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ChickFlows in Maputo, Mozambique: High-risk Behaviors, Management Practices, and Pathways for Childhood Exposure to Enteropathogens from Chickens

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Abstract

ChickFlows in Maputo, Mozambique: High-risk Behaviors, Management Practices, and Pathways for Childhood Exposure to Enteropathogens from Chickens

By: Frederica G. Lamar

Pathogens transmitted in animal feces account for 28% of diarrheal deaths in children <5 years old. Small-scale poultry production is ubiquitous and increasing in LMICs, yet the containment and management of poultry-associated fecal waste is minimal. This dissertation sought to provide data to inform potential interventions to reduce child exposures to enteropathogens carried by chickens.

We conducted a mixed methods study, using a triangulation convergence model design, in Maputo, Mozambique to understand high-risk pathways for child exposures to chicken-sourced enteropathogens. The first aim employed a value chain approach to map and characterize the broiler, layer, and indigenous chicken value chains. The second aim quantified microbial hazards along each value chain to determine carriage of enteropathogens and contamination of chicken meat at key settings. We collected chicken feces (*N=136*) and carcass samples (*N=75*) to detect *C. jejuni/coli*, *Salmonella* spp., and *Cryptosporidium* spp. and analyzed a subset of child stool samples (*N=64*) from the study area for *C. jejuni/coli*. The third aim used a time-series approach to assess the accumulation of *C. jejuni/coli*, *Salmonella* spp., and *E. coli* during chicken processing at informal markets. We collected rinse water (*N=70*) and broiler carcass (*N=60*) samples. Samples were analyzed using quantitative polymerase chain reaction (qPCR) assays and the Colilert-18 method.

High-risk exposure pathways are present along each value chain. We detected *C. jejuni/coli* in 84(76%) fecal and 52(84%) carcass samples, and *Salmonella* spp. in 13(11%) fecal and 16(21%) carcass samples in Aim 2 sampling. *Cryptosporidium* spp. was not detected. Children(92%) are infected with *C. jejuni/coli*. In Aim 3 sampling, *C. jejuni/coli* and *E. coli* were detected in 100% of samples, and *Salmonella* spp. were detected in 42% of rinse water and 48% of carcass samples, excluding baseline. *C. jejuni/coli* concentrations increased as more chickens were processed.

These findings illuminate food safety issues and highlight the need for properly managed poultry feces along each value chain. Informal markets are high risk for purchasing contaminated meat, which has the potential to seed household transmission. Our results provide the framework necessary to inform and design strategies to mitigate child exposures to enteropathogens carried by chickens.

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Introduction

Motivation

Zoonotic enteropathogens cause disease, and the highest burden of disease is in children.

Zoonotic diseases (transmissible from animals to humans) are a global public health threat, causing an estimated 2.7 million deaths annually.[1] Zoonotic enteropathogens are commonly transmitted in animal feces and cause diarrheal disease in humans.[2] Diarrhea contributes 74.4 million disability adjusted life years (DALYs) worldwide and is the eighth leading cause of death among all ages.[2] Though zoonotic enteropathogens cause disease in all age groups, diarrhea disproportionately affects children <5 years old as the fifth leading cause of death,[2] thus creating a focus on understanding the disease burden in this specific age group Pathogens transmitted in animal feces account for 28% of diarrheal deaths in children < 5 years old.[3] Although diarrheal deaths in children have decreased considerably since 2000, poor water, sanitation, and hygiene (WASH) remain a major contributor to mortality.[2] Exposures to animal feces along WASH-related pathways contribute to the remaining burden of diarrheal disease.[4]

The burden of enteric disease is further complicated by the asymptomatic carriage and transmission of zoonotic enteropathogens, which is common in children.[5–7] Persistent enteric infections in children are associated with environmental enteropathy,[5] stunting[8] or impaired growth,[5] malnutrition,[9] and poor cognitive development.[9] Household assessments have relied on self-reported cases of child diarrhea to determine risks associated with animal exposures,[10] but self- reported diarrhea has limitations as it is subject to bias.[11,12]A recent study in Ecuadorian children found that animal ownership was not a significant risk factor for child diarrhea with 7- day recall; however, the majority of child stool samples that were positive for enteric pathogens were solid (not loose stool), suggesting asymptomatic carriage.[10] The burden of asymptomatic carriage of enteric pathogens in children is unknown, but is hypothesized to cause environmental enteropathy and has been associated with linear growth faltering and impaired vaccine response, among other long-term health impacts.[13–16] Studies that do not perform microbial testing of child stool samples when assessing the influence of animal exposures on child health outcomes

may overlook asymptomatic infections, thus limiting our understanding of the complex relationship between animal exposures and enteric infections in children.

Poorly managed animal feces disproportionality affects populations in LMICs.

The overall burden of disease from poorly managed animal feces is unknown but likely substantial.[17] Annual global production of feces from animals (primarily cattle, chickens and sheep) is estimated at $29.7x10^9$ kg, approximately four times the amount of human fecal biomass, and ratios of animal feces to human feces are increasing.[18] Though exposures to animal feces are not limited to low and middle income countries (LMICs) and may occur in high income countries as well,[19,20] exposure risks associated with poorly managed animal feces are highest in LMICs,[18] where domestic livestock ownership is common.[4,21] In addition to livestock feces contaminating the environment, manure may be applied as organic fertilizer to crops or used as cooking fuel.[22] Even beyond the domestic setting, enteropathogens are highly prevalent in the environment in public areas where animal feces is common, and children play in these highly contaminated settings.[23,24] Proper animal fecal waste management has the potential to reduce human exposures to animal feces and prevent the contamination of water sources and the surrounding environment.[25]

Evidence assessing the effectiveness of animal husbandry interventions on reducing human health risks is scarce, and long-term evaluations of these interventions is limited. Interventions have generally focused on separating children from feces and corralling animals. Clean play spaces have reduced children's ingestion of soil and feces.[26] Improvements in household flooring have reduced parasitic infections and diarrhea prevalence in children, resulting in improvements in child cognitive development.[27] Studies have provided extensive training and technical assistance in communities to encourage corralling poultry but found short-term success.[28] Low-resource communities are sometimes resistant to corralling poultry due to the resources required to feed chickens.[29] Additional barriers to improving livestock hygiene include

fear of theft, cost of animal welfare supplies, time required to feed corralled animals, and preference for free- range chicken and eggs.[30]

Small-scale poultry production is increasing in LMICs.

Small- scale poultry production is already prevalent and is increasing in resource-limited areas as a means of providing nutrition, income, and food security for households.[31] Poultry, specifically village chickens, provide access to income for vulnerable populations who are at a higher risk of food insecurity.[31] Due to their small size , short production cycles, and minimal care requirements, poultry fulfill the availability dimension of food security where households can slaughter poultry in times of need.[31]

Despite its benefits, poultry carry highly pathogenic bacteria that pose a risk to child health. Scavenging poultry roam within and around the household searching for food waste, but also defecating in the environment.[31] Children may ingest chicken feces in the household setting directly or via contaminated soils and spaces.[29,32,33] Evidence suggests that children with household exposure to poultry are at an increased risk of diarrhea[4,34] and anemia.[35] Measures to limit exposures to chicken feces, such as corralling chickens, have not been sufficient in preventing exposures and ultimately decreasing negative child health outcomes.[36,37] Studies have reviewed the benefits of smallholder livestock, such as production, income, and empowerment, against the risks to child health, including enteric pathogen infections, impaired gut health, and undernutrition.[25] Overall, the relationship between poultry production and human health is complex and warrants further research.

One challenge to improving child health outcomes is that primary barriers to controlling exposures to animal feces have been largely overlooked in traditional WASH interventions.[17,38–40] Along the modified F- diagram,[17] household exposure assessments have focused primarily on contaminated fluids, fields, fingers, and fomites with less attention on fecal exposures via contaminated foods.[17,29,32,33] While household exposures to chicken feces are well-documented, investigation of exposure pathways beyond

the domestic setting is limited. Specifically, there is a gap in understanding the direct contamination of food from poorly managed animal feces in informal production spaces and exposures to contaminated foods coming home.[17]

Informal food systems may be a major contributor to enteropathogen exposures.

Informal food systems may play a major role in transmitting zoonotic enteropathogens to humans. *Campylobacter*, non-typhoidal *Salmonella* and *Cryptosporidium*, all carried by poultry,[41–44] are of high concern for their burden of disease and transmission in animal feces.[3] *Campylobacter spp*. is the second leading cause of global foodborne disease, and *Salmonella* and *Cryptosporidium spp*. are also important contributors.[45,46] Where informal production and marketing of poultry is common, contamination of chickens has been well documented.[47–54]

Each stage of poultry production presents opportunities for pathogen transmission.[42,55,56] NTS can persist in the farm environment and throughout the poultry value chain with contamination at all levels, including primary breeder and broiler farms, feed production, transportation, slaughter house operations, and processing.[57] With *Campylobacter* being ubiquitous in the environment, biosecurity measures alone have not been successful at preventing farm to processing contamination.[58] Vertical transmission of *Salmonella* and horizontal transmission of *Salmonella* and *Campylobacter* can occur within and between broiler flocks.[59,60] Reuse of poultry by-products, such as the application of chicken litter to produce on farms, may introduce contamination.[61] Beyond the farm environment, colonization in live chickens increases during transportation and holding before slaughter.[58,62] Slaughter and subsequent processing can contaminate chickens and the surrounding environment with *C. jejuni/coli* and *Salmonella* [49,51,52,63] present in the intestinal tracts of chickens.[43,64] Each of these stages favors the risk of contaminating the final food product[65].

Both *Campylobacter* and *Salmonella* are able to form biofilms on foods, allowing them to persist through the food supply chain.[66] Biofilm formation enables these organisms to better tolerate hot and cold temperatures, low pH, various environmental conditions, and antibiotics[67,68] and can only be removed following proper cleaning, disinfection, and sometimes mechanical force (i.e. scaping or chemical treatment).[69] At optimal production temperature for broilers and layers, *Salmonella* display their highest potential for biofilm formation in poultry house environments.[68] Critical surfaces for *Salmonella* and *Campylobacter* biofilm formation include common household items (i.e. refrigerators and cutting boards),[66] which suggests that the formation of biofilms from contaminated poultry meat and meat juice residues[70] entering the home may present risks of persistent household exposures.

