71.4%, respectively, among cases. Conversely, 25% of isolates from controls exhibited non-susceptibility to amoxicillin, ampicillin, and cefazolin. Similar to other pathogens, carbapenems showed the lowest resistance rate in cases. Results are summarized in Figure S2.

Regarding multidrug resistance (MDR) and extensively drug resistance (XDR), the majority of the DEC (111/117), *Salmonella spp.* (17/19) *and Shigella spp.* (16/18) isolates were MDR. Only, 17/117 of DEC, 4/18 of *Shigella spp.* and 2/19 of *Salmonella spp.* were XDR.

Gene characterization of ESBL- and Carbapenemase-producing isolates

In phenotypic analysis, isolates of DEC, *Salmonella spp*., and *Shigella spp*. confirmed as ESBL- and carbapenemase-producing pathotypes underwent conventional PCR for further confirmation. The ESBL resistance gene $bla_{\text{CTX-M-15}}$ was found in 64.3% (9/14), 48.5% (48/99), and 42.9% (9/21) of *Shigella spp*., DEC, and *Salmonella spp*. cases, respectively. In the control group, $bla_{\text{CTX-M-15}}$ was harbored by 21.4% (3/14), 18.2% (18/99), and 14.3% (3/21) of *Shigella spp*., DEC, and *Salmonella spp*., respectively. Regarding carbapenem resistance genes, the different pathotypes showed nearly similar rates of $bla_{\text{NDM-5}}$, with 16% in *Salmonella spp*. and 14% each in DEC and *Shigella spp*. cases. In the control group, only 7% and 5% of *Salmonella spp*. and *Shigella spp*. carried $bla_{\text{NDM-5}}$. The $bla_{\text{OXA-48}}$ gene was exclusively detected in cases, with 16% in *Salmonella spp*., and 14% each in DEC and *Shigella spp..*

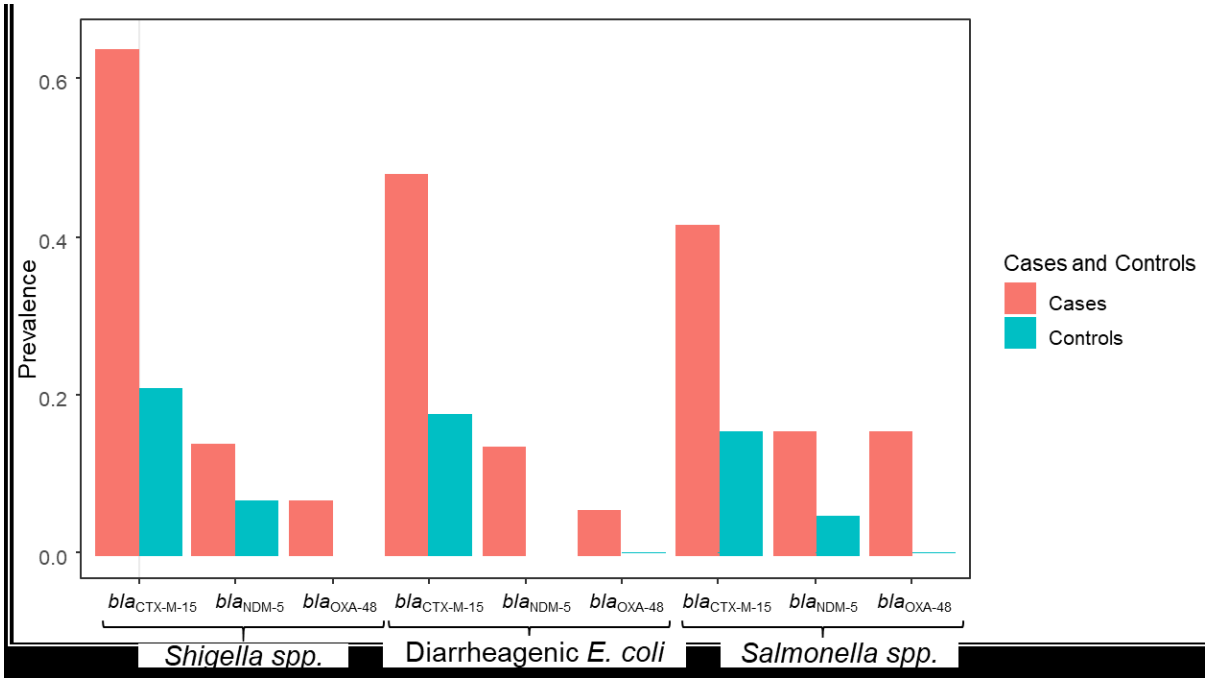

Figure 14. Prevalence of ESBL and Carbapenem resistance genes among diarrheagenic *E. coli, Salmonella spp. and Shigella spp.* isolates from Jigjiga, Somali region, Ethiopia

5.5 Discussion

Despite the ongoing preventive strategies, diarrhea remains one of the leading causes of illness and death in children under five, especially in LMICs, where access to advanced diagnostic facilities and thus to an effective, targeted treatment is limited. The high antibiotic consumption resulting from this is a major precursor of antimicrobial resistance (Cohen et al., 2022; Rogawski et al., 2017). In order to develop effective strategies and policies to address this issue, it is important to have knowledge about the antibiotic resistance landscape within pathogens in a specific setting. Therefore, here we provide results of the first comprehensive molecular characterization of the etiology of diarrhea and resistance genes in the Somali Region of Ethiopia.

In this study, the overall detection of at least one potential pathogen including DEC, *Campylobacter jejuni*, *Salmonella spp*., *Shigella spp*., and rotavirus, was higher among cases than controls. This is expected and aligns with results obtained by other studies conducted in Global Enteric Multicenter Study (GEMS) along with Ethiopia, China, and Central African Republic (Breurec et al., 2016; J. Liu et al., 2016; Mekonnen, Mengistie, Sahilu, Kloos, & Mulat, 2019; S. X. Zhang et al., 2016). *Rotavirus*, known as the leading cause of diarrheal morbidity and mortality in young children globally, attributing to 25%–30% of fatalities, emerged as the least discerned pathogen in our study, detected in only 4% of the cases. However, a systematic review conducted in Ethiopia showed a higher prevalence of rotavirus (23%) (Damtie et al., 2020). Possible explanations for this disparity could include differences

in the methods used for rotavirus detection. Additionally, the geographical context of our study, conducted in an urban setting where children are more likely to receive vaccination, may have influenced the lower incidence observed (Asmare, Madalicho, & Sorsa, 2022).

Among the identified enteric pathogens, *Campylobacter jejuni, Salmonella spp.,* STEC, and ETEC were significantly associated with diarrhea cases. Similarly to a recent study in Ethiopia, our study found different pathotypes of DEC, which are the main cause of diarrhea in children, with different prevalence rates: EPEC (34%), ETEC (25%), EAEC (22%), STEC (21%) and EIEC (4%). The prevalence of each pathotype was higher in children with diarrhea compared to the control group, aligning with prior studies (Mulu et al., 2024; A. Wolde, Deneke, Sisay, & Mathewos, 2022). In the Global Enteric Multicenter Study (GEMS), ETEC was highlighted as a one of the major causes of moderate to severe diarrhea (Kotloff et al., 2013). The diverse clinical manifestations associated with STEC infections, ranging from mild diarrhea to life-threatening hemolytic uremic syndrome (HUS), emphasize the critical importance of prompt diagnosis and management (Nataro & Kaper, 1998). Both STEC and ETEC showed significant associations with diarrhea in our study, reaffirming their roles as important contributors to diarrheal diseases.

The genomic versatility and plasticity of *E. coli* enables it to persist in several niches within both the host and the environment, allowing for the development of novel strains capable of harboring multiple virulent genes from different DEC pathotypes simultaneously (Braz, Melchior, & Moreira, 2020). This study revealed the emergence of hybrid pathotypes exhibiting a virulent combination of DEC. Among these hybrids, the fusion of EPEC/ETEC, EPEC/EAEC and EPEC/STEC were found in 23%, 13.4% and 7.7% of isolates, respectively. These hybrid strains were found globally, including in children with diarrhea in Ethiopia, Madagascar, Nigeria, India and Brazil (Collard et al., 2022; Dutta, Pazhani, Nataro, & Ramamurthy, 2015; Liebchen et al., 2011; Mulu et al., 2024; Ogunbiyi, Fayemi, Akanni, Ayolabi, & Hald, 2023), indicating the potential severity of these infections and another layer of concern to the already burdensome issue of childhood diarrhea in developing nations. The reach of these hybrid strains extends beyond human hosts. Instances of EPEC/STEC were detected in other reservoirs such as pet birds, cattle, and water sources across Mexico, Iran, and South Africa, suggesting a broader impact (Badouei, Morabito, Najafifar, & Mazandarani, 2016; Bolukaoto, Singh, Alfinete, & Barnard, 2021; Chiacchio et al., 2018). Perhaps most alarmingly, the infamous hybrid STEC/EAEC strain, implicated in a deadly outbreak in Germany that claimed 54 lives, was found in this study (Rasko et al., 2011).

Interestingly, multiple virulent pathotypes with more than two combinations of hybrids have been shown in children with diarrhea in Pakistan and Mexico (Patzi-Vargas et al., 2015; Zil-e-

Huma et al., 2019). Similarly, our study has shown the combination of three virulent pathotypes of EPEC/STEC/ETEC in 13.4% isolates, EPEC/STEC/EAEC 11.5%, EPEC/ETEC/EAEC in 5.7% and STEC/ETEC/EAEC in a single isolate. Notably, a four-way of hybrid of virulent pathotypes, EPEC/STEC/ETEC/EAEC, was also identified in 7.7% of the children, underscoring the circulation of highly virulent strains within the local population. Subsequent confirmatory tests such as sequencing and comparative genomics are imperative to delineate the characteristics and ascertain the phylogenetic positioning of these strains. Further analysis of phylodynamics and phylogenetics are needed to understand whether the variants were imported from other regions or originated within country. Additionally, integrated surveillance adopting a One Health approach is essential to elucidate the genetic dissemination of these hybrid DEC strains beyond the human host (Osman et al., 2023).

Campylobacter jejuni, Shigella spp. and *Salmonella spp.,* are prominent pathogens recognized as leading cause of diarrhea in children less than five, constantly reporting their association with diarrhea in several case-control studies (Hendrickson et al., 2023; Kaakoush, Castaño-Rodríguez, Mitchell, & Man, 2015; Kotloff et al., 2013; Krumkamp et al., 2015). Our study corroborates these findings, revealing a higher prevalence of pathogens in children with diarrhea compared to those without. Adjustments for potential contributing pathogens further highlighted the significant association of *Campylobacter jejuni* and *Salmonella spp.* with diarrhea, consistent with observations in previous studies (Kariuki et al., 2006; Murugesan, Abraham, Samuel, & Ajjampur, 2022). Moreover, the detection rate of *Campylobacter jejuni* was 8% in diarrheal cases, nearly consistent with recent systematic reviews conducted in Ethiopia (10%), South Africa (13%) and Nigeria (8%) (Diriba et al., 2021; Ohanu & Offune, 2009; Samie et al., 2022). Additionally, the detection rates of *Salmonella spp.* (12%) and *Shigella spp.* (10%) in our study align with other studies conducted in Ethiopia (Ameya, Tsalla, Getu, & Getu, 2018; Mastewal Balew & Mulugeta Kibret, 2023; Dessale et al., 2023). Conversely, studies in Ethiopia reported a high prevalence in *Shigella spp.* (16%) and a lower prevalence in *Salmonella spp.* (6%) in Gambella and Southern Ethiopia (Hayamo, Alemayehu, Tadesse, Mitiku, & Bedawi, 2021; Mekonnen et al., 2019). These differences could be explained by the unique socio-environmental context of our study area, where livelihoods heavily depend on livestock and dairy products. This underscores the importance of further researches into pathogen detection across various environmental compartments such as water, milk, and manure. Moreover, implementing educational initiatives targeting breastfeeding practices and Water, Sanitation, and Hygiene (WASH) interventions emerge as promising strategies to mitigate diarrhea incidence among children.

Moreover, studies have reported that infections caused by *Campylobacter,* DEC and *Shigella spp.* are correlated with reduced weight gain and impaired linear growth, often resulting in

mortality (Haque et al., 2019; Hossain et al., 2023; G. Lee et al., 2013; Tickell et al., 2020). These pathogens have shown to induce increased inflammation or exploit nutrients from the gut lumen, exacerbating malnutrition (Haque et al., 2019). Although our study did not find an association between enteric pathogens and nutritional status, enteric bacteria such as *Campylobacter jejuni, Salmonella spp. Shigella spp*. and most of DEC were predominately detected in children experiencing stunting, underweight and wasting along with diarrhea. Considering the connection of these pathogens with increased inflammation or nutrient utilization from the gut, further research would be essential for a better understanding of the relationship between enteric pathogen-induced inflammation and impaired linear growth in children.

This study is the first study to assess antimicrobial resistance in clinical isolates of the Somali Region. Isolates of DEC, *Salmonella spp.,* and *Shigella spp*. in our study and others in the country showed high resistance to most of the first- and second-line antibiotics including penicillin, cephalosporin, fluoroquinolone, azithromycin, tetracycline, trimethoprim-sulfamethoxazole (Ararsa, Wolde, Alemayehu, Bizuwork, & Eguale, 2023; Ayako Wendy Fujita et al., 2022; Mulu et al., 2024; Zelelie et al., 2023). Of note, diarrheic children from Ambo and Addis Ababa exhibited lower resistance to fluoroquinolones compared to our findings (B. Ayele et al., 2023; Tosisa, Mihret, Ararsa, Eguale, & Abebe, 2020). This discrepancy may be attributed to differences in antibiotic usage patterns, differences in healthcare practices, or regional differences in bacterial strains and their resistance profiles. Furthermore, despite azithromycin being a first-line drug for *Salmonella spp*., a significant proportion of isolates showed resistance, similar to *Shigella spp*., consistent with another study conducted in Ethiopia (Dessale et al., 2023). Fortunately, carbapenem and amikacin showed lower resistance rates, offering potential treatment options (Hayamo et al., 2021; Tosisa et al., 2020). However, these results raise concerns as they suggest that many antibiotics recommended by the country's guidelines are ineffective on the isolates gathered through this study (Sisay, Mengistu, Molla, Amare, & Gabriel, 2017). These findings emphasize the urgent need for enhanced surveillance and stewardship efforts to address the rising rates of antibiotic resistance in diarrheal pathogens within the region.

ESBL and carbapenemase resistance poses significant challenges to public health globally (Tamma et al., 2021). A growing concern lies in the increasing prevalence of these highly problematic resistance genes within both healthcare settings and communities. These genes make beta-lactamase antibiotics ineffective, which are often the first line for treating infections, especially in developing countries where they are more affordable and easily accessible compared to alternative antibiotics. The $bla_{\text{CTX-M-15}}$ gene, one of the widely distributed ESBL resistance genes found in most of the human infections globally, was also observed in high

prevalence among DEC, *Shegilla spp.* and *Salmonella spp*. in both symptomatic and asymptomatic children in our study. Similarly, other research and systematic reviews in Ethiopia have identified *bla*$_{\text{CTX-M-15}}$ as the dominant ESBL gene in *E. coli* and *Salmonella* (Tafese B Tufa et al., 2020; D. Wolde et al., 2024; Zenebe et al., 2023). In Kenya and Tanzania, *bla*$_{\text{CTX-M-15}}$ is also prevalent in *E.coli* among children in both hospital and community settings (S. Karambwe, A. N. Traoré, & N. Potgieter, 2024; Kibwana et al., 2022; Moremi et al., 2017).

Furthermore, there has been a significant rise in *Shigella spp*. carrying the *bla*$_{\text{CTX-M-15}}$ gene, reported in both developed and developing countries (Campos-Madueno et al., 2020; Kiros et al., 2021; Sangeetha, Parija, Mandal, & Krishnamurthy, 2014). In Ethiopia, *Salmonella* Concord has been reported, representing a notable public health concern due to its frequent multidrug resistance (MDR) and the presence of the *bla*$_{\text{CTX-M-15}}$ gene (Cuypers et al., 2023; Fabre et al., 2009). This highlights the widespread presence of the *bla*$_{\text{CTX-M-15}}$ resistance gene across different pathogens in both symptomatic and asymptomatic children, intensifying the challenge of treating infections with commonly used drugs. Furthermore, comprehensive genetic characterization using whole-genome sequencing is crucial to understanding the sub-species of *Salmonella* and *Shigella spp.* and determining the location of resistance genes on either plasmids or chromosomes, which can inform intervention strategies.

Carbapenem serves as the last option against infections caused by ESBL-producing MDR *Enterobacteriaceae*, which are listed as critical pathogens (Kopotsa, Mbelle, & Osei Sekyere, 2020). Our study, along with others, has identified the presence of *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$ carabapenem resistance genes in DEC in symptomatic children (Han et al., 2020; Legese et al., 2022; Medugu et al., 2022; Zenebe et al., 2023). Previous research conducted in hospital settings in Djibouti and Uganda reported a lower incidence of *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$ compared to our findings (Legese et al., 2022; Okoche, Asiimwe, Katabazi, Kato, & Najjuka, 2015). Additionally, *bla*$_{\text{OXA-48}}$ was found at a higher prevalence in children in China (Han et al., 2020). These genes have also been found in *Salmonella spp.* strains in China (Gao et al., 2020; Wei et al., 2024). Notably, regional disparities in resistance patterns, as evidenced by variations between Djibouti, Uganda, Ethiopia, and China, emphasize the complex interplay of factors influencing antimicrobial resistance.

Moreover, our study discloses intriguing insights into the prevalence of carbapenem resistance genes in *Shigella spp*. strains. While *bla*$_{\text{NDM-5}}$ was more frequently found in symptomatic children than in the community, *bla*$_{\text{OXA-48}}$ was exclusively detected in hospitalized children. This is particularly concerning given that the presence of *bla*$_{\text{OXA-48}}$ in *Shigella spp.* was initially reported in a single isolate from India (Ralte et al., 2022; Walsh, Weeks,

Livermore, & Toleman, 2011). To our knowledge, our study is the first to report the presence of *bla*$_{\text{NDM-5}}$, and *bla*$_{\text{OXA-48}}$ in *Shigella spp* in Ethiopia. Remarkably, *bla*$_{\text{NDM-5}}$ has been shown to exhibit a broad host range and can transfer across different bacterial phyla (Q. E. Yang et al., 2024), highlighting the escalating challenge posed by carbapenem resistance, particularly in children. The detection of *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$ in the vulnerable demographic highlights a concerning trend, as they confer resistance to all beta-lactamase antibiotics (Hornsey, Phee, & Wareham, 2011). The spread of carbapenem resistance and a broad variety of transferrable resistance plasmids, raises concerns about the future reliability of carbapenems. Continued surveillance and concerted efforts to mitigate antimicrobial resistance are imperative. Effective infection control measures and prudent antibiotic usage are critical components of strategies aimed at addressing this pressing public health issue.

This study has several limitations, including the inability to perform whole genome sequencing on enteric pathogens due to resource constraints. Additionally, the lack of diagnostic tools hindered the detection of certain pathogens like *Cryptosporidium* and *Adenovirus*. This restriction curtailed our capacity to fully elucidate the genetic landscape and evolutionary dynamics of the target pathogens. Despite deploying the rapid Xpect *Rotavirus* kit, the study refrained from leveraging more advanced diagnostic modalities, thus potentially losing nuanced insights into the prevalence and genomic diversity of *Rotavirus* strains within the study. Furthermore, susceptibility and resistance genes were only tested for some pathogens, excluding *Campylobacter*. These limitations stress the imperative need for enhanced resource allocation and diagnostic competence in future research endeavors, facilitating a more comprehensive understanding of diarrheal disease etiology and antimicrobial resistance patterns. The study missed to differentiate clearly between nutritional, sanitation and animal exposure as main risk factors because of a limited sample size.

In summary, to the best of our knowledge, this study represents the first comprehensive molecular characterization of the etiology of diarrhea and resistance genes in Ethiopia. Our findings indicate a higher prevalence of potential pathogens such as *Campylobacter*, DEC, *Salmonella spp*., *Shigella spp*. and rotavirus in diarrheal cases compared to controls. Specifically, *Campylobacter jejuni*, *Salmonella spp*., ETEC, and STEC were significantly associated with children suffering from diarrhea. Moreover, we observed a complex landscape of co-infections, including the emergence of hybrid pathotypes within DEC combining two to four virulent combinations, and the co-occurrence of DEC with other pathogens such as *Campylobacter jejuni*, *Salmonella spp*. or *Shigella spp*. DEC, *Salmonella spp*., and *Shigella spp*. predominantly carried the bla$_{\text{CTX-M-15}}$ gene, particularly in children with diarrhea. Moreover, carbapenem resistance genes, namely *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$, were predominantly present in children with diarrhea. Remarkably, our study is the first to report the presence of *bla*$_{\text{NDM-5}}$ and

bla$_{OXA-48}$ in *Shigella spp* in Ethiopia, which is rarely reported globally. Further research to elucidate the spread of *Enterobacteriaceae* and the localization of resistance genes, employing whole genome sequencing, is needed. Additionally, given their broad host range, it is imperative to expand researches beyond human populations to include other compartments such as the environment, livestock, and livestock products. Establishing integrated surveillance frameworks under the One Health approach will be essential for addressing the complex interplay between pathogens, antimicrobial resistance, and environmental factors, ultimately guiding effective prevention and control strategies.

Declaration

Ethics approval and consent to participate

Ethical approval was obtained from the Swiss Ethics Committee of Northwest and Central Switzerland (Ethikkommision Nordwest- und Zentralschweiz; REQ-2020-00608), the Review Committee of Armauer Hansen Research Institute in Addis Ababa, Ethiopia (AF-10-015), the Review Committee of the University of Jigjiga in Ethiopia (JJU-RERC030/2020), and National Research Ethics Review Committee (NRERC) (D2/152/533/4). A written consent was obtained from the parent or legal guardian of all participating children before study enrolment (signed or finger print). Data was recorded using the Open Data Kit and securely stored on a server at the Swiss TPH in Basel. All personally identifiable information was maintained by the local study team in Ethiopia and securely stored in a closed cupboard.

Consent for Publication: Not applicable

Availability of data and materials: On reasonable request, the corresponding author will provide the datasets used or analyzed during the current study.

Competing interest: The authors declare no competing interests.

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Authors' contributions: AM, RT, JZ, GC and PV developed the research questions. AM, AA, YO, and TS conducted laboratory work, supervised by PV. JH contributed to data analysis. AM wrote the first draft of the manuscript and all authors (AA, YO, RT, TS, JH, JZ, GC, and PV) revised and approved the final text.

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5.6 Supplementary

Table S1: Clinical symptoms presented along with diarrhea cases in Jigjiga, Somali region, Ethiopia

Characteristic	N = 272[1]
Stool had blood	
No	129 (94.9%)
Yes	7 (5.1%)
Had vomiting	
No	25 (18.4%)
Yes	111 (81.6%)
Had cough	
No	67 (49.3%)
Yes	69 (50.7%)
Had fever	
No	36 (26.5%)
Yes	100 (73.5%)
Had edema	
No	129 (94.9%)
Yes	7 (5.1%)
General condition of dehydration	
Lethargic	32 (12.7%)
Restless	64 (23.5%)
Sunken eye	38 (13.9%)
Unable to drink	64 (23.5%)
Thirsty	39 (14.3%)
Dry eyes	44 (16.2%)
Dry mouth	55 (20.2%)
Took medication before coming to the hospital	
No	106 (77.9%)
Yes	30 (22.1%)

Characteristic	N = 272[1]
History of previous hospitalization	
No	235 (86.4%)
Yes	37 (13.6%)
Previously treated for malnutrition	
No	261 (96%)
Yes	11 (4.0%)

[1]n (%)

Table S2: Distribution of enteropathogens among diarrhea cases and controls by nutritional status in Jigjiga, Somali region, Ethiopia

Diarrheagenic *E.coli*	N = 52[1]
EPEC/ETEC	12 (23%)
EPEC/EAEC	7 (13.4%)
EPEC/STEC/ETEC	7 (13.4%)
EPEC/STEC/ETEC/EAEC	6 (11.5%)
EPEC/STEC	4 (7.7%)
EPEC/STEC/EAEC	4 (7.7%)
EPEC/ETEC/EAEC	3 (5.7%)
EAEC/EIEC	1 (1.9%)
EPEC/EIEC	1 (1.9%)
EPEC/ETEC/EIEC	1 (1.9%)
ETEC/EAEC	1 (1.9%)
ETEC/EIEC	1 (1.9%)
STEC/EAEC	1 (1.9%)
STEC/ETEC	1 (1.9%)
STEC/ETEC/EAEC	1 (1.9%)
STEC/ETEC/EIEC	1 (1.9%)

[1]n (%)

Table S3: Distribution of enteropathogens among diarrhea cases and controls by nutritional status in Jigjiga, Somali region, Ethiopia

Variables	Cases n (%)	Controls n (%)	Overall n (%)
Normal children			
Campylobacter jejuni	2 (4.8%)	2 (2.8%)	4 (3.5%)

EAEC	9 (21%)	10 (14%)	19 (17%)
EIEC	1 (2.4%)	0 (0%)	1 (0.9%)
EPEC	15 (36%)	12 (17%)	27 (24%)
ETEC	12 (29%)	3 (4.2%)	15 (13%)
STEC	10 (24%)	2 (2.8%)	12 (11%)
Salmonella spp.	4 (9.5%)	4 (5.6%)	8 (7.0%)
Shigella spp.	4 (9.5%)	2 (2.8%)	6 (5.3%)
Rotavirus	4 (9.5%)	0 (0%)	4 (3.5%)
Stunted children			
Campylobacter jejuni	3 (9.1%)	0 (0%)	3 (4.8%)
EAEC	6 (18%)	4 (14%)	10 (16%)
EIEC	1 (3.0%)	1 (3.4%)	2 (3.2%)
EPEC	11 (33%)	5 (17%)	16 (26%)
ETEC	11 (33%)	1 (3.4%)	12 (19%)
STEC	9 (27%)	0 (0%)	9 (15%)
Salmonella spp.	5 (15%)	0 (0%)	5 (8.1%)
Shigella spp.	5 (15%)	0 (0%)	5 (8.1%)
Rotavirus	0 (0%)	0 (0%)	0 (0%)
Underweight children			
Campylobacter jejuni	2 (9.1%)	1 (5.9%)	3 (7.7%)
EAEC	7 (32%)	3 (18%)	10 (26%)
EIEC	0 (0%)	0 (0%)	0 (0%)
EPEC	7 (32%)	4 (24%)	11 (28%)
ETEC	4 (18%)	2 (12%)	6 (15%)
STEC	5 (23%)	2 (12%)	7 (18%)
Salmonella spp.	4 (18%)	0 (0%)	4 (10%)
Shigella spp.	1 (4.5%)	1 (5.9%)	2 (5.1%)
Rotavirus	0 (0%)	0 (0%)	0 (0%)
Wasted children			
Campylobacter jejuni	4 (10%)	0 (0%)	4 (7.0%)
EAEC	8 (21%)	3 (17%)	11 (19%)
EIEC	3 (7.7%)	0 (0%)	3 (5.3%)
EPEC	13 (33%)	2 (11%)	15 (26%)
ETEC	7 (18%)	3 (17%)	10 (18%)
STEC	5 (13%)	0 (0%)	5 (8.8%)
Salmonella spp.	4 (10%)	0 (0%)	4 (7.0%)

Shigella spp.	3 (7.7%)	0 (0%)	3 (5.3%)
Rotavirus	1 (2.6%)	0 (0%)	1 (1.8%)

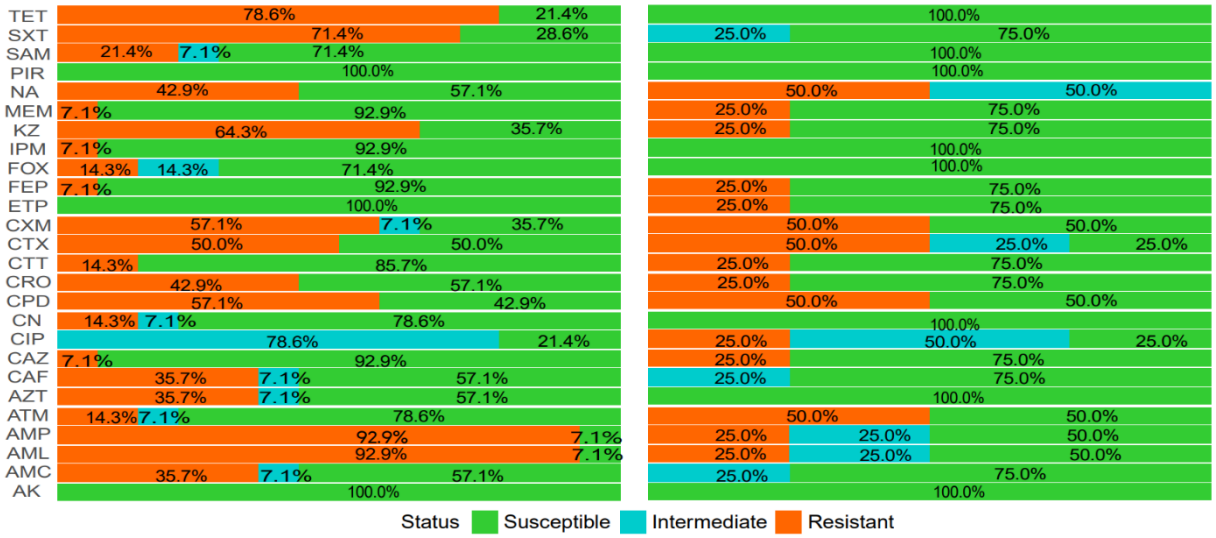

Figure S1: Antimicrobial resistance pattern for *Salmonella spp,* isolates from children with and without diarrhea in Jigjiga, Somali region, Ethiopia. TET (Tetracycline), SXT (trimethoprim-sulfamethoxazole), SAM (*Ampicillin-sulbactam)*, PIR (Piperacillin/tazobactam), NA (Nalixidic Acid), MEM (Meropenem), KZ (Cefazolin), IMP (Imipenem), FOX (Cefoxitin), FEP (Cefepime), ETP (Ertapenem), CXM (Cefuroxime), CTX (Cefotaxime), CTT (Cefotetan), CRO (Ceftriaxone), CPD (Cefpodoxime), CN (Gentamycin), CIP (Ciprofloxacin), CAZ (Ceftazidme), CAF (Chloramphenicol), AZT (Azithromycin), ATM (Aztreonam), AMP (Ampicillin), AML (Amoxicillin), AMC (Amoxicillin-clavulanic acid), AK (Amikacin).

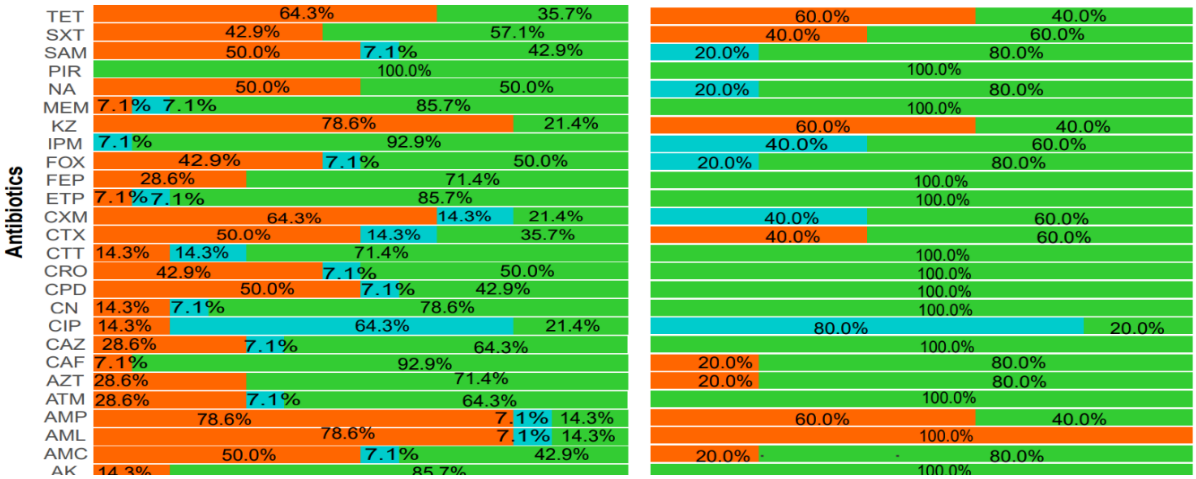

Figure S2: Antimicrobial resistance pattern for *Shigella spp.* isolates from children with and without diarrhea in Jigjiga, Somali region, Ethiopia

Methods

1. Identification of the controls

To identify the controls, the caregiver of each case was asked to provide the details about their residence, including their address, and if they know any children of the same age and sex living nearby. If the caregiver did not know such children, they were demanded to provide the phone number of a neighbor (only a willing neighbor) or local leader (zonal or kebele leader). Once the contact details were obtained, we collaborated with the zonal and kebele leaders to conduct a household-to-household survey in the setting until a suitable control was identified. In case no suitable control was found in the initial zone, the search was extended to neighboring zones.

2. Data collection procedure and Anthropometric measurements

The questionnaire administration and anthropometric measurements were done by trained health professionals composed of medical doctors, nurses, nutritionists and epidemiologists. Height measurements were taken with children standing against a WHO standard wooden measuring board, ensuring the correct posture and position. Weights were recorded using a WHO standard weight scale, either the child alone or with the mother by adjusting the weight scale (Onis, 2008). MUAC measurements were performed using a WHO standard tape measure. To ensure the accuracy of height, weight and MUAC measurements, each anthropometric measurement was repeated at least twice or until the measurements were within 1 mm, 100 g, or 1 mm of one another, respectively.