Lack of formal food system regulations and enforcement is a challenge to food safety in LMICs,[47] and national-level data monitoring microbial hazards in food are scarce, leaving in question baseline food safety hazards associated with poultry in informal production contexts. Cross- over between informal and formal sectors adds to the complexity of food systems and is especially prominent in urban settings.[71,72] Longer value chains associated with informal production to distribution can cause challenges with traceability.[47,65,73] While consumers may be aware of food safety risks associated with purchasing from informal suppliers, lower-income consumers often do not have a choice in what they can afford to consume.[65]

Value chain frameworks are fundamental to understanding disease transmission risks within animal food systems.

A better understanding of value chains for food animals could illuminate where to target mitigation efforts and additional ways contamination can enter the domestic environment, including via contaminated animal source foods (ASF) and food products. Value chain frameworks can be implemented in informal settings to map, understand governance, and highlight sanitary risks along animal food systems.[74] There are numerous methodologies for mapping value chains.[65,75–77] Generally, mapping value

chains can include mapping the following: a) core processes; b) key stakeholders; c) product, knowledge, and geographical flows; d) volumes of products and monetary value; e) relationships and linkages; f) services; and g) constraints and potential solutions. [77] The process may end with a matrix to summarize key information into one table.[77] Mapping animal food systems exceptionally important in settings with limited foodborne surveillance and outdated, inadequate, and unregulated food safety policies.[65] Limited disease surveillance in LMICs may result in an underestimation of the foodborne disease burden and makes it difficult to disentangle the proportion of disease transmitted by food, water and the environment.[45]

Understanding food value chains in LMICs – apart from some exceptions[71,72,74,78–80] – has not been widely conducted, but could provide actionable information on the locations and risks of exposure to animals and animal feces. For example, mapping beef, sheep, and goat food systems in Nairobi allowed for the identification of potential sources of environmental contamination, meat contamination, and disease transmission and provided a foundation for investigating pathogen flows and exposure risks along animal food systems.[71] Mapping dairy food systems in Nairobi revealed the importance of improving food safety education in both the formal and informal sectors.[72] Poultry value chain studies in Asia and Africa were key to identifying entry points for highly pathogenic avian influenza (HPAI) outbreak intervention and control[81]. Without identifying potential risk-hotspots along food systems, we are limited in our abilities to implement successful public health strategies.[65] Investigating pathways of enteropathogen transmission along animal food systems is critical to understanding animal contributions to the burden of diarrheal disease in children. Value chain mapping provides the framework necessary to inform and design mitigation strategies.

Strategies to end food insecurity focus on food access, leaving the safety aspect of food security under-addressed

Food security is defined by all people having physical, social, and economic access to sufficient, safe, and nutritious foods.[82] Sustainable Development Goal Target 2.1 aims to end hunger and ensure access to safe and sufficient food throughout the year by 2030;[83] yet, indicators of food insecurity do not measure access to safe foods.[84] Strategies to reduce the burden of foodborne disease from animal-source foods may have far-reaching effects if food security efforts include a specific focus on access to *safe* foods. The above sections describe human health impacts, primarily in children, from exposures to enteropathogens carried in animal feces and how exposure risks may increase with increasing livestock production. Value chain frameworks can guide the identification of disease transmission risks along these animal food systems, and their findings may push for greater attention to food safety along these value chains.

Dissertation Research

This dissertation research is part of the Chicken Exposures and Enteric Pathogens in Children Exposed through Environmental Pathways (ChEEP ChEEP) study in Maputo, Mozambique. ChEEP ChEEP is a mixed methods study, using a triangulation convergence model design to understand exposure risks to poultry- associated pathogens and identify potential mitigation strategies to reduce and eliminate exposure risks in children. Given the majority of poultry production in Mozambique is from small- scale operations with low biosecurity[85], we believe there is no single location or behavior that potentially exposes children to chicken- related pathogens, but instead a network of high- risk interactions along the entire poultry value chain that could lead to transmission events. The purpose of this dissertation was to provide actionable information to inform potential interventions to reduce exposure to chicken-sourced fecal pathogens in children under five years old. Our study maps the chicken value chain in Maputo and documents management practices, microbial hazards, and potential exposure pathways along the chicken production value chain that potentially put children at risk of exposure to chicken-related enteropathogens. Novel to the WASH field, we take a food systems approach to characterize sanitary risks along chicken production and marketing processes. This work contributes to discourse on food insecurity as small-scale poultry is being encouraged as a means of providing access to food, with the

safety aspect of food security being largely overlooked. The findings have implications for child health in other LMIC settings where small-scale livestock production is widely practiced and presses the safety component of food security in these settings.

Dissertation Aims

The overall goal of this dissertation was to understand risks to child health along the informal chicken production food system in Maputo, Mozambique. This dissertation fulfills three core research aims. Research aims are outlined in **Figure 1** to show the integration of the mixed methods triangulation convergence model design.

Aim 1: Characterize and map the chicken value chain (ChickFlows) for broilers, layers, and indigenous chickens in Maputo, Mozambique, highlighting potential high- risk pathways for exposure.

Aim 2: Quantify food safety hazards (*C. jejuni/coli*, *Salmonella*, and *Cryptosporidium* spp.) along the chicken value chain.

Aim 2a. Update ChickFlows to reflect microbial hazards at key settings along each chicken value chain.

Aim 2b: Identify which chicken has the highest carriage between broilers, layers, and indigenous.

Aim 2c: Identify the setting of highest carriage.

Aim 3: Assess the time-dependent accumulation of *C. jejuni/coli*, *Salmonella* and *Escherichia coli* on broiler chicken carcasses and in processing water at informal markets.

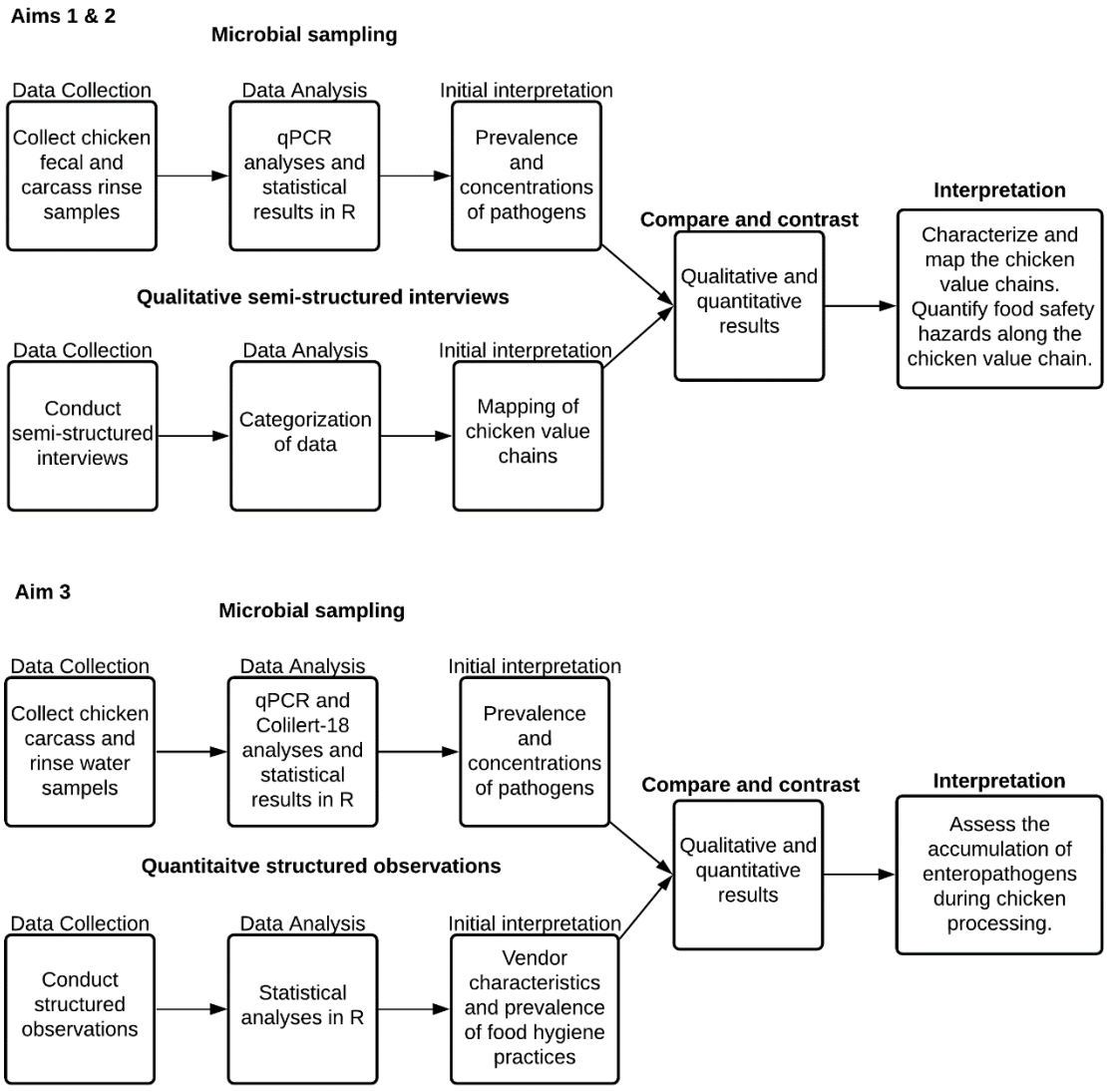

Figure 1. Diagram of Aims 1-3 showing the mixed methods triangulation convergence model study design.

Aim 1 maps and characterizes the chicken value chains (ChickFlows) for broilers (raised for meat production), layers (raised for egg production), and indigenous chickens (free- roaming chickens raised for meat and egg production), highlighting potential high-risk pathways for exposure to enteropathogens carried by chickens. There is a paucity of data highlighting both childhood exposures to chicken feces in low-income settings and differences in potential exposure pathways between broilers, layers, and indigenous chickens. Chapter 1 answers unknowns associated with childhood exposures to chicken-related enteropathogens. First, mapping chicken value chains identifies additional locations beyond the domestic setting where children can become exposed to chicken-related enteropathogens. Second, understanding the sourcing and distribution of chickens, eggs, and chicken production byproducts allows for a better understanding of where to target exposure mitigation efforts. Chapter 1 provides evidence of potential enteropathogen exposures via the food system and a value chain framework that can be adapted to other contexts and implemented as a tool to first understand food-safety hazards and risks before developing interventions.

Aim 2 investigates microbial hazards along the chicken value chain to identify which chicken types and key settings have the highest risk and carriage of chicken- related enteropathogens, which is currently unknown. Children in Maputo have *Campylobacter* and *Salmonella* infections[86,87] bringing into question where along the chicken value chain chicken- related enteropathogens are most likely to be detected and where they are detected in the highest concentrations. This data informs potential risks at locations, identified in Chapter 1, where children are most likely to be exposed from Chapter 1. Chapter 2 investigates pathogen carriage in all three chicken types and goes beyond reporting pathogen prevalence to also quantify microbial loads on chicken meat and in feces. Understanding where shedding occurs in the highest concentrations provides relative information on which points of contact pose the greatest exposure risk. This baseline information is necessary for targeting appropriate exposure pathways and management practices for potential interventions. Chapter 2 also provides evidence of *C. jejuni/coli*

infections in children, making a plausible link between exposures to chicken feces and acquiring infections with the specific *Campylobacter* spp. species carried by chickens.

Aim 3 examines the accumulation of chicken-related enteropathogens during chicken processing at informal markets. In Chapter 2, we found considerable contamination at informal markets; *C. jejuni* DNA was detected in 100% of carcasses sampled. This result prompted the question of how WASH and food hygiene practices contribute to the cross-contamination of chicken meat as chickens are processed and cleaned at informal markets. To our knowledge, investigating the time-dependent contribution of processing activities, particularly the reuse of rinse water, on contaminated meat allows for a better understanding of the role informal market hygiene plays in enteropathogen risk. Studies monitoring *Campylobacter* and *Salmonella* during chicken processing have largely been conducted in commercial or high-income settings with formal hazard analysis critical control points (HACCP) protocols.[88,89] Our study answers how processing activities impact the quality of raw chicken meat in a population where 90% of low-income households buy food from informal suppliers.[90] We contribute evidence of additional sources of potential transmission of enteropathogens, via foods entering the household, which are not typically considered in WASH studies.

Study Setting

Maputo City, having a population of approximately 1.1 million,[91] was selected based on its peri-urban context and growing poultry sector as development agencies in the region actively promote chicken production.[92–94] Maputo province is the leading producer of chicken meat in Mozambique.[93] Poultry production in Mozambique varies by scale: small, medium, and large.[85] Domestic production of chicken meat in Mozambique is exceeded by consumer demand. As a result, eggs and chickens are imported legally and illegally from neighboring countries.[85,95] Mozambique's poultry industry has grown mainly as a result of government and NGO cooperation.[85,93] Small- scale production and informal marketing are

essential to Mozambique's poultry sector with 70% of production capacity contributed by informal, small-scale farmers raising up to 5,000 chickens.[95] Additionally, 71% of Maputo households are food insecure, and the majority of households rely on markets and small shops for purchasing chickens.[96] There is minimal veterinary oversight and enforcement of regulations and microbial standards for poultry production and marketing.[85]

Child health remains a major challenge in Mozambique with diarrhea accounting for 6% of childhood (<5 years) deaths in 2019.[97] In a recent controlled before-and-after trial to evaluate sanitation impacts on child health in in Maputo, *Salmonella*, *Campylobacter*, and *Cryptosporidium* were detected in 21% (at baseline, N = 759), 9% (at 24-month follow up, N = 921), and 3% (at 24-month follow up, N = 921) of children's stools < 48 months old, respectively.[86,87]

Study Design

The ChEEP ChEEP study is a three-year mixed methods study using a triangulation convergence model design. ChEEP ChEEP was designed to be iterative with subsequent phases and research questions developed and informed by the results of the previous phase. Dissertation data were collected from June 2018 through September 2021.

Aims 1 and 2 are cross- sectional. Semi-structured surveys were administered to determine sourcing and selling of chickens and eggs, characterize management practices, and understand where children have the potential to come into direct and/or indirect contact with chicken feces (Aim 1). Observations were performed at informal markets to understand food hygiene practices (Aim 1). Chicken carcass (n=75) and fecal samples (n=136) were collected from broilers, layers, and indigenous chickens at key settings identified during formative research: depots selling broiler chicks and farming supplies, small-scale farms, informal markets, grocery stores, corner stores, and households. Carcass and fecal samples were analyzed by quantitative polymerase chain reaction (qPCR) for *Campylobacter* spp., *C. jejuni/coli,*

Salmonella, and *Cryptosporidium* spp. to quantify prevalence and microbial concentration across settings and chicken and sample types (Aim 2). Value chains were updated to highlight the accumulation of enteropathogens as chickens move along value chains and to compare exposure risks across settings.

Aim 3 consists of collecting time-series data from broiler vendors at informal markets. Matched chicken carcass and rinse water samples, starting with the first broiler chicken that was processed, were collected at 75-minute increments. The main outcome of interest was time-dependency of pathogen concentrations in samples as chickens were processed. Concurrent with sample collection, observations checklists were conducted to record specific vendor stand characteristics and hygiene practices. Carcass and rinse water samples were analyzed by qPCR to quantify prevalence and microbial concentration of *C. jejuni/coli* and *Salmonella* and by IDEXX Colilert-18 to quantify *E. coli* and capture contamination with additional fecal bacteria.

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Chapter 1. ChickFlows: A value chain approach to characterizing risks of chicken-related enteropathogen exposure for children in Maputo, Mozambique[1]

Abstract

Food production animals are ubiquitous in low-and middle-income countries within the domestic environment and formal and informal food systems. Small-scale poultry farming is a growing development strategy, providing nutrition and income, but also yielding increased biohazardous waste. With limited hygiene protections in place, exposure to zoonotic pathogens can increase risks to human health. This study examines the value chain associated with chickens and their byproducts and childhood exposure to chicken-related enteropathogens in Maputo, Mozambique. Semi-structured interviews were conducted at depots, small-scale farms, live informal markets, grocery stores, corner stores, and households to map broiler, layer, and indigenous chicken value chains, understand management practices, and characterize opportunities for childhood exposure to chicken fecal contamination. Food safety hazards and microbiological risks were present throughout each value chain. This mapping approach can be broadly applied to animal value chains in other settings to first understand sanitary risks from animal production and associated exposures to enteropathogens to inform mitigation strategies.

[1] This chapter is a manuscript formatted for submission to PLOS Global Public Health. The structure is consistent with journal requirements.

Introduction

Diarrhea caused by enteropathogen infections kills approximately 446,000 people annually,[1] predominantly young children under 5 years. The burden of disease attributable to zoonotic pathogens is unknown, but potentially substantial,[2,3] and our understanding of the dominant exposure pathways is limited[4]. Domestic animals are ubiquitous in low- and middle-income countries (LMICs), and annual global production of animal feces is estimated at 29.7×10^9 kg, approximately four times human fecal biomass[5]. Children have frequent contact with animals and their feces in highly contaminated domestic settings[2], and through unhygienic disposal and reuse of animal byproducts and via the food system. The water, sanitation, and hygiene (WASH) sector has focused on containment of human waste, but has yet to develop effective strategies for the containment and safe use of animal waste within domestic environments or informal food systems.[5,6] By contrast, the agriculture sector primarily focuses on the access element of food security,[7] while food safety remains an underrepresented element, especially in LMICs.[8]

Small-scale and family poultry farming provide income, food, and nutrition, contributing to multiple Sustainable Development Goals,[9,10] but can only improve health outcomes if foods are safe to eat.[8] Poultry farming is the fastest growing livestock subsector, with a 250% increase globally in the past 30 years.[11] Poorly managed poultry feces presents microbiological risks, especially within shared settings between poultry and children.[12] Poultry meat is the primary exposure route for foodborne campylobacteriosis and a major exposure route for foodborne salmonellosis.[3,13] *Campylobacter* and non-typhoidal *Salmonella* infections caused an estimated 78,264 deaths globally in children under 5 in 2016.[1]

Human and animal health may be linked through socio-economic, nutritional, and zoonotic disease transmission pathways.[14] Value chain frameworks[15] are therefore useful for characterizing food systems and are an emerging tool for animal health assessments.[16,17] Studies to understand food value chains in LMICs – apart from some exceptions[18-22] – have not been widely conducted, but could provide actionable

information on the locations and risks of exposure to animals and animal feces. In Mozambique, 89,000 tons of chicken were produced in 2017,[23] and 29% of children enrolled in a recent trial had *Salmonella* or *Campylobacter* infections at baseline.[24] Yet there is limited characterization of poultry production and potential childhood exposures, a research gap that extends throughout sub-Saharan Africa and is an important element of food security – safety.

This novel, multi-disciplinary approach to WASH and food security, notably safety, examines childhood exposure risks to enteropathogens via zoonotic transmission, using data from peri-urban Maputo, Mozambique. We take a food systems approach to understand "ChickFlows", the value chain of chickens and eggs from importation through consumption. The objectives of this study were to: 1) Map ChickFlows for broilers (raised for meat), layers (raised for eggs), and indigenous chickens (free-range and raised for meat and eggs); 2) Understand chicken management practices affecting food hygiene risks; and 3) Characterize opportunities for childhood exposure to chicken-sourced enteropathogens. This approach can serve as a model for future articulation of risks and hazards to child health and food security and as a way to prioritize interventions to reduce exposure to zoonotic enteropathogens.