3. DNA extraction method

Three to five pure colonies from nutrient agar (Oxoid, UK) were suspended in 300µl of 1x TE buffer and vortexed for 15 seconds in an Eppendorf tube. Then, the tube was boiled for 15 minutes at 94°C in a water bath for 15 minutes. Immediately afterwards, the tube was placed in a freezer at -20°C for 10 minutes. Subsequently, the tube was left at room temperature for one minute, and then centrifuged for 5 minutes at 14000 rpm. 100µl of supernatant were carefully transferred to a clean Eppendorf tube. Finally, a Thermo Scientific Nano Drop was used to measure the quality and quantity of DNA.

4. ESBL screening and confirmatory test

According to CLSI, any strain showing resistance against cefotaxime (CTX) 30µg, ceftazidime (CAZ) 30µg, or ceftriaxone 30 µg was considered as a potential extended-ESBL producer. Confirmatory tests were performed utilizing a disc of cefotaxime (CTX) 30µg and one of ceftazidime (CAZ) 30µg alone, and a disc of cefotaxime and one of ceftazidime combined with clavulanic acid 10 µg. Strains were considered ESBL-producers if an increase of inhibition zone diameter of 5 mm or greater was observed in the discs of CTX or CAZ combined with clavulanic acid compared to the inhibition observed in the CTX or CAZ alone discs (Oxoid, United Kingdom). *E. coli* ATCC 25922 and *E. coli* ATCC BAA-2326 were used as negative and positive controls (Humphries et al., 2018). For carbapenem confirmatory tests, isolates resistant or intermediate to imipenem, ertapenem or meropenem were assessed for carbapenemase production using the modified carbapenem inactivation method (CIM), per the CLSI guidelines. Briefly, 1µL loop containing a full colony of the test isolates from the overnight nutrient agar plate was suspended in 2 mL of Oxoid UK Tryptosoya broth. Subsequently, a 10µg meropenem disc was introduced and completely submerged in the Tryptosoya broth. The tubes were then incubated for four hours at 37°C in an incubator. After the incubation period, the meropenem discs were extracted using a 10 µL inoculation loop and placed on Mueller-Hinton agar plates (Oxoid, UK) that had been freshly inoculated with a 0.5 McFarland suspension of a carbapenem-susceptible strain (Escherichia coli ATCC® 25922) incubated at 35°C ± 2°C for 18-24 hours. Carbapenemase producing strains will inactivate the meropenem disc, leading to grown of bacteria (CLSI, 2022)

Table S4: Primer Sequences and the Expected Amplicon Sizes for the Polymerase Chain Reaction

DEC Pathogen	**Primers**	**Sequence (5'-3')**	**Target gene**	**Base pair(bp)**	**Reference**
EPEC	eae-F	GACCCGGCACAAGCATAAGC	*eae*	384	(Panchalingam et al., 2012)
	eae-R	CCACCTGCAGCAACAAGAGG			
	bfpA-F	GGAAGTCAAATTCATGGGGG	*bfpA*	300	(Panchalingam et al., 2012)
	bfpA-R	GGAATCAGACGCAGACTGGT			
STEC	stx1-F	ATAAATCGCCATTCGTTGACTAC	*stx1*	180	(Panchalingam et al., 2012)
	stx1-R	AGAACGCCCACTGAGATCATC			
	stx2-F	GGCACTGTCTGAAACTGCTCC	*stx2*	255	(Panchalingam et al., 2012)
	stx2-R	TCGCCAGTTATCTGACATTCTG			
ETEC	LT-F	CACACGGAGCTCCTCAGTC	*elt*	508	(Panchalingam et al., 2012)
	LT-R	CCCCCAGCCTAGCTTAGTTT			
EAEC	CVD432F	CTGGCGAAAGACTGTATCAT	*aatA*	630	(Panchalingam et al., 2012)
	CVD432R	CAATGTATAGAAATCCGCTGTT			
EIEC	ipaH-F	CTC GGC ACG TTT TAA TAG TCT GG	*ipaH*	933	(Vidal et al., 2005)
	ipaH-R	GTG GAG AGC TGA AGT TTC TCT GC			
Resistance genes					
	Primer	Sequence	Gene	Base pair(bp)	Reference

ESBL	CTX-M F	AGACTGGGTGGCATTGAT	*bla*$_{CTX-M-15}$	676	(Murugan, Malathi, Therese, & Madhavan, 2018)
	CTX-M R	TTAGGTTGAGGCTGGGTGAAGT			
CARBA	NDM-F	GGTTTGGCGATCTGGTTTTC	*bla*$_{NDM-5}$	621	(Poirel, Walsh, Cuvillier, & Nordmann, 2011)
	NDM-R	CGGAATGGCTCATCACGATC			
	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	*bla*$_{KPC}$	232	(Poirel et al., 2011)
	KPC-Rm	CTTGTCATCCTTGTTAGGCG			
	OXA-R	CATCAAGTTCAACCCAACCG	*bla*$_{OXA-48}$	438	(Poirel et al., 2011)
Real time PCR primers and probes for pathogens					
Pathogens	Primers	Sequence (5'-3')	Target genes		Reference
Campylobacter jejuni	cadF-F	CTGCTAAACCATAGAAATAAAATTTCTCAC	*cadF*		(J. Liu et al., 2013)
	cadF-R	CTTTGAAGGTAATTTAGATATGGATAATCG			
	cadF-P	CATTTTGACGATTTTTGGCTTGA			
Shigella spp.	ipaH-F	CCTTTTCCGCGTTCCTTGA	*ipaH*		(J. Liu et al., 2013)
	ipaH-R	CGGAATCCGGAGGTATTGC			
	ipaH-P	CGCCTTTCCGATACCGTCTCTGCA			
Salmonella spp.	invA-F	TCGGGCAATTCGTTATTGG	*invA*		(J. Liu et al., 2013)
	invA-R	GATAAACTGGACCACGGTGACA			
	invA-P	AAGACAACAAAACCCACCGC			

Chapter 6 Knowledge, Attitudes, and Practices of Rural Communities Regarding Antimicrobial Resistance and Climate Change in Adadle District, Somali Region, Ethiopia: A Mixed-Methods Study

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6.1 Abstract

There is an urgent need for interventions in addressing the rapid and disproportionate impact of antimicrobial resistance (AMR) and climate change (CC) on low- and middle-income countries. Within this context, it is important to understand indigenous knowledge in rural communities, which are highly affected. This study examined knowledge, attitude, and practices (KAP) regarding AMR and CC in the Adadle district, Somali region, Ethiopia, utilizing mixed methods, including 362 surveys and 12 focus group discussions among rural communities. Findings showed that 39% and 63% of participants were familiar with AMR and CC, respectively. Of those surveyed, 57% attributed AMR to inappropriate antimicrobial use in animals and humans, while CC was often associated with Allah/God. Multivariable analysis indicated that males exhibited superior knowledge and a positive attitude towards AMR and CC. Additionally, individuals aged 26–35 and 36–45 years showed heightened awareness of AMR and CC, respectively. Moreover, participants who were government employees, pastoralists, and business owners showed better knowledge on CC compared to family caretaker. Religious education and households with more than six members were linked to lower AMR knowledge. This study underlines a greater awareness of CC than AMR and highlights gender-based disparities, recommending integrated educational AMR programs targeting different demographics through a One Health lens, actively involving females, and incorporating local beliefs and practices.

6.2 Introduction

Globally, antimicrobial resistance (AMR) and climate change (CC) pose serious threats to public health systems and economics (Roberta Magnano San Lio et al., 2023). It is projected that 10 million lives will be lost by 2050 due to AMR, with an additional annual toll of 250,000 lives between 2030 and 2050 due to CC (O'Neill, 19 May 2016; Organization, 2014), and specifically its effects on malnutrition, malaria, diarrheal diseases and heat-stress (Organization, 2014). AMR and CC share the commonality of having unpredictable consequences and necessitating urgent measures for control and mitigation (Harring & Krockow, 2021). Furthermore, there is a profound interconnection between them, with CC exacerbating and amplifying the issues associated with AMR (Kaba, Kuhlmann, & Scheithauer, 2020).

Climate change, driven by greenhouse gas emissions from fossil fuels and human activity, directly and indirectly impacts human health (Lelieveld et al., 2019). Higher temperatures lead to heat-related mortality and create conditions favorable for the spread of microbes, including those with resistance genes, contributing to the rise of AMR (Kaba et al., 2020; MacFadden et al., 2018). CC projections anticipate an increase in both floods and droughts, which have

indirect implications for human health (Patz & Olson, 2006; Zinsstag et al., 2018). In fact, population displacement due to these climatic shifts elevates the potential for zoonotic disease transmission as individuals come into closer contact with animals (Zinsstag et al., 2018). Furthermore, water scarcity from migration may result in shared water sources, leading to inadequate sanitation and hygiene practices, consequently heightening the risk of waterborne diseases like diarrhea (G. Cissé, 2019). These multifaceted challenges, combined with food shortages during migration, can contribute to malnutrition (Lake et al., 2012; Springmann et al., 2016). As infectious diseases become more prevalent under such circumstances, it leads to the heightened utilization of antibiotics, a well-established precursor of AMR (Roberta Magnano San Lio et al., 2023). Therefore, an increase in infectious diseases, living in crowded conditions, or experiencing malnutrition, all consequences of CC, increase the risk of acquiring antimicrobial resistant pathogens (Roberta Magnano San Lio et al., 2023). Recent studies in Europe have also confirmed the association between CC, specifically increased temperature, and AMR (Kaba et al., 2020; Roberta Magnano San Lio et al., 2023).

Ethiopia has experienced recurring droughts and floods in recent years, leading to tragic loss of human lives and livestock (Wassie, 2020). These environmental changes have also triggered outbreaks of acute watery diarrhea and malnutrition, especially in the Somali region (Simane et al., 2016). These challenges not only contribute to increased antibiotic misuse but also elevate the risk of acquiring AMR pathogens. Several studies in Ethiopia have indicated that rural communities often possess limited knowledge about AMR (Simegn & Moges, 2022; Tesfaye, 2017). Other studies have shown that rural communities have a better understanding of CC, which can be attributed to the direct impact it has on their livelihoods (Abrham & Mekuyie, 2022; Z. Y. Amare, Ayoade, Adelekan, & Zeleke, 2017; Belay et al., 2022). Remarkably, the knowledge and perspectives of these communities regarding CC may sometimes differ from the scientific consensus.

Given the rapid and disproportionate impact of AMR and CC on low- and middle-income countries (LMICs), such as Ethiopia, there is a critical necessity for intervention. One of the key elements in controlling AMR and adapting to CC is the indigenous knowledge held by rural populations, which plays a pivotal role in determining their adaptive capacity and resilience. Understanding how these communities perceive, engage with, and respond to these challenges is instrumental in crafting effective interventions and policies. This, in turn, paves the way for comprehensive strategies to mitigate the adverse impacts of AMR and CC.

To achieve this goal, we employed a mixed-method approach to simultaneously assess the knowledge, attitude, and practice (KAP) on AMR and CC among rural communities in the Somali region of Ethiopia.

6.3 Results

Quantitative Results

The characteristics of the participants in the survey are summarized in Table 1. The settlements were evenly represented with roughly half of the participants being pastoralist (50.6%) and half agro-pastoralist (49.4%).

Table 4. Demographic characteristic of participants in Adadle district, Somali region, Ethiopia.

Variables	N = 362 (%)
Settlement	
Pastoralist	183 (50.6%)
Agro-pastoralist	179 (49.4%)
Sex	
Female	257 (71.0%)
Male	105 (29.0%)
Age group (in years)	
<30	141 (39.0%)
31–40	112 (30.9%)
>40	109 (30.1%)
Marital status	
Single	25 (6.9%)
Married	327 (90.3%)
Divorced	3 (0.8%)
Widowed	7 (1.9%)
Educational status	
Illiterate	235 (64.9%)
Primary school	58 (16.0%)
Religious learning	63 (17.4%)
College and above	6 (1.7%)
Occupation	
Housewife	212 (58.6%)
Government employee	19 (5.2%)
Pastoralist	56 (15.5%)
Farmer	23 (6.4%)
Business	52 (14.4%)
Numbers of person per household	

<5	129 (35.6%)
6–8	121 (33.4%)
>8	112 (30.9%)
How long they have lived in the area	
<10 years	153 (42.3%)
>10 years	209 (57.7%)

Knowledge and Attitude on Antimicrobials and AMR

Most of the study population (95.9%) correctly agreed with the definition of antibiotics. However, less than half of the study participants (10-42%) accurately stated the indications for antibiotic use in common illnesses. Most of them reported that antibiotics can be used to treat watery diarrhea (89.5%), fever (71%), common cold (68.2%), and viral infections (57.7%). Moreover, most of the participants perceived that using antibiotics as injection (85.9%), costly antibiotics (92.5%) or using multiple antibiotics (85.1%) are the most effective ways to treat infections.

Among the participants, 39% had heard of AMR, with the primary sources of information being health professionals (75.8%) and radio (63.8%). Regarding AMR transmission, it was observed that more than half of the participants reported that AMR could not be transmitted between humans (74.3%), between animals and humans (54.7%), and between the environment and humans (63.3%). Notably, only 4% had heard of the antimicrobial stewardship program. The summarized results are available in table S1.

In terms of AMR attitudes, less than half of the participants agreed that AMR is a problem locally and globally (Figure 1). Nearly 57% of the participants agreed that self-medication practices, non-adherence, and over-the-counter antimicrobial sales contribute to AMR. Additionally, 36.7% associated AMR with high antimicrobial usage in society, and 38.4% implicated animals. The measures agreed upon by the majority to reduce AMR include adherence (86.1%), proper handwashing (67.9%), and government restrictions on antimicrobial sales (61.4%). The results are summarized in Figure 15.

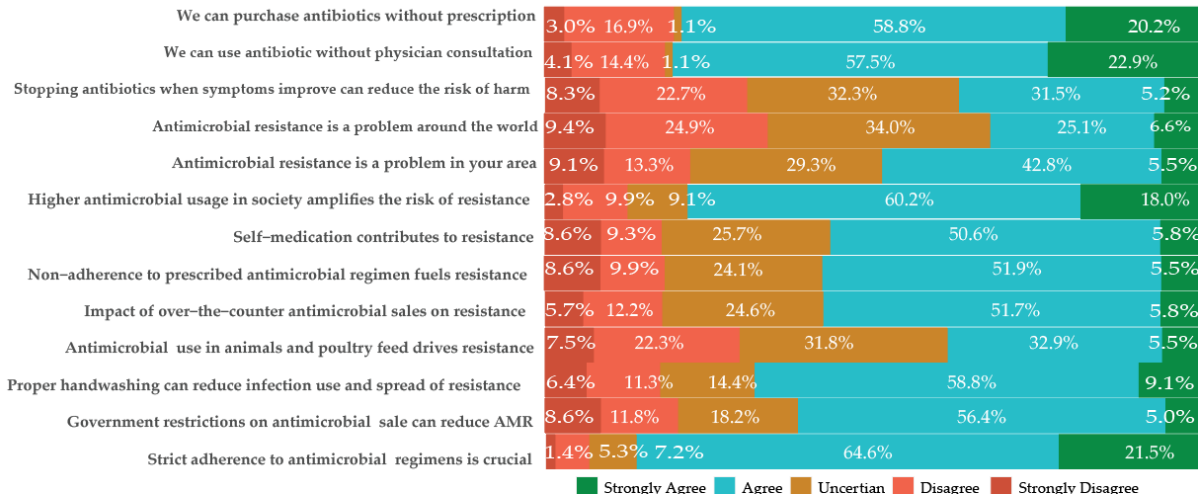

Figure 15. Attitude on antimicrobial use and resistance (AMR) in Adadle district, Somali region, Ethiopia.

Practice on Antimicrobial Use

Among the study participants, 52.4% had used antibiotics in the past month, 17.1% in the past six months, 10.5% in the past year, and 6.3% more than a year before this study commenced. The most common diseases for which antibiotics were reported to have been used included upper-respiratory infection (75.5%), urinary tract infection (UTI, 72.7%), diarrhea (71.5%), malaria (66.6%), common cold (52.5%), and acute febrile illness (47.5%), while tuberculosis (TB, 7.2%) was the least frequently associated with reported antibiotic use. Amoxicillin (79%), ampicillin (36.5%), tetracycline (22.9%), cloxacillin (21.2%), metronidazole (19%), cotrimoxazole (11.6%), and ciprofloxacin, azithromycin, and amoxicillin with clavulanic acid (8%) constituted the antimicrobials most frequently identified by the participants as their commonly used antibiotics, as determined by their appearance (summarized in Figure S1). These antibiotics were mostly purchased from the drug store (69.3%).

Most participants (67.4%) did not have a prescription from an authorized healthcare professional when purchasing antibiotics. The main reasons for self-medication practice included limited access to healthcare services and medication supply (65.7%), purchasing the same antibiotic when their symptoms closely resembled those of a previous illness (53%), the presence of mild symptoms (40.3%), willingness to share antibiotics with family members who exhibited similar symptoms (31%), geographical distance to healthcare facilities (26.2%), costs of antibiotics (13.3%), and limited time (10.5%) (Figure 16A). Moreover, most of the study population (62.7%) reported discontinuing medication once their symptoms had resolved.

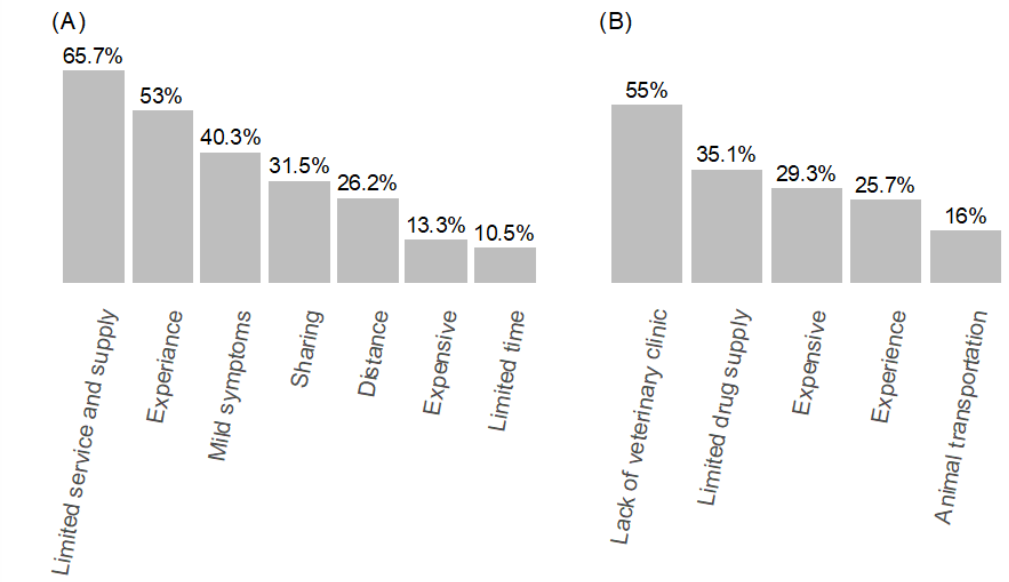

Figure 16. Participants' reported reasons for not seeking health professionals (**A**) and veterinary professionals (**B**) for their livestock.

A total of 306 participants (85%) possessed animals. The most frequently reported diseases among these animals were pasteurellosis (47.8%), diarrhea (45.6%), sheep and goat pox (43.1%), tick intoxication (22.1%), pneumonia (15.2%), anthrax (13.3%), black leg (9.1%), trypanosomiasis (8.3%), rabies (4.7%), and mastitis (3.9%). The three most used medications for these animal diseases, as cited by the participants, were oxytetracycline (83.1%), albendazole (60.8%), and procaine-streptomycin (32.6%) (summarized in figure S1). Notably, most of the study participants (87.7%) purchased these medications without consulting veterinarians or community animal health workers (CAWS). The main reason (55%) for not visiting veterinary clinics was absence of veterinary services in their village. Participants cited several other reasons for not seeking professional veterinary care, including resource constraints (35.1%), medication affordability (29.9%), their own experience in treating and selecting medications for their livestock (25.7%), and animal transportation (16%). Results are summarized in Figure 16B.

Knowledge and Attitude on Climate Change

In the community, most of the participants (63.5%) had heard of CC. Most participants (68.5%) attributed climate change to God's will, while half of the community linked it to deforestation. Conversely, frequent droughts were the most frequently reported CC challenges (88.1%), followed by deforestation (28.7%), excessive temperatures (23.5%), changes in rainfall patterns (23.3%), and erosion (22.4%). Flooding was the least frequently reported challenge, with only 8% of participants mentioning it (Figure 17).

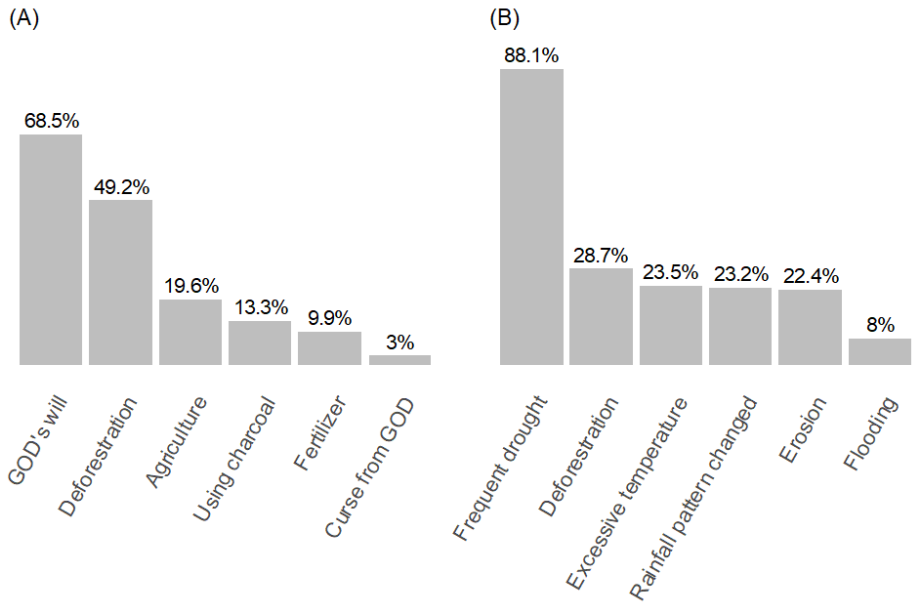

Figure 17. Participants' reported causes of climate change (CC) (**A**) and emerging challenges (**B**) in the past decade

Among the study population, 72.4% perceived an increase in disease trends over the past five to ten years, while only 12% reported no change. The diseases reported during this period included upper respiratory infections (84.8%), diarrhea (80.1%), UTIs (76.8%), malaria (71.3%), pneumonia (67.1%), and malnutrition (53%). Less than half of the participants reported dermatological problems (22.7%), cardiovascular disease (18%), asthma (11.6%), and TB (6.6%) (Figure S2). In addition to the diseases, nearly all participants (94.5%) revealed that, due to CC, particularly drought, they were compelled to leave their residences in search of water and grazing areas for their livestock. Additionally, the other problems they faced were shortage of food (33.9%), loss of livestock (33.4%), water scarcity (30.4%), difficulty in getting access to clean water (28.4%) and health problems (26.2.%). The summarized results are available in table S2.

Only 120 participants (33.1%) acknowledged the existence of a relationship between CC and AMR, with 21.2% perceiving that CC plays a role in the rise of AMR. Most of the community (71.8%) believed that there is a need for awareness and knowledge regarding the relationship between the two topics. Encouragingly, a substantial proportion (69.6%) of respondents expressed a keen interest in receiving updates and information pertaining to the mitigation strategies concerning both CC and AMR. Notably, a significant majority (77.3%) of these individuals favored obtaining such updates through media channels such as radio and television, while a smaller yet noteworthy portion (15.7%) indicated a preference for social media platforms, as detailed in Table S2.

Knowledge, Attitudes, and Practices Scores of Study Participants in different settings

The mean scores for K, A and P regarding CC among agro-pastoralists were 55, 60, and 20, respectively. In contrast, for AMR, the scores were 50, 56, and 40. When assessing overall KAP scores for climate change among pastoralists, we found the averages to be 51.8, 55, and 23 for K, A, and P, respectively. Comparatively, for AMR, the scores were 47, 55, and 42. Based on the mean score cut-off points, 52 and 40 of the participants demonstrated limited knowledge regarding AMR and CC, respectively. The result is summarized in Table S9.

Mean Knowledge and Attitude Scores of Participants Regarding AMR and CC across Demographics

The mean knowledge score for AMR and CC was higher among males than females (70 vs. 47 for CC and 63 vs. 60 for AMR). In general, older participants had highest knowledge scores. In the case of CC, participants aged 36–45 years, 25–35 years, >46 years and <25 years demonstrated a mean knowledge score of 59.1, 57.1, 50.1, and 41.6, respectively. Conversely, for AMR, the mean knowledge scores differed slightly by age group: 26–35 years (62.4), 36–45 years (61.3), and participants aged >46 and <25 years showed similar means (59). Participants with a college education or higher, those with religious education, illiterate individuals, and those with primary school showed mean scores of 73, 56, 53, and 50, respectively, in CC. In AMR, the highest mean knowledge score was shown by participants with a college and above education level (66), followed by those with religious education (61), primary school (61) and illiterate (59). In terms of marital status, the mean knowledge scores for CC were 57, 54, and 38 for widowed, married, and single individuals, respectively. For AMR, married and widowed participants showed similar mean scores (61 and 61), while singles scored the lowest mean score (58). Families composed of five or fewer members exhibited a better mean knowledge score in both CC (56) and AMR (62) compared to families with more than six members, where both groups showed similar mean scores (60). Furthermore, mean knowledge scores for both CC and AMR were consistent regardless of whether the family had lived in the area for less or more than ten years.

Regarding attitude, males, exhibited an elevated mean attitude score in CC and a slightly higher mean attitude score in AMR compared to females (66 vs. 54 of CC and 57 vs. 55 of AMR). Participants aged 35–46 and >46 years showed a higher mean attitude score, followed by those aged 26–35 years, while those <25 years registered the lowest mean score in CC. On the other hand, in AMR, almost all age groups registered similar means, between 55 and 56 (Table S1). Those with religious teaching and those with an educational level of college and above demonstrated the highest mean attitude scores of 67 and 65, respectively, whereas those with primary school education and illiterate participants showed equal mean attitude

scores in CC. In AMR, only those with religious teaching showed a marginally higher mean score (58), while the rest of the educational levels showed similar mean scores (55). The detailed results are presented in table S3-S4.

Comparing the Mean Knowledge and Attitude Scores of AMR and CC

Figure 18 illustrates the relationship between the mean knowledge scores of AMR and CC. In Figure 18A, the scatter plot depicts a notable positive trend wherein an increase in the mean knowledge score of AMR corresponds with a concurrent increase in the mean knowledge score of CC. Similarly, Figure 18B portrays a similar positive correlation, wherein higher mean attitude scores of AMR align with elevated mean attitude scores of CC.

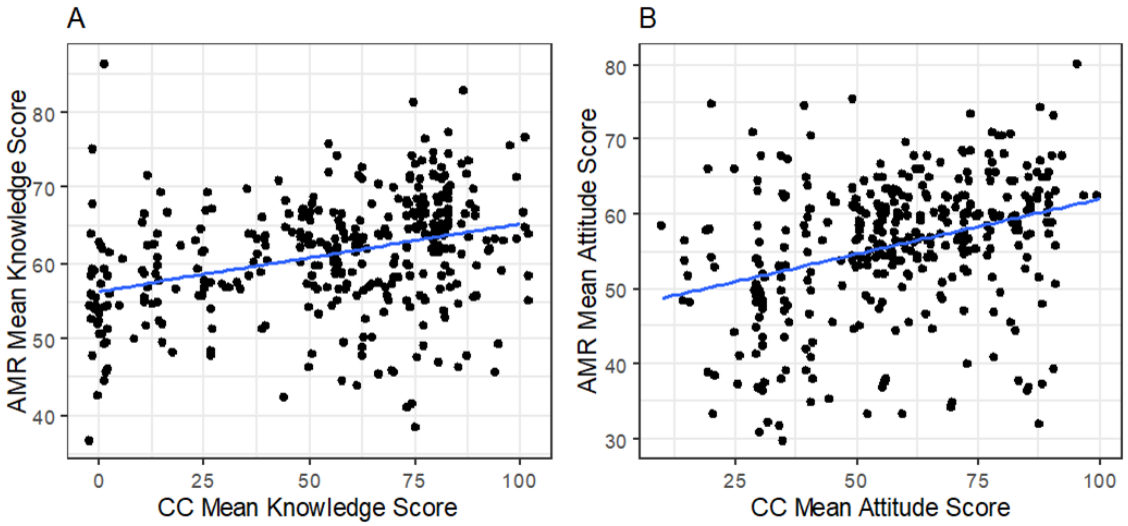

Figure 18. Comparison of participants' AMR mean knowledge score and CC mean knowledge score (**A**), and participants' AMR mean attitude score and CC mean attitude score (**B**) in Adadle district, Somali region, Ethiopia.

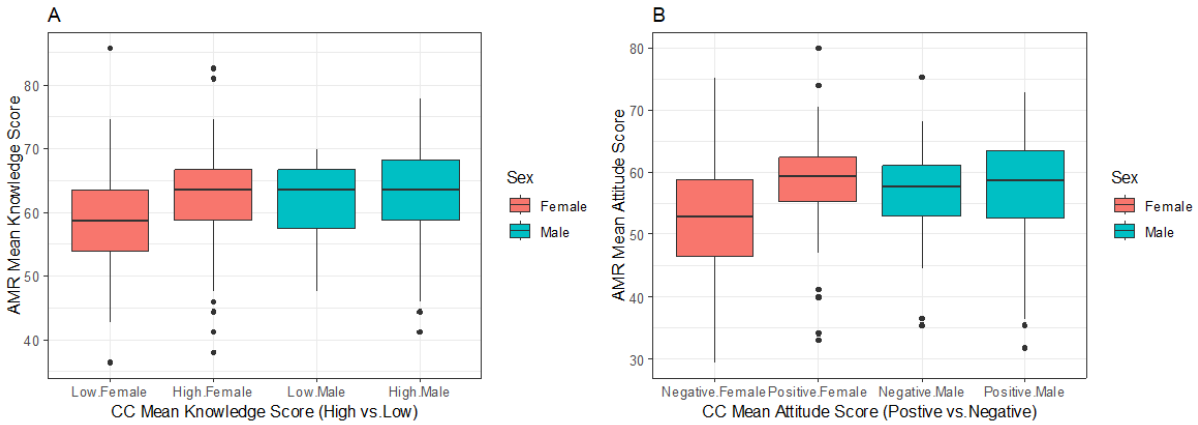

Figure 19. Comparing participants AMR mean knowledge score and high/low CC knowledge (**A**), and participants AMR Mean attitude score and positive/negative CC attitude (**B**) across sex in Adadle district, Somali region, Ethiopia.

In terms of sex, females who demonstrated a strong comprehension of CC showed a notably high median score in the “AMR Mean Knowledge Score.” Conversely, when their understanding of CC was limited, their median score in the “AMR Mean Knowledge Score” was correspondingly low. In contrast, for males, their median “AMR Mean Knowledge Score” remained relatively constant regardless of their level of comprehension of CC (Figure 19A). Furthermore, both females and males displayed high median scores in the “Mean AMR attitude score” when they expressed a positive attitude towards CC. Conversely, when participants exhibited a negative attitude towards CC, the median “AMR Attitude Mean Score” was low for both genders (Figure 19B).

Factors Associated with Knowledge and Attitudes (Multivariable Analysis)

In the multivariable analysis, we identified significant associations between specific demographic factors and knowledge levels and attitudes towards AMR and CC. Notably, being male was associated with better knowledge levels, with odds ratios (OR) of 5.48 (CI: 2.4–12.5) for AMR. Participants aged 26–35 years showed twice the odds of possessing better knowledge on AMR (OR: 2.39; CI: 1.17–4.89). Likewise, individuals in the 36–45 age group demonstrated similar odds of having twice the knowledge on CC (OR: 2.3; CI: 1.04–5.11), compared to those aged 18–25 years old. Moreover, participants whose occupations were government employees, pastoralists, and business owners, respectively, showed better knowledge on CC, with odds of (OR: 6.45; CI: 1.48–28), (OR: 5.18; CI: 2.33–11.5), and (OR: 3.5; CI: 1.5–7.92), compared to housewives.

Additionally, participants with only religious education, compared to those who were illiterate, and those living in households with more than six people, compared to households with fewer than five people, were also associated with lower knowledge levels on AMR (OR: 0.49, CI: 0.25–0.95) and (OR: 0.56, CI: 0.32–0.97).

Regarding attitudes, males had twice the odds of positive attitude towards AMR (OR: 2.64, CI: 1.28–5.47) and three times the odds of positive attitude towards CC (OR: 3.14, CI: 1.44–6.88). Participants with a religious education had positive attitude towards AMR (OR: 1.99, CI: 1.06–3.74) and CC (OR: 4.13, CI: 2.03–8.41). Conversely, participants in pastoralist and business-related occupations showed significant association with negative attitudes towards AMR, with ORs of 0.37 (CI: 0.16–0.87) and 0.33 (CI: 0.15–0.71), respectively. Additionally, being a farmer had a negative association with CC attitudes, with an OR of 0.19 (CI: 0.06–0.62). The summarized results are reported in Tables S5–S8.

Qualitative Results

In the focus group discussion (FGD), most of the Kebeles had an equal number of participants, of which 50 (53.1%) were male and 46 (47.9%) were female. Most participants (77) were illiterate (80.2%). In total, 11 (11.4%) went to college, 6 (6.3%) reached high school, and 2 (2.1%) went to primary school. Participants' characteristics are presented in Table 4.