Methods

Ethics statement. The Institutional Review Board at Emory University (IRB00108546) and the Research Council to the Veterinary Faculty at Eduardo Mondlane University determined that this research is exempt from further human subjects review, and the Municipality of Maputo (Reference number 754/SG/426/GP/2019) authorized this research. Prior to each interview, the study's purpose and participant rights were explained in Portuguese, and participants provided verbal informed consent.

Study setting. Maputo City, having a population of approximately 1.1 million,[25] was selected based on its peri-urban context and growing poultry sector as development agencies in the region actively promote chicken production.[26-28] Mozambique's agricultural sector contributes 24% to its gross domestic product

(GDP).[29] Nationally, chickens make up nearly half of small and medium farms for livestock production.[30] The government's investment in hatcheries, production of day-old chicks, and increased control over imported chickens has contributed to the growth of Mozambique's poultry sector.[31] Nine in-country hatcheries provide 46.5 million day-old chicks annually[26]. Chicken consumption and production has grown from approximately 56,000 tons of production and 61,000 tons of consumption in 2013 to 89,000 tons of production and 91,000 tons of consumption in 2017.[23] Maputo leads poultry meat and egg production in Mozambique.

Practices are highly variable between the formal and informal chicken production sectors, depending on the scale of operations, level of expertise, and capital to invest in process controls, with direct implications for disease transmission.[31] Few regulations exist and enforcement is scarce. Commercial producers follow HACCP guidelines and test for bacterial agents, such as *Salmonella* and *Escherichia coli*. Smaller, informal operations do not have resources to monitor quality. The formal and informal sectors overlap,[31] with the reselling of commercial goods at informal markets and corner stores. The National Directorate of Agricultural Health and Biosafety monitors imported chicken products, and the National Inspection of Economic Activities inspects commercial activities. The National Institute for Standards and Quality (INNOQ) provides norms for chicken production,[32] but these are available only for a fee, thus limiting access among farmers with less resources. Regulatory oversight of the informal sector is minimal.

This study is the first phase of a larger multi-phase and triangulation convergence model mixed methods study- ChEEP ChEEP (Chicken Exposures and Enteric Pathogens in Children Exposed through Environmental Pathways) - to identify potential intervention strategies to mitigate risks to children associated with exposure to enteropathogens of poultry origin. Identifying these key additional points of exposure represents an important opportunity to design additional targeted strategies. This first phase was implemented to generate evidence of potential hazards and risks to children.

Formative research. A formative study of Maputo's chicken value chain was executed between June 2018 – September 2019 whose goal was to identify key settings along the chicken value chain that potentially posed a high risk for exposing children to chicken fecal contamination. Forty key informant interviews were conducted with 18 small-scale farmers, two commercial producers, two processing plant clerks, two veterinarians, five market vendors, two street vendors, one feed mill clerk, one hatchery clerk, two depot clerks providing day old chicks, two restaurant owners, one grocery store clerk, and three administrators from local foundations promoting village poultry production and poultry farmer associations. Open-ended questions were asked to understand the flow of chicken products, opportunities for direct and indirect exposures to children, and challenges that affect management practices and hygiene. A preliminary "ChickFlows" map was developed from the formative research. Low biosecurity measures at small-scale farms, butchering and food hygiene practices at markets, free-roaming indigenous chickens and the application of chicken-sourced compost to gardens at households were noted as potential high-risk scenarios for chicken fecal contamination. These key findings informed the identification of key settings for additional data collection. Small-scale farms, markets, and households were identified as key settings for direct exposure to chicken feces or indirect exposure via cross-contamination of foods or an accumulation of chicken fecal enteropathogens downstream the chicken value chain.

Study design. To refine the preliminary ChickFlows map and associated unconventional exposure risks to children, we conducted qualitative interviews and observations at poultry supply depots, small-scale farms, markets, grocery stores, corner stores, and households.[21,33-37] In-depth interviews and observations were suitable for this study to learn about a range of experiences and complex behaviors and opinions[33] and to directly observe specific behaviors [38]. Both methods have been used extensively in WASH and agricultural research.[21,34-37] We conducted 77 semi-structured interviews with two depot store clerks, 18 broiler farmers, six layer farmers, two farmers raising both broilers and layers, 20 live informal market (market) vendors, six grocery store clerks, four corner store clerks, and 19 household respondents.

Survey objectives were to understand: 1) sourcing, pricing, and seasonality of chickens and eggs, 2) waste management procedures, 3) chicken management, disease prevention, and food hygiene practices, 4) governance and regulatory oversight, and 5) interactions between children and chickens. Additional setting-specific questions were developed as detailed below. We conducted eight structured observations at markets. Observations aided to capture WASH and food hygiene behaviors and interactions between children and chickens or eggs.

Depots. Depots are extensions of commercial producers, supplying broiler chicks, farming supplies (feeders and drinkers), medicines, vaccinations, and feed. Day-old chicks are ordered in advance and arrive on 1-2 pre-specified days each week in boxes of 80-100 chicks each. Interviews included specific questions about the types of feed, medicines, and vitamins sold to farmers, usage of antibiotics, advice given to farmers, and waste management practices.

Small scale farms. Small-scale farmers greatly outnumber commercial producers, producing five to eight cycles per year. A majority (56%) of chicken farms in Maputo are small-scale, producing 100-2,000 birds per cycle. Small-scale farmers operate individually, selling mature birds at markets.[31] Interviews included specific questions about management and hygiene practices, disease prevention measures, and types of feed administered to chickens.

Informal markets. Market vendors sell live, butchered, and cooked broilers, layers, and indigenous chickens. Vendors purchase chickens to sell from local producers. Eggs are sold by separate vendors. Egg vendors purchase eggs to sell from local producers and neighboring countries. Interviews included specific questions about any feed or medicines given to chickens while at the market, and food hygiene and sanitation practices. Direct observations of food hygiene, chicken handling, and butchering practices were also carried out at markets. Same-day, morning (~9:00AM) and afternoon (~3:00PM) observations were conducted for each market at 30-minute intervals for one hour. Enumerators used a pre-defined

checklist to record WASH and food hygiene actions and interactions between children and chicken products.

Grocery stores. Grocery stores receive chickens and eggs from commercial producers. Interviews included specific questions about chicken meat and egg storage conditions, food hygiene practices, and any measures taken to preserve meat and egg quality.

Corner stores. Corner stores import frozen whole chickens from neighboring countries, and buy chicken parts from informal markets. They function separately from grocery stores and have a license administered by the municipality for commercial activity. Corner stores were asked the same questions as grocery stores.

Households. Households own indigenous chickens, as well as broilers, layers, and other poultry, such as ducks and geese. Interviews included specific questions about the knowledge of diseases passing from chickens to humans, chicken husbandry practices, and feed and medicines, if any, given to indigenous chickens.

Data collection and management. Observations and responses to interviews were recorded using pen and paper by one male and one female enumerator. Enumerators were trained over a period of seven days. Training included (1) a review of research ethics and informed consent, (2) study protocols, (3) group back translation, reframing and review of interview questions for each survey instrument and observations, and (4) piloting and revising surveys. Each question was back translated to English to check translation accuracy, and then translated back into Portuguese for the final tools. Enumerators reviewed surveys for completion daily. Data entry errors were checked, validated against paper survey responses, and corrected. Field staff and the field supervisor debriefed regularly to identify key themes as data was

collected. Open-ended responses and data were entered into a spreadsheet weekly. Interview responses were written in Portuguese, and then translated into English.

Selection of study districts, sites, and participants

Districts. Data were collected in six of seven Maputo City districts (Nlhamankulu, KaMaxaquene, KaMavota, KaMubukwana, KaMpfumo, and KaTembe) to capture variability across socioeconomic levels, population densities, and small-scale chicken production processes; we did not collect data in the seventh district, KaNyaka, because it is an island representing 0.5% of the population, having no broiler farms and very low production of indigenous chickens. Chicken production varies between districts, thus influencing which sites and the number of sites included in each district. There were no limitations on size of operations for inclusion.

Depots. Poultry supply depots were defined as stores that sell broiler chicks, medicines, feed, equipment, and other supplies needed for poultry raising. Poultry supply companies that supply depots were contacted to compile census of 10 depot locations. The two depots included in our study represent leading poultry distributors in Mozambique.

Informal markets. Markets were outdoor, open-air markets that sell various goods, including chickens and eggs. Field staff compiled and confirmed a census of 18 markets. Each market was visited to confirm if they sold chickens. Only markets selling chickens were included. Following the 2019 construction of a bridge connecting KaTembe to the inner city, sellers have shifted to selling at markets within inner-city district and no markets were identified in KaTembe.

Households. Households were defined as a group of people sharing a common living space that have children under five years old and who do not raise chickens for sale. However, some may have owned or raised chickens for personal consumption. Households were included if they owned indigenous chickens.

Grocery stores. Grocery stores are enclosed shops or supermarkets that sell goods, including prepackaged chickens from commercial farmers in Maputo or imported from other countries. A Google Maps search of grocery stores was used to generate a census of 24 grocery stores, in which the majority of locations were chain supermarkets; local staff members confirmed that no major supermarkets were overlooked. The six grocery stores included represent the major supermarkets within each district. No grocery stores were identified from our census in KaMaxaquene and KaTembe. Prior to data collection, grocery stores were visited to receive permission to return for survey administration.

Corner stores. Corner stores are neighborhood convenience shops that sell household goods and chicken products. Corner stores were added as a study setting after learning that they are a popular and cheaper alternative to grocery stores, especially in districts with fewer grocery stores. Corner stores were convenience sampled in three districts having a limited number of grocery stores based on the knowledge of local team members.

Small-scale farms. Small-scale farms were defined as individual farms raising broilers or layers for sale directly to customers or resellers, who do not function as contract farmers by returning the flock back to commercial abattoirs for slaughter. Since no formal farming registries were available, and for logistical reasons related to obtaining permissions, neighborhood secretaries were consulted to provide a list of known small-scale farmers raising broilers and layers, and households keeping indigenous chickens. Small-scale chicken farming is practiced most widely in KaMubukwana, but is not allowed within KaMpfumo, the city center, due to noise and smell concerns. We did identify one small-scale farm within KaMpfumo; however, no households owning indigenous chickens were identified.