Table 5. Background characteristics of the community members taking part in the Focus group discussion (FGD) (N = 96) in Adadle district, Somali region, Ethiopia.

Variable	**N = 96 (%)**
Sex	
Female	46 (47.9%)
Male	50 (53.1%)
Age	
18–25	22 (22.9%)
26–35	35 (36.5%)
>36	39 (40.6%)
Level of education	
Illiterate	77 (80.2%)
Primary school	2 (2.1%)
Secondary school	6 (6.3%)
College and above	11 (11.4%)
Occupation	
Farmer	30 (31.2%)
Housewife	22 (22.9%)
Government	15 (15.6%)
Business owner	12 (12.5%)
NGOs	17 (17.8%)
Village of residence	
Bursaredo	17 (17.7%)
Gabal	16 (16.6%)
Malkasalah	16 (16.6%)
Harsug	15 (15.6%)
Dabafayd	16 (16.6%)
Todob	17 (17.7%)

Knowledge on Antimicrobials and Antimicrobial Use

Most participants of the FGD were able to associatiote antimicrobials based on the color of the capsules, particularly red and black, which are commonly used for ampicillin capsules known as “qoormadoobe” in the Somali language. Moreover, some participants demonstrated the ability to identify specific antimicrobials by their names, such as amoxicillin and ampicillin.

> *Aaah’ whenever we go to the health facility or pharmacy, they always say it is an infection and give us Amoxicillin, which I believe is not as effective as Ampicillin (qoormdoobe, or black neck). The two most common antibiotics that we use are amoxicillin and ampicillin (qoormadoobe, or black neck). [CM: Woman: Age: 42 years].*

> *They are drugs that can treat any diseases or alleviate pain, and without them, I believe life would have been very difficult for us. [CM: Man: Age: 54 years].*

Knowledge on AMR

Most participants had a limited knowledge of AMR, only few participants were able to share their ideas and draw from personal experiences with antibiotic use after being prompted and provided with a simple explanation of antimicrobial resistance.

> *Yeah, we often hear that, if we do not take the medication correctly or misuse it, our bodies can adapt to it, which means it may not work in the future. [CM: Man: Age: 52 years]*

Regarding gender, women were reported to be more susceptible to diseases and tend to rely on antimicrobial drugs more than men. They often expressed dissatisfaction with the effectiveness of current medications, attributing it to the lack of diagnostic facilities and a mismatch between the disease and the prescribed treatment. Furthermore, there is a prevailing belief among some women that older medications are more effective than those currently available. In fact, some have gone as far as to claim that “both people and medications from the past were of better quality”.

> *I have heard several times that people complain about antibiotics like amoxicillin not working. I have experienced this myself—I took another antibiotic, and it did not work either. However, I never considered it might be due to resistance; I simply thought maybe the disease and the medicine didn't match. There are many such cases. [CM: Woman: Age: 49 years]*

Most participants had poor knowledge regarding the source and spread of resistant pathogens between humans, animals, and the environment. Some participants knew resistant pathogens

can spread from humans to humans, such as drug-resistant TB, but not to/from animals or the environment.

> *Yes, it can be transmitted, for example, if one person is infected with TB, he/she can transmit it to the other family members who live with them or share food or are in close proximity to them. We used to take them outside the house and keep them away from the family. Because we know that, they can transmit to the other family member. If he gets close to the family member. [CM: Female: Age: 49 years].*

Overall, community participants had never heard about the AMR stewardship program.

Attitude and Practices on Antimicrobial Use

Most participants found it difficult to adhere to the prescribed antibiotic regimen. The most common habit was not finishing the prescribed antimicrobials, either because of a deliberate decision to stop taking the medication after feeling better or forgetting it because of workload. Severe illness was reported as one of the main drivers of adherence.

The participants agreed upon sharing antimicrobials with neighbors or family members, particularly if they have similar symptoms. A small number of community participants had differing opinions on not using leftover antimicrobials. Overall, participants reported the disposal of leftover/unused medicine as part of household waste.

> *Yes, we ask and share medicine within ourselves. For example, if we share the same signs and symptoms with a family member or neighbor, like coughing, I will share the antibiotic with them. Sometimes, I keep it for future use in case somebody gets sick. [CM: Woman: Age: 51 years]*

According to most participants, self-medication is extensively practiced in the region. Most participants stated that it was easy to purchase antibiotics without prescription from pharmacies or drug stores. This is especially true for symptoms such as coughing or mild diarrhea, which are often considered minor illnesses. Most participants stated that they also self-medicated in cases in which the symptoms were the same as those of a prior disease.

Other common characteristics associated with self-medication include insufficient drug supply at the health facility, time, cutting the costs of doctor consultation, education, medical staff behavior, fear of being diagnosed with another disease, and patient behavior.

> *I would like to go to the health facility, but I have to take care of the children, the house, and other family activities. Therefore, it is easier and quicker to get the drugs from the pharmacy instead of going to the hospital, which takes all morning. [CM: Female: Age: 45 years]*

We prefer the health center because the medications there are cheaper and of good quality compared to outside pharmacies. However, insufficient drug supply, inappropriate diagnosis, lack of laboratory services, and a limited number of health professionals often compel us to go to the pharmacy instead. This is because pharmacies may have a better drug supply than the health center. Additionally, the health professionals who work in the health center often own the pharmacy. The services they provide are essentially the same since both lack basic investigation. [CM: Female: Age: 37 years]

Livestock Antimicrobial Use and Practice

The community reported that the most observed livestock diseases were runny nose, diarrhea, pasteurellosis, sheep and goat pox, and pneumonia. It was noted that most of the community used antibiotics such as oxytetracycline and penstrep without seeking advice from community animal health workers.

One of the challenges we face is the limited availability of medication for livestock in our area. Throughout the year, we receive only a small number of vials of tetracycline and penstrep. Tetracycline is the only drug accessible for treating livestock, and we utilize it whenever it is available or when there are remaining doses. In situations involving severe conditions, we consult animal health workers. However, their capacity to provide comprehensive assistance is constrained by the limited availability of animal health services. Regardless of whether we consult with professionals or not, the situation remains unchanged. Consequently, we continue to rely on our experiences to address the health needs of our livestock. [CM: Community animal health worker: Age: 45 years]

Climate Change and Antimicrobial Resistance

The terms “CC” and “weather” were widely misunderstood by respondents. After a brief explanation of CC, most of the community acknowledged it and recounted their experiences with recurring droughts.

The weather is getting worse year after year. Well… let me tell you. In the past eight to ten years, we have been tussling with severe droughts. For ten years now, we used to name the droughts because they occurred one at a time for extended periods. However, in the last eight years, droughts have been happening consecutively. Due to their recurrence, we no longer give them names. [CM: Female: Age: 41 years]

Over the past eight years, we have lost most of our livestock to drought rather than diseases. To save the remaining livestock, most of the community migrated from the

Kebele in search of water and food. Whenever we face severe drought, we usually migrate to find water and food, but during these migrations, we lose some of our livestock due to hunger and diseases. [CM: Male: Age: 54 years]

Well … we do not have the technology or materials to measure the weather, only God knows the change of the weather. [CM: Female: Age: 43 years]

Participants commented on how the disease pattern has changed in their families or communities over the last five years. Most people in the community said that there was a link between droughts and infectious illnesses such as diarrhea, upper respiratory infections, and malnutrition.

6.4 Discussion

AMR and CC are important, current issues that affect the entire world population, and will increasingly do so in the future (Kaba et al., 2020). Multiple aspects of biological, economical, socio-cultural and political nature must be taken into account when studying and addressing them (Roberta Magnano San Lio et al., 2023). Furthermore, their interconnection and the effect the one has on the other must also be considered (Roberta Magnano San Lio et al., 2023). In fact, CC may have an impact on AMR, as drought and flooding—and the living conditions resulting from them—may increase infection rates, leading to an increase in misuse of antimicrobials (Nyoni, Grab, & Archer, 2019), which is the main driver of AMR (Ayukekbong, Ntemgwa, & Atabe, 2017b). Vulnerable, rural populations in the Global South are likely highly impacted by both AMR and CC (Ayukekbong et al., 2017b; Lelieveld et al., 2019), but often lack knowledge or means to understand and address them. Therefore, in this study we assessed the knowledge, attitudes and practices of rural communities in Adadle district, Ethiopia, regarding AMR and CC, with the aim of increasing awareness and laying the foundation to support these communities in controlling AMR and living with CC in the future.

In general, respondents demonstrated a lack of knowledge concerning both AMR (52.5%) and CC (40%). Our findings are in alignment with recent systematic reviews conducted in Ethiopia (Woldegeorgis, Kerbo, Obsa, & Mokonnon, 2023) and communities in Dessie (Simegn & Moges, 2022), which reflect the same trends for AMR awareness. Moreover, other studies conducted in different rural communities in Ethiopia, Kenya, and South Africa similarly reported a commendable level of knowledge regarding CC (Greibe Andersen, Kallestrup, Karekezi, Yonga, & Kraef, 2023; Melore & Nel, 2020). This confirms our predictions of rural communities lacking knowledge about these important issues, and highlights the need for interventions in this regard. Notably, in both qualitative and quantitative analyses, participants

were found to be more knowledgeable on CC than on AMR, suggesting that the direct impact of CC on rural livelihoods may contribute to their heightened sensitivity and awareness to it.

Our results, combined with other results from Ethiopia and other East African countries (Musoke et al., 2021; Ndaki et al., 2022; Simegn & Moges, 2022; Sindato et al., 2020; Tafa, Endale, & Bekele, 2017), link the cause of AMR to three main factors: (1) self-medication practice, (2) over-the-counter sale of antibiotics, and (3) inappropriate use of antibiotics in humans and animals. All of these were found to be highly practiced in the community and livestock in our study, and could be attributed to their precarious socio-economic conditions, limited accessibility to adequate infrastructure and lack of knowledge about correct antimicrobial use.

Indeed, the primary drivers of self-medication practices among humans in our study and other studies encompass limited access to healthcare services and supplies, prior personal experiences, and the severity of the ailment (Amaha, Alemu, & Atomsa, 2019; Ayalew, 2017); while for livestock, the major factors include the absence of veterinary clinics, limited drug availability, cost considerations, and prior experience. Additionally, the financial burden of medical visits play a significant role in promoting self-medication (Z. Zheng et al., 2023). This practice is further facilitated by the accessibility of over-the-counter antibiotics, which are most often sold without a prescription. This sheds light on the challenging implementation of government regulations on antimicrobial use and sale in rural communities.

These observations emphasize the necessity for of developing integrated educational and stewardship programs on antimicrobial use in both humans and animals and of strengthening regulatory measures regarding over-the-counter sale of antimicrobials. Additionally, there is an overall need for improving healthcare infrastructure and accessibility, making services more affordable, and expanding veterinary clinics to ensure proper animal care.

The third main driver of AMR, namely the inappropriate use of antimicrobials, has been previously attributed to stopping treatment after signs and symptoms faded or simply forgetting to take them (Ayukekbong et al., 2017b; Cambaco et al., 2020). Unused antimicrobials might be given to a family member or neighbor exhibiting similar signs and symptoms, stored for future use, or discarded as household waste (Y. Ayele & Mamu, 2018; E. Gebeyehu et al., 2015; Muhie, 2019). A recent review in an East African pastoralist setting reported that misuse of antimicrobials in humans and animals significantly contributes to antimicrobial resistance (AMR) in these regions (Hussein, Abdi, & Ahad, 2023). These findings align with our qualitative and quantitative results. This illustrates how essential counseling by health professionals is when prescribing medicines, particularly antimicrobial drugs, to ensure that patients

understand the importance of taking the medication as directed and the risks of not doing so. This can improve health outcomes and reduce the risk of developing antibiotic resistance.

Regarding CC, most our respondents acknowledged CC, with a particular emphasis on the frequent droughts, deforestation, excessive heat, and a reduced predictability of rainfall. However, these communities often attributed these changes to Allah or God (Artur & Hilhorst, 2012; Bryan, Deressa, Gbetibouo, & Ringler, 2009; S. Gandure, S. Walker, & J. Botha, 2013; Mandleni & Anim, 2011; Shackleton, Ziervogel, Sallu, Gill, & Tschakert, 2015), as most of our study participants also reported. This perspective is deeply entrenched in cultural and religious beliefs, often transmitted across generations. While these beliefs offer solace and explanations for the inexplicable, they can impede the comprehension of CC as a scientific phenomenon driven by human activities, including the combustion of fossil fuels, deforestation, and industrial processes (Rouleau et al., 2022). Addressing this aspect requires a delicate, transdisciplinary approach that respects local beliefs while also introducing scientific knowledge. For instance, the Bidirectional Emic-Etic tool (BEE) has been employed to bridge the gap between traditional beliefs and scientific understanding concerning intercultural differences (Berger-Gonzalez, Stauffacher, Zinsstag, Edwards, & Krütli, 2016). This approach has proven effective in addressing societal challenges related to environmental sustainability and is recommended for addressing issues related to CC (Hadorn, Bradley, Pohl, Rist, & Wiesmann, 2006; Stålne & Pedersen, 2021).

Additionally, deforestation was another reason that our participants attributed to CC, which aligns with studies in Nigeria and Bangladesh (Asekun-Olarinmoye et al., 2014; Kabir et al., 2016). This highlights the pressing need for education and awareness campaigns in rural communities, which heavily rely on forests for their livelihoods and energy. These campaigns should not only emphasize the environmental consequences of deforestation but also promote practical alternatives, like stoves powered by solar energy (H.-Y. Liu, Skandalos, Braslina, Kapsalis, & Karamanis, 2023).

Despite the critical interplay between CC and AMR, our study revealed a stark lack of awareness among the majority of respondents regarding this crucial connection. Less than half of those surveyed acknowledged the correlation between CC and AMR, highlighting a significant gap in understanding within the public domain. Interestingly, despite this lack of awareness, there was widespread recognition among participants of the escalating trend in disease prevalence over the past decade. This trend can be largely attributed to the escalating challenges posed by climate change, including the frequent droughts and flooding (Dorosh & Rashid, 2013; Simane et al., 2016). Among the challenges highlighted by respondents, recurrent droughts emerged as particularly profound, exacerbating issues such as food

insecurity, water scarcity, and the loss of livestock (Dorosh & Rashid, 2013; Simane et al., 2016). The severity of these challenges often forces individuals to flee from their homes in search of more sustainable livelihoods (Cochrane & Singh, 2017). This displacement frequently results in increased proximity between humans and animals, as well as overcrowding at water sources (Zinsstag et al., 2018). Consequently, these conditions create fertile ground for the spread of infectious pathogens within these populations (Seiler & Berendonk, 2012; Singer, Shaw, Rhodes, & Hart, 2016). Consistent with findings from numerous other studies, our research underscores a notable surge in infectious diseases among displaced populations, including respiratory infections, diarrhea, and vector-borne diseases (G. M. Cissé, R.; Adams, H.; Aldunce, P.; Bowen, K.; Campbell-Lendrum, D.; Clayton, S.; Ebi, K.L.; Hess, J.; Huang, C.; , 2022; Greibe Andersen et al., 2023). This surge in infectious diseases is often compounded by the misuse of antibiotics, a significant contributing factor to the development of AMR (Malik & Bhattacharyya, 2019).

When analyzing the impact of the climate crisis on the proliferation of infectious diseases and drug-resistant bacteria, the nexus between AMR and CC becomes indisputably apparent. Addressing this knowledge gap is essential for developing integrated educational programs to mitigate the intertwined challenges of climate change and antimicrobial resistance. Remarkably, rural communities have demonstrated a keen interest in acquiring knowledge and awareness pertaining to the correlation between CC and AMR, as well as strategies for their mitigation though radio/TV and social media. Leveraging innovative communication platforms such as radio, television, and mobile health applications can serve as effective channels for disseminating educational programs tailored to address these pressing issues (Cecchini & Scott, 2003). By integrating information on climate change and antimicrobial resistance into such communication channels, educational initiatives can reach rural communities and facilitate greater understanding and adoption of mitigation measures (Cecchini & Scott, 2003).

In the multivariable analysis, it was determined that males exhibited significantly higher levels of awareness and a more positive attitude toward AMR and CC than females. Similar results were reported in Ethiopia, Tanzania, and Nigeria for AMR (Simegn, Dagnew, Weldegerima, & Dagne, 2022; Simegn & Moges, 2022; Sindato et al., 2020), and in Ethiopia and Bangladesh for CC (Kabir et al., 2016; Maja, Idiris, Terefe, & Fashe, 2023). Conversely, recent reviews have indicated that females tend to be more knowledgeable about AMR (Pham-Duc & Sriparamananthan, 2021). This has been attributed to the fact that females are more often exposed to antimicrobials throughout their lifetimes, which leads to greater awareness about antibiotics and AMR. This discrepancy in findings could be attributed to the cultural distinctions in the study setting, particularly in Ethiopia, where a significant gender gap exists in higher

education enrollment, favoring males (Simegn & Moges, 2022). This enrollment discrepancy may explain the greater knowledge demonstrated by males regarding AMR and climate change compared to females, as previous research has consistently shown a positive association between higher levels of education and a better understanding of AMR and CC (Erku, Mekuria, & Belachew, 2017; Ojomo, Elliott, Amjad, & Bartram, 2015; Tesfaye, 2017; Wibeck, 2014). Additionally, in rural communities, male are often seen as the head of households, facilitating their involvement in meetings, training sessions, media exposure, and information sharing, which is culturally accepted to primarily occur among men (D. T. Gebeyehu, Bekele, Mulate, Gugsa, & Tintagu, 2021). This underscores the importance of an integrated educational program addressing AMR and CC, with a particular focus on involving female participants. WHO and other studies have stressed that achieving gender equity holds practical significance in tackling both AMR and CC (Organization, 2018; Y. Zhang, Huang, Chao, Yang, & Chen, 2021).

Our study, along with other findings, highlights that adults generally possess a better understanding of AMR and CC compared to young adults (Deressa, Hassan, & Ringler, 2011; Rouusounides et al., 2011; Sindato et al., 2020). This could be attributed to the fact that adults' increased vulnerability to both infectious and chronic diseases might contribute to their AMR knowledge, as it could lead to greater exposure to health information from healthcare professionals, thus enhancing their understanding (Alduhaimi et al., 2022; Simegn & Moges, 2022). This finding suggests a potential correlation between age and knowledge proficiency, potentially attributable to accrued life experiences, prolonged exposure to informational resources, as well as increased societal recognition and leadership roles within communities, facilitating easier access to information dissemination channels of both AMR and CC, whether through governmental initiatives or specialized training programs (Wiernik, Ones, & Dilchert, 2013).

In addition, our FGD, participants shared experiences regarding naming drought events over the last ten years. They mentioned ceasing to name them due to the increased frequency of drought occurrences. This observation also highlights how being adults has heightened their awareness of CC. This potential correlation between age, knowledge acquisition, and societal standing underscores the multifaceted nature of the dynamics shaping awareness and comprehension of critical issues such as CC. In contrary, in a study conducted in Singapore, adults exhibited illusory knowledge on CC (X. Yang, Wei, & Su, 2020). This underlines the complex interplay of cultural, social, and contextual factors in shaping attitudes and behaviors related to CC. These findings emphasize the importance of considering local contexts and generational perceptions when planning interventions or communication strategies aimed at addressing these issues.

Participants whose occupations were government employees, pastoralists, and business owners showed better knowledge on CC compared to housewives. This discrepancy can be attributed to the fact that the government employees generally have better access to information and training opportunities related to CC provided by different stakeholders. Additionally, their level of education emerges as a significant predictor of CC awareness (Hess & Collins, 2018). Regarding business owners, the observed difference in knowledge can be elucidated by the nature of their establishments, often serving as hubs for community discussions. These interactions facilitate information accessibility and contribute to a deeper understanding of CC. Additionally, the typically higher-income levels of business owners may contribute to their better understanding of CC, as observed in previous studies linking higher income with better knowledge on CC (Destaw & Fenta, 2021; X. Yang et al., 2020). This finding advocates empowering women in both educational, political, and economic spheres, as they are main components for mitigating climate change (Alston, 2014).

In summary, our study concurrently assessed the KAP pertaining to AMR and CC. Our findings revealed that participants exhibited a greater level of familiarity with CC in comparison to AMR. This discrepancy is attributed to the direct impact of CC on rural livelihoods. However, it is crucial to note that this CC knowledge did not consistently align with established scientific understanding. Furthermore, our analysis indicated a notable gender-based disparity, with males exhibiting a higher level of comprehension in both AMR and CC-related domains. Given the multidimensional nature of these issues and their intricate interplay, we propose implementing community-based educational programs or policy interventions to promote responsible antimicrobial use and environmental conservation. These interventions should target different demographic strata, including farmers and pastoralists, employing a comprehensive transdisciplinary approach. It is imperative to emphasize the active involvement of females in educational initiatives, while being sensitive to and incorporating local beliefs and practices into the pedagogical framework. Furthermore, we recommend that future research adopting a One Health approach involve a broader spectrum of expertise, including health professionals, veterinarians, environmental scientists, and social scientists.

6.5 Materials and Methods

Study Area

This study was conducted in Adadle, a district located within the Shebele zone of the Somali Regional State in Eastern Ethiopia (Figure 20).

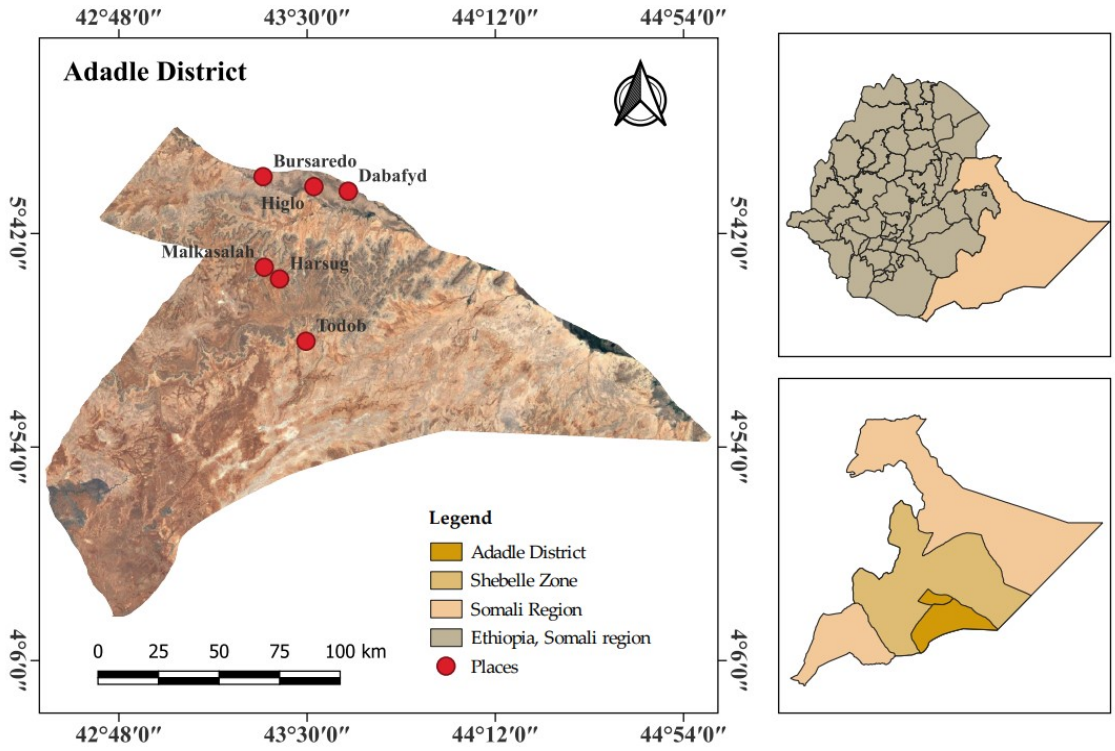

Figure 20. Map of study area, Adadle district, Somali region, Ethiopia

Study Desing

A mixed-methods approach was employed to assess the knowledge, attitude, and practice (KAP) of AMR and CC among pastoralist and agro-pastoralist populations.

Sample Size Calculation

Based on a previous study conducted in Bahardar, Ethiopia, we expected the prevalence of the knowledge level of AMR to be 42% (Tesfaye, 2017) and assumed an intercluster correlation coefficient of 0.15. We calculated a sample size of 360 participants, which would be sufficient to estimate the prevalence of knowledge, with a margin of error of 10% at the 95% confidence level.

Data Collection Instrument

After reviewing the published literature, a semi-structured interview guide and survey questionnaire were developed (Irawati, Alrasheedy, Hassali, & Saleem, 2019; M. S. Rahman, Karamehic-Muratovic, et al., 2021; M. S. Rahman, Overgaard, et al., 2021; Russom et al.,

2021). A pilot study was conducted to ensure the clarity of the questionnaire and amendments were made accordingly. The interview guide and survey questionnaire were translated into the local language (Somali) and administered to the participants, whose responses were translated back to English (Chen & Boore, 2010). Most of the questions were open-ended, with sequential prompts as needed, to enable free discussion. This encouraged the participants to elaborate and share different examples on the topic, aiding in collecting a comprehensive dataset. The interview guide for the survey and focus group discussions comprised five sections: (i) socio-demographic characteristics; (ii) knowledge of antimicrobials and antimicrobial use; (iii) KAP of AMR; (iv) knowledge of antimicrobial use in animals; (v) KAP of CC and its relation to AMR.

Sampling Technique and Data Collection

Out of the 13 kebele (villages) of the Adadle district, six were randomly selected. Community leaders and elders in Adadle district were engaged in the selection of households included in this study. In this survey, we conducted interviews with 362 participants in June, 2023, from three pastoralist communities (Malkasalah, Todab, Harsug) and three agro-pastoralist communities (Bursaredo, Dabafyd, Higlo). For household selection, the kebele leaders provided a list of households in the kebele. Based on this list, we generated a random number using R to select households. In cases where a selected household was not available during data collection, it was immediately substituted with the next household that was not previously selected.

Twelve focus group discussions (FGD) with community members were conducted with 8–9 participants to better understand the community KAP on AMR and CC. The selection of participants considered having an equal representation of the different social strata, such as gender, community health worker, community animal worker, community leaders, and religious leader. The moderators ensured that the participants felt free to express their views and experiences to uncover the degree of consensus or variety on the topic. Each FGD lasted from 60 to 90 min, and audio recordings and notes were taken with the participants' consent. The interviewees continued to interview until the point of saturation (Curry, Nembhard, & Bradley, 2009; Saunders et al., 2018). By the tenth interview, saturation was achieved for the FGD, but two additional interviews were conducted to confirm saturation.

Data Analysis

R statistical software version 4.1.3 was used to perform the statistical analysis. Descriptive analysis was carried out initially for all the variables to gain an overview of the data using the gtsummary package. For the knowledge, attitude, and practice responses, a scoring scheme was employed. For binary responses, a score of "1" was assigned to correct responses, and

“0” to incorrect responses. In the case of questions with multiple responses, we split each response into binary format using the grepl function in R. A score of “1” was assigned for each correct response, and “0” for incorrect responses. Similarly, for Likert scale questions, answers were assigned a range from 1 to 5 based on the selected response. A sum score above the mean was categorized as “good knowledge”, while a sum score below the mean was categorized as “poor knowledge”. Likewise, a sum score greater than the mean assigned a “favorable attitude”, and a sum score less than the mean assigned an “unfavorable attitude”.

Multivariate analysis was performed to determine the association between binary outcome (good vs. poor knowledge and favorable vs. unfavorable attitude) and independent variables using a logistic regression. The logistic regression analysis was conducted using the ‘glmer’ package. Initially, an inclusive model was created, encompassing all variables (age, sex, education, marital status, occupation, number of people per household, and years lived in the area), based on prior literature, while adjusting the cluster. In a stepwise manner, we iteratively improved the model by removing variables that did not significantly contribute ($p > 0.2$) while considering their impact on the overall model fitness. Variables with a *p*-value less than 0.2 were retained in the model. We used the likelihood ratio test, AIC (Akaike Information Criterion), and adjusted R-squared to assess the model’s goodness of fit. Variables with a *p*-value less than 0.05 were deemed statistically significant.

For the qualitative analysis, all interviews were audio-recorded and transcribed verbatim. The thematic analysis was performed by multiple independent analysts (Braun & Clarke, 2006). Before analysis, we familiarized ourselves with the data to have an overview of all collected data. Independent researchers (A. Muhummed and Y. Osman) coded the documents simultaneously using inductive process with Atlas.ti (version 8.4). Each researcher shared and discussed the meaning of the codes. Subsequently, the codes underwent a cross-check for intercoder reliability, calculated using a simple percent agreement method (O’Connor & Joffe, 2020), resulting in a 92% agreement. In instances of notable disagreement, an additional coder with expertise in qualitative analysis was consulted to address and resolve the outstanding discrepancies (A. Kaiser-Grolimund). The generated codes were grouped and converted into themes. The researchers (A. Muhummed, A. Kaiser-Grolimund, and Y. Osman) then reviewed these themes to ensure they accurately reflected the meaning and nuances of the coded data (Castleberry & Nolen, 2018). Following the review, a consensus was reached on naming the themes, along with their corresponding codes and supporting evidence from the dataset (Castleberry & Nolen, 2018).

6.6 Supplementary

Table S1: The knowledge of antimicrobials and antimicrobial resistance in Adadle district, Somali region, Ethiopia. Table S2: The knowledge and attitude of climate change and AMR in Adadle district, Somali region, Ethiopia. Table S3: Predictors of knowledge towards climate change in Adadle district, Somali region, Ethiopia. Table S4: Knowledge and attitude scores of the participants by socio-demographic variables regarding climate change in Adadle district, Somali region, Ethiopia. Table S5: Knowledge and attitude scores of the participants by socio-demographic variables regarding antimicrobial resistance in Adadle district, Somali region, Ethiopia. Table S6: Predictors of attitude towards climate change in Adadle district, Somali region, Ethiopia. Table S7: Predictors of knowledge towards antimicrobial and antimicrobial resistance in Adadle district, Somali region, Ethiopia. Table S8: Predictors of attitude towards antimicrobial and antimicrobial resistance at multivariable level in Adadle district, Somali region, Ethiopia. Table S9: the percentage of householders' knowledge, attitude, and practice (KAP) scores in Adadle district, Somali region, Ethiopia. Figure S1: Participants reported common human (A) and animal diseases (C), as well as antimicrobial medications used by the participants (B) and for their livestock (D) in Adadle district, Somali region, Ethiopia. Figure S2: Participant's reported illnesses that they have experienced over the past five to ten years.

Authors contributions: A.M., G.C., J.Z. and R.T. developed the conception and design. A.M. and Y.O. independently coded the transcribed documents. A.M., Y.O. and A.A. generated categories and themes from the documents. J.H. and A.M. analyzed the quantitative date. A.M. took the lead in writing the first draft of the manuscript, and Y.O., A.A., R.T., P.V., R.T., J.Z. and G.C. contributed to the final text. All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: According Declaration of Helsink, ethical approval was obtained from the Swiss Ethics Committee of Northwest and Central Switzerland (Ethikkommision Nordwest- und Zentralschweiz; REQ-2020-00608), the Review Committee of Armauer Hansen Research Institute in Addis Ababa, Ethiopia (AF-10-015), the Review Committee of the University of Jigjiga in Ethiopia (JJU-RERC030/2020), and National Research Ethics Review Committee (NRERC) (D2/152/533/4). The zone and district administrations were also informed and given a letter from the university regarding the planned survey.

Informed Consent Statement: All the participants were given informed consent written in the local language, and after the respondents agreed, they were asked to sign. Illiterate respondents were asked about their fingerprints. To maintain participants' confidentiality, all data were coded. The codes used for the focus-group participants were "CM".

Data Availability Statement: On reasonable request, the corresponding author will provide the datasets used or analyzed during the current study.

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Conflicts of Interest: All authors declare no competing interests.

Table S1. The knowledge of antimicrobials and antimicrobial resistance in Adadle district, Somali region, Ethiopia.