All censes were confirmed by local enumerators and a local agricultural consultant. Censes were randomized for selection within each district. GPS coordinates were recorded for all locations.

Data analysis. Consolidation of data and mapping of value chains were adapted from Carron et al. (2017).[21] Interview and observation data were entered into Excel and translated by enumerators. Interview responses were categorized in Word according to the five survey objectives. Additional data from field notes taken during the interviews were written in English. This categorization of data enabled the mapping of separate value chains for broilers, layers, and indigenous chickens, including eggs. First, key settings (depots, small-scale farms, markets, grocery stores and corner stores, and households) in the farm to fork framework were identified. Next, branches were connected to show ChickFlows throughout this framework. Flows from commercial production were included to display the intersection of the commercial and informal production sectors. External sourcing was included to highlight the importance of international trade of chickens and chicken products to the chicken value chain. While each setting along the chicken value chains have the potential for pathogen accumulation, settings with the greatest potential for pathogen accumulation were shaded gray. Grocery stores, corner stores, and restaurants should have adequate temperature controls to prevent spoilage, and chicks do not stay at depots for extended periods of time (same-day delivery and pick up).

Three overarching themes emerged from a full review of the data: human health, animal health, and governance. Subthemes emerged, were mapped to major themes, and were categorized for each setting: chicken management practices,[39-42] human health hazards,[43,44] exposure risks to children[45-47]and risk reduction measures.[48-50] Governance and regulatory oversight were also included as drivers of management practices and food hygiene. Market observation data were categorized by WASH measures, food hygiene, and interactions with children. Observed direct contact with chickens and chicken products and potential indirect contact with chicken fecal contamination were summarized for each setting.

Results

Summary of data collection sites. A total of 77 semi-structured interviews and eight observations were conducted at depots, small-scale farms, live informal markets (markets), grocery stores, corner stores, and households in six districts of Maputo (**Tables 1 and 2**).

Table 1. Number of sites, per district, where semi-structured interviews and observations were conducted in the study.

District	Depots	Small-scale farms	Live, informal markets	Grocery stores	Corner stores	Households	Total
Nlhamankulu	-	2	4	1	1	3	11
KaMaxaquene	-	4	1	-	2	3	10
KaMavota	-	4	1	1	-	4	10
KaMubukwana	1	10	3	2	-	4	20
KaMpfumo	1	1	1	2	-	-	5
KaTembe	-	5	-	-	1	5	11
Total	2	26	10	6	4	19	67*

*Multiple interviews were conducted at markets. Therefore, there are 67 sites, but 77 interviews.

Table 2. Flock size for each setting and characteristics of respondents.

Setting	Size of flock, if applicable	Gender (Average age) of respondents
Depots	Broiler chicks available for pick-up - Depot 1: 7300 chicks - Depot 2: 640 chicks	1 Female (22) 1 Male (23)
Small-scale farms	Broiler farmers - <500 broilers: 13 - 500-1000 broilers: 6 - >1000 broilers: 1 Layer farmers - <100 layers: 3 - 100-200 layers: 2 - 200-300 layers: 3	12 Females (50.5) 14 Males (38.2)
Live, Informal markets	Broiler vendors - <100 broilers: 6 - 100-210 broilers: 2 Layer vendors - 1-50 layers: 3 Indigenous chicken vendors - 1-15 chickens: 4 Egg vendors - 15 dozen: 2 - 10 boxes with 15 dozen: 1	12 Females (40.5) 8 Males (27.5)
Grocery stores	NA	6 Males (26.5)
Corner stores	NA	4 Males (37.3)
Households	Indigenous chickens - 1- 15 chickens:19	9 Females (40.4) 10 Males (40.8)

Two small-scale farms had both layers and broilers. One market vendor had broilers and indigenous chickens, and one vendor had layers and indigenous chickens.

ChickFlows: Broiler, Layer, and Indigenous Value Chains

Three chicken types were identified, broilers, layers, and indigenous, and value chains were mapped (**Figures 1-3**). Commercial broiler producers import parent flock from neighboring countries (primarily South Africa) to produce day-old chicks at hatcheries. Broilers and layers are raised both formally and informally. For broilers, fully integrated companies own all stages of production. Commercial broiler producers often employ medium-scale farmers as contract growers, returning market-ready broilers to commercial abattoirs for processing and distribution. Approximately 90% of layer egg production was external to Mozambique in 2013[31]. While domestic production has grown, eggs remain largely imported from South Africa and Swaziland. Indigenous chickens are raised informally, and their distribution is primarily to households and markets. They contribute to income and household food and are important for local cultural practices.

Management, Hazards, Risk Reduction, and Childhood Exposures

Exposure risks and biohazard measures were identified via structured observation and surveys at each point along the value chain and are described below. Key themes identified from participants' interview responses, categorized by governance, animal health, and human health, are displayed in **Figure 4**.

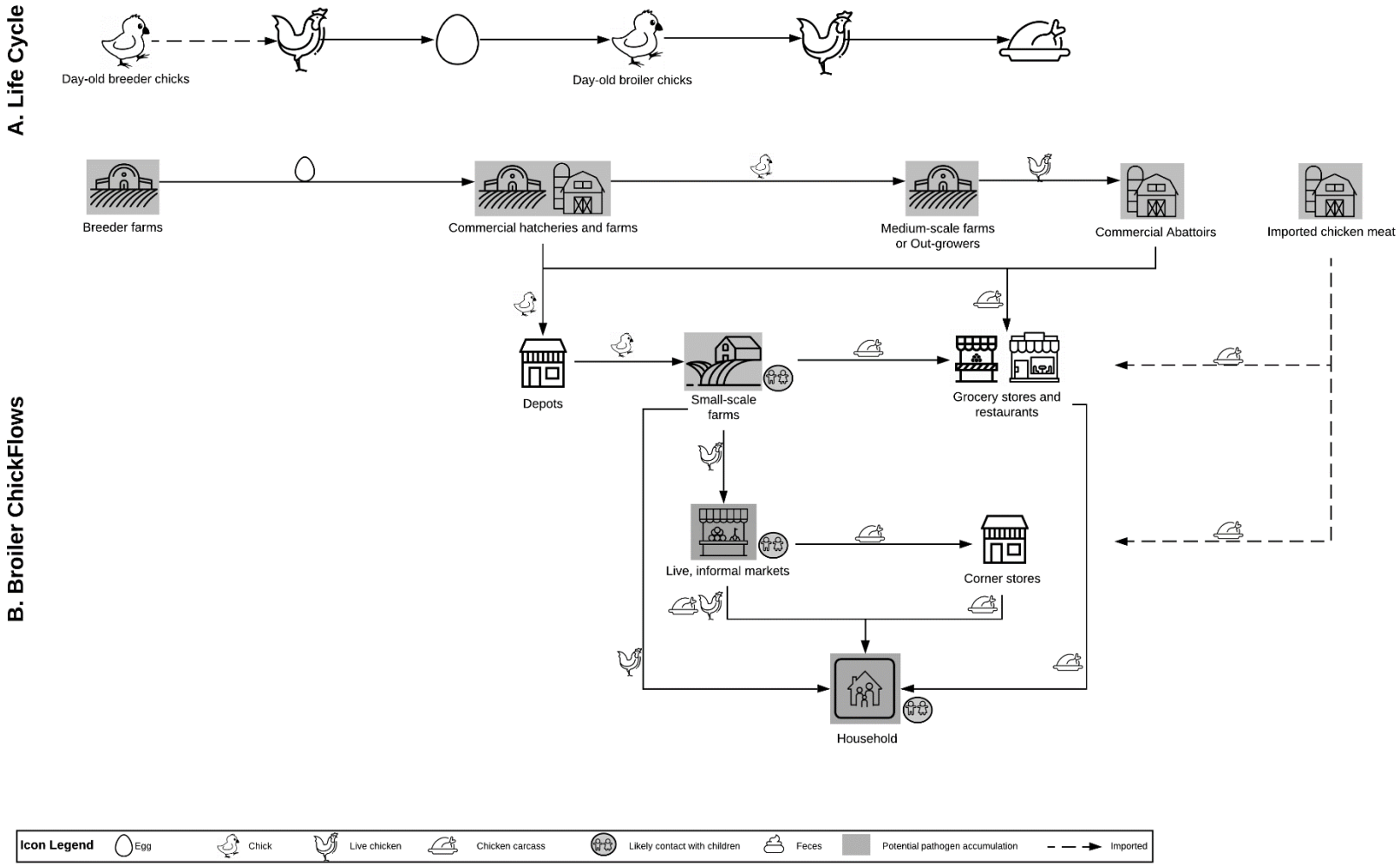

Figure 1. **Broiler ChickFlows value chain.** This map outlines broiler ChickFlows from importation of day-old breeder chicks from Zimbabwe, Zambia, and South Africa to household consumption, for both commercial and small-scale production. Day-old broiler chicks are also imported from neighboring countries, and frozen chicken meat can be imported from Brazil, South Africa, and Portugal. Grayscale icons represent settings with the greatest potential for pathogen accumulation, based on observed and reported hygiene and management practices and scale of operations. Child icons represent settings where children are likely to come into contact with chicken fecal enteropathogens, based on observed and reported contact with chickens and chicken products.