Variables	N = 362[1]
Antibiotics can treat common cold	
No	115 (31.8%)
Yes	247 (68.2%)
Antibiotics can treat diarrhea	
No	38 (10.5%)
Yes	324 (89.5%)
Antibiotics can kill virus	
No	150 (41.8%)
Yes	209 (58.2%)
Antibiotics should always be used to treat fever	
No	105 (29.0%)
Yes	257 (71.0%)
Combination antibiotics accelerate infection cure	
No	39 (10.8%)
Yes	323 (89.2%)
Injectable antibiotics are more potent than oral ones	
No	51 (14.1%)
Yes	311 (85.9%)
Costly antibiotics are powerful	
No	27 (7.5%)
Yes	335 (92.5%)
Participant has heard about AMR	
No	221 (61.0%)
Yes	141 (39.0%)
Human-to-human AMR transmission	
No	269 (74.3%)
Yes	93 (25.7%)
Human-to-animal AMR transmission is possible	
No	198 (54.7%)
Yes	164 (45.3%)

Human-to-environment AMR transmission is possible	
No	229 (63.3%)
Yes	133 (36.7%)
Participant has heard about stewardship programs	
No	345 (95.3%)
Yes	17 (4.7%)

[1]n (%),

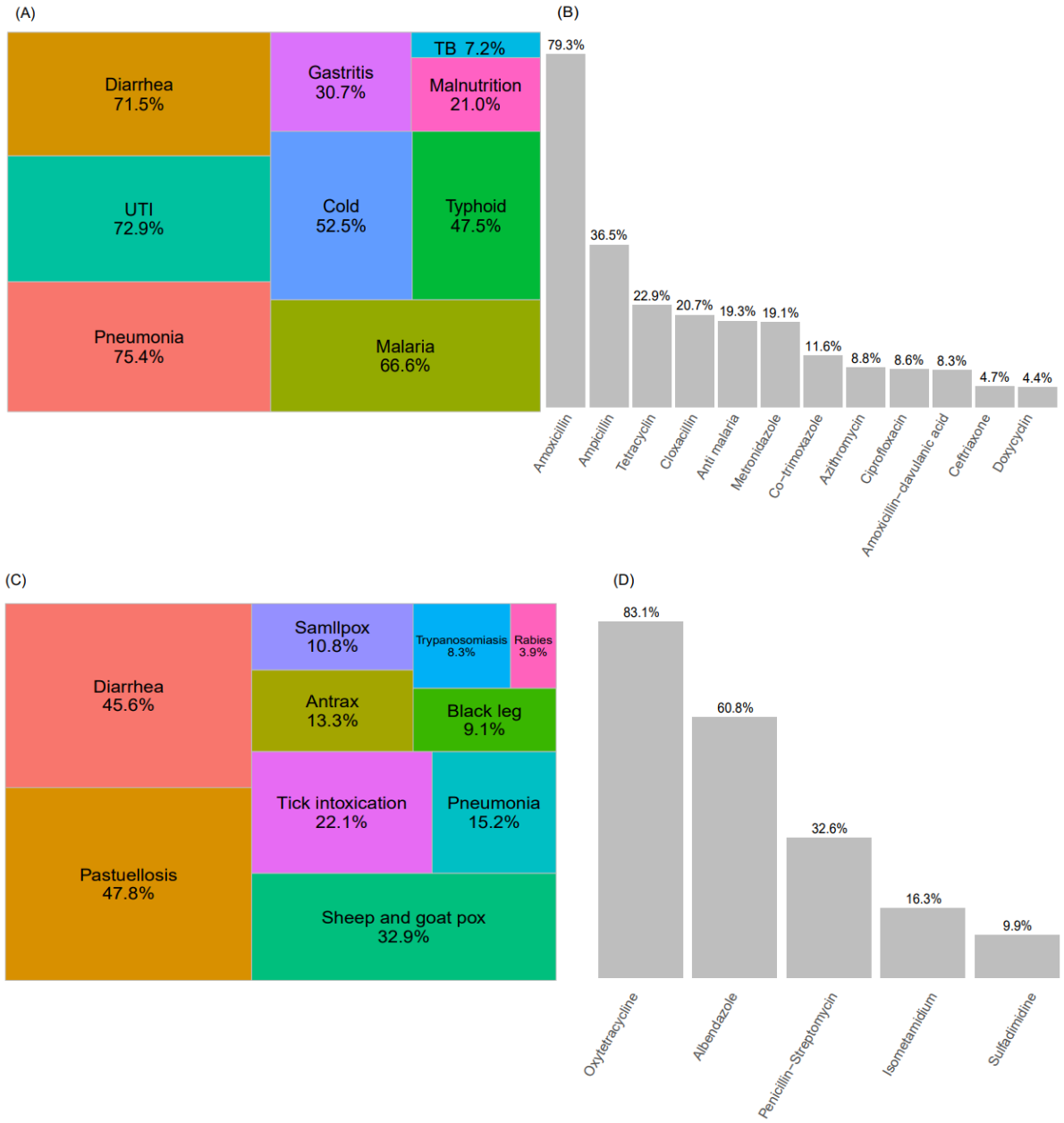

Figure S1. Participants reported common human (A) and animal diseases (C), as well as antimicrobial medications used by the participants (B) and for their livestock (D) in Adadle district, Somali region, Ethiopia.

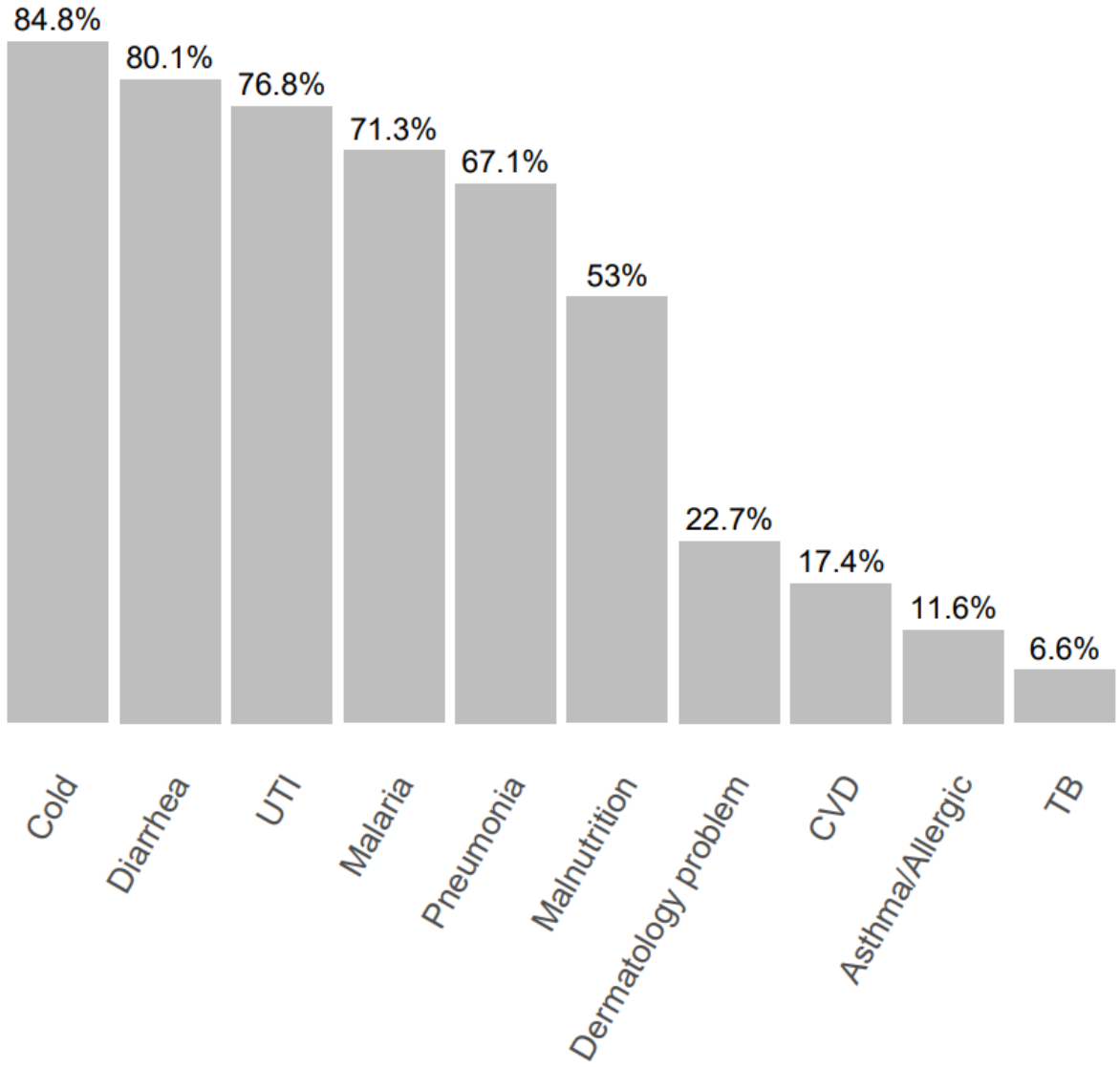

Figure S2. Participant's reported illnesses that they have experienced over the past five to ten years.

Table S2. The knowledge and attitude of climate change and AMR in Adadle district, Somali region, Ethiopia.

Variables	N = 362[1]
Participants has heard climate change	
No	132 (36.5%)
Yes	230 (63.5%)
Challenges faced due to climate change	
Forced to move out from the shelters	342 (94.5%)
Lack of food due to drought	123 (33.9%)
Loss of livestock	121 (33.4%)
Water scarcity	110 (30.4%)
Access to clean water	103 (28.4%)
Health problems	95 (26.2%)

Climate change and AMR	
Relationship between climate change and AMR	
No	242 (66.9%)
Yes	120 (33.1%)
How serious Climate change and AMR at your area?	
Not serious	71 (19.3%)
Moderately serious	131 (36.2%)
Extremely serious	36 (9.9%)
Don’t know	125 (34.5%)
Should the government take responsibility for controlling the spread of AMR due to climate change?	
No	169 (46.7%)
Yes	193 (53.3%)
Need of awareness of climate change and AMR	
No	102 (28.2%)
Yes	260 (71.8%)
Desire to receive updated information on mitigation measures related to climate change and AMR	
No	110 (30.4%)
Yes	252 (69.6%)
Preferred way to receive updated information	
TV/Radio	280 (77.3%)
Social media	57 (15.7%)

[1]n (%),

Table S3. Knowledge and attitude scores of the participants by socio-demographic variables regarding climate change in Adadle district, Somali region, Ethiopia.

Variables	Pastoralist		Agro-pastoralist		Overall	Overall
	Knowledge Mean (SD)	Attitude Mean (SD)	Knowledge Mean (SD)	Attitude Mean (SD)	Knowledge Mean (SD)	Attitude Mean (SD)
Sex						
Female	46.1 (29.3)	51.4 (19.8)	48.7 (31)	57.6 (20.3)	47.3 (30.1)	54.4 (20.2)
Male	67.7 (21)	64.8 (16)	69.9 (21.7)	67.7 (17.3)	68.9 (21.3)	66.3 (16.7)
Age groups (years)						
≤ 25	38.4 (33.6)	47.7 (23.1)	45.7 (33.8)	57.3 (22.5)	41.6 (33.6)	51.9 (23.1)
26-35	53.1 (26.8)	54.3 (17.7)	62.1 (25.8)	60.3 (16.8)	57.2 (26.6)	57.1 (17.5)
36-45	61.9 (25.1)	58.9 (18.1)	56.6 (31.1)	62.1 (19.8)	59.1 (28.3)	60.5 (19)
≥ 46	48.8 (27.9)	58.4 (20.6)	51.1 (30.2)	61.9 (22.5)	50.1 (29.1)	60.4 (21.6)
Educational level						
Illiterate	49.8 (28.7)	51.8 (18.5)	56.7 (30.3)	59.4 (19.4)	53.3 (29.7)	55.7 (19.4)
Primary school	48.2 (32.6)	52.7 (22)	52.5 (33)	59.6 (22.4)	49.9 (32.5)	55.4 (22.2)
Religious education	61.8 (22.8)	67.9 (15)	51.7 (27.3)	66.8 (18.8)	56.1 (25.7)	67.3 (17.2)
College and above	70 (20.9)	72.5 (19.6)	87.5 (NA)	30 (NA)	72.9 (20)	65.4 (24.7)
Marital status						
Single	39.2 (39.4)	49.5 (29.1)	37.5 (37.5)	54.6 (22.6)	38.3 (37.5)	52.2 (25.3)
Married	52.5 (28.4)	54.7 (18.9)	57.4 (28.2)	61.5 (19.4)	54.9 (28.3)	58.1 (19.4)
Widowed	53.1 (13)	74.4 (12.3)	62.5 (54.5)	65.0 (30.4)	57.1 (33.2)	70.3 (20.2)
How long they have lived in the area						
< 10 years	52.9 (28.6)	53.3 (17.1)	53.5 (29.5)	59.8 (20.7)	53.2 (28.9)	56.5 (19.2)
> 10 years	51 (29.3)	56.2 (21.4)	56.7 (30.5)	61.4 (19.4)	53.8 (29.9)	58.8 (20.5)
Numbers of person per household						
≤ 5 person	53.2 (27.0)	51.9 (14.5)	61.5 (30.2)	57.3 (15.7)	56.7 (28.5)	54.1 (15.2)
6-8 person	50.9 (29.8)	55.8 (20.6)	52.6 (28)	59.9 (20.2)	51.7 (28.7)	57.9 (20.3)
> 8 person	51.0 (31.1)	58.7 (24.5)	52.8 (31.6)	64.6 (22.5)	52 (31.2)	61.9 (23.5)

Table S4. Knowledge and attitude scores of the participants by socio-demographic variables regarding antimicrobial resistance in Adadle district, Somali region, Ethiopia

Variables	Pastoralist		Agro-pastoralist		Overall	Overall
	Knowledge Mean (SD)	Attitude Mean (SD)	Knowledge Mean (SD)	Attitude Mean (SD)	Knowledge Mean (SD)	Attitude Mean (SD)
Sex						
Female	59.2 (8.1)	54.9 (10.3)	61.5 (6.9)	55.7 (7.5)	60.3 (7.6)	55.2 (9.1)
Male	61 (7.5)	56.3 (9.2)	64.1 (7.5)	57.4 (9.3)	63 (7.6)	56.9 (9.2)
Age groups (years)						
≤ 25	57.7 (10.8)	54.6 (9.7)	61.5 (7)	56.5 (7.4)	59.4 (9.4)	55.5 (8.8)
26-35	60.5 (7.4)	55.3 (10.2)	64.5 (5.9)	57.6 (6)	62.4 (7)	56.3 (8.6)
36-45	61.1 (6.1)	56 (10.2)	61.5 (6.8)	55.9 (7.7)	61.3 (6.4)	55.9 (8.7)
≥ 46	58.4 (7.9)	55 (10.5)	60.9 (8.6)	54.5 (11.1)	59.8 (8.3)	54.7 (10.7)
Educational level						
Illiterate	59.8 (7.7)	54.5 (9.7)	62.7 (7.6)	55.9 (8)	59.4 (7.8)	55.2 (8.9)
Primary school	58.3 (8.5)	55.6 (11.1)	61.5 (7.2)	54.9 (8.3)	61.3 (8.1)	55.3 (9.1)
Religious education	60.1 (8.2)	58.4 (9.6)	61.8 (5.3)	57.9 (8.3)	61.1 (6.7)	58.1 (8.8)
College and above	67 (6.4)	55.7 (8.5)	61.9 (NA)	54.1 (NA)	66.1 (6.2)	55.4 (7.6)
Marital status						
Single	55.4 (11.2)	55.4 (9.4)	61.2 (6.4)	55.1 (7.6)	58.7 (9.1)	55.2 (8.3)
Married	60.1 (7.8)	55.3 (10.1)	62.5 (7.1)	56.4 (7.9)	61.3 (7.6)	55.8 (9.1)
Widowed	58.3 (3.2)	58.5 (7.3)	65.1 (7.2)	52.4 (17.1)	61.2 (5.9)	55.9 (11.6)
How long they have lived in the area						
< 10 years	60.1 (8.5)	54.3 (9.9)	61.8 (6.7)	55.5 (7.6)	60.9 (7.7)	54.8 (8.9)
> 10 years	60 (7.6)	56.1 (10)	62.7 (7.4)	56.7 (8.5)	61.1 (7.6)	56.4 (9.2)
Numbers of person per household						
≤ 5 person	60.5 (7.4)	55.5 (8.2)	65.2 (6.1)	57.7 (6.9)	62.4 (7.2)	56.4 (7.8)
6-8 person	59.3 (8.7)	54.3 (11.5)	61.5 (7.4)	56.1 (8.2)	60.4 (8.1)	55.2 (9.9)
> 8 person	59.4 (8.1)	56.2 (10.5)	60.7 (7.2)	55 (8.9)	60.1 (7.6)	55.5 (9.7)

Table S5. Predictors of knowledge towards climate change in Adadle district, Somali region, Ethiopia.

Variables	Event Rate	OR[1]	95% CI[1]	*p*-value
Age group				
< 25	30 / 63 (48%)	—	—	
26-35	75 / 124 (60%)	1.51	0.72, 3.19	0.3
36-45	71 / 102 (70%)	2.30	1.04, 5.11	0.040
> 46	41 / 73 (56%)	0.99	0.42, 2.35	>0.9
Educational status				
Illiterate	137 / 235 (58%)	—	—	
Primary school	34 / 58 (59%)	1.46	0.68, 3.14	0.3
Religious learning	42 / 63 (67%)	1.54	0.80, 2.96	0.2
College and above	4 / 6 (67%)	0.84	0.12, 6.05	0.9
Marital status				
Single	11 / 25 (44%)	—	—	
Married	201 / 327 (61%)	5.92	1.66, 21.1	0.006
Divorced	1 / 3 (33%)	1.03	0.04, 30.1	>0.9
Widowed	4 / 7 (57%)	3.45	0.44, 27.2	0.2
Occupation				
Housewife	113 / 212 (53%)	—	—	
Government employee	12 / 19 (63%)	6.45	1.48, 28.0	0.013
Pastoralist	44 / 56 (79%)	5.18	2.33, 11.5	<0.001
farmer	12 / 23 (52%)	0.73	0.28, 1.92	0.5
Business	36 / 52 (69%)	3.45	1.50, 7.92	0.003

[1]OR = Odds Ratio, CI = Confidence Interval

Table S6. Predictors of attitude towards climate change in Adadle district, Somali region, Ethiopia.

Variables	Event Rate	OR[1]	95% CI[1]	*p*-value
Sex				
Female	122 / 257 (47%)	—	—	
Male	72 / 105 (69%)	3.14	1.44, 6.88	0.004
Age				
< 25	27 / 63 (43%)	—	—	
26-35	64 / 124 (52%)	1.54	0.75, 3.16	0.2
36-45	60 / 102 (59%)	1.76	0.80, 3.85	0.2
> 46	43 / 73 (59%)	1.59	0.68, 3.70	0.3
Occupation				
Housewife	107 / 212 (50%)	—	—	
Government employee	10 / 19 (53%)	0.85	0.24, 2.99	0.8
Pastoralist	33 / 56 (59%)	0.62	0.25, 1.53	0.3
Farmer	11 / 23 (48%)	0.19	0.06, 0.62	0.006
Business owner	33 / 52 (63%)	0.81	0.36, 1.84	0.6
Educational status				
Illiterate	111 / 235 (47%)	—	—	
Primary school	30 / 58 (52%)	1.55	0.76, 3.17	0.2
Religious learning	50 / 63 (79%)	4.13	2.03, 8.41	<0.001
College and above	3 / 6 (50%)	1.21	0.19, 7.75	0.8
How long they have lived in the area				
< 10 years	73 / 153 (48%)	—	—	
> 10 years	121 / 209 (58%)	1.46	0.90, 2.38	0.13

[1]OR = Odds Ratio, CI = Confidence Interval

Table S7. Predictors of knowledge towards antimicrobial and antimicrobial resistance in Adadle district, Somali region, Ethiopia.

Variables	Event Rate	OR[1]	95% CI[1]	*p*-value
Sex				
Female	106 / 257 (41%)	—	—	
Male	66 / 105 (63%)	5.48	2.40, 12.5	<0.001
Educational status				
Illiterate	122 / 235 (52%)	—	—	
Primary school	22 / 58 (38%)	0.79	0.38, 1.64	0.5
Religious learning	23 / 63 (37%)	0.49	0.25, 0.95	0.035
College and above	5 / 6 (83%)	14.0	0.87, 225	0.062
Age				
< 25	19 / 63 (30%)	—	—	
26-35	71 / 124 (57%)	2.39	1.17, 4.89	0.017
36-45	52 / 102 (51%)	1.98	0.91, 4.31	0.086
> 46	30 / 73 (41%)	1.49	0.63, 3.50	0.4
Occupation				
Housewife	96 / 212 (45%)	—	—	
Government employee	6 / 19 (32%)	0.25	0.05, 1.19	0.081
Pastoralist	32 / 56 (57%)	0.40	0.16, 1.01	0.052
Farmer	8 / 23 (35%)	0.23	0.07, 0.73	0.013
Business owner	30 / 52 (58%)	1.07	0.48, 2.39	0.9
Numbers of person per household				
< 5	75 / 129 (58%)	—	—	
6–8	53 / 121 (44%)	0.56	0.32, 0.97	0.038
> 8	44 / 112 (39%)	0.51	0.28, 0.95	0.035

[1]OR = Odds Ratio, CI = Confidence Interval

Table S8. Predictors of attitude towards antimicrobial and antimicrobial resistance at multivariable level in Adadle district, Somali region, Ethiopia.

Variables	Event Rate	OR[1]	95% CI[1]	*p*-value
Sex				
Female	139 / 257 (54%)	—	—	
Male	65 / 105 (62%)	2.64	1.28, 5.47	0.009
Educational status				
Illiterate	125 / 235 (53%)	—	—	
Primary school	32 / 58 (55%)	1.17	0.59, 2.34	0.6
Religious learning	44 / 63 (70%)	1.99	1.06, 3.74	0.033
College and above	3 / 6 (50%)	1.17	0.20, 6.74	0.9
Age				
< 25	34 / 63 (54%)	—	—	
26-35	74 / 124 (60%)	1.10	0.56, 2.16	0.8
36–45	57 / 102 (56%)	0.90	0.44, 1.84	0.8
> 46	39 / 73 (53%)	0.86	0.39, 1.87	0.7
Occupation				
Housewife	126 / 212 (59%)	—	—	
Government employee	10 / 19 (53%)	0.48	0.14, 1.58	0.2
Pastoralist	30 / 56 (54%)	0.37	0.16, 0.87	0.022
Farmer	14 / 23 (61%)	0.48	0.16, 1.43	0.2
Business owner	24 / 52 (46%)	0.33	0.15, 0.71	0.005

[1]OR = Odds Ratio, CI = Confidence Interval

Table S9. The percentage of householders' knowledge, attitude, and practice (KAP) scores in Adadle district, Somali region, Ethiopia.

	Agro pastoralist (N=179)	**Pastoralist (N=183)**	***p*-value**
Knowledge about Climate Change			
Mean (SD)	55.3 (30.1)	51.9 (28.9)	0.265
Median [Min, Max]	62.5 [0, 100]	56.3 [0, 100]	
Attitude about Climate Change			
Mean (SD)	60.7 (20.0)	55.0 (19.7)	0.00623
Median [Min, Max]	60.0 [20.0, 90.0]	55.0 [10.0, 100]	
Climate Change practice			
Mean (SD)	20.4 (24.1)	23.6 (25.2)	0.218
Median [Min, Max]	16.7 [0, 100]	33.3 [0, 100]	
Knowledge about AMR			
Mean (SD)	50.1 (20.5)	47.2 (15.2)	0.131
Median [Min, Max]	53.3 [6.67, 93.3]	46.7 [6.67, 100]	
Attitude about AMR			
Mean (SD)	56.2 (8.13)	55.3 (10.0)	0.354
Median [Min, Max]	57.6 [30.6, 75.3]	56.5 [29.4, 80.0]	
Antibiotic use and AMR practice			
Mean (SD)	40.1 (16.7)	42.8 (14.1)	0.103
Median [Min, Max]	42.9 [0, 71.4]	42.9 [14.3, 75.0]	

Chapter 7 Discussion

Summary of findings

The evidence outlined in this thesis highlights the concerning spread of antimicrobial resistance, particularly focused on the extended-spectrum β-lactamase (ESBL) and carbapenemase genes among children and livestock, alongside the prevailing low knowledge of AMR and climate change in the Somali region of Ethiopia. Prior studies to this thesis have underscored the alarming spread of AMR genes in humans, animals and the environment (Katale et al., 2020) indicating the potential exchange of clones or flow of resistance genes across the sectors via mobile genetic elements. Despite the insights given in this review, none of the studies involved truly implemented a One Health approach using genetic characterization of AMR. In Ethiopia, a few studies have attempted a One Health approach based on phenotypic methods only, and they reported a high prevalence of resistance to third-generation cephalosporin. However, evidence-based research on genotyping methods is scarce hindering the understanding of the role resistance genes and their exchange between different sectors play in Ethiopia calling for a concerted effort and additional research to curb the issues faced in this area of research.

To annul these inequities, the goal of the research presented here was to examine the molecular studies on AMR using a One Health approach in Africa, assess the genetic profile of AMR genes in children and livestock, study diarrheagenic pathogens and their resistance genes, and assess the communities' knowledge regarding AMR and climate change.

The first section of the thesis (systematic review) found very limited molecular studies on AMR using a One Health approach in Africa. Specifically, we found very few studies that connected AMR in humans, animals, water and the environment in a spatio-temporal way, allowing an assessment of the distribution and spread of drug-resistant bacteria. Therefore, to contribute to the gap in existing knowledge, we performed a genetic characterization of faecal carriage of ESBL-producing *E. coli* in children and livestock in the Somali region of Ethiopia (chapter 4). This study showed a high prevalence of ESBL-producing *E.coli* in children, and a low prevalence in livestock, predominantly harboring $bla_{\text{CTX-M-15}}$. Additionally, whole genome sequencing revealed a high diversity of sequence types (ST), with ST-2353 being among the most prevalent, harbouring multiple resistance genes. Notably, this is the first reported identification of ST-2353 in Ethiopia. Moreover, we found a significant association between ESBL carriage in children, being both wasted and stunted and drinking treated water with chlorine. Possessing chickens also showed a significant relationship with ESBL-producing E. coli, with the odds of colonization being five times higher for chicken owners.

In the subsequent chapter (chapter 5), we examined diarrheagenic pathogens and their resistance genes in children with and without diarrhoea, where we detected ESBL (*bla*$_{CTX-M-15}$) and carbapenemase genes (*bla*$_{NDM-5}$ and *bla*$_{OXA-48}$). Interestingly, *Shigella spp.* harbouring *bla*$_{NDM}$ and *bla*$_{OXA-48}$, which have been rarely described to date, and a DEC hybrid of more than two virulent combinations were revealed in this study.

In chapter 6, we assessed the rural communities' knowledge, attitude and practice regarding AMR and climate change. This chapter revealed the limited knowledge of AMR and climate change in the communities of the rural Somali region. Furthermore, our analysis revealed a significant gender difference, with females showing lower levels of knowledge towards AMR and climate change compared to males. This can be linked to our findings in chapter 4 (Faecal carriage of ESBL-producing *E. coli* in children and livestock in the Somali region of Ethiopia), where we observed that children whose mothers were illiterate had higher odds of carrying ESBL-producing *E.coli.*

AMR in humans, animals and the environment: establishing an Integrated AMR Surveillance System

In LMICs, growing evidence suggests the spread of ESBL- and carbapenemase-producing *Enterobacteriaceae* in humans, animals and the environment. This thesis shed light on the spread of AMR genes, specifically ESBL (*bla*$_{CTX-M-15}$) and carbapenemase (*bla*$_{NDM-5}$ and *bla*$_{OXA-48}$) encoding genes in *E. coli, Salmonella spp.* and *Shigella spp.* isolates from children under five years of age residing in the Somali region of Ethiopia. *bla*$_{CTX-M-15}$ gene was also dominantly found in livestock. Moreover, reports of *Shigella spp.* harbouring *bla*$_{NDM}$ and *bla*$_{OXA-48}$ have been rarely found, where only a few isolated instances have been recorded (Ralte et al., 2022; Walsh et al., 2011), making us the first to report this in Ethiopia. Alarmingly, these strains confer resistance to a wide range of commonly used antimicrobials, including last-resort options for treating infections caused by EBSL-producing *Enterobacteriaceae*.

The high prevalence of ESBL- and carbapenemase-producing *Enterobacteriaceae* in these children is not only due to early antibiotic exposure in the first years of life, but it could also be attributed to mother-to-offspring transmission, ingestion of contaminated water or food, or coming into contact with contaminated objects (in the hospital or daycare centers) or humans. Additionally, interaction with animals, which is common in pastoralist communities, can also increase the transmission of AMR. For instance, this thesis found a significant association between possessing chickens and ESBL-producing *E. coli*. Prior studies in Egypt and Kenya have also highlighted chickens as a primary source of ESBL-producing *E. coli* transmission to humans (Aworh et al., 2020; Badr et al., 2022). This could be explained by the fact that chickens are easily handled by children, or come into contact with dishes or resources used

for food preparation, thereby increasing the chance of interaction and transmission. This is exacerbated in LMICs, where access to clean water, poor sanitation and hygiene are lacking. AMR is therefore an issue of humans, but also animals and the environment.

Several studies have attempted to compare the molecular profile of ESBL-plasmids or clones between humans, animals and the environment (Kluytmans et al., 2013; Leverstein-van Hall et al., 2011). These studies have demonstrated similar genetic backgrounds, with sequence types of CTX-M-producing *E. coli*, such as ST410, ST38 or ST10, found in both human and animal sources (Diab, Hamze, Madec, & Haenni, 2017). Additionally for ST131, the most widely spread multidrug-resistant *E. coli* sequence type in humans, has shown a high degree of similarities observed between humans, companion animals, and poultry based on resistance profiles and genetic characteristics (Platell, Johnson, Cobbold, & Trott, 2011).

Therefore, it is imperative to establish an integrated AMR surveillance system that incorporates whole genome sequencing (WGS) data from isolates simultaneously collected from humans, animals and the environment, providing a holistic understanding of the dynamics of AMR across different sectors. This approach enables early detection and intervention, preventing the spread of resistant strains. The generated data can lead to inform policies and standardized practices, fostering intersectoral coordination through the One Health approach. Moreover, it can contribute to the global and national AMR control and prevention strategy.

AMR and the microbiome: early childhood AMR prevention and surveillance at community level

Despite its crucial role in maintaining individuals healthy, the human microbiome serves as a main reservoir of AMR (Ducarmon et al., 2022). Resistance genes are usually selected for by the use of antimicrobials. Interestingly, the gut microbiome of healthy infants and children, even in the absence of antibiotic exposure, has been previously found to carry *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Streptococcus* spp., *Escherichia coli*, and *Shigella* spp. with resistance genes (Casaburi et al., 2019; Moore et al., 2013; L. Zhang et al., 2011). Additionally, healthy individuals and children residing in remote areas with limited access to antimicrobials, including those in our study, have been shown to harbor the ESBL-producing commensal *E. coli* (Bartoloni et al., 2009; Nji et al., 2021; Woerther et al., 2013). Therefore, additional factors other than use of antimicrobials may impact the development of AMR within the microbiome.

One's microbiome is significantly shaped in the first days of life, mainly influenced by mode of delivery during birth (Dominguez-Bello et al., 2010; Penders et al., 2006), primal environment

(Azad et al., 2013) and food stimuli (De Filippo et al., 2010; Koenig et al., 2011). During this period of maturation and development, bacteria of the human microbiome can acquire drug resistant genes via horizontal gene transfer from resistant microbes coming from the mother or the environment. For instance, pregnant women with or without symptoms were found to harbour ESBL-producing *E. coli* in their feces, urine and vagina (Moradi, Eshrati, Motevalian, Majidpour, & Baradaran, 2021). Evidence also showed the presence of genes resistant to aminoglycoside and β-lactam antibiotics in infants' meconium samples (Fouhy et al., 2014; Gosalbes et al., 2016; W. Li et al., 2021). These findings support the hypothesis of vertical mother-to-child transmission of resistant pathogens.

Furthermore, the environment in which the child is born may also play a role in shaping the microbiome. Indeed, nosocomial pathogens (Hourigan et al., 2018) but also pathogens found in the environment and animals (food-producing or pets) have been proven to impact the development of the microbiome and possibly transfer resistance genes to the infant (Gómez-Gallego et al., 2021; Pal et al., 2016; Tun et al., 2017; Y. J. Zhang et al., 2019). Additionally, not only direct but also indirect exposure of the infant to antimicrobials may further foster the development of AMR. For example, intrapartum antimicrobial prophylaxis (IAP) is a common procedure carried out during delivery that increases exposure to antimicrobials and has been demonstrated to enhance the presence of bacteria harbouring resistance genes in the developing microbiome of the infant (Arboleya, Saturio, & Gueimonde, 2022; Van Dyke et al., 2009).

Due to the intricate interplay between early-life exposure and the long-term health consequences of a resistant microbiota, it is essential to enhance prudent antimicrobial use in maternal and early-life care. This highlights the importance of addressing the spread of AMR outside of health settings, which is especially important for rural communities. Indeed, most of the current surveillance systems focus on clinical settings only, leaving a crucial gap in AMR surveillance and intervention strategies by overlooking the transmission pathway in the community, especially in early life.

Ethiopia's revised AMR strategy encompasses both the human and animal sectors, particularly focusing on urban centers where more advanced laboratories are accessible for surveillance. While this represents a huge step forward for halting AMR, it leaves out the rural communities that could also contribute to the emergence and spread of AMR through the misuse of antimicrobials in humans and animals as well as others factors mentioned above, along with limited sanitation and hygiene practices. Hence, we recommend the inclusion of the integrated surveillance system in rural communities.

An exemplary model illustrating the importance and feasibility of a One Health Surveillance and Responses System in rural communities has been established in Ethiopia. In this system, the Community Animal Health Workers (CAHWs), Community Health Workers (CHWs), and both human and animal health district staff and regional experts are coached. A Community-Based Emergency Fund (CBEF) and CAHWs cost recovery mechanisms are in place, to ensure the active engagement of communities in the surveillance response system. With this approach, unified public and animal health surveillance response system have been achieved, enabling joint interventions for disease outbreaks (Osman et al., 2023). This existing system could be complemented to include AMR surveillance. By providing training and capacity building programs for CHWs and CAHWs on AMR surveillance, judicious antibiotic prescription, and infection prevention and control measures during maternal and early-life care and within the community, the risk of resistance development and spread may be reduced.

AMR at molecular level: complementing the integrated surveillance system with molecular techniques

Next generation sequencing (NGS) has revolutionized the genetic characterization of microbes found in clinical, community and environmental settings. It has become an essential tool in the field of infectious diseases like AMR research and public health. This technique not only allows to detect resistance genes, but can also be used to perform bacteria relatedness analyses, identify bacterial clusters in populations or settings, track the origins of pathogens with a specific genetic background, or make other genomics-related analyses.

In this thesis, we successfully employed whole genome sequencing (WGS) to analyze bacterial isolates from rural children isolates, and reported ST2353 *E. coli* carrying multiple resistance genes, representing the first reported instance of this in Ethiopia. Further, we reported the presence of the bla_{NDM-5} and bla_{OXA-48} genes in *Shigella spp.*. These findings were based on PCR assays, but more in-depth analyses employing WGS, which are currently planned, will shed light on the origins and spread of these resistance genes.

Furthermore, cross-border infections are a primary contributor of AMR transmission and spread globally (Bokhary, Pangesti, Rashid, Abd El Ghany, & Hill-Cawthorne, 2021; C. J. von Wintersdorff et al., 2014). The Somali region is the only region in Ethiopia to border with three other countries: Djibouti, Somalia and Kenya. In this region, cross-border travel is frequent for various reasons, such as business exchange, health reason and visits, and this increases the spread of resistant pathogens across regions and countries.

Therefore, because of the importance of in-depth laboratory analyses in understanding AMR and pathogen relatedness in humans, animals and the environment, and their rapid spread across regions, we recommend complementing the Integrated AMR Surveillance System with

molecular surveillance with NGS. One tool that can be used for WGS is the MinION by Oxford Nanopore Technologies (ONT) sequencer, which represents a cost-effective portable device solution capable of generating long reads and thus allows whole-genome sequencing (Leggett et al., 2020). This technique offers the fast detection of pathogens and their corresponding resistance profiles within hours.

We recommend establishing NGS techniques in local laboratories to support AMR surveillance in the country. This will require strengthening local capacity on laboratory and bioinformatics skills, which will contribute to sustainability and country-ownership in AMR research. By including the power of genomics within surveillance systems, we can unravel the multifaceted web of interactions that drive AMR emergence and spread, enabling proactive and evidence-based interventions to protect both human and animal populations.

First initiatives by the Jigjiga One Health Initiative (JOHI) launched a molecular diagnostic center in the Somali region, which served as the sole laboratory catering to a population of approximately 8 million during the COVID-19 pandemic. JOHI's objective is to implement the NGS technology utilizing the MinION platform by ONT. This strategic move aims at streamlining and enhancing the generation of high-quality AMR data, both on a national and international scale.

AMR and malnutrition: improving nutritional status to boost immunity and reduce antimicrobial use

Antibiotic treatment is recommended by the WHO for severe acute malnutrition and has demonstrated benefits in improving nutrition recovery and reducing mortality (Trehan et al., 2013). However, this practice enhances the concern about the potential increase in antibiotic misuse, especially in LMICs, where diagnostic services are limited (Trehan et al., 2013; World Health Organization, 2013). This often leads to improper prescription practices, contributing to the emergence and spread of AMR. For instance, a randomized control trial conducted in Niger revealed that severe acute malnutrition children treated with antibiotics had a two-fold increase in the fecal carriage of ESBL-producing *E. coli* (Maataoui et al., 2020). Additionally, there was an increased rate of these bacteria in the feces of individuals sharing the same household as those who received antibiotics (Maataoui et al., 2020).

There is increasing evidence indicating that malnourished children are at high risk of infections caused by ESBL-producing *E. coli*, which are independent of initiation of antimicrobial treatment, as showed in this thesis along with other studies in Tanzania and Senegal (Ahmed et al., 2015; Ndir et al., 2016). This could also be explained by the fact that undernourished children often have dysbiotic microbiome, which may enhance the transfer of resistance or virulent genes via HGT in the gut microbiota (Vonaesch et al., 2018). This doubles the burden

faced by malnourished children, making it difficult to treat the opportunistic infections in these vulnerable children. Therefore, it is important to prioritize nutritional rehabilitation, ensuring access to therapeutic food, micronutrient supplementation, and breastfeeding support to improve the nutritional position and support immune functions in children, in order to reduce the need for antibiotics.

It is also necessary not to overlook the nomadic communities such as the Somali pastoralists in Ethiopia, who are often affected by adverse effects of climate change, particularly droughts. These changes often exacerbate food insecurity, potentially enhancing malnutrition within these vulnerable communities. Additionally, the shortage of water resulting from drought impairs access to clean water, sanitation and hygiene, thereby heightening the susceptibility of these communities to infectious diseases (Guéladio Cissé et al., 2022). These infectious pathogens could potentially harbour resistance genes, complicating the management of affected individuals. Hence, it is essential to establish adaptation and mitigation strategies to address malnutrition related to extreme weather events. For instance, among Somali pastoralists in Ethiopia, the camel, which is resilient to droughts, holds a significant cultural value. Camel milk and meat, in particular, are perceived as health profits, believed to confer strength and safeguard against disease within the community. Muleta et al. reported that camel milk consumption has been associated with low stunting and underweight in children (Muleta, Hailu, Stoecker, & Belachew, 2021). Therefore, a recommended strategy is to dry camel milk into a shelf-stable powder, enabling these communities to store it and consume it during the droughts. This could safeguard against malnutrition and infections, resulting in reduced antimicrobial use.

Other suggestions obtained during two JOHI stakeholder meetings on food preservation with the community could be a potential solution, for instance, the food preservation practice of sun-drying sliced meat and then combining it with butter, locally called “colab/muqmady”. These strategic interventions are essential to mitigate food insecurity during drought periods and reduce the malnutrition within these susceptible populations.

AMR, sanitation and hygiene: implementing WASH practices among the communities

Drinking unsafe water, poor sanitation and hygiene practices are contributing factors to the global burden of infectious diseases (G. Cissé, 2019). Consequently, they also contribute to the emergence and spread of AMR, particularly in LMICs. In the light of the results presented here, the lack of basic hygiene in both rural and urban areas, coupled with limited sanitation infrastructure, likely facilitated the spread of diarrheagenic pathogens such as DEC, *Campylobacter jejuni, Salmonella spp.*, *Shigella spp.*, and DEC hybrid (two-four virulent combinations) in the communities. Most of these pathogens also carried resistance genes,

making these infections difficult to treat with the available antibiotics. Over decades, significant efforts have been directed towards improving water, sanitation and hygiene (WASH) conditions in LMICs. A recent review underscores the success of these efforts, demonstrating a substantial reduction in the risk of diarrheal diseases through sanitation and hygiene interventions (Wolf et al., 2022).

Therefore, we recommend implementing a community-led WASH program in the Somali region to improve sanitation and hygiene practices. One effective approach is utilizing community-led total sanitation (CLTS) interventions. This approach works by triggering the community sense and educating its members on the crude truths about open defecation and its repulsive consequences (Zinsstag et al., 2020). The collective realization within the community stimulates the members to take ownership of the issue and develop solutions. Subsequently, community members are stirred to construct toilets and stop open defecation, thereby promoting sustainable improvement in sanitation (Kar & Chambers, 2008). Additionally, waste management strategies should be introduced. These include disposing waste safely and treating human and animal feces before using them as fertilizer, to combat the spread of microbial threats to and from the environment.

Moreover, several methods including chemical disinfection, solar disinfection, boiling, and filtering have been employed to enhance water safety in rural communities in LMICs. For instance, chlorination is one of oldest and most widely used methods in rural and marginalized communities at both community and household level (E. Mintz, Bartram, Lochery, & Wegelin, 2001; E. D. Mintz, Reiff, & Tauxe, 1995). It has been proven successful in preventing and controlling waterborne disease outbreaks and effectively reducing AMR (B. F. Arnold & Colford Jr, 2007; Khan, Beattie, & Knapp, 2016; Wolf et al., 2022). Interestingly, in this thesis, we observed an association between children who drank water treated with chlorine and being infected with ESBL-producing *E. coli*. This association could be explained by the inappropriate storage and insufficient dosing of chlorine in rural communities, which might be due to a lack of understanding of instructions or incompetence to accurately measure the volume of water to be treated. Furthermore, the unpleasant chlorine taste may lead to hesitation in adding the recommended dose, as some individuals may believe that adding less chlorine will result in a less noticeable alteration in the taste of the water (Rothstein et al., 2015). These factors could reduce the efficacy of chlorine, resulting in unsafe water and further dissemination of pathogens and AMR. Hence, we recommend enhancing the knowledge of rural families on how to handle and use chlorine for water treatment.

Other studies have also reported that, despite its anticipated purpose of diminishing or eliminating AMR, chlorine appears to have inadvertently contributed to the enrichment of AMR

(S.-S. Liu et al., 2018). Additionally, Jin et al. noted that chlorination fosters the emergence and exchange of resistance genes among bacterial groups (Jin et al., 2020). Thus, it is crucial to perform regular water quality checks and further investigate the dynamics and mechanisms of chlorine in increasing AMR. Exploring alternative options such as filtration or boiling is necessary, as both were proven to be effective in improving the quality of water and reducing the AMR.

AMR and gender: enhancing inclusive education with a One Health approach

While the risk of AMR is generally shared by males and females, the latter are more vulnerable to infections (Dias, Brouwer, & van de Beek, 2022). Indeed, especially in LMICs, women are at higher risk of acquiring infections, for example during childbirth or menstruation (Barinov et al., 2022; P. Das et al., 2015). For instance, during menstruation and pregnancy, women are at higher risk of contracting urinary tract infections (UTIs), which may have an increased AMR potential (Elkady, Sinha, & Hassan, 2019; Muhammed, 2015).

Moreover, in contrast to men, women in rural areas are responsible for the household chores, and the care and wellbeing of children and of adults who need special care. As the primary caregivers for children, women handle their feces, often with limited hygiene and sanitation measures, which may increase the risk of exposure to pathogens harboring resistance genes (Aluko et al., 2017; Bauza et al., 2020). In rural areas, their role often extends beyond the household to the care of small livestock and maintenance of the garden (Lynch et al., 2024). Additionally, women take care of sick animals. When these sick or injured animals show no improvement, they are always slaughtered, with men usually responsible for this chore (Barasa, 2019). Then, women are tasked with butchering the meat and preparing the food, which includes not only meat but also the preparation of vegetables. These tasks collectively increase the risk of exposure to resistance pathogens, intensifying AMR in women (Bisholo, Ghuman, & Haffejee, 2018; Godijk, Bootsma, & Bonten, 2022).

Inadequate access to education further enhances the challenge of AMR in rural communities, particularly for women. As highlighted earlier, women often bear a large burden of biological factors and household roles, increasing their exposure to resistant pathogens. However, their lower literacy rates can impede their comprehension of the importance of sanitation and hygiene and proper antimicrobial use for themselves and their families (Bosley, Henshall, Appleton, & Jackson, 2018; Jones et al., 2022). Indeed, 75% of women in Ethiopia's Somali region are illiterate (Hussen & Workie, 2023). In this thesis, we report a strong correlation between the limited knowledge of caregivers regarding AMR and higher infection rates from ESBL-producing *E. coli* observed in their children. Women generally have limited AMR knowledge because control and prevention trainings are primarily targeted at males or

household heads (Ström et al., 2018). Also, cultural norms often require women to seek permission from their husbands to participate in such trainings, public awareness campaigns, and health information, limiting their access to education, and impeding their capability of travelling and participating in AMR activities and trainings (Bikaako et al., 2022).

To address the issue of AMR in these communities, it is imperative to prioritize educational programs and awareness campaigns particularly tailored for women. These programs should not only address their limited knowledge but also account for cultural context. Targeting women's education is therefore crucial for AMR mitigation, due to their extensive role in the household and community. Such programs should empower women with the knowledge on antimicrobial stewardship and skills necessary to make informed decisions regarding antimicrobial use for themselves, their children, elderly people, livestock, and the community as a whole. Moreover, it is important to draw attention to the women's role in taking care of animals and preparing food, in order to equip them with proper skills to handle animal waste and improve their hygiene practices while handling food. This could reduce the risk of transmitting infections, potentially carrying resistance genes, from animals and food to themselves, the children, elders, and the broader community.

Due to the specific cultural context, males should also take part in the training. Indeed, males in rural areas are the primary decision-makers in households, and their opinion is highly valued within the community. Educating them on these topics will allow them to better understand the vulnerability of women to AMR, and the role that they can play in control and mitigation, and thus facilitate them to encourage women's participation in the educational programs and campaigns.

Need for enhanced antimicrobial stewardship strategies using a One Health approach

In LMICs, the unnecessary use of antimicrobials in humans, animals and agriculture accelerates the emergency of antimicrobial resistance (Dadgostar, 2019). The misuse and overuse of antimicrobials are due to poor or non-existing regulatory frameworks in LMICs (Bonna, Pavel, Ferdous, Khan, & Ali, 2022; Mshana, Sindato, Matee, & Mboera, 2021). The lack of a definite treatment protocol and the consequent antimicrobial use for the COVID-19 pandemic has further fueled inappropriate antimicrobial use, expediting the emergence of AMR (Nandi, Pecetta, & Bloom, 2023). Additionally, the focus on COVID-19 has redirected the resources away from AMR surveillance and research, hindering endeavors to address this global health threat (Nandi et al., 2023). The growing demand for animal protein in developing nations instigated intensive farming practices, leading to the persistence of antimicrobial residues in livestock products, and consequently contributing to selecting resistant pathogens (Manyi-Loh, Mamphweli, Meyer, & Okoh, 2018). These pathogens can be easily transmitted

to humans via the food chain, making infections more challenging and costly to treat, and potentially leading to increased hospital stays and even fatalities (Manyi-Loh et al., 2018; Naylor et al., 2018). It is indisputable to protect and safeguard the effectiveness of these antimicrobial agents (Schuts et al., 2016).

With this thesis, we demonstrated concerning practices known to contribute to AMR in Ethiopia, such as self-medication practices, over-the-counter sale of antimicrobials and inappropriate use of antimicrobials in humans and animals. In Ethiopia, the majority of the population are pastoralists who depend on livestock and its products for sustenance. In some areas like the Somali region, antibiotics are used to preserve milk, preventing spoilage before it reaches the market for sale. Additionally, the consumption of raw meat is deep-rooted in the culture, famous as one of its culinary treasures. These practices could clearly have an effect on AMR. Indeed, studies have shown the presence of antimicrobial residuals in both milk and meat (Abdeta, Tafesse, & Bacha, 2024; Agmas & Adugna, 2018; Mohamed et al., 2020). While the existing antimicrobial stewardship program in Ethiopia aimed at improving patient outcomes via the rational use of antimicrobials, especially within human medicine, it overlooked the essential role of animal medicine in AMR. Hence, it is indispensable to urgently strengthen the existing stewardship program and integrate antimicrobial stewardship (AMS) within the animal health sector, as AMR is a complex issue that transcends one sector and cannot be managed in isolation.

The importance of this integrated approach was highlighted during the first One Health AMS Conference, where Canadian leaders and subject matter experts in both animal and human health sectors convened to comprehend each other's challenges in addressing AMR (McCubbin et al., 2022). In LMICs, a transdisciplinary approach including laypersons, policymakers and scientists with the AMS program has shown promising results in reducing antimicrobial use in animal health and the development of AMR pathogens in both animals and humans (Eagar & Naidoo, 2017). This collaborative approach exemplifies the importance of an integrated disciplinary approach through the lens of One Health, promoting a sense of ownership among stakeholders to ensure the long-term sustainability of public health initiatives.

Thus, we recommend the AMR resistance prevention and containment committee to include surveillance of antimicrobial use and sale in animals. Behavioural change strategies are also necessary, involving targeted engagement and sensitization of livestock farmers regarding the benefits of accountable antimicrobial use in animals and strictly adhering to withdrawal periods after treatment (McKernan, Benson, Farrell, & Dean, 2021; Regan et al., 2023). This will reinforce the attainment of the objectives outlined in the "Ethiopia antimicrobial resistance

prevention and containment strategy", thereby reaffirming the government's dedication to tackle AMR (EFDA, 2021).

AMR and climate change: increasing community awareness

This thesis, being the first to simultaneously assess the rural communities' knowledge regarding climate change and AMR, has shown the limited knowledge on both climate change and AMR. Similar findings were reported from studies assessing the two topics separately in Ethiopia (Belay et al., 2022; Woldegeorgis et al., 2023). Additionally, several studies have shown that the general public in LMICs may lack awareness of the causes and consequences of AMR and climate change, leading to limited responses and increased susceptibility to their effect (Greibe Andersen et al., 2023; Hundera et al., 2019; Ofori et al., 2023; Woldegeorgis et al., 2023). Interestingly, rural communities acknowledge the occurrence of climate change due to its impact on their livelihoods, such as drought and other related phenomena. However, our study, along with several others conducted in Mozambique, Ghana, and Ethiopia, revealed that rural communities attribute climate change to God (Artur & Hilhorst, 2012; Bryan et al., 2009; S. Gandure, S. Walker, & J. J. Botha, 2013). This illustrates the interplay of cultural, social, and contextual factors in shaping attitudes and behaviors related to climate change. These beliefs impede the comprehension of climate change as a scientific phenomenon driven by human activities, including the burning of fossil fuels, deforestation, and industrial processes (Rouleau et al., 2022). On the other hand, rural communities often face difficulties to comprehend the impact of AMR on their livelihoods due to their limited knowledge about it and it not being as practical as the impact of climate change.

Furthermore, other studies in LMICs showed that despite the direct and indirect impact of climate change on health, there remains a knowledge gap in health professionals as well as in health science students about these intertwined issues (Nigatu, Asamoah, & Kloos, 2014; Shrikhande et al., 2023). This knowledge gap in these different stakeholders, indicates the lack of integrated educational programs on climate change and health in both communities and health professionals. This is mainly caused by the lack of data on climate change and health, which was also a major limitation of our study and of further studies or other quantitative analyses. Additionally, the scarcity of research on climate change and health, relevant policies and laws, and the perceptions of actors and institutions at central level and financial challenges also play a role (Guéladio Cissé et al., 2022; Simane et al., 2016).

These two intertwined crises necessitate urgent action to protect public, animal, and environmental health (Zaman, 2022). For instance, climate warming creates favorable conditions for the growth of resistant microbes. MacFadden et al. showed the association between increased temperature and AMR (MacFadden et al., 2018). Additionally, extreme

weather events such as droughts and flooding exacerbate the dissemination of infectious diseases (Guéladio Cissé et al., 2022), leading to higher misuse of antimicrobials, a key driver of AMR (Roberta Magnano San Lio et al., 2023). These interconnected issues, fueled by humans, exemplify the “tragedy of the commons” (Hardin, 1968). Addressing these challenges needs a multidisciplinary approach, with knowledge being a key strategy to contain and mitigate AMR and climate change, thereby protecting the shared resources to reduce the impact and threats to vulnerable populations globally.

Given the ramifications, multifaceted, and cross-sectoral dimensions of AMR and climate change, along with their pervasive nature, one-off campaigns such as the World Antimicrobial Resistance Awareness Week (WAAW) and climate change annual campaigns are insufficient to initiate sustainable behavioral change or yield enduring impact (WHO, 2022b, 2024). Moreover, these initiatives often disregard rural communities that lack access to education to understand the campaign, as well as communities’ perspective and indigenous knowledge. Therefore, there is a need to employ a transdisciplinary approach, an approach that integrates indigenous and scientific knowledge and respects local perspectives and beliefs. For instance, a study conducted with health sector representatives and pastoralists used a transdisciplinary approach to address not only the different concerns and interests of the parties involved, but also their diverging worldviews and distinct forms of communication (Zinsstag et al., 2011). In Guatemala, a Bidirectional Emic-Etic tool (BEE) has been employed to bridge the gap between traditional Mayan beliefs and scientific medical oncology understanding, taking into consideration intercultural differences (Berger-Gonzalez et al., 2016). This approach has demonstrated effectiveness in addressing societal challenges related to environmental sustainability and is recommended for addressing issues related to climate change (Stålne & Pedersen, 2021). Using this approach, culturally sensitive integrated education programs can be developed to address both AMR and climate change in rural communities. These educational programs on climate change and its effects on health can be included in school curricula. They can also be disseminated though workshops, training sessions, multimedia products and regular campaigns for the communities and health professionals that highlight the interconnectedness of these issues and their impact on health, livestock, agriculture and environment.

AMR control using One Health in Social-Ecological Systems (OHSES)

For over five decades, Garrett Hardin’s concept of the “tragedy of the commons” has been highly controversial. In this concept, Hardin argues that without mutual coercion, conflict over the use of finite shared resources is inevitable as individual interests override the common good (Hardin, 1968). Recent issues of natural resource overexploitation evident in global

challenges such as biodiversity loss and climate change, and attendant human and animal health threats bring this concept into renewed focus. Boyd and colleagues suggest that the emergence and spread of AMR is another prominent example of “tragedy of the commons” (Boyd et al., 2018). The overuse of antimicrobials in humans, animal, agriculture and the environment contribute to the rise of AMR, posing serious threats to human and animal health globally (Velazquez-Meza, Galarde-López, Carrillo-Quiróz, & Alpuche-Aranda, 2022).

A game theory approach, utilizing mathematical modeling to predict the strategic engagement and responses of multi-sectoral stakeholders, has been recently proposed to tackle shared health problems. The One Health socio-ecological system (OHSES) model developed by Zinsstag et al. suggests that practical applications of game theory can ensure sustainable access to commonly shared resources by fostering considerate use for the benefit of all (Zinsstag, Meyer, Bonfoh, Fink, & Dimov, 2024). A game-theoretical analysis of rabies elimination strategy demonstrated that coordinated mass dog vaccination could lead to the elimination of canine rabies in Africa, with projected overall welfare gain of 9.5 million USD in the next 30 years, highlighting the added value of maintaining a functional balance in ecological systems (Bucher et al., 2023).

By predicting the reaction of different actors to strategic engagements in an AMR control strategy, this approach could play a crucial role in global efforts to address AMR through the responsible use of antimicrobials across relevant sectors within a harmonized regulatory framework. Without cooperation across sectors and regions in the world, the rapid development and spread of AMR will undermine antibiotics effectiveness, leading to massive losses of human and animals lives and resources. This comprehensive approach enables a deeper understanding of the complex dynamics of AMR, facilitating coordinated intervention mechanisms.

Future research

The intricate nature of AMR poses a significant challenge in understanding its acquisition pathways across humans, animals, and the environment (K. E. Arnold et al., 2024). This complexity has called for the adoption of a One Health approach, which enlightened the interconnectedness of AMR resistance genes between these sectors (WHO, 2023). For instance, several studies conducted in countries in the Global South have demonstrated similarities in resistant gene profiles across these sectors (Abukhattab et al., 2023). This has contributed to a partial understanding of AMR genes in these regions. However, it is necessary to also understand their acquisition pathways and transmission dynamics, which is essential to develop novel interventions to combat AMR effectively. Therefore, we recommend a One Health research approach incorporating WGS aiming at elucidating the links and transmission

of AMR genes in humans (from early life to adulthood), food-producing animals, and the environment (hospitals, waste facilities and residence areas).

Further, it is essential to trace bacteria carrying resistance genes circulating within the community. Hence, we advocate for research on the microbiome and its relationship to AMR using a One Health approach. This will not only enlighten our comprehension of microbiome composition and their resistance genes, but it will also allow us to compare the healthcare settings-based research with community settings-based studies to understand the dynamic nature of resistance genes in both settings. Furthermore, it will help us to grasp how the human microbiome is shaped by other sectors like environment, food, and food-producing animals, to develop more comprehensive interventions to combat AMR.

This thesis demonstrated that antimicrobial stewardship programs, a key strategy in the optimization of antimicrobial use recommended by the WHO, are either poorly applied or completely unknown in many areas (Eagar & Naidoo, 2017). To address this knowledge gap and foster judicious antimicrobial use is necessary to reduce the emergence and spread of AMR. Therefore, we propose to conduct a pre-survey to gauge the level of comprehension of healthcare workers regarding antimicrobial stewardship. Based on these baseline findings, we recommend introducing antimicrobial stewardship programs and training workshops and establishing a framework to support and ensure the sustainability of these programs. Moreover, we suggest conducting a cluster randomized controlled trial research using digital algorithms to guide antibiotic prescription and reduce inappropriate prescriptions. By integrating novel technologies into antimicrobial stewardship efforts, we can heighten the precision and efficiency of antimicrobial prescribing practices, ultimately contributing to the control of AMR.

Evidence is growing regarding the potential relationship between climate change and the emergence and dissemination of AMR (MacFadden et al., 2018; Roberta Magnano San Lio et al., 2023). However, this critical issue is not receiving enough attention as a priority research agenda at the global and national levels. The lack of knowledge about the relationship between climate and AMR is prominent among the most affected populations, as emphasized in this thesis. As our study design took an etic approach, future research should consider deploying an emic approach to obtain a deeper understanding of knowledge, attitudes, and practices of climate change and AMR among study populations. Additionally, due to the limited availability of data, the relationship between these two issues remains poorly understood. Therefore, a research initiative starting at the regional level to explore the association between meteorological parameters and AMR surveillance will enhance the comprehension of their link and inform adaptive actions.

Chapter 8 General Conclusions

The research conducted in this thesis aimed to contribute to a better understanding of the prevalence of resistance genes in rural and urban children and animals, alongside knowledge and attitudes towards AMR and climate change in rural communities.

Given the multidimensional nature of these issues and their intricate interplay, we propose implementing a holistic One Health approach to understand the acquisition and dynamics of AMR genes. Establishing integrated surveillance frameworks under the One Health approach will be essential to address the complex interplay between pathogens, antimicrobial resistance, and environmental factors, including climate change impacts, ultimately guiding effective prevention and control strategies. Further, we recommend comprehensive community-based educational programs, particularly tailored for women to promote responsible antimicrobial use and environmental conservation.

AMR in humans, animals, and the environment, as a prime One Health issue, fits perfectly into the Jigjiga One Health initiative (JOHI) vision as a building block into integrated Surveillance-Response Systems and community-based AMR stewardship programs. Training human and animal health professionals on AMR and fostering SRS competence in genomics through the introduction of next-generation sequencing, will have important benefits for the aetiological diagnosis of infectious diseases in general.

Chapter 9 References

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Appendix

Under the appendix, I have attached two projects that I was involved in during my PhD, entitled:

1. “Prevalence and associated risk factors of intestinal parasitic infections among children in pastoralist and agro-pastoralist communities in the Adadle woreda of the Somali Regional State of Ethiopia”
2. “Gut microbiomes of agropastoral children from the Adadle region of Ethiopia reflect their unique dietary habits”

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Prevalence and associated risk factors of intestinal parasitic infections among children in pastoralist and agro-pastoralist communities in the Adadle woreda of the Somali Regional State of Ethiopia

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Abstract

Background

Intestinal parasitic infections (IPIs) can cause illness, morbidity, and occasional mortality in children. Agro-pastoralist and pastoralist children in the Somali Regional State of Ethiopia (ESRS) are especially at risk for IPIs, as access to safe water, sanitation, and health services is lacking. Minimal data on the prevalence of IPIs and associated risk factors exists in this region.

Methodology

We assessed the prevalence of IPIs and associated risk factors during the wet season from May-June 2021 in 366 children aged 2 to 5 years in four agro-pastoralist and four pastoralist *kebeles* (wards) in Adadle *woreda* (district) of the Shebelle zone, ESRS. Household information, anthropometric measurements, and stool samples were obtained from included children. Parasites were identified microscopically using Kato-Katz and direct smear methods. Risk factors were assessed using general estimating equation models accounting for clustering.

Principal findings

Overall prevalence of IPIs was 35%: 30.6% for single infections and 4.4% for poly-parasitic infections. Intestinal protozoan prevalence was 24.9%: 21.9% *Giardia intestinalis*, and 3.0% *Entamoeba* spp.. Intestinal helminth prevalence was 14.5%: 12.8% *Ascaris lumbricoides*, 1.4% hookworm (*Ancylostoma duodenale* / *Necator americanus*.), and 0.3% *Hymenolepis*

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nana. *G. intestinalis* infection was associated with drinking water sourced from the river (aOR 15.6, 95%CI 6.84, 35.4) and from collected rainwater (aOR 9.48, 95%CI 3.39, 26.5), with toilet sharing (aOR 2.93, 95%CI 1.36, 6.31) and with household ownership of cattle (1–5 cattle: aOR 1.65, 95%CI 1.13, 2.41; 6+ cattle: aOR 2.07, 95%CI 1.33, 3.21) and chickens (aOR 3.80, 95%CI 1.77, 8.17). *A. lumbricoides* infection was associated with children 36 to 47 months old (aOR 1.92, 95%CI 1.03, 3.58).

Conclusions/significance

Improving access to safe water, sanitation, and hygiene services in Adadle and employing a One Health approach would likely improve the health of children living in (agro-) pastoralist communities in Adadle and the ESRS; however, further studies are required.

Author summary

Intestinal parasitic infections remain a silent threat to the health and life-trajectories of children living in areas with inadequate access to clean water, proper sanitation, and hygiene facilities, including the Somali Regional State of Ethiopia. A large majority in this region live as pastoralists (semi-mobile animal herders), in close contact with their animals and nature, at risk for climate-related threats like drought and flooding, and at risk for infectious agents like intestinal parasites. We assessed the prevalence of intestinal parasitic infections in pastoralist children in the Adadle district of the Somali Regional State of Ethiopia (ESRS), and the individual and household-level factors associated with these infections. We found that locally collected water, shared toilets, along with ownership of cows and chickens increased the risk for having an intestinal parasitic infection with *Giardia intestinalis*, which can cause diarrhea and is transmitted through water, food, and soil that have been contaminated by the feces of infected humans and animals. If access to clean water, sanitation, and hygiene infrastructure is not improved, these infections remain recurrent in these communities and their animals, continually affecting the health of children. This study is one of few involving pastoralists in this region, hopefully lending guidance to regional public health policies.

Introduction

Intestinal parasitic infections (IPIs) remain one of the most common infectious diseases in humans and animals globally with a high burden of disease and occasional mortality [1,2]. Children are especially at risk for IPIs due to changes in nutrition, age-specific behaviours around play and hygiene, and their developing immune systems [1]. Additional risk factors include lack of access to clean water, inadequate sanitation and hygiene, lack of knowledge on transmission pathways and lack of epidemiological surveillance [2]. While the morbidity of specific IPIs varies, children chronically infected can develop iron deficiency (anemia), vitamin A, and other micronutrient deficiencies, become undernourished, experience prolonged diarrheal episodes (dysentery), develop cognitive delays, become stunted, and may even die as a result of acute or chronic infection [1,3]. The majority of IPIs are transmitted via the faecal-oral route, either through faecally contaminated hands, food, water, vegetation, other surfaces, and aerosolized particles [2,4–6]. Many human intestinal parasites are also zoonotic and can

be transmitted to or from other animals, some of which can also cause illness and occasional death in the animal, and as well as economic loss for the animal's owner [7–10].

In Ethiopia, prevalence of IPIs has been found to be between 42% and 53% in children under five years of age [6,11–17] and school-aged children [18–23], from which associated risk factors were unsafe or contaminated drinking water [6,13], no sanitation facilities and open defecation [6,11,12], poor hygiene and knowledge on transmission [6,11,13], living in crowded homes [12], eating raw vegetables [6,12,17], and coming into contact with contaminated soils [6,17]. However, a 2019 systematic review found that in several regions of Ethiopia, including the Ethiopian Somali Regional State (ESRS), no prevalence estimates of IPIs in children were published or available [24]. Indeed, to date, there is a single study assessing undernutrition and prevalence of IPIs in pastoralist children in the ESRS during the 2019 drought season [25]. There is no data on prevalence in the wet season nor information on associated risk factors of IPIs, leaving a knowledge gap regarding IPIs in pastoralist communities in the ESRS.

Pastoralist and agro-pastoralist communities make up the majority of the total population of the ESRS [26,27]. Communities are tight-knit and often blood related, often sharing child-care, water and food resources, and medicines. They rely mainly on their animals for food (milk and meat) and livelihood, are semi-mobile and lack access to safe water and sanitation as well as health services [25,28]. As pastoralists live in close connection and proximity to one another, their animals, and their environment year-round, it is expected that the sharing of microbial species, including intestinal parasites, is high. This study assessed the prevalence of intestinal parasitic infections and associated risk factors in children aged 2 to 5 years in pastoralist and agro-pastoralist communities in the Adadle *woreda* (district) of the Shabelle zone of the Ethiopian Somali Regional State (ESRS). This study is part of the Jigjiga One Health Initiative to improve the health of humans, animals, and their environments among pastoralist communities in the ESRS, with a specific focus on improving the health of children in the ESRS.

Methods

Ethics statement

Ethics approval for this study was obtained from the Review Committee of the University of Jigjiga in Ethiopia (JJU-RERC030/2020) and the Review Committee of Armauer Hansen Research Institute in Addis Ababa, Ethiopia (AF-10-015), and from the Swiss Ethics Committee of Northwest and Central Switzerland (Ethikkommision Nordwest- und Zentralschweiz; REQ-2020-00608). Oral or written consent was obtained from the parent of all participating children before study enrolment. If a child was found to have an intestinal parasitic infection, the child's mother was given anti-parasitic medications (mebendazole, albendazole or metronidazole) to administer to her child. Data was recorded on the Open Data Kit and stored on a secured server at the Swiss TPH in Basel. All identifying information was kept with the local study team in Ethiopia and is securely stored in a closed cupboard.

Study design

Study area. The study was a cross-sectional study in the Adadle *woreda* (district) in the Shabelle zone of the Somali Regional State of Ethiopia and was carried out from May 2021 until June 2021 during the wet season, known locally as *gu-'ga*. Adadle is located 17 km from the city of Gode in the lowlands of the Wabi Shabelle River subbasin and experiences a mean annual temperature of 32˚C and a mean annual rainfall of 300 mm. The altitude is 300–500 meters above sea-level and 80% of the land is flat, while 20% is undulated. The pastoralist and agro-pastoralist communities in ESRS and Adadle rely mainly on their animals for food and livelihood and live largely outside of modern systems.