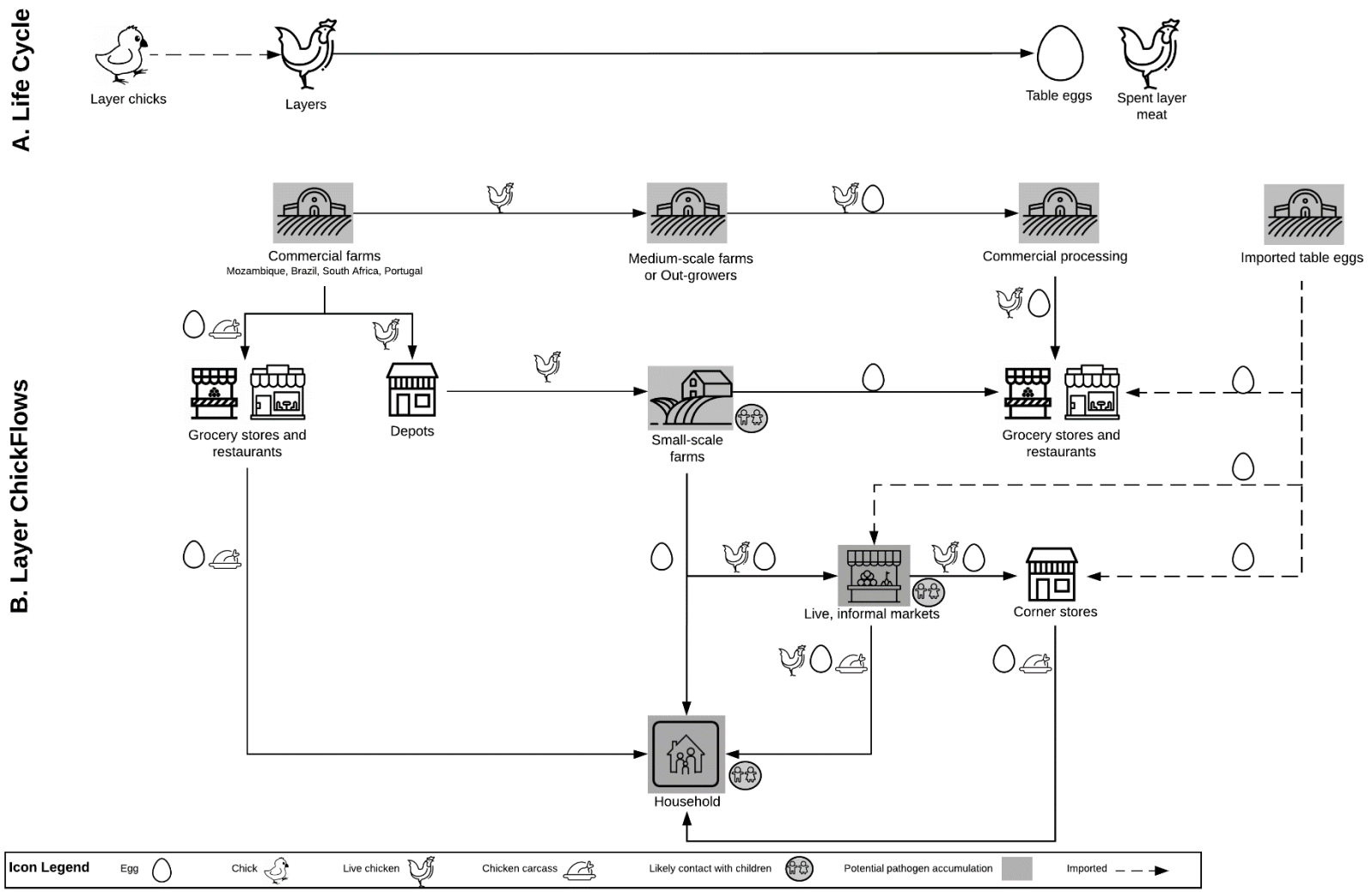

Figure 2. Layer ChickFlows value chain. This map outlines layer ChickFlows, from importation of layers to household consumption of spent layer meat and eggs, for both commercial and small-scale production. Layers are imported from neighboring countries one to two weeks before they start laying. Grayscale icons represent settings with the greatest potential for pathogen accumulation, based on observed and reported hygiene and management practices and scale of operations. Child icons represent settings where children are likely to come into contact with chicken fecal enteropathogens, based on observed and reported contact with chickens and chicken products.

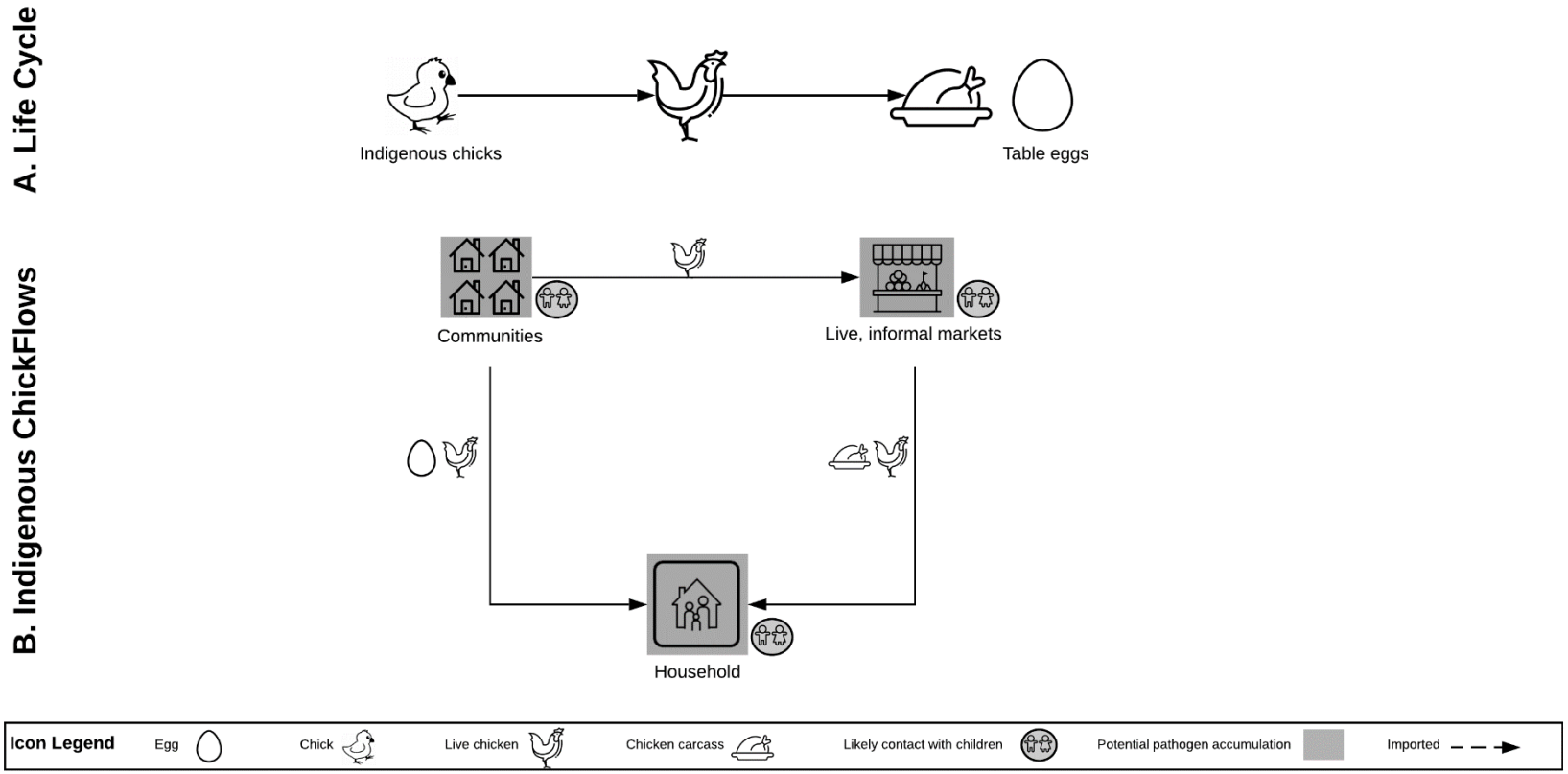

Figure 3. Indigenous ChickFlows value chain. This map outlines indigenous ChickFlows, which primarily involves chickens roaming in the community, exchange of chickens between family members, and sale at informal markets. These scavenging chickens are owned by households. Grayscale icons represent settings with the greatest potential for pathogen accumulation, based on observed and reported hygiene and management practices and scale of operations. Child icons represent settings where children are likely to come into contact with chicken fecal enteropathogens, based on observed and reported contact with chickens and chicken products.