Sample size. The sample size was determined based on several components, as this study was part of a larger project studying antimicrobial resistance and the microbiome from a One Health perspective. A previous study in children aged 2 to 5 years in this region showed a prevalence of intestinal parasitic infections of 42% [25]. As we expected clustering on the *kebele* (ward) level, the intra-cluster correlation coefficient was assumed to be 0.15. We chose a 95% confidence interval, power of 80%, alpha of 0.05, and a margin of error of 10%. Based on these factors and to achieve the necessary sample size for all aims of the larger research project, we calculated a sample size of 360 eligible children, 180 each in pastoralist and agro-pastoralist communities.

Selection criteria. Stakeholders (community leaders and health officials) in Adadle were approached at each level (district, ward, sub-ward) and invited to help determine which kebeles in Adadle could be included in the present study. From the 15 kebeles in Adadle, four pastoralist (Malkasalah, Todob, Harsug, Kulmis) and four agro-pastoralist (Bursaredo, Gabal, Higlo, Boholhagare) kebeles were randomly selected (**Fig 1**). A pre-enrolment screening was carried out at the local health centre in each kebele, for all children aged 2 to 5 years. Medication and hygienic supplies were given as incentive to each household to attend the pre-screening, regardless of further participation in the study. All children were screened for stunting (height for age) and wasting (weight for height), according to the 2006 WHO growth standards [29]. All screened children who were severely wasted based on their weight for height z-score (WHZ < -3) or severely stunted based on their height for age z-score (HAZ < -3) were automatically included in the study. Screened children not presenting as severely stunted and/or severely wasted were randomly selected (random number generation) from an excel file containing all pre-screened households/children in the community, until the maximum sample

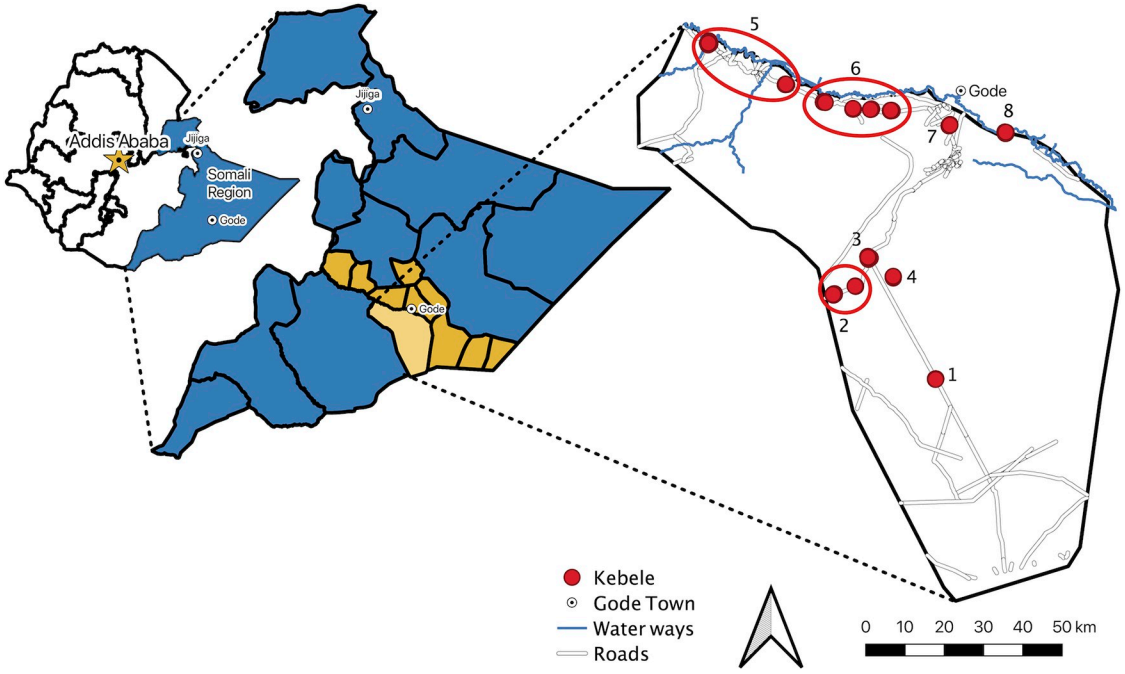

Fig 1. Map showing included kebeles in Adadle woreda in the Shabelle zone of the Somali Regional State, Ethiopia. Kebele locations are based on geopoints collected during the study. Kebeles 1–4 are pastoralist communities (1: Todob; 2: Kulmis; 3: Malkasalah; 4: Harsug) and kebeles 5–8 are agro-pastoralist communities (5: Gabal; 6: Bursaredo; 7: Higlo; 8: Boholhagare). All public geographic data files (borders, cities, roads, and waterways) were downloaded from the open source Humanitarian Data Exchange (HDX) [39]. Map created in QGIS3 [38].

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size was reached for each kebele and the overall study. Children were excluded if they were older than 5 years, younger than 2 years and/or had taken antibiotics in the last 14 days.

Anthropometric measurements

Anthropometric measurements of height, weight, and mid-upper arm circumference (MUAC) were measured at least twice, or until the measurements were within 1 cm, 100 g, or 1 mm of one another, respectively. The measurements were then averaged for each child. Height was measured by having the child stand up straight against a WHO-standard wooden measuring board [29] set on flat ground. Attention was given that the child was looking forward, had relaxed shoulders, straight legs, no shoes on, and arms at their side. Weight was measured by having the child stand alone in the middle of a WHO-standard scale [29] without shoes, and with light clothing. If the child was not willing or able to be weighed alone, then the mother was first weighed alone and then weighed again with the child in her arms. The final weight of the child was therefore the weight of the mother alone subtracted from the weight of mother and child together. The scale was calibrated daily against known weights to ensure accuracy on all study days. The mid-upper arm circumference (MUAC) was measured using a WHO-standard tape measurer [29]. In the pre-screening, z-scores for height-for-age (HAZ; stunting) and weight-for-height (WHZ; wasting) were calculated using the 2006 WHO growth standard tables [29]. Following the end of data collection, HAZ, WAZ, and WHZ were recalculated using the R package 'zscorer' [30]. Stunting, wasting, and underweight were defined as HAZ, WHZ, WAZ $<$ -2, while acute malnutrition (low MUAC) was defined as MUAC $<$ 12.5 cm.

Data collection

A detailed questionnaire using the Open Data Kit (ODK) software [31] was administered in the local language by field workers to the mother of the child. The questionnaire was divided into several sections: (1) child anthropometric measures, current health, and health history of the child, (2) household characteristics and assets, and the type and number of household animals, (3) WASH behaviours of the child and household, (4) a nutritional survey of the child, and finally (5) breastfeeding and birth history of the mother and child. Birth records often do not exist in pastoralist communities; therefore, age in months was estimated based on group discussions with the child's mother, other family, and community members to determine seasonal (floods, month of year, drought) and festive events (Ramadan, other religious events) that occurred before or after the birth of the child. Geo-reference points for spatial visualizations were collected for most households.

Stool sample collection and parasitological analyses

Stool samples were collected by the mothers in sterile specimen cups after detailed instruction by a trained field worker in the local language. The samples were aliquoted and then immediately transferred to a cold box containing ice. All parasitology analyses were performed in the field in each kebele health clinic by trained parasitologists from Jigjiga University. The aliquot for parasitology was divided into four portions. One portion was used for the quantification of helminth ova using the thick-smear Kato-Katz detection method [32] under a microscope at 10x and 40x magnification, which allows for the detection of common helminth species such as hookworm (*Ancylostoma duodenale* /*Necator americanus*), *Hymenolepis nana*, *Trichuris trichiura*, *Ascaris lumbricoides*, and *Schistosoma mansoni*. Another two portions were used for the detection of protozoan cysts and trophozoites, such as *Giardia intestinalis* and *Entamoeba* spp. using the direct smear method in duplicate. Briefly, a drop of normal saline and a drop of Lugol's iodine were each placed on one half of a single sterile glass slide. Then, using a cotton

swab, a small amount of stool sample was smeared onto each drop, covered with a glass slip, and inspected under a microscope at 10x magnification. Although microscopy can only be used to identify *Giardia* at the genus level, *Giardia intestinalis* is the only species of *Giardia* that has been found to infect humans. Therefore, all *Giardia* identified through microscopy in the human faecal samples are referred to as *Giardia intestinalis* throughout the manuscript. The final portion of stool was fixed and stored in SAF (sodium acetate–acetic acid-formalin) at 4˚C as a backup [33]. The consistency of each stool sample was determined by a trained parasitologist.

Statistical analysis

All statistical analyses were performed in R Statistical software version 4.0.4 [34]. Tables were generated using the gtsummary package [35]. Briefly, the ODK questionnaire data (including anthropometric data) and parasitology data were merged according to each child's identification number. We used the available case population for all analyses. For household-level metrics, missing data was addressed by applying the completed questionnaires of siblings in the same household. Missing data for individual metrics (such as anthropometric metrics) were not able to be recovered and these observations were therefore left out of analyses. Individual observations where the mother answered "don't know" are listed as "Unknown" in descriptive tables. Categorization of numerical variables, such as ownership of cattle, were based on the median value as a cut-off point. Univariable and multivariable analysis were carried out using the general estimating equation logistic regression model for binary outcomes using the geepack package [36], to account for clustering on the kebele level. Variables in the multivariable model were pre-selected based on the published literature and complemented with variables associated in the univariable analysis ($p < 0.2$) [37]. Spatial visualizations were performed on QGIS3 [38] and public geographic data (borders, cities, roads, and waterways) was downloaded from open source Humanitarian Data Exchange (HDX) [39].

Results

A total of 366 children aged between two to five years from 270 households spread between eight kebeles in the Adadle woreda (district), Shabelle zone of the Ethiopian Somali Regional State (ESRS) were included in the present study, of which 184 children were from pastoralist and 182 from agro-pastoralist communities. During the screening process, 7 children presented as severely stunted, 27 as severely wasted, and 3 as severely stunted and severely wasted, all of whom were included in the study. For all included children, parasitology reports were documented. However, for twenty-one children, a questionnaire was not recovered or completed, due to logistical issues with the software and field activities. Therefore, 345 children completed both a questionnaire and parasitology report (**S1 Fig**). By applying household-level data from completed questionnaires of siblings in the same household, 13 of the 21 children with a missing questionnaire were able to be recovered for the household-level descriptive analyses (N = 358; **S1 Fig**).

Participant characteristics

Participant characteristics are summarized in **Table 1**. The age and sex of enrolled children were distributed evenly, including between pastoralist and agro-pastoralist communities. Only 10.9% of children had a complete vaccination at the time of sampling. Only 8.4% of children were exclusively breastfed (EBF) for the first 6 months. The median duration of breastfeeding was 12 months (Range: 2–28 months). Supplementary milk from household animals (cows, goats, camels) was given in the first 6 months of life to 57.5% of children, while 21.7% received

Table 1. Characteristics of (agro-) pastoralist children aged 2 to 5 years living in Adadle woreda, Shabelle zone, Somali Regional State, Ethiopia.

Characteristic	N = 345
Age	
23–35 months	120 (34.8%)
36–47 months	106 (30.7%)
48–60 months	119 (34.5%)
Sex	
Female	169 (49.0%)
Male	176 (51.0%)
Vaccination status	
Complete vaccination	37 (10.9%)
Incomplete vaccination	301 (89.1%)
Unknown	7
Exclusively breastfed for 6 months	29 (8.4%)
Complementary milk starting when	
Before 6 months	196 (57.5%)
After 6 months	74 (21.7%)
Never gave other milk	71 (20.8%)
Unknown	4
Complementary food starting when	
Before 6 months	80 (23.9%)
After 6 months	255 (76.1%)
Unknown	10

EBF, Exclusive breastfeeding; Complementary food included mostly soft staple foods cooked in milk. Overall statistics are shown, as group differences were minimal. Data collected in the wet season 2021.
Data are presented as n (%).

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supplementary milk after 6 months, and 20.8% were never given supplementary milk prior to weaning. Supplementary animal milk was given as early as 1 month and as late as 28 months, with the median start time at 1 month old. Supplementary foods (mostly soft staple foods like porridge, rice, potatoes, and injera) were started in 23.9% of children in the first 6 months of life, while the rest (76.1%) received supplementary food after 6 months of age. Supplementary food was given as early as 1 month but as late as 40 months, with a median start time at 6 months.

Height, weight, and mid-upper arm circumference (MUAC) were similarly distributed between pastoralist and agro-pastoralist children (Table 2). Of the included children with anthropometric metrics (N = 345), 10 (3%) were severely stunted (HAZ < -3) and 30 (9%) were severely wasted (WHZ < -3), of which 3 children were both severely stunted and wasted. The overall stunting (HAZ < -2), wasting (WHZ < -2), underweight (WAZ < -2) and low MUAC (MUAC <12.5 cm) were 14%, 30%, 17%, and 2.3%, respectively. Only 5% (17/345) of children were both wasted and stunted.

Household and Kebele characteristics

The included agro-pastoralist kebeles all reside along or near the Shebelle River (Fig 1); two kebeles (7: Higlo; 8: Boholhagare) lived along the Shebelle River and were approximately 16 km downstream from Gode town, one kebele (6: Bursaredo) was near an agricultural zone 20–40 km from Gode banking the Shebelle River, and the last kebele (5: Gabal) banked the

Table 2. Anthropometric characteristics of agro-pastoralist and pastoralist children aged 2 to 5 years living in Adadle woreda, Shabelle zone, Somali Regional State, Ethiopia.

Characteristic	Agro-pastoralist N = 168	Pastoralist N = 177	Overall N = 345
Height (cm)	96 (90, 102)	98 (90, 105)	97 (90, 104)
Weight (kg)	12.60 (11.38, 13.90)	13.30 (11.60, 15.00)	12.80 (11.40, 14.50)
HAZ	-0.37 (-1.45, 0.41)	0.02 (-0.97, 0.78)	-0.18 (-1.21, 0.59)
Stunted	26 (15%)	21 (12%)	47 (14%)
WHZ	-1.55 (-2.18, -0.98)	-1.33 (-2.03, -0.65)	-1.41 (-2.15, -0.82)
Wasted	53 (32%)	49 (28%)	102 (30%)
WAZ	-1.34 (-1.95, -0.72)	-0.83 (-1.62, -0.22)	-1.06 (-1.78, -0.40)
Underweight	39 (23%)	19 (11%)	58 (17%)
MUAC (cm)	14.00 (13.50, 14.72)	14.20 (13.60, 14.90)	14.10 (13.50, 14.90)
Low MUAC	5 (3.0%)	3 (1.7%)	8 (2.3%)

HAZ, height for age z-score; Stunted: HAZ < -2 SD; WHZ, weight for height z-score; Wasted: WHZ < -2 SD; WAZ, weight for age z-score; Underweight: WAZ < -2 SD; MUAC, mid-upper arm circumference; Low MUAC: MUAC <12.5 cm.

Data are presented as median (IQR) or n (%) unless otherwise stated.

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Shebelle River 50–70 km upstream of Gode town. The included pastoralist kebeles (1: Todob; 2: Kulmis; 3: Malkasalah; 4: Harsug) did not reside near any rivers, but did flank road systems, and were between 48–75 km from Gode town (Fig 1). Households (regardless of if pastoralist or agro-pastoralist) spoke exclusively the Somali language (100%), were of the Muslim faith (100%), were majority illiterate (head of household, 74.3%; mother of child, 76.0%), and a majority owned a mobile phone (62.3%) (S1 Table).

Household water, sanitation, and hygiene (WASH) characteristics showed some differences between pastoralist and agro-pastoralist communities and are summarized in Table 3. The majority (85.3%) of agro-pastoralist communities sourced their water from the Shebelle River, while the majority (89.0%) of pastoralist households sourced their water from rain collected in open shallow wells or birkads. Only a small minority sourced their water from a borehole, tank truck or natural spring (agro-pastoralist: 5.6%, pastoralist: 9.4%). More agro-pastoralist (46.3%) than pastoralist (14.9%) treated their water, through chlorination. Agro-pastoralists almost exclusively practiced open defecation (99.4%), while pastoralists practiced open defecation 92.8% of the time and 7.2% indicated they used a type of pit latrine. Toilet sharing, meaning the sharing of a latrine or defecation spot, was shared by a minority in both agro-pastoralist (12.4%) and pastoralist (5.5%) communities. Waste disposal was similar between agro-pastoralists and pastoralists; the majority dumped their waste (84.1%), either in their compound, river or in the open, while few burned their waste (15.9%). Household ownership of soap was also quite low, with 56.0% indicating they do not usually have soap in the house. Finally, hygienic characteristics of the households were similar, with most mothers reporting that they washed their child's hands with water only (92.3%), and few with water and soap (7.7%).

Agro-pastoralists and pastoralists in Adadle and the ESRS depend greatly on livestock animals as a means of food and livelihood. Household ownership of domestic animals, which includes in the present study cattle, camel, goats, sheep, donkeys, and chickens, varied in quantity between the agro-pastoralist and pastoralist communities in Adadle (Fig 2 **and** S2 Table**).** Agro-pastoralists owned more cattle, sheep, donkeys, and chickens, while pastoralists owned more goats and camels and no chickens. Livestock animal ownership ranged from zero to 149 animals, with a median of 25 animals, and more animals were kept inside the house or compound of pastoralist households (78.5%) than agro-pastoralist households (57.6%) (S2 Table).

Table 3. Household WASH characteristics of agro-pastoralist and pastoralist children aged 2 to 5 years living in Adadle woreda, Somali Regional State, Ethiopia.

	Characteristic	Agro-pastoralist N = 177	Pastoralist N = 181	Overall N = 358
WATER	**Source of drinking water**			
	Borehole/Spring/Tank truck	10 (5.6%)	17 (9.4%)	27 (7.5%)
	Rainwater in Birkads	16 (9.0%)	161 (89.0%)	177 (49.4%)
	River water	151 (85.3%)	3 (1.7%)	154 (43.0%)
	Treatment of water (Yes)	82 (46.3%)	27 (14.9%)	109 (30.4%)
SANITATION	**Toilet type**			
	Pit latrine	1 (0.6%)	13 (7.2%)	14 (3.9%)
	Outdoor	176 (99.4%)	168 (92.8%)	344 (96.1%)
	Shared toilet (Yes)	22 (12.4%)	10 (5.5%)	32 (8.9%)
	Waste disposal			
	Burned	29 (16.4%)	28 (15.5%)	57 (15.9%)
	Dumped	148 (83.6%)	153 (84.5%)	301 (84.1%)
HYGIENE	**Household owns soap**			
	Yes	83 (47.2%)	74 (40.9%)	157 (44.0%)
	No	93 (52.8%)	107 (59.1%)	200 (56.0%)
	Unknown	1	0	1
	Child hand washing method			
	With water	151 (88.3%)	172 (96.1%)	323 (92.3%)
	With water and soap	20 (11.7%)	7 (3.9%)	27 (7.7%)
	Unknown	6	2	8

Birkads: open shallow wells or ditches; Treatment of water was mostly through chlorination.
Data are presented as n (%).

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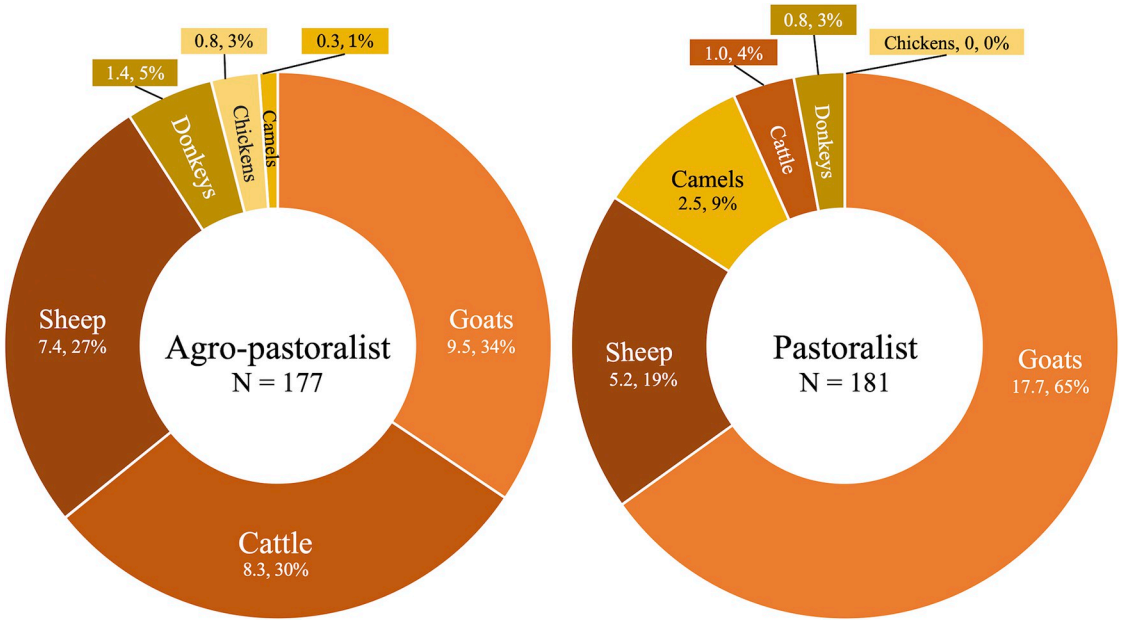

Fig 2. Average household animal herd makeup of agro-pastoralist and pastoralist children aged 2 to 5 years living in Adadle woreda, Somali Regional State, Ethiopia. Numbers are the mean and mean percentage of each type of animal owned per participant per group (pastoralist or agro-pastoralist). N = 358.

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Table 4. Prevalence of intestinal parasitic infections during the 2021 wet season in agro-pastoralist and pastoralist children aged 2 to 5 years living in Adadle woreda, Somali Regional State, Ethiopia.

Characteristic	Agro-pastoralist N = 182	Pastoralist N = 184	Overall N = 366
Overall			
Parasite free	115 (63.2%)	123 (66.8%)	238 (65.0%)
Single parasite	57 (31.3%)	55 (29.9%)	112 (30.6%)
Poly-parasite	10 (5.5%)	6 (3.3%)	16 (4.4%)
Protozoa			
Giardia spp.	44 (24.2%)	36 (19.6%)	80 (21.9%)
Entamoeba spp.	3 (1.6%)	8 (4.3%)	11 (3.0%)
Helminths			
Ascaris lumbricoides	26 (14.3%)	21 (11.4%)	47 (12.8%)
Hookworm (*Ancylostoma duodenale /Necator americanus*)	4 (2.2%)	1 (0.5%)	5 (1.4%)
Hymenolepis nana	0 (0.0%)	1 (0.5%)	1 (0.3%)

Data are presented as n (%).

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Intestinal parasitic infection prevalence

The overall prevalence of intestinal parasitic infections (IPIs) was 35%, with a prevalence of 30.6% for single parasitic infections and 4.4% for poly-parasitic infections (coinfection with protozoan and helminth species). The infection pattern is summarized in Table 4. Intestinal protozoan infections were detected in 24.9% of children, while intestinal helminth infections were detected in 14.5% of children. The prevalence of protozoan infections was 21.9% *Giardia intestinalis* and 3.0% *Entamoeba* spp.. Of the *G. intestinalis* infections detected, 65% were in the cystic stage and 35% in the trophozoite stage. *G. intestinalis* prevalence was slightly higher in agro-pastoralist communities (24.2%) compared with pastoralist communities (19.6%), although this difference was not significant in univariate or multivariate analyses (Table 5). The overall prevalence of helminth infections was 12.8% *Ascaris lumbricoides*, 1.4% hookworm (*Ancylostoma duodenale /Necator americanus*), and 0.3% *Hymenolepis nana*. Both *A. lumbricoides* and hookworm (*A. duodenale /N. americanus*) had a slightly higher prevalence in agro-pastoralist communities compared with pastoralist communities, although this difference was not significant in univariate or multivariate analyses (Table 6). Poly-parasitic infections (4.4%) were all found to be a co-infection of a protozoan with a helminth species, of which 25% (4/16) were *Entamoeba* spp. with the helminth *A. lumbricoides* and 75% (12/16) *G. intestinalis* with a helminth (*A. lumbricoides* 9/16; *H. nana* 1/16; hookworm 2/16).

Factors associated with *Giardia intestinalis* infection

In univariate analysis, *G. intestinalis* infection was associated with children aged 36 to 47 months, source of drinking water, ownership of cattle and ownership of chickens. In multivariate analysis, *G. intestinalis* infection was associated with water source, toilet sharing, ownership of cattle and ownership of chickens (Table 5). Sourcing drinking water from rainwater stored in birkads (open shallow wells/ditches) had an aOR of 9.48 [3.39, 26.5], p-value < 0.001, while sourcing from river water had an aOR of 15.6 [6.84, 35.4], p-value < 0.001, when compared to sourcing water from a spring, tank truck, or borehole. Toilet sharing had an aOR of 2.93 [1.36,6.31], p-value = 0.006. Household ownership of cattle revealed an aOR of 1.65 [1.13 2.41], p-value = 0.009, when owning 1–5 cattle, and an aOR of 2.07 [1.33, 3.21], p-value = 0.001, when owning more than 6 cattle, compared with owning no cattle. Similarly, owning chickens had an aOR of 3.8 [1.77, 8.17], p-value < 0.001. All further

Table 5. Univariate and multivariate analysis of *Giardia intestinalis* in agro-pastoralist and pastoralist children aged 2 to 5 years living in Adadle woreda, Somali Regional State, Ethiopia.

	Univariate				Multivariate		
Characteristic	**n/N (% Positive)**	**OR**	**95% CI**	**p-value**	**aOR**	**95% CI**	**p-value**
Group							
Agro-pastoralist	41 / 168 (24)	—	—		—	—	
Pastoralist	35 / 177 (20)	0.76	0.36, 1.63	0.49	2.52	0.73, 8.73	0.14
Sex							
Female	38 / 169 (22)	—	—		—	—	
Male	38 / 176 (22)	0.95	0.62, 1.45	0.81	0.99	0.63, 1.56	0.97
Age							
23–35 months	21 / 120 (18)	—	—		—	—	
36–47 months	30 / 106 (28)	1.86	1.08, 3.21	**0.025**	1.68	0.90, 3.16	0.11
48–60 months	25 / 119 (21)	1.25	0.81, 1.94	0.31	1.19	0.75, 1.91	0.46
Source of drinking water							
Borehole/Spring/Tanktruck	1 / 27 (3.7)	—	—		—	—	
Rain water	36 / 171 (21)	6.93	2.41, 19.9	**<0.001**	9.48	3.39, 26.5	**<0.001**
River water	39 / 147 (27)	9.39	2.64, 33.4	**<0.001**	15.6	6.84, 35.4	**<0.001**
Shared toilet							
No	66 / 315 (21)	—	—		—	—	
Yes	10 / 30 (33)	1.89	0.94, 3.79	0.074	2.93	1.36, 6.31	**0.006**
Number of cattle							
No	23 / 130 (18)	—	—		—	—	
1–5 cattle	29 / 128 (23)	1.36	0.74, 2.52	0.32	1.65	1.13, 2.41	**0.009**
6+ cattle	24 / 87 (28)	1.77	1.12, 2.80	**0.014**	2.07	1.33, 3.21	**0.001**
Owns chickens							
No	67 / 327 (20)	—	—		—	—	
Yes	9 / 18 (50)	3.88	2.63, 5.73	**<0.001**	3.80	1.77, 8.17	**<0.001**

OR = Odds Ratio; aOR = adjusted Odds Ratio; CI = confidence interval; Univariate and multivariate analyses performed using a general estimating equation logistic regression model for binary outcomes. N = 345.

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variables included in multivariate analysis were non-significant. Univariate and multivariate analyses were not possible for *Entamoeba* spp., due to low positive numbers.

Factors associated with *Ascaris lumbricoides* infection

In univariate analysis, *Ascaris lumbricoides* was associated with children aged 36 to 47 months and with owning more than 6 cattle (Table 6). In multivariate analysis, *A. lumbricoides* infection remained associated with children aged 36 to 47 months (aOR 1.92 [1.03, 3.58], p-value = 0.040) compared to children aged 23 to 35 months. All further variables included in multivariate analysis were non-significant. Univariate and multivariate analyses were not possible for the other helminth parasites due to low positive numbers.

Discussion

To our knowledge, this is the first study assessing the impact of livestock animal keeping and WASH characteristics on the risk of intestinal parasitic infections (IPIs) in children 2 to 5 years of age living in pastoralist and agro-pastoralist communities in the ESRS. In addition, this study adds to the limited body of research on the health of pastoralist communities living

Table 6. Univariate and multivariate analysis of *Ascaris lumbricoides* in agro-pastoralist and pastoralist children aged 2 to 5 years living in Adadle woreda, Somali Regional State, Ethiopia.

	Univariate				Multivariate		
Characteristic	**n/N (% Positive)**	**OR**	**95% CI**	**p-value**	**aOR**	**95% CI**	**p-value**
Group							
Agro-pastoralist	26 / 168 (15)	—	—		—	—	
Pastoralist	20 / 177 (11)	0.70	0.35, 1.39	0.30	2.03	0.62, 6.71	0.24
Sex							
Female	21 / 169 (12)	—	—		—	—	
Male	25 / 176 (14)	1.17	0.69, 1.97	0.56	1.31	0.73, 2.38	0.37
Age							
23–35 months	14 / 120 (12)	—	—		—	—	
36–47 months	22 / 106 (21)	1.98	1.12, 3.53	**0.020**	1.92	1.03, 3.58	**0.040**
48–60 months	10 / 119 (8.4)	0.69	0.23, 2.09	0.52	0.68	0.23, 2.01	0.48
Source of drinking water							
Borehole/Spring/Tanktruck	4 / 27 (15)	—	—		—	—	
Rain water	18 / 171 (11)	0.68	0.24, 1.87	0.45	0.78	0.24, 2.52	0.67
River water	24 / 147 (16)	1.12	0.36, 3.52	0.84	1.39	0.38, 5.01	0.62
Shared toilet							
No	39 / 315 (12)	—	—		—	—	
Yes	7 / 30 (23)	2.15	0.64, 7.30	0.22	2.31	0.68, 7.88	0.18
Number of cattle							
No	13 / 130 (10)	—	—		—	—	
1–5 cattle	16 / 128 (12)	1.29	0.68, 2.44	0.44	1.19	0.58, 2.46	0.64
6+ cattle	17 / 87 (20)	2.19	1.01, 4.71	**0.046**	1.85	0.76, 4.51	0.18
Owns chickens							
No	41 / 327 (13)	—	—		—	—	
Yes	5 / 18 (28)	2.68	0.77, 9.39	0.12	2.98	0.58, 15.2	0.19

OR = Odds Ratio; aOR = adjusted Odds Ratio; CI = confidence interval; Univariate and multivariate analyses performed using a general estimating equation logistic regression model for binary outcomes. N = 345.

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in the ESRS and in the horn of Africa, the latter geographic zone encompassing an estimated 20–30 million humans living a pastoral lifestyle [26,27].

A recent study performed in the same district with pastoralist communities during the dry (drought) season showed slightly higher overall prevalence of IPIs (47%) than in our study (35%), although the prevalence of *G. intestinalis* and *A. lumbricoides* were comparable [25]. This difference is likely due to the effect of seasonality on transmission and prevalence of IPIs [40,41] as well as on nutritional status [42,43], which is known to have synergistic interactions with IPIs. In other regions of Ethiopia, prevalences of IPIs in pre-school aged children varied (between 17% and 58%), with most studies conducted in non-pastoralist communities, at hospitals or schools, and in wet or mountainous zones [6,11–17]. Pastoralists in Chad were found to have a higher prevalence of IPIs (60%) [44]. Ecological and cultural differences, such as behaviours around hygiene, animals, and food, as well as child rearing, weaning and outdoor play, may all impact the prevalence of IPIs in each study setting, making comparisons difficult.

Among the intestinal parasites detected in this study, *Giardia intestinalis* infection was the highest and was found to be associated with source of drinking water, toilet sharing, ownership of cattle, and ownership of chickens. Sourcing from river water or from rainwater collected in shallow open wells or ditches (birkhads) both showed a higher odds of *G. intestinalis* infection

compared to sourcing from a borehole, spring, or tank-truck, indicating a potential contamination of the water sources and/or where they are stored. Both water sources (river or rain) represent much higher chances of environmental and animal/human contamination, especially during rainy (flood) seasons, when soils contaminated with human and animal feces, due to high levels of open defecation, are carried into nearby rivers, streams, and open wells/ditches. Other studies have demonstrated that poor water quality or feces contaminated water contributes to *G. intestinalis* (and other parasites) transmission and prevalence in humans and animals [6–9,40]. In addition, water security in the region is a recurrent problem, with drought-intensity increasing every year due to climate change [45,46]. Therefore, in combination with drought-related water shortages and contamination of crucial and limited water sources, the water security situation in this region for pastoralists may worsen and impact human and animal health even more [47]. Our results therefore represent a strong indication for priority to be given to constructing and maintaining improved water sources for agro-pastoralists and pastoralists in Adadle woreda and the wider ESRS to reduce the transmission of *G. intestinalis* and other water-borne pathogens.

Household sharing of the toilet area was found to be associated with *Giardia intestinalis* infection, indicating that this may be a potential transmission point between households. Indeed, studies in Ethiopia, the UAE, and India all found sharing of the toilet area with multiple people or between households increased the individual risk for IPIs [48–50]. As *G. intestinalis* is transmitted via the faecal-oral route, behaviours surrounding defecation, such as style of latrine, maintenance of latrine, disposal of human waste, personal methods used to clean oneself, and handwashing behaviours, are all important factors that may contribute to transmission in shared toilets [48–50]. Paired with our finding that many households (56%) did not own soap, most mothers (92%) washed their child's hands with only water, and most households (84%) dumped their waste in either the compound, the river, or the open, appropriate sanitation and hygiene measures should be developed in cooperation with (agro-) pastoralist communities in Adadle, to reduce transmission of *G. intestinalis*.

Ownership of cattle was also found to be significantly associated with *Giardia intestinalis* infection. With increasing numbers of cattle, categorically evaluated, the odds of infection also increased. Similarly, ownership of chickens was significantly associated with *G. intestinalis* infection, although this result is limited by the small number of participants living in households owning chickens (N = 18) and the fact that no pastoralist participants owned chickens. The association of *G. intestinalis* with chicken ownership is therefore more applicable to agro-pastoralist communities. Our results are supported by the fact that *G. intestinalis* is a zoonotic parasite, with the ability to infect and be transmitted to and from many mammals, including in cattle, chickens, and other livestock [7,51–54]. Indeed, a recent study found frequent cases of animal syndromes in pastoralist livestock in Adadle woreda during a seven month study period, with cattle and sheep showing high rates of gastrointestinal diseases (42.8%) [28,55]. Transmission of *G. intestinalis* from cows or chickens to children could therefore occur through several indirect pathways: children playing around household animals may become exposed to animal feces containing parasitic cysts, eggs, or larva; animals and humans defecating in similar spots, including near shared water sources, may allow for cross-over contamination; and if proper hygiene measures (handwashing with soap) are not observed following handling of animals, then food, water and milk can be cross-contaminated [56–59]. In our study, many households reported having animals "inside" their household or compound (66%), which could increase the amount of animal faeces near cooking, sleeping, and water/food storage areas. In addition, free-range chickens may also be apt disseminators of *G. intestinalis* cysts throughout the communal environment, other households, and to other animals, as they are often not bound or fenced in [60,61]. Given that pastoralists live in close connection

with their animals and their shared environment, we recommend future studies of IPIs in pastoralist communities consider a One Health approach [9]. Taken together, novel water, sanitation, and hygiene (WASH) methods for semi-mobile (agro-) pastoralist humans and their animals must be considered in order to reduce transmission in these settings.

Children aged 36 to 47 months old had a higher odds of being infected with *A*. *lumbricoides*. Indeed, a study of children in the Southern Ethiopian region found a peak in infection for *A*. *lumbricoides* in the 36 to 47 months old age group [16]. However, several other studies have found peaks in younger or in older age groups [17,44], indicating that the specific social and ecological context likely plays a role. As *A*. *lumbricoides* is transmitted via the faecal-oral route [2,6,9], age-specific changes such as initiation of exploratory behaviour outside of the home, playing with faeces-contaminated soil, and loss of immunologic protection from breastmilk following the weaning period may contribute to a heightened risk in the 36 to 47 month old group [16,44]. In addition, the low rate of exclusive breastfeeding until 6 months (8.4%) and early introduction of animal milks (as early as 1 month) in our study may impact the immune health and nutritional needs of pastoralist children, putting them at greater risk for both IPIs and malnutrition [25,62]. Further qualitative and ethnographic studies are warranted to elucidate the specific factors leading to this result.

There are some limitations in the present study. In this study, severely stunted or severely wasted children were automatically included, while children receiving antibiotics for any type of illness were excluded (which may include symptomatic IPIs). It is therefore possible that we overestimated wasting and stunting and underestimated IPIs in the study population. Further, due to logistical constraints, only one stool sample was able to be analysed per child. Since the diagnostic techniques we used lack perfect sensitivity for some intestinal parasites [33,63], the true prevalence of IPIs may have been underestimated. Given the strong associations of water source, toilet sharing, and cattle and chicken ownership with *G*. *intestinalis* infection in this study, future studies should include environmental and animal parasitology when assessing parasite prevalence and risk factors in communities in which there is a high rate of contact between humans, animals, and their environment. In addition, the One Health approach in parasitology and pathogenic microorganism research is applicable beyond the few parasites analysed in this study; indeed, there are many parasites and microorganisms of human and animal health importance that are transmitted among and between humans and animals through environmental channels (water, soil, food, plants, air) [6,7,9,40,64,65]. Transmission events between humans, animals, and their environment would best be addressed using genomic techniques [66], and supported with qualitative information to place the transmission events in context and develop appropriate interventions in the given context [67,68]. Taking these approaches would likely improve the health of humans and their animals, as well as reduce the loss of food and income to illness [64,69].

Conclusions

The prevalence of IPIs in agro-pastoralist and pastoralist children (aged 2–5 years), especially of *G*. *intestinalis*, are of regional public health concern, given the immediate and long-term health impacts of these types of infections in children. Source of drinking water (rain and river), toilet sharing, and household ownership of cattle and chickens were found to be important risk factors for *G*. *intestinalis* infection in these communities. Children aged 36 to 47 months were associated with *A*. *lumbricoides* infection. We recommend additions and improvements to water, sanitation, and hygiene (WASH) infrastructure for use by semi-mobile pastoralists, with attention given to the unique relationship pastoralists have with their animals and environment. Any intervention should be implemented in a transdisciplinary

manner with pastoralists and seek to involve actors from governmental agencies for humans, animals, and the environment, for the improved health of children in pastoralist communities.

Supporting information

S1 Fig. Methodological and analytical flow of study.
(DOCX)

S2 Fig. Household WASH characteristics of agro-pastoralist and pastoralist children aged 2–5 years living in Adadle woreda, Somali Regional State, Ethiopia. Alternative informational figure to Table 3, with Somali translations.
(PDF)

S1 Table. Sociodemographic household characteristics of agro-pastoralist and pastoralist children aged 2–5 years living in Adadle woreda, Somali Regional State, Ethiopia.
(DOCX)

S2 Table. Household animal herds of agro-pastoralist and pastoralist children aged 2–5 years of age living in Adadle woreda, Somali Regional State, Ethiopia.
(DOCX)

S1 File. Completed STROBE checklist.
(DOCX)

S2 File. Manuscript data. Anonymized study data for reproducing results.
(CSV)

S3 File. R Master File. R Markdown master file for reproducing results.
(DOCX)

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Gut microbiomes of agropastoral children from the Adadle region of Ethiopia reflect their unique dietary habits

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The composition and function of the intestinal microbiota are major determinants of human health and are strongly influenced by diet, antibiotic treatment, lifestyle and geography. Nevertheless, we currently have only little data on microbiomes of non-westernized communities. We assess the stool microbiota composition in 59 children aged 2–5 years from the Adadle district of Ethiopia, Somali Regional State. Here, milk and starch-rich food are predominant components of the local diet, where the inhabitants live a remote, traditional agropastoral lifestyle. Microbiota composition, function and the resistome were characterized by both 16S rRNA gene amplicon and shotgun metagenomic sequencing and compared to 1471 publicly available datasets from children living in traditional, transitional, and industrial communities with different subsistence strategies. Samples from the Adadle district are low in *Bacteroidaceae*, and *Prevotellaceae*, the main bacterial representatives in the feces of children living in industrialized and non-industrialized communities, respectively. In contrast, they had a higher relative abundance in *Streptococcaceae*, *Bifidobacteriaceae* and *Erysipelatoclostridiaceae*. Further, genes involved in degradation pathways of lactose, D-galactose and simple carbohydrates were enriched. Overall, our study revealed a unique composition of the fecal microbiota of these agropastoral children, highlighting the need to further characterize the fecal bacterial composition of human populations living different lifestyles.

Abbreviations

CAR	Central African Republic
VANISH	Volatile and/or associated negatively with industrialized societies of humans
BloSSUM	Bloom or selected in societies of urbanization/modernization
Primer set 1	16S rRNA gene primer v4.SA501-v4.SA508 and v4.SA701-v4.SA712 targeting the V4 region
Primer set 2	16S rRNA gene primer 515F and 806R targeting the V4 region
ASVs	Amplicon sequence variants
PD	Faith's phylogenetic diversity
PCoA	Principal coordinates analysis
WUF	Weighted UniFrac
mOTUs	Metagenomic-based operational taxonomic units
AMR	Antimicrobial resistance

The human gastro-intestinal tract microbiota plays a crucial role in immunity, brain development, metabolism and general health of human beings[1–4]. For the last two decades, the composition and function of the microbiome

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has been an area of intense and dynamic research facilitated by the advancement in sequencing methods and data analysis tools[5]. However, despite large-scale efforts in the characterization of the intestinal microbiota, many unknowns remain in our understanding of the colonization of our intestinal tract by microorganisms, their functionalities and their associations with non-communicable diseases[4,6,7].

Factors, such as birth-mode, breast-feeding, diet, antibiotic treatment, diseases, and proximity with animals, have been shown to strongly influence the intestinal microbiota and vary widely among populations[8–11]. Such factors have led to significant variations in the composition of what is considered a "healthy microbiome". The definition of a eubiotic community is crucial to develop microbiota-targeted interventions. Nevertheless, societies that live traditional lifestyles and communities currently undergoing a transition towards industrialization and urbanization remain understudied in comparison to populations from industrialized northern-American and European countries[12,13]. It is therefore crucial to better characterize the composition and function of the microbiome in diverse communities across the globe.

In recent years, studies on the intestinal microbiota of hunter-gatherer communities such as the Hadza from Tanzania or the Matses from Peru and Brazil, as well as other traditional populations such as agriculturalists from Malawi or Venezuela, showed an enrichment in members of the *Prevotellaceae, Spirochaetaceae* and *Succinivibrionacea*[14–17]. In contrast, the intestinal microbiota in subjects from industrialized societies has been associated with increased relative abundance of *Bacteroidaceae* and *Akkermensiaceae*[18–20]. The terms VANISH (volatile and/or associated negatively with industrialized societies of humans) and BloSSUM (bloom or selected in societies of urbanization/modernization) have been proposed to describe these taxa shared between populations with similar lifestyles[18]. While VANISH taxa are associated with a characteristic high-fiber diet of traditional communities, BloSSUM taxa correlate with the higher consumption of animal fat and protein in industrialized societies[18,21,22].

Although mostly reported in adults, lifestyle has an equally important role in shaping the fecal microbiota composition in children[23,24]. During the first two years of life, the maturation of the intestinal microbiota is strongly influenced by factors including birth mode, breastfeeding, and diet[8,25]. Children's gut microbiota continues to develop during childhood to stabilize towards an adult-like phylogenetic distribution later in life[26]. Growing evidences suggest that compositional alterations during this dynamic maturation and developmental period might have long-lasting effects on the health of an individual[8].

In light of the important contribution of lifestyle and diet on the intestinal microbial community, the intestinal microbiota composition and microbial functional potential need to be studied and characterized in populations from across the globe with differing subsistence strategies, lifestyles and dietary preferences. Here, we assessed the intestinal microbiota in agropastoral children from the Adadle *woreda* (district) in the Somali regional state of Ethiopia. We used both16S rRNA gene amplicon as well as whole-genome shotgun metagenomic sequencing to compare these children to other children living in geographically distant sites and living different lifestyles. Due to their unique way of life and their specific diet, we hypothesized that these agropastoral children harbor a distinct microbiome profile compared to children living any other traditional lifestyle.

This study is part of the Jigjiga University One Health initiative (JOHI), aiming at the improvement of health and livelihoods of mobile pastoralists and their animals in the Somali Region of Ethiopia. It primarily aimed at assessing the nutritional status and health care of children[27,28]. In parallel, the status of antimicrobial resistance and the health status of animals are assessed, aiming towards an integrated surveillance-response system for human and animal health[29].

Results

Description of study population

The Ethiopian population studied were agropastoralists from the Adadle *woreda* (district) in the Shabelle zone of the Somali Regional State. This region is mostly inhabited by pastoral and agropastoral communities that rely mainly on animals for food and livelihood (Fig. 1). This study included feces from children aged 2–5 years, living in traditional agropastoral communities in the Adadle *woreda*. Samples were collected in the context of a previous cross-sectional study on parasitic infection and micronutrient status conducted in this region in the dry season between July and September 2016[27]. Overall, 54 children were included in the final analysis using the first primer set (V4 region 501-508/701-712), 13 in the study using the second primer set (V4 region 515/806) and 15 children using shotgun metagenomic sequencing. Of the 54 children (primer set 1), 41% (22/54) were girls and 59% (32/54) were boys. Children were between 2 and 5 years old with the median age being 4 years of age (Table 1). In the 24-h dietary recall (Table 1), the main staple food consumed by the children included whole wheat (20% of the children) or wheat flour (15%), maize (29%), rice (19%), sorghum (4%) and potato (2%). Only few children were reported as having consumed tomato (15%) and onions (13%) but none had other vegetables, fruits, meat or fish. Additionally, 44 out of 54 children (82%) consumed animal milk (from camels, goats, sheep or cows) or tea with milk in the 24 h before sampling[27]. The metadata for primer set 1, 2 and shotgun metagenomic sequencing groups are shown in Table 1.

Composition of the fecal microbiota of children from the Adadle region, Ethiopia

Using primer set 1, we generated a total of 3,832,363 reads and an average of 70,970 ± 34,438 reads per subject. Negative control samples had an average of 173 ± 40 reads, ruling out any potential contamination. Out of the 1490 identified ASVs, 1294 were assigned to *Bacteria* or *Archaea* and were retained to explore the composition of the fecal microbiota of these children (Supplementary data 3, 4). In the 54 stool samples, 125 bacterial families from 21 different phyla were detected (Supplementary data 6), without applying any prevalence filter. After filtering at 10% prevalence, 12 phyla and 69 bacterial families with low prevalence were removed in the dataset. Filtering did not influence any conclusions from downstream analysis (Supplementary Fig. S1).

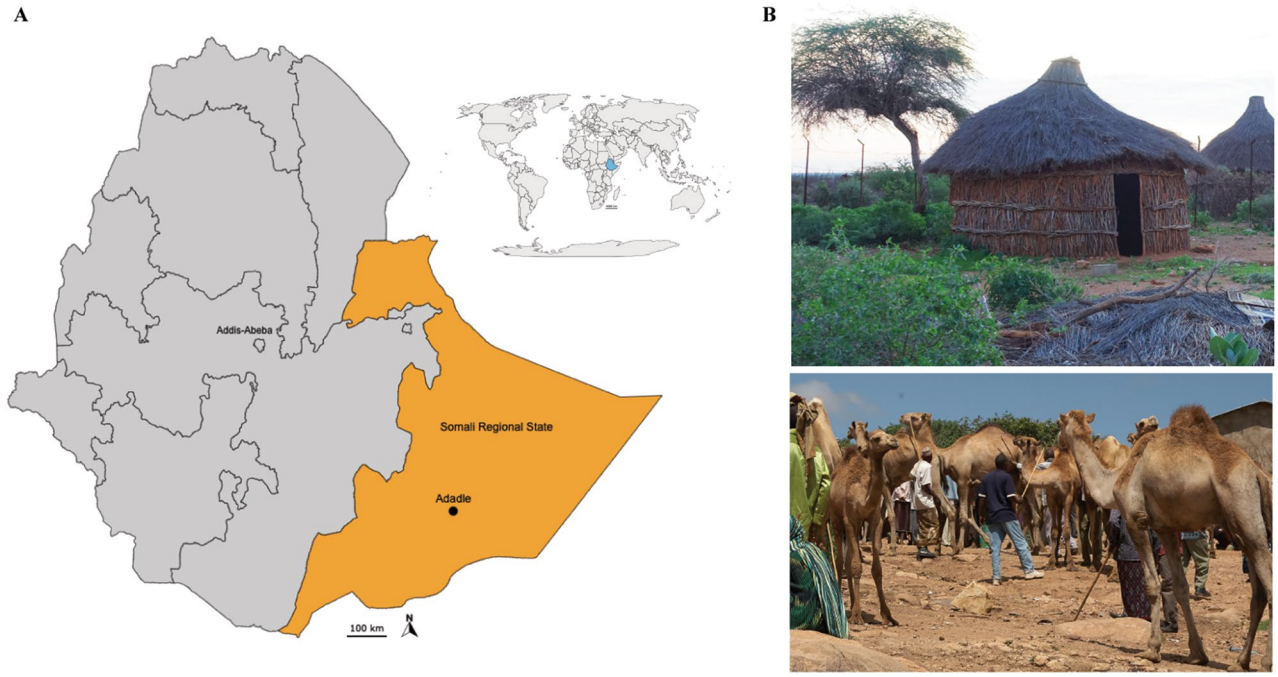

Figure 1. Sampling location and habitats of the studied agropastoral population. (**A**) Map of Ethiopia with the Somali Regional State highlighted in orange and Adadle *woreda* pinpointed. Upper right: map of the African continent with Ethiopia highlighted in blue. The maps were generated with GADM data (gadm.org, v4.0.4) and the magrit application (magrit.cnrs.fr, v0.8.14). (**B**) Habitats of the population. Top picture, Adadle *woreda*, Ethiopia. Bottom picture, camel market in Ethiopia (Photos courtesy of Pascale Vonaesch).

Dataset	Primer set 1	Primer set 2	Shotgun metagenomic
N	54	13	15
Sequencing method	16S rRNA gene amplicon	16S rRNA gene amplicon	Shotgun metagenomic
Sex			
Female	41% (22/54)	31% (4/13)	47% (7/15)
Male	59% (32/54)	69% (9/13)	53% (8/15)
Age			
Median	4 years old	4 years old	4 years old
2–3 years	15% (8/54)	0% (0/13)	0% (0/15)
3–4 years	31% (17/54)	31% (4/13)	33% (5/15)
4–5 years	54% (29/54)	69% (9/13)	67% (10/15)
Food consumption			
Whole wheat	20% (11/54)	23% (3/13)	13% (2/15%)
Wheat flour	15% (8/54)	8% (1/13)	0% (0/15)
Maize	28% (15/54)	15% (2/13)	40% (6/15)
Rice	19% (10/54)	8% (1/13)	0% (0/15)
Sorghum	4% (2/54)	0% (0/13)	0% (0/15)
Potato	2% (1/54)	8% (1/13)	0% (0/15)
Tomato	15% (8/54)	8% (1/13)	27% (4/15)
Onions	13% (7/54)	8% (1/13)	7% (1/15)
Animal milk	82% (44/54)	77% (10/13)	100% (15/15)

Table 1. Description of the study population.

The samples were low in relative abundance of the phylum *Bacteroidota* (formerly known as *Bacteroidetes*, relative abundance: 3.5 ± 6.3%, prevalence: 54/54), including mostly the *Prevotellaceae* family (relative abundance: 3.1%, prevalence: 52/54) (Fig. 2). There was a high percentage of *Actinomycetota* (formerly known as *Actinobacteria*, relative abundance: 16.8 ± 15.6%, prevalence: 54/54), especially of *Bifidobacteriaceae* (relative abundance: 10.8%, prevalence: 54/54) (Fig. 2). The samples were high in both prevalence and relative abundance

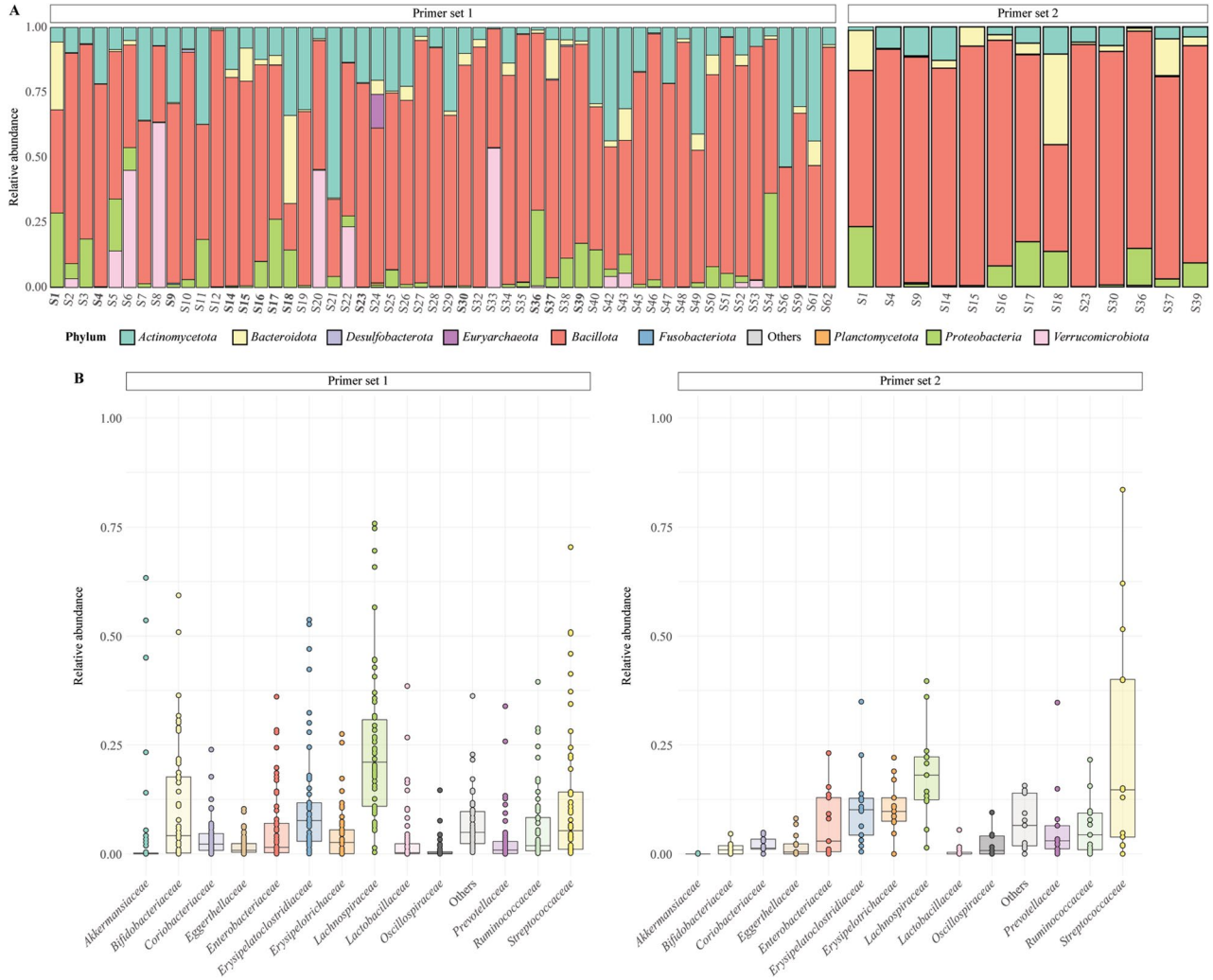

Figure 2. Composition of the fecal microbiota of children living in the Adadle region. Primer set 1 is targeting the V4 region 501–508/701–712, N = 54. Primer set 2 is targeting the V4 region 515/806, N = 13. (**A**) Relative abundance of the most abundant phyla for samples from the Adadle *woreda*. Less abundant phyla are grouped in the Others category. Samples in common in both datasets are highlighted in bold in the primer set 1 plot. (**B**) Box plot of the relative abundance of the most abundant bacterial families for samples from the Adadle *woreda*. The less abundant families are grouped in the Others category. Primer sets' relative abundance and prevalence are compared using Wilcoxon rank test at a significance threshold of 0.05 with Bonferroni correction for multiple comparisons.

of *Erysipelatoclostridiaceae* (relative abundance: 11.3%, prevalence: 54/54), *Streptococcaceae* (relative abundance: 12.3%, prevalence: 54/54), *Erysipelotrichaceae* (relative abundance: 4.3%, prevalence: 52/54) and *Lactobacillaceae* (relative abundance: 3.3%, prevalence: 54/54). Most strikingly, the level of *Akkermansiaceae* (relative abundance: 4.9%, prevalence: 44/54) were high in several samples (Fig. 2, Supplementary data 6).

Using primer set 2, with the 13 samples that passed quality control, we generated 98,908 reads with an average of 7608 ± 2421 reads per samples and 1,197 identified ASVs assigned to *Bacteria* (Supplementary data 3, 5). Negative control samples for primer set 2 failed the DADA2 pipeline due to low read count, ruling out potential contamination. When assessing for the composition of the microbiota in the reduced dataset shared between both primer set, we noted that the composition of the samples was largely similar in terms of the main taxa recovered as well as their relative abundance (Fig. 2B, Supplementary Fig. S2, Supplementary data 6). A notable exception was the *Akkermansiaceae* bacterial family, whose prevalence was significantly lower (p value = 0.041) in the 515F/806R samples (2/13) compared to the primer set 2 dataset (11/13) (Fig. 2B, Supplementary data 6). Overall, these observations showed a commonly shared microbiome in the agropastoral children dominated by *Bacillota* (formerly known as *Firmicutes*) and *Actinomycetota* and low relative abundance of different members of *Bacteroidota*.

Fecal samples from agropastoral children from the Adadle region are distinct compared to children from other geographic locations

To test whether the intestinal microbiota of the children from the Adadle *woreda* is different from other traditional communities, we compared the microbiota composition between these children and data from previously published studies around the globe (Table 2). We first explored the species diversity within communities using Faith's phylogenetic diversity (PD) and found that agropastoral children from the Adadle *woreda* have a similar species diversity than children from Madagascar and Central African Republic (CAR) (Fig. 3A). Using the primer set 2, we found that samples from the Adadle *woreda* have significantly lower phylogenetic diversity than children from other countries, except for children coming from the transitional population of Lima, Peru (Fig. 3A). Moreover, we found the same PD results on both primer set when applying a 0.25% filter on the taxa abundance (Supplementary Fig. S3A, C) as well as when rarefying multiple times and calculating the mean PD (Supplementary Fig. S3E–F). Overall, these results suggest that children from the Adadle *woreda* have a lower species diversity than children coming from traditional and industrial communities yet remains comparable to children from transitional populations from Africa and Peru.

Next, we applied Principal Coordinate Analysis (PCoA) of WeightedUniFrac (WUF) distance at the species level to assess for overall taxonomic composition of the samples. The ordination on the first, second and third components showed that samples from the Adadle *woreda* formed a clearly separated cluster compared to samples from Madagascar and CAR in the primer set 1 dataset (PERMANOVA p value < 0.005) (Axes 1 & 2: Fig. 3B, Axes 1 & 3, Axes 2 & 3: Supplementary Fig. S4). The ordination of the primer set 2 dataset showed that samples from the Adadle *woreda* clustered away from samples coming from industrial and traditional populations (PERMANOVA p value < 0.005). Further, even though samples from the Adadle *woreda* clustered more closely to samples from transitional communities, their microbiota composition was still significantly different (PERMANOVA p value < 0.005) (Fig. 3B). The same trend was confirmed using the Bray–Curtis, Jaccard and Generalized UniFrac distance metrics (Supplementary Figs. S4, S5). Moreover, we observed the same clustering of samples when applying a 0.25% abundance filter instead of removing singletons and using the Generalized UniFrac distance (Supplementary Figs. S3B, D).

Last, we used the Euclidean distance and the Ward's linkage method for hierarchical clustering. We identified two clusters (*P9+* and *P9–*), with the relative abundance of *Prevotella_9_copri* (Primer set 1 p value = $3.38e^{-68}$, Primer set 2 p value = $2.07e^{-56}$) and *Unassigned_Prevotella_9* (Primer set 1 p value = $8.46e^{-60}$, Primer set 2 p value = $1.84e^{-65}$) being the most significantly different between the two clusters (Wilcoxon rank test with Bonferroni correction for multiple comparisons) and the main driver separating the two clusters. In primer set 1 dataset, 50 out of the 54 samples and, in primer set 2 dataset, 11 out of 13 samples from the Adadle *woreda* clustered in *P9-*. Samples from Madagascar (328/431) and CAR (194/274) mostly clustered in *P9+* (Fig. 3C). While the samples' cluster repartition between Madagascar and CAR was not significantly different (χ^2 test p value > *0.05*), the repartition of samples from the Adadle *woreda* significantly differs from the two African countries (χ^2 test p value < *0.05*) (Fig. 3C). Additionally, samples from industrial (422/484) and transitional (86/88) communities clustered mostly in *P9–* similar to samples from the Adadle *woreda* (χ^2 test p value > 0.05) (Fig. 3C). Finally, 68 out of the 107 samples from traditional population clustered in *P9+* with a repartition significantly different from samples from the Adadle *woreda* (χ^2 test p value < 0.05). More specifically, most samples from Cameroon, China, Peru, and Tanzania clustered in *P9+* and most samples from Malawi and Venezuela clustered in *P9–* (Fig. 3C). Thus, in conclusion, samples from the Adadle *woreda* cluster more closely to samples from transitional communities than with samples from populations adopting a traditional lifestyle.

Analysis of enriched and depleted species in different communities

We further compared the relative abundance of specific bacterial families to assess for the compositional differences between samples from communities adopting different lifestyles. Samples from industrialized countries had high relative abundance of *Akkermansiaceae* and *Bacteroidaceae* (BloSSUM taxa) (Fig. 3D) whereas samples from traditional populations were high in the relative abundance of *Prevotellaceae* and *Succinivibrionaceae* (VANISH taxa) (Fig. 3D). Children from the Adadle region were found to have a significantly lower relative abundance of both BloSSUM and VANISH taxa compared to children from industrialized countries and traditional communities, respectively (Fig. 3D, p value < 0.05). Additionally, we observed a significantly higher relative abundance of *Erysipelatoclostridiaceae* and *Streptococcaceae* in samples from the Adadle *woreda* compared to any of the other samples included in the analysis (Fig. 3D, p value < 0.05). In the primer set 1 dataset, samples coming from the Adadle *woreda* had a higher abundance of *Bifidobacteriaceae* and *Lactobacillaceae* compared to samples coming from CAR and Madagascar (Fig. 3D, p value < 0.05). Using SIAMCAT[30] and LefSe[31] analysis, we confirmed the association between the higher abundance of the bacterial families and lifestyle (Supplementary Fig. S6).

Altogether the 16S rRNA gene amplicon sequencing data indicate that children living in the Adadle *woreda* have a distinct fecal microbiota composition compared with children of the same age living in different regions of the world. Children from the Adadle region are closer to children coming from transitional communities with lower alpha diversity and lower relative abundance of *Prevotellaceae* than children adopting a similar traditional lifestyle or children from industrialized countries.

Shotgun metagenomic sequencing confirms distinct fecal microbiota composition

To confirm the amplicon sequencing taxonomic composition trends and account for any primer bias, we used mOTUs2[32] and MetaPhlan3[33] taxonomic profilers on the 15 samples sent for shotgun metagenomic sequencing. A total of 2,698,693,772 reads passed fastp[34] filtering, with an average of 179,912,918 ± 72,371,443 reads per samples. Using mOTUs2, for the Adadle *woreda* dataset, 787 metagenomic-based operational taxonomic units (mOTUs) were assigned to the kingdom of *Bacteria* and accounted for 95.6% of the mapped reads while 4.3% of

Figure 3. Children's fecal microbiota composition from the Adadle *woreda* compared with children living on other subsistence strategies. Primer set 1 is targeting the V4 region 501–508/701–712, N = 759. Primer set 2 is targeting the V4 region 515/806, N = 692. (**A**) Alpha diversity measure as Faith's phylogenetic diversity at species level. Pairwise comparisons done using Wilcoxon rank test with Bonferroni correction for multiple comparisons (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (**B**) First and second coordinates of dimension reduction for WeightedUniFrac distance with the values indicating the amount of total variability explained by the coordinates. All pairwise comparisons were significant using PERMANOVA at a significance threshold of 0.05 using Benjamini–Hochberg correction for multiple comparisons. (**C**) Heatmap of the most abundant genera with significantly different relative abundance between the two clusters (P9+ and P9–). Relative abundance difference significance tested with Wilcoxon rank test at a significance threshold of 0.05 with Bonferroni correction for multiple comparisons and samples distribution tested using χ^2-test at significance threshold of 0.05. (**D**) Boxplot of the log10 of the relative abundance of enriched or depleted taxa in the different communities. BloSSUM: Bloom or selected in societies of urbanization/modernization. VANISH: Volatile and/or associated negatively with industrialized societies of humans. Relative abundance test using Wilcoxon rank test at a significance threshold of 0.05 with Bonferroni correction for multiple comparisons (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

the reads were unmapped to any species and less than 1% of the reads were assigned to unknown cellular organisms. The 787 mOTUs belonged to 387 known and 56 unknown bacterial species divided in 14 phyla, 82 families and 165 genera (Supplementary data 3). Using MetaPhlAn3 for profiling the microbial community, 349 species were assigned to *Bacteria*, divided in 8 phyla, 62 families and 129 genera (Supplementary data 3). We observed no major differences between MetaPhlAn3 and mOTUs2 profiles at different taxonomic levels (Supplementary Fig. S7A, B). Further, we observed the same trends in the taxonomic composition at the family level of the 6 samples sequenced using both primer sets and by shotgun metagenomic sequencing (Supplementary Fig. S8).

Moreover, using the number of assigned reads in mOTUs2 profiler, we compared the bacterial composition of the samples from the Adadle *woreda* with samples from other communities adopting differing lifestyles (Table 2, Supplementary Fig. S9A, Supplementary data 3). Notably, using PCoA of Bray–Curtis's distance, we confirmed the previous results from amplicon sequencing that samples from the Adadle *woreda* clustered away from all the other samples on the first and second components (Supplementary Fig. S9C, Supplementary data 7). In addition, Ward's linkage method for hierarchical clustering at species level resulted in the same two clusters (*P*+ and *P*−) (Supplementary Fig. S9D). Samples from the Adadle *woreda* clustered uniquely in the low *Prevotella* abundance cluster (*P*−), similarly to samples from Lima, Peru (100% in *P*−) and the USA (89.7% in *P*−). Moreover, the clusters repartition was significantly different (χ^2 test p value < 0.05, Supplementary data 7) from samples from Tanzania (54.5% in *P*+), traditional Peruvian communities (60% in *P*+), Zimbabwe (78.9% in *P*+) and El Salvador (80% in *P*+). Finally, we observed lower relative abundance of both BloSSUM and VANISH taxa compared to children from industrialized countries and traditional communities, respectively (Supplementary Fig. S9B, p value < 0.05) and high relative abundance of *Lactobacillaceae, Bifidobacteriaceae, Erysipelotrichaceae* and *Streptococcaceae* (Supplementary Fig. S9B).