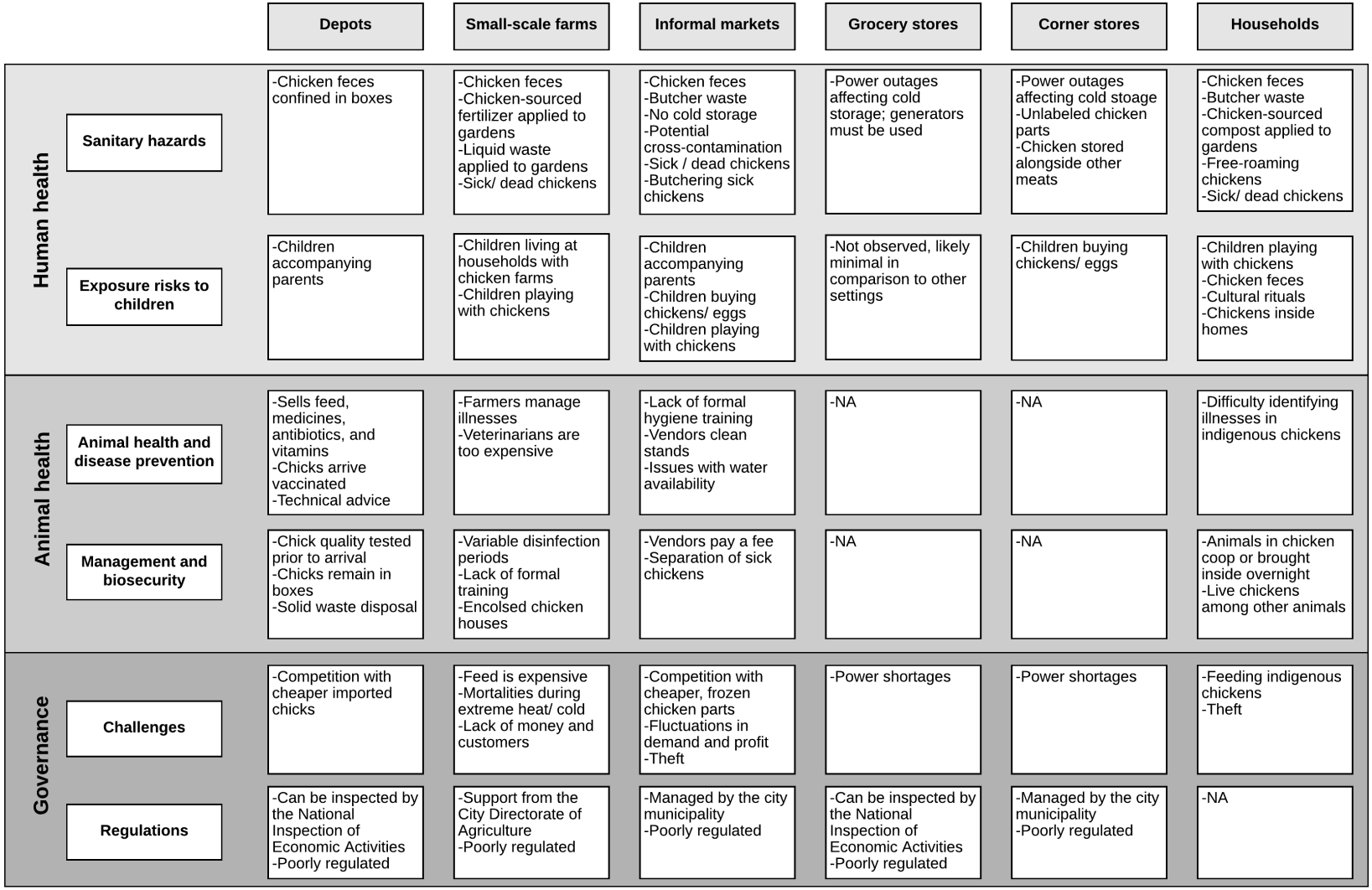

Figure 4. Framework summarizing human health, animal health, and governance themes for each setting.

Depots (n=2). Depots function as distribution centers for broiler chicks, feed, medicines, and supplies. Depots perform basic waste management practices, including trash disposal (paper waste, boxes). Chicken fecal waste is the primary hazard at depots. Chicks arrive and remain in boxes. No liquid waste from cleaning is generated on site to prevent moisture in feed stockpiles. Newcastle (a viral respiratory disease) and Gumboro (a viral bursal disease) vaccines are administered to chicks prior to arrival at depots. Staff advise new farmers on good management practices and preventing illnesses in chickens. Children were observed at each site, but no child contact with chicks was observed.

Small-scale farms (n=26). Small-scale farms raise layers and/ or broilers for profit. These farms have no registration requirements or production and marketing guidelines, and 96% of farmers reported that governmental organizations do not supervise operations. The Poultry Association of Maputo (ADAM) provides funding opportunities and trainings on good management practices and business fundamentals. Farmers are responsible for sourcing water and managing waste, and some experience occasional issues with water availability. Solid waste (feed bags, dead chickens) is disposed in nearby dumpsters or burned. When chickens reach market weight, farmers (63%) reported packaging the wood chip bedding to sell as fertilizer for vegetable gardens, and applying it directly to their own produce gardens (19%), potentially introducing foodborne disease agents if insufficiently desiccated prior to application.

Human health risks include exposure to chicken feces, chicken-sourced compost, sick and dead birds, and rodents. Farmers (11%) reported repurposing liquid waste and applying it to their gardens. Farmers (27%) reported raising multiple flocks within the same chicken house, which can introduce diseases from one flock to another.[48] Veterinarians are considered expensive and typically not consulted. Farmers (96%) administered antibiotics prophylactically to prevent disease and stimulate growth. Measures are taken to prevent the spread of illness, including culling and separating ill chickens, treating chickens with traditional or commercial medications, and changing the bedding when chickens have diarrhea. There was inconsistent implementation of biosecurity measures, including disinfection periods between flock,

designated boots or shoe coverings for the chicken house, and having enclosed chicken houses to limit unwanted human and animal access. Farmers (41%) had children who live at the household where the farms are present. Farmers (48%) were aware that illnesses can pass from chickens to humans and cited limiting children from entering chicken houses, washing their hands, and proper disinfection and hygiene as exposure prevention strategies.

Informal markets (n=20 vendors; 10 markets). Markets sell wet and dry products, including eggs and fresh and live chickens. Market vendors receive live chickens from farmers and sell them live or butcher (and cook) on-site. The City Agricultural Directorate is responsible for implementing regulations regarding animal welfare and health; however, only 30% of vendors had previous visits by government officials. Vendors (70%) reported no formal hygiene training. Inadequate hygiene measures were observed at each market, as market infrastructure did not support hygienic operations. Vendors did not have cold storage for butchered chicken meat, and flies were observed around butchered chickens and remaining parts. Vendors (30%) reported issues with fresh water availability for butchering; the same water is often used throughout the day. Butcher waste and wastewater are stored in large bowls and thrown out at the vending stand or in nearby drains. Vendors (15%) were observed cleaning leafy greens at the same stands where chickens were butchered, creating a cross-contamination risk. Vendors (10%) reported butchering sick birds. Measures to clean the surrounding area and chicken feces included changing the bedding, sweeping, and washing holding crates with soap and water.

Young children were observed buying freshly butchered chickens and eggs from market vendors. Children came to the market with parents, walked through butchering areas, and were seen playing with chicken cages. Vendors (25%) reported children playing with or touching chickens.

Grocery stores (n=6). Grocery stores sell food products, including eggs and frozen, fresh, and ready-to-eat chicken. Raw and ready-to-eat chickens have temperature-controlled storage. Store clerks reported

that eggs arrive at grocery stores designated as "*Salmonella*-free", and are monitored by the National Directorate of Agricultural Health and Biosafety. Grocery stores use generators during power shortages. Store clerks (50%) reported having inspections by the National Institute of Economic Activities (INAE). Children were not observed at grocery stores.

Corner stores (n=4). Corner stores are small convenience shops that sell limited food products, including eggs and frozen chicken parts. Chicken parts are unwrapped, stored alongside other unwrapped meats, and unlabeled, providing no information on the source or expiration date. Frozen chickens are commonly transported in vans with no temperature controls and can thaw during transport, presenting a contamination risk. When power shortages occur, corner stores do not have the capacity to make special provisions. Two clerks reported having inspections by the INAE. Children were observed at three of the four corner stores at time of visit.

Households (n=19). Households raise indigenous chickens for eggs, meat, and income and also purchase live, fresh and frozen layer and broiler chickens and parts from farmers, grocery and corner stores, and markets. Most households (84%) prefer purchasing live or freshly butchered broiler chickens, due to lower cost and better taste. The source, age, and breed of indigenous chickens are variable and typically unknown, and managing illnesses is difficult. Chickens were often sheltered among other poultry (ducks). Chickens free-roam in the community (32%) or are kept in chicken houses during the day or at night (74%). Most households (68%) report that chickens enter the home. The City Agriculture Directorate and The Kyeema Foundation administer Newcastle vaccinations to indigenous chickens, free of charge. Indigenous chicken parts and blood are used for traditional ceremonies and treatments. Chicken feces is swept from the yard or washed away with water. Solid waste is collected in an open pit, placed in a bag and thrown out, or disposed of in a hole.

Children have the potential for contact with live chickens brought home from markets and free-roaming indigenous chickens. Chickens roamed in gardens (11%), potentially contaminating crops. Child play

areas had visible waste, including chicken feces (53%) and feathers (21%). Chickens roamed in child play areas (63%) and were corralled inside the home, sharing spaces with children. Respondents reported children playing with (68%), helping to feed (32%) and helping to pluck (32%) chickens.

Discussion

We mapped value chains for broilers, layers, and indigenous chickens in Maputo, Mozambique and characterized management and food hygiene practices that potentially contribute to childhood exposures to enteropathogens from chickens. We targeted settings upstream of the end user to identify hazards and characterize risks of direct and indirect exposures within the chicken production system. The risk of child exposure to chicken fecal waste at depots and grocery and corner stores is likely minimal, yet cross contamination at these sites may lead to downstream exposures. Children are most likely to encounter chicken-related fecal pathogens in domestic settings, through contact with indigenous chickens or via ingestion of chicken meat, eggs, and produce from small scale farms and markets with poor hygiene and hazard management. Poor hygiene control by market vendors and small-scale producers is a potentially substantial contributor to fecal pathogen exposure to adults, and could seed transmission within households.

Children were observed making direct contact with live and butchered chickens, which could counteract the benefits of poultry ownership as a child nutritional strategy. While we did not quantify risks of this exposure, this framework provides the foundation for further measurement of dominant exposure routes. Our findings are consistent with a growing body of literature investigating childhood exposures to domestic livestock. Children less than 5 years old have frequent hand-to-mouth contact while simultaneously encountering fecal-oral vectors, such as chicken feces in soil,[51] and can directly ingest soil in areas where poultry defecate,[52] thus putting children at an increased exposure risk.[45] While small-scale livestock ownership has its exposure risks and increases the prevalence of diarrheal infections in children,[45] poultry also has nutritional benefits and is associated with less stunting in children.[53] This

highlights possible trade-offs between the advantages of chickens providing nutrition and the disadvantages of chickens carrying human enteropathogens. Given widespread food insecurity in Maputo,[54] households value having chickens available for consumption; however, simply having and consuming chickens does not ensure security due to potential food safety risks. Studies have tested child play spaces to minimize exposure to chicken feces,[55] and investigated corralling chickens to improve health outcomes,[12,56] but have shown mixed results. Our results are important for understanding food security, as they describe multiple points of potential exposure to chicken feces and variability in hazard mitigation strategies across value chains.