Overall, these results show that the observed fecal microbiota composition was robust across all sequencing methods and taxonomic assignment tools and confirmed the distinctive bacterial composition of the fecal samples of children from the Adadle *woreda* in Ethiopia.

Samples from the Adadle *woreda* enriched in pathways reflecting dietary habits

To explore the functional profile of the children's fecal microbiota, we used HUMAnN3 to predict the abundance of microbial metabolic pathways present in our shotgun metagenomic dataset[33]. HUMAnN3 detected 1,400,457 evolutionary-related protein-coding sequences grouped in gene families which mapped to 490 known microbial pathways (Supplementary data 3). The total abundance of genes that contributed to a pathway accounted on average for 5.97% while the ones that did not contribute to any known pathways accounted for 69.57%. Additionally, the total abundance of reads unmapped to any known gene accounted on average for 24.46% (Supplementary Fig. S10). Out of the 490 detected pathways, 26 were uniquely found in samples from the Adadle *woreda*. In samples from other communities, we found 23 additional pathways not observed in samples from the Adadle *woreda*. Finally, 268 out of 490 pathways were detected in every sample from the Adadle *woreda*, among these pathways 95 were detected in all samples from both agropastoralists from the Adadle *woreda* and all other populations. Metabolic pathways were grouped in 7 superclass categories 1, with biosynthesis being the most abundant superclass (4.35 ± 0.0044%), followed by degradation/utilization/assimilation (0.96 ± 0.0021%) and generation of precursor metabolites and energy (0.55 ± 0.00062%) (Supplementary Fig. S10, Supplementary data 7). Further, pathways were classified in 46 superclass categories 2, with the first 5 most abundant being amino acid biosynthesis (1.20 ± 0.0016%), nucleoside and nucleotide biosynthesis (0.84 ± 0.0014%), cofactor, carrier, and vitamin biosynthesis (0.77 ± 0.00079%), carbohydrate biosynthesis (0.43 ± 0.00052%), followed by carbohydrate degradation (0.38 ± 0.00091%) (Supplementary Fig. S10, Supplementary data 7).

In addition, we noticed different species contributing to metabolic pathways in the feces of geographically distant communities. Amongst the 95 detected species, 20 contributed to a metabolic pathway in all communities, such as *Escherichia coli,* or *Ruminoccoccus torques*. While 39 were involved uniquely in samples from the Adadle *woreda*, including *Bifidobacterium catenulatum, Bifidobacterium longum* and *Lactobacillus ruminis,* 25 species, notably *Blautia obeum* and *Treponema succinifaciens,* were not involved in any of the metabolic pathways found in the samples from the Adadle *woreda* (Supplementary Fig. S11). Additionally, we noted a high diversity of *Streptococcus* species contributing to metabolic pathways in samples from the Adadle *woreda*. These species were notably involved in carbohydrate degradation pathways, including starch (PWY-6731), lactose (LACTOSECAT-PWY) and galactose (PWY-6317), (Fig. 4) as well as stachyose (PWY-6527), sucrose (PWY-5384, PWY-621), and glycogen (GLYCOCAT-PWY, PWY-5941) (Supplementary Fig. S12, Supplementary data 3).

Last, we used Ward's linkage method for hierarchical clustering of the samples and identified two clusters (*Clust1* and *Clust2*) (Supplementary Fig. S13). Samples from the Adadle *woreda* (14/15) and from the transitional community of Lima, Peru (8/8) clustered mostly in *Clust2* and samples from the USA (29/29), El Salvador (9/10), and the traditional populations of Tanzania (10/11), Zimbabwe (18/19) and Peru (10/10) clustered mostly in *Clust1* (Supplementary data 7). Out of the 513 pathways, we identified 228 that showed significant differences in abundance between the two clusters (Wilcoxon rank test at a significance threshold of 0.05 with Bonferroni correction for multiple comparisons, Supplementary data 7). Of these, 6 were enriched in *Clust*1 and 222 were enriched in *Clust2*.

Amino acid biosynthesis superclass 2 was significantly different between the two clusters (p value = $1.272e^{-10}$) but contrasting results were observed at the pathway level with 37 out of 47 pathways related to amino acid biosynthesis enriched in *Clust2* (Supplementary Fig. S14). Out of the 27 carbohydrate degradation pathways, only mannan degradation (PWY-7456, p value = $7.367e^{-4}$) was enriched in *Clust1* while 12 pathways were enriched in *Clust2*, including the degradation of lactose (LACTOSECAT-PWY, p value = $3.696e^{-5}$), galactose (PWY-6317, p value = $2.221e^{-5}$), D-arabinose (DARABCATK12-PWY, p value = $4.17e^{-8}$), and stachyose (PWY-6527,

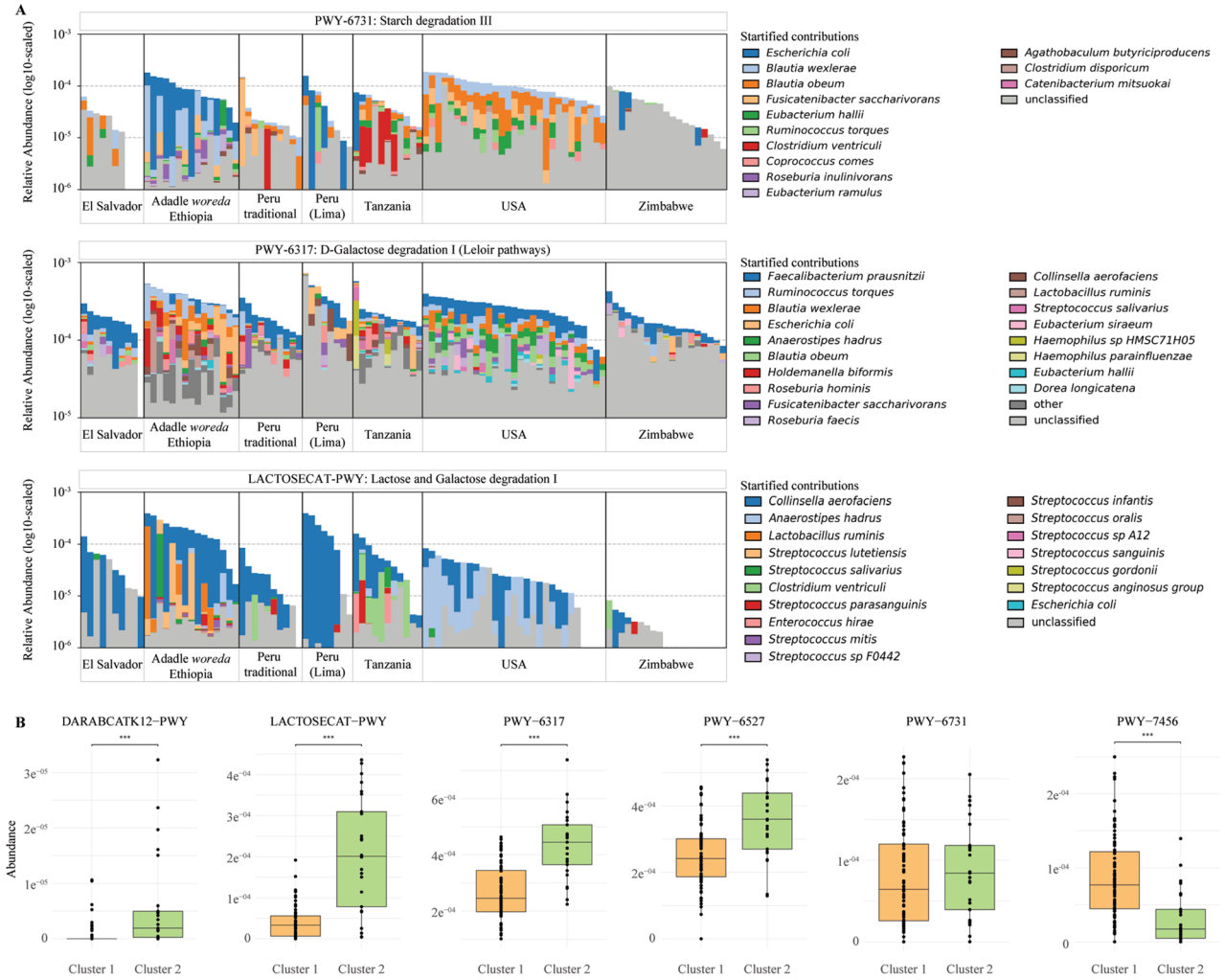

Figure 4. Enrichment of carbohydrate degradation pathways in the different clusters. (**A**) Stacked bar plots of the log10 of the relative abundance of species contributing to pathways PWY-6731, PWY-6317 and LACTOSECAT-PWY. (**B**) Carbohydrate degradation pathways enriched in the different clusters. DARABCATK12-PWY: D-arabinose degradation I, LACTOSECAT-PWY: lactose and galactose degradation I, PWY-6317: galactose degradation I, PWY-6527: stachyose degradation, PWY-6731: starch degradation III, PWY-7456: mannan degradation. Hierarchical clustering of the samples using Ward's linkage method. Pathways enrichment in the clusters tested with MaAsLin2 at q-value < 0.05. (***q < 0.05). N = 102.

p value = 2.143e^{-5}). Starch degradation was not enriched in either of the clusters (PWY-6731, *p* value > 0.05) (Fig. 4, Supplementary data 7).

Resistome

Finally, to assess for the presence of putative resistance genes in the gut microbiome of children from the Adadle *woreda*, we quantified the antibiotic resistome by mapping genes to the reference database CARD[35]. Among the 15 samples, we found 166 putative antimicrobial resistance (AMR) genes, potentially conferring resistance to 29 functional drug classes (Supplementary data 3). Antibiotic efflux was the most frequently detected encoded resistance mechanism, followed by antibiotic target protection, antibiotic target replacement, antibiotic target alteration, antibiotic inactivation, and reduced permeability (Fig. 5). We observed that AMR genes predicted to confer resistance against tetracycline were the most common, followed by AMR genes related to resistance against fluoroquinolones, penams (penicillin), and macrolides (Fig. 5). The most abundant genes were *tet(O)*, followed by *dfrF*, *tet(W)*, *tet(40), and Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin (Fig. 5).

In samples from other communities, genes, such as *tet(O)*, *tet(Q),* and *tet(W),* conferring resistance against tetracycline were consistently the most commonly detected resistance genes. Resistance against macrolide and streptogramin antibiotics and specific AMR genes, such as *cfxA6* and *cfxA2,* related to resistance to cephamycin were also frequent in samples from all communities. Additionally, in samples from the transitional community of Lima, resistance against rifamycin and mupirocin-like antibiotics conferred by *Bifidobacterium adolescentis rpoB* and *Bifidobacterium bifidum ileS*, respectively, were the most common resistance genes detected (Supplementary data 7). While samples from the Adadle *woreda* seem to cluster away from the other communities on the x-axis of the PCoA of the Jaccard distance, our data suggests that the position of the samples is correlated

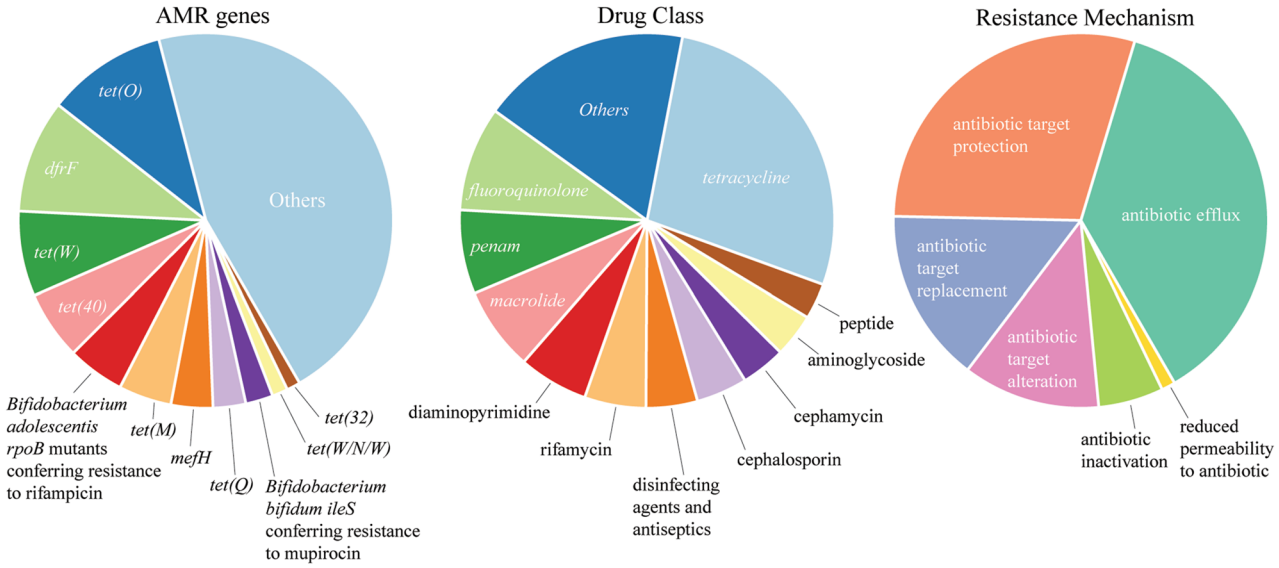

Figure 5. Overview of the resistome in the feces of children living in the Adadle *woreda*. From left to right: Antimicrobial Resistance genes (AMR). Drug classes to which AMR genes confers resistance. Resistance mechanism given by the AMR genes. *tet* tetracycline, *dfr* dihydrofolate reductase, *rpoB* rifamycin-resistant beta-subunit of RNA polymerase, *mef* major facilitator superfamily antibiotic efflux pump, *ileS* isoleucyl-tRNA synthetase, *penam* penicillin. N = 15.

Country	Population/region	Age range (years)	Lifestyle	N	Study
16S rRNA gene amplicon sequencing samples					
Primer set 1				705	
Central African Republic	Bangui	2–5	Transitional	274	[55]
Madagascar	Antananarivo	2–5	Transitional	431	[55]
Primer set 2				679	
Bangladesh	Dhaka (Mirpur)	3–4	Transitional	51	[56]
Cameroon	Baka, Bantu	4–5	Traditional	5	[57]
China	Nagqu, Hongyuan, Gangcha, Lhasa, Tianzhu, Gannan (Tibet)	2–5	Traditional	24	[58]
El Salvador	South of San Salvador	2–5	Transitional	29	[59]
Malawi	Mayaka, Mbiza	2–3	Traditional	13	[16]
Peru	Matses, Tunapuco	2–5	Traditional	11	[15]
	Lima	2–5	Transitional	8	[59]
Sweden	Halmstad	3–4	Industrial	335	[26]
Tanzania	Hadza (Sengeli, Hukamako)	3–5	Traditional	35	[42]
USA	St Louis, Philadelphia, Boulder	2–5	Industrial	8	[16]
	Los Angeles	2–5	Industrial	141	[60]
Venezuela	Platanillal, Coromoto	2–5	Traditional	19	[16]
Shotgun metagenomic sequencing samples				87	
Peru	Matses, Tunapuco	2–5	Traditional	10	[15]
	Lima	2–5	Transitional	8	[59]
Tanzania	Hadza	2–4	Traditional	11	[23]
El Salvador	South of San Salvador	2–5	Transitional	10	[59]
Zimbabwe	Chihuri, Mupfure	2–5	Traditional	19	[61]
USA	Rhode Island	2–5	Industrial	29	[62]

Table 2. Additional sequences sourced from previously published studies.

with the sequencing depth (Supplementary Fig. S15). Additionally, the presence and absence of the putative AMR genes and the drug classes were tested using generalized linear models, but none were significantly different between the communities.

Discussion

Here, we characterized the fecal microbiota composition and function of 59 agropastoral children, aged 2–5 years, from the Adadle *woreda* of the Somali Regional State of Ethiopia. With the use of 16S rRNA gene amplicon and shotgun metagenomic sequencing, our data suggest that these children harbor a specific microbiome. This community composition may reflect their dietary habits and that their microbiota is closer to that of children from transitional communities than to that of children living similar traditional lifestyles.

The observed difference in the microbiota composition between the agropastoralist children from the Adadle *woreda* and children from other communities is in line with the diet adopted by the population of the Adadle region. Their diet has an extremely low variety and consists mainly of milk and to a lower extent of starch-rich foods such as rice and wheat. None of the children had meat or fish in the last 24 h prior to sampling. Only a few children consumed tomatoes or onions but no other vegetables or fruits were reported as being consumed the day prior sample collection[27]. This is reflected in the composition of the fecal microbiota with, notably, a higher abundance of *Streptococcaceae*, *Bifidobacteriaceae*, *Lactobacillaceae* as well as *Akkermansiaceae* and a lower abundance of *Bacteroidaceae, Prevotellaceae, Succinivibrionaceae*, and *Spirochaetes*.

The higher relative abundance *Akkermansiaceae*, *Bifidobacteriaceae* and *Lactobacillaceae* is most likely due to the high consumption of milk in our study group. Indeed, *Akkermansiaceae* was recently shown to thrive on milk oligosaccharides in vitro[36] and *Bifidobacteriaceae* and *Lactobacillaceae* are well known to be boosted by the consumption of dairy products[11]. *Streptococci* thrive on simple sugars[37,38] and their high abundance might therefore be associated with the consumption of wheat and rice, one of the main food items consumed by the agropastoralists besides milk[27]. Additionally, metagenomic analysis of the bacterial community of Ethiopian traditional fermented camel milk[39], a commonly consumed milk in the Adadle region alongside milk from other livestock[40], revealed that species of *Streptococci* were amongst the most abundant and most prevalent detected bacteria. This may further explain their high abundance in fecal samples from the Adadle *woreda*. Interestingly, two *Bifidobacterium* species, *Lactobacillus ruminis,* and diverse *Streptococcus* species were found to be contributing to the degradation of carbohydrates. This suggests a primordial role of *Streptococci* in overall community metabolism in the samples from the Adadle *woreda*. Pathways for the degradation of lactose, one of the main constituent of milk[41], and D-galactose, one of the mono-saccharides forming lactose and stachyose, as well as simple carbohydrates such as D-arabinose and fucose were enriched in fecal samples from the Adadle *woreda*. These pathways likely reflect the abundant consumption of milk and food products composed of simple sugars in this community.

Species of the *Bacteroidaceae* family have been previously associated with a higher consumption of animal fat and protein in westernized societies[18,19,42]. The very low levels in *Bacteroidaceae* observed in our study group are likely linked to the low consumption of these food items. The enrichment of metabolic pathways related to amino acid biosynthesis observed in our study might be linked to the low protein consumption by the agropastoral children from the Adadle *woreda*. However, little is known on this subject and more investigations would be needed. In contrast with earlier studies[15,42–44], we did not observe an increased abundance of *Prevotellaceae, Succinivibrionaceae*, or *Spirochaetes,* which were previously associated with a traditional lifestyle[17]. This findings suggest that these taxa are likely dependent on the fiber-rich vegetables and fruit-based diets often observed in other traditional communities[18]. In our study, we observed an extremely low abundance of *Prevotella*, which contrasts with other studies of the fecal microbiome of populations with a traditional lifestyle[14–16,19,20,45]. Interestingly, *Treponema succinifaciens*, a member of the *Spirochaetes* family, was not found to be involved in any metabolic pathway in samples from the Adadle *woreda* but found to be involved in the degradation of D-galactose in samples from El Salvador and traditional communities of Peru, Tanzania, and Zimbabwe. This virtual absence of *Prevotella*, *Succinivibrionaceae* and *Spirochaetes* is probably a result from the adaptation of the microbiota to a diet poor in fiber and complex carbohydrates in the agropastoral children from the Adadle *woreda*.

The high abundance of *Erysipelotrichaceae* and *Erysipelatoclostridiaceae* is of surprise, as these families have been shown to increase upon consumption of a high-fat, westernized diet in mice[46]. Additionally, members of these families have been found in the gut microbiota of cattle, notably in Mongolia[47]. We hypothesize that the higher level of these taxa might be due to the closeness of the children with cattle. Further, the consumption of camel milk, in which fat matter is one of the main component,[41] could promote the growth of these taxa.

Additionally, we assessed the presence of putative antibiotic resistances genes in the feces of children living in the Adadle region. AMR genes mapped to the CARD database and predicted to confer resistance to antibiotic such as tetracycline, fluoroquinolones, penams (penicillin) and macrolides were notably detected, and we noticed variations in the pool of putative AMR genes in the different communities, but these differences were not statistically significant. The observed AMR genes were predicted by mapping against the CARD database, representing known genes. Other complementary machine learning methods as well as structural approaches should be used in future studies to predict putative AMR genes. Additionally, the expression of the observed AMR genes would need to be evaluated experimentally to confirm the resistance potential found in the feces of children from the Adadle *woreda*.

Even though the children from the Adadle *woreda* follow a traditional agropastoral lifestyle, we observed that their fecal microbiota composition and function was significantly different than the one in children from other traditional communities. In recent years, numerous studies have highlighted the associations between bacterial taxa and specific lifestyles[14–16,19,20,45,48–50]. In accordance with these studies, we showed that the agropastoral way of living of the children from the Adadle region shapes their microbiome. However, we observed different bacterial taxa being more prevalent and abundant than the usual taxa found to be associated with traditional communities. These differences are likely due to the high specificity and limited variety in the diet consumed by the children of the Adadle *woreda*. These findings highlight the importance of including dietary information in studies aimed

to characterize the intestinal microbiota. Further, additional factors such as the presence of parasitic infections or periods of dietary restrictions might also influence the microbiota composition in the Adadle region[27,28].

Our study has several notable strengths: to the best of our knowledge, it is the first study to describe the fecal microbiota in the Somali Regional State of Ethiopia. Further, the specific diet, dominated by milk products and starch-rich foods, is widely different from the diet of children previously studied. Last, using both 16S rRNA gene amplicon sequencing using two different primer pairs as well as shotgun metagenomic sequencing allow us to robustly profile the microbiota composition of the children from the Adadle *woreda*. However, as expected, using different sequencing methods and profiling tools revealed slightly differing results depending on the chosen method and tools. While the comparison between groups were not influenced by the profiling tools and sequencing methods, the description of the microbiota composition varied in abundance. As any study including secondary data analysis, our study has a few limitations. These include bias introduced by the fact that we were not able to control for sampling, storage, and DNA extraction methods in the data retrieved from public repositories. Further, the small sample size in our study group could influence the observation made on the microbiota composition and function of children from the Adadle *woreda*. This point should be addressed in future studies with larger sample sizes.

In conclusion, this study reveals a unique fecal microbiota composition and function of agropastoral children living in the Adadle *woreda* in the Somali regional state of Ethiopia. This unique microbial profile is likely influenced by their specific and low-diversity diet. Our study highlights the need to further understand the microbial composition of communities with different lifestyles and geographic origins in a bid to improve our knowledge on microbiota dynamics and the associated health outcomes. Such advances could ultimately be used to develop personalized and effective treatments for dysbiosis-associated diseases. This study sets a baseline for further research assessing dysbiotic microbiota which may occur during regular periods of malnutrition in the Somali regional state. Future research may also characterize livestock microbiota, as agropastoral communities live in very close proximity to their livestock and under poor sanitation and hygiene conditions. A One Health approach characterizing the microbiome in an interconnected manner will be crucial to better understand the specific profile found in this population.

Methods

Cohort/study population

This study included feces from 59 children aged 2–5 years, sampled in the context of a previous study on parasitic infection and micronutrient status conducted in the Adadle *woreda* (district) of the Somali regional state of Ethiopia, in the dry season between July and September 2016[27]. This region is mostly inhabited by pastoral and agropastoral Muslims. The original cross-sectional cohort study included 387 subjects from pastoral and agropastoral households, but only a small fraction corresponded to the age group selected for (2–5 years), had a height for age z-score score above − 1.5, and had a fecal sample available for DNA extraction and microbiota analysis (Supplementary Fig. S16). In total, 59 samples from children living in 3 different kebele (municipalities), Gabal, Higlo and Buursaredo were sent for 16S rRNA amplicon sequencing and 15 for whole-genome shotgun metagenomic sequencing (Supplementary Fig. S15).

Sample collection, DNA extraction and sequencing

Stool samples were collected as described by Osman et al.[27]. Briefly, plastic containers with detailed instructions for collection of fresh stool sample were given to mothers or caregivers and collected the same day of sample preparation and freezing at the local health facility. DNA was extracted using a commercial kit (QiaAmp DNA Mini Kit, Qiagen) with an additional bead-beating step according to a pre-established protocol[51]. In brief, 100 mg of feces were homogenized by bead-beating with 0.7–1.2 mm Granat beads (BioLabProducts GmbH) in 250 µl 2% Polyvinylpolypyrrolidone (PVPP) buffer (Sigma Aldrich). Then DNA extraction steps were conducted as indicated by the DNA extraction kit's manufacturer.

Extracted DNA samples were shipped to two different sequencing service providers (Microbiome Insights, Vancouver, Canada and Integrated Resource, Dalhousie University, Halifax, Canada) where library generation and sequencing were performed. At Microbiome Insights, library preparation was performed using the primer set v4.SA501-v4.SA508 (forward) and v4.SA701-v4.SA712 (reverse) (referred as primer set 1), targeting the 16S V4 region[52]. The amplicon library was sequenced on a MiSeq using the MISeq 500 Cycle V2 Reagent Kit (2 × 250 bp paired-end). At Dalhousie University, library preparation was performed using the 515F/806R primer pair (referred as primer set 2) which amplifies the V4 region of the 16S rRNA gene[53,54]. The amplicon library was sequenced on Illumina MiSeq (2 × 300 bp paired-end) using V3 chemistry.

Whole-genome shotgun metagenomic sequencing was performed by Eurofins Genomics (Eurofins Genomics Europe Sequencing GmbH, Konstanz, Germany) using the Illumina HiSeq (Sequence mode NovaSeq 6000 S2 PE150 XP).

Secondary data analysis of previously published studies

Additional sequences for reference groups were sourced from either the Afribiota project[55] (Table 2, Supplementary data 2, using primer set 1 with primers v4.SA501-v4.SA508/v4.SA701-v4.SA712) as well as several additional published studies (Table 2, Supplementary data 2, using primer set 2 with primers 515F/806R). The final 16S rRNA amplicon sequencing dataset included in addition to the 59 sequences from the Adadle *woreda*, 705 fecal samples from the Afribiota project (Primer set 1) and 679 fecal samples from other previously published studies (Primer set 2) described in Table 2. The shotgun metagenomic dataset included in addition to the 15 samples from the Adadle *woreda*, 87 samples from previously published studies (Table 2, Supplementary data 2).

Lifestyle classifications of the different populations are based on the original publications. Briefly, hunter-gatherers, pastoralists, agropastoralists and agriculturalists were classified as traditional; populations living in rural, peri-urban and urban area of low- and middle-income countries as transitional; populations from urban North American and European cities as industrial. Samples from the Adadle *woreda* are classified in this manuscript as Adadle agropastoralism in order to separate our samples from samples from other traditional communities.

Bioinformatic analysis of the 16S rRNA gene amplicon sequences

Bioinformatic analysis was performed using DADA2 (v1.22) according to a previously well-described standard pipeline[63]. Briefly, retrieved sequences were filtered and trimmed based on the sequencing quality (240nt forward reads, 220nt reverse reads) and paired-end reads were merged after dereplication and sample inference (Supplementary data 2). Taxonomy was assigned by matching the sequences to the Silva reference database (v138.1)[64]. Sequences alignment and phylogenetic tree construction were performed using DECIPHER (v2.22.0)[65] and phangorn (v2.10.0)[66] packages. Samples with less than 5′000 reads were excluded from the analysis. Further, mitochondrial DNA, chloroplasts as well as sequences with an assignment not belonging to the kingdom of *Bacteria* and *Archaea* were removed. Out of the 59 samples sent for amplicon sequencing, 54 using primer set 1 and 13 using primer set 2 passed quality filtering and inclusion criteria for analysis. Each primer dataset was processed separately, and the final taxonomy and sequence count tables were then joined for the final analysis (Supplementary data 3, 4, 5).

Raw sequences sourced from other studies were processed as described above in DADA2[63]. Individual studies were processed independently until merging of the sequence tables for joint chimeras' removal and taxonomy assignment. The sequences that passed the quality control are summarized in Supplementary data 2. Resulting amplicon sequence variants (ASVs) tables and taxonomy tables were filtered and processed as described above.

To correct for differences in sequencing depth for alpha and beta diversity analysis, samples were rarefied to 5′000 reads (Supplementary Fig. S17). Alpha diversity (species diversity) was measured using the Faith's phylogenetic diversity. For β-diversity analysis, singletons were removed, and logarithmic transformation was applied for principal coordinates analysis (PCoA) of WeightedUniFrac, Bray–Curtis, Jaccard and Generalized UniFrac distances at species level. Hierarchical clustering was performed using Euclidian distance and Ward's linkage method. The Calinski–Harabasz's index was calculated to obtain the optimal number of clusters to split the dendrogram resulting from the hierarchical clustering. Differential abundance analysis was performed using SIAMCAT (v1.14.0)[30] and LefSe[31] from the microbiomeMarker package (v1.0.2)[67].

Bioinformatic analysis of whole-genome shotgun metagenomic sequences

Shotgun metagenomic data were first treated with fastp (v0.20.1)[34] for quality control, trimming of adapters and quality filtering. Taxonomic assignation was performed using mOTUs profiler version 2[32] with the output in number of reads (Supplementary data 3) to corroborate the 16S rRNA taxonomic profile. Mitochondrial DNA, chloroplasts, as well as sequences with an assignment not belonging to the kingdom of *Bacteria* and *Archaea* were removed from the abundance table.

Profiling of microbial metabolic pathways was performed with HUMAnN 3.0 (v3.0.1)[33] using the taxonomy abundance table obtained with MetaPhlAn3 taxonomic profiler[33], the full ChocoPhlAn database (v296_201901b) from the BioBakery3[33] and the UniProt database (UniRef90_annotated_v201901b_full)[68]. The utility script humann_renorm_table with output in relative abundance was used to normalize the default HUMAnN's output reads per kilobase (RPK) and correct for different samples sequencing depths (Supplementary data 3). Metabolic pathways were classified using the MetaCyc Metabolic Pathway Database (MetaCyc 19.1) at the superclass 1, superclass 2 and pathways levels[69].

To identify antimicrobial resistance genes, the Resistance Gene Identifier (RGI *bwt* v6.0.0) was used to map reads on the Comprehensive Antibiotic Resistance Database's protein homolog model (CARD, v3.2.5, Supplementary data 3)[35]. Results were filtered for genes with at least 100 mapped reads and 80% coverage. Further, reads per kilobase million (RPKM) was used to correct for gene length and sequencing depth efforts.

Biostatistics analysis

Biostatistical analysis was performed in the R environment and language (v4.1.2, R Core Team, 2021) using the packages phyloseq (v1.38.0)[70], vegan (v2.5-7)[71], microbiomeMarker (v1.0.2)[67], microbiome (v1.6.5)[72], ape (v5.6-1)[73], picante (v1.8.2)[74] and clusterCrit (v1.2.8)[75]. Data visualizations were realized with packages RColorBrewer (v1.1-3)[76], ComplexHeatmap (v2.13.1)[77] and ggplot2 (v3.3.5)[78]. The detailed R-scripts can be found on github (https://github.com/VonaeschLabUNIL/Pastobiome).

Relative abundance differences and Faith's phylogenetic diversity differences were tested using Wilcoxon rank test at a significance threshold of 0.05 with Bonferroni correction for multiple comparisons. Differential abundance was analyzed using SIAMCAT (v1.14.0)[30] and LefSe[31] from the microbiomeMarker package (v1.0.2)[67]. A pseudo-count of $1e^{-4}$% was added to relative abundances of 0 for logarithmic transformation. Analysis of variance using distance matrix was performed using ADONIS2 from vegan (v2.5-7)[71] package with Benjamini–Hochberg correction for multiple comparisons. Enrichment of pathways was analyzed using MaAsLin2 (v1.8.0)[79] and SIAMCAT (v1.14.0)[30].

Ethical approval

The study was conducted according to the declaration of Helsinki, and ethical clearance was obtained from the Review Committee of the University of Jigjiga in Ethiopia (JJU-RERC 002/2016) and the Swiss Ethics Committee of Northwest and Central Switzerland (Ethikkommision Nordwest- und Zentralschweiz; EKNZ BASEC UBEreq. 2016-00204). A material transfer agreement was established by the Food, Medicine and Health Care

Authority of Ethiopia for the shipment of fecal samples from Ethiopia to Switzerland. All the parents/caregivers of the participating children gave oral and written consent prior to the study enrollment of their children.

Data availability

All raw data included in this study have been uploaded to NCBI Sequence Read Archives under accession number PRJEB61656. The datasets analyzed during the current study are available in the NCBI Sequence Read Archives repository under accession numbers: PRJEB48119, PRJNA547591, PRJNA392012, PRJNA381333, PRJEB13051, PRJEB3079, PRJEB38986, PRJNA300541, PRJEB27068, PRJEB27517, PRJNA521455, or on figshare repository, https://doi.org/10.6084/m9.figshare.7011272.v3.

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Author contributions

Designed the study: P.V., J.Z.; Acquired data: K.O., A.M., C.C., R.T.; Performed wetlab analyses: P.V.; Analyzed the data: S.Y., J.R.G., P.S., P.V.; Wrote the initial manuscript draft: S.Y., P.V.; all authors read and revised the final manuscript.

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Competing interests

The authors declare no competing interests.

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