Our findings provide evidence in support of the recent call to monitor WASH at wet markets and reiterate the potential for enteric pathogen transmission resulting from hygiene and sanitation conditions.[57] In African subregions with high mortality, food contamination is responsible for a significant proportion of illnesses from *Campylobacter spp*. (57%) and non-typhoidal *Salmonella spp*. (46%).[13] Cleaning leafy greens at chicken stands, selling live, raw, and cooked chickens, and having limited water availability all present potential childhood exposures to chicken fecal enteropathogens. There are additional opportunities for exposure, including purchasing chickens, when live chickens, chicken meat or eggs are brought home, or when adults become infected and seed household transmission events.

Our work can support planning for strategies (interventions, policies, or enforcement) to mitigate childhood exposure to fecal pathogens. Lack of government regulation and sanctions, coupled with economic and technical constraints, may be responsible for excessive hazards and minimal biosecurity measures.[58] Insufficient biosecurity measures at small-scale farms, including failure to use a disinfection footbath, inadequate disinfection periods between flocks, and presence of other animal species, are significant risk factors for *Campylobacter* and non-typhoidal *Salmonella* colonization.[59,60] Contamination at farms is associated with contamination downstream during processing.[61] Small-scale farmers and market vendors are not required to – and thus did not – institute preventative food safety guidelines, such

as hazard analysis critical control point (HACCP), International Organization for Standardization (ISO), or Codex Alimentarius standards. Our findings align with that of the UN Food and Agriculture Organization,[31] and echo the vulnerabilities and challenges among broiler and egg food systems in Kenya[21,22] and poultry value chains in two Mozambican provinces.[26]

Our work provides insights about health hazards from the informal chicken production sector, adding to what is already known about health risks from commercial poultry farms. Commercial companies can afford to invest in hazard mitigation programs, but also concentrate large volumes of waste, providing opportunities for pathogen accumulation that can then propagate into the distribution system and community.[62] We observed an accumulation of chicken waste at small-scale farms, yet, there were no formal hazard mitigation programs. Like commercial farms, informal poultry farms also have antibiotic inputs that contribute to the emergence and spread of antimicrobial resistance.[63] Neighborhoods along poultry transport routes are at risk of exposure to antibiotic-resistant bacteria.[64]

Our collaboration between human infectious disease and veterinary scientists supports insights on animal-WASH interactions[65] across multiple interfaces and associated threats to food security – safety. To date, there is a paucity of research investigating enteropathogen transmission pathways of importance to children while highlighting food safety hazards and risks. As encouraged by the FAO, combining an understanding of livestock production systems with an evaluation of disease risks are instrumental to plan for disease mitigation.[66] Global efforts to create healthy diets and more sustainable food systems, such as the EAT-*Lancet* Commission,[67] should also consider animal-related pathogens in foods and food waste when creating strategies if the intention is ultimately healthy people. Mapping value chains and interviewing stakeholders along these value chains allowed us to observe and identify exposure risks that may have been overlooked had we taken an end-user approach, focusing solely on household exposures. Collecting data across six of Maputo's seven districts allowed for capturing variability across interviewer responses. Our study characterizes value chains for broilers, layers, and indigenous chickens, which are

commonly studied separately. The production system for layers was previously undescribed for Mozambique[31].

Our selection of participants from depots, markets, and grocery stores relied on a self-generated census, and we were forced to rely on the knowledge of neighborhood secretaries to identify small-scale farms and households, leading to potential selection bias. While our value chains provide a foundational framework for future risk analyses, future work will collect data on consumption and purchasing habits to more specifically quantify risks.

Our findings suggest three distinct opportunities for future research. First, quantifying contamination levels across settings would support intervention strategies. Second, quantification of pathogen accumulation and opportunities for cross-contamination with produce at markets would help identify contamination pathways. Third, quantification of chicken purchasing and consumption habits would provide data on the relative consumption of each type of chicken to better define risks. This additional research can contribute to the emerging body of data generated and monitored by the development of the Africa Food Safety Index[68,69] to inform policy initiatives to prioritize food safety and reduce foodborne illnesses in African countries.

Enteropathogen infections in young children are responsible for high levels of morbidity and mortality in LMICs; the role of exposure vis-à-vis the food system and through animal contact is understudied and potentially significant, and requires greater attention by those engaged in food security. Developing and implementing interventions to mitigate animal exposures are needed, and understanding exposure pathways is a critical next step. The ChickFlows value chain framework can be implemented as a tool across contexts to first understand food-safety hazards and risks along animal value chains before developing infrastructural, behavioral, and policy interventions. Our work suggests that the Mozambican chicken production sector is susceptible to microbiological risks to children, highlights the need for a

greater focus on foodborne pathogens in LMICs, and characterizes settings that can be targeted to inform local food-safety policies.

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Author Contributions

F.L., K.L., and M.C.F. designed the research, with contributions and feedback from H.N.M., E.F, J.A.O.S. and J.M.F. F.L. led data collection, and H.N.M. contributed to its execution. F.L. analyzed the data with support from K.L. and M.C.F. F.L. wrote the initial manuscript draft, with writing contributions from K.L. and M.C.F. B.A.C. contributed to the presentation of results and the organization and framing of the manuscript. All authors reviewed and provided feedback on the final draft.

Competing Interests

The authors declare no competing interests.

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Chapter 2. Quantifying microbial hazards along the chicken value chain in an urban low-income country setting[1]

Abstract

Small-scale poultry production as a means of income and nutrition is ubiquitous and increasing in low- and middle-income countries (LMICs), yet the containment and management of poultry-associated fecal waste is minimal. Exposure to *Campylobacter* spp., nontyphoidal *Salmonella* spp., and other human enteropathogens in poultry feces may put children at risk for diarrheal disease. We conducted a study of microbial hazards along the broiler, layer, and indigenous chicken value chains in Maputo, Mozambique to identify points along the value chain that may pose a health risk to children. Chicken carcasses (*N*=75) and fecal samples (*N*=136) were collected from broilers, layers, and indigenous chickens at veterinary depots, small-scale farms, grocery stores, corner stores, informal markets, and households. Samples were analyzed by qPCR for *C. jejuni/coli*, *Salmonella* spp., and *Cryptosporidium* spp. We detected *C. jejuni/col*i in 84 (76%) fecal samples and 52 (84%) carcass rinses, and *Salmonella* spp. in 13 (11%) fecal samples and 16 (21%) carcass rinses from fully grown chickens. Median concentrations of *C. jejuni/coli* in feces and carcasses from fully grown chickens were $4.3x10^4$ and $3.9x10^2$, respectively, and of *Salmonella* spp. in feces and carcasses from fully grown chickens were $2.0x10^2$ and $1.6x10^1$, respectively. *C. jejuni/coli* and *Salmonella* spp. were not detected in broiler chick feces. *Cryptosporidium* spp. was not detected. 100% of carcasses sampled from informal markets were contaminated with *C. jejuni/coli*, making informal markets particularly high-risk settings for the cross-contamination of chicken meat. A subset of child stool samples (*N*=64) from the study area were analyzed for *C. jejuni/coli* to confirm childhood infections with specific *Campylobacter* species carried by poultry. *C. jejuni/coli* were detected in 59 (92%) child stool samples. High prevalence and concentration of pathogen contamination along chicken value chains coupled with *C. jejuni/coli* in child stool suggests a high risk of childhood exposure

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to chicken-sourced enteropathogens along chicken production and marketing processes. Food hygiene at informal markets should be prioritized for the development of intervention strategies to reduce microbial hazards associated with processing chickens for sale. Mitigation strategies to reduce enteropathogen exposures in children should include food safety measures for animal-sourced foods, as well as animal fecal waste management.

Introduction

Foodborne illnesses resulted in an estimated 420,000 deaths globally in 2010, 40% of which occurred in children <5 years.[1] Over half of these deaths are attributable to pathogens causing diarrhea, a particular outcome of foodborne infections. *Campylobacter spp*. is the second leading cause of global foodborne disease, and non-typhoidal *Salmonella enterica* and *Cryptosporidium spp*. are also important contributors.[1,2] These three enteropathogens are among the top five pathogens of concern for the burden of disease through transmission in animal feces, causing close to one million deaths annually, and invasive non-typhoidal *Salmonella* caused over half of these deaths.[3] The highest burden of diarrhea from foodborne hazards is observed in WHO African subregions.[1] Microbial food safety is a challenge in low and middle- income countries (LMICs), because of an increase in the consumption of fresh and perishable foods relative to high income settings, lack of cold storage and access to water for hygiene, complex food value chains, informal markets, lack of regulations, and limited capacity to enforce the regulations that do exist.[4,5]

Small- scale poultry production is already prevalent and is increasing in resource-limited areas as a means to concurrently provide nutrition, income, and food security for households, available for food when needed.[6] Though poultry production is associated with economic and nutritional gains, such as income generation, liquid assets, and providing essential macro- and micronutrients,[6] evidence weighing the advantages of small-scale poultry husbandry against the public health risks has been limited.[7] While much work has investigated food safety in rural settings, previous studies have found that poultry-associated human infections with *Campylobacter* and *Salmonella* are more likely in urban areas.[8,9] The risks may be even higher in contexts where poor water and sanitation can impact food hygiene practices.[10] Evidence suggests that children with household exposure to poultry are at an increased risk of diarrhea[11,12] and anemia.[13] *Campylobacter*, non-typhoidal *Salmonella* and *Cryptosporidium*, all carried by poultry,[14–17] are of high concern for their burden of disease and transmission in animal feces.[3]

Each stage of poultry production can play a role in pathogen transmission.[8,15,18] Where informal production and marketing of poultry is common, contamination of chickens at live markets and small-scale farms has been well documented.[4,19–25] The farm environment introduces various opportunities for both horizontal transmission of pathogens between members of a flock and vertical transmission between parent and offspring.[15,18,26,27] Minimal biosecurity measures at small-scale operations within low-resource areas[28] are compounded by the possibility of subclinical or undetected colonization with *Cryptosporidium, Campylobacter,* and *Salmonella* within flocks.[26,29,30] Lack of formal food system regulations and enforcement is a challenge to food safety[4], and national-level data monitoring microbial hazards in food are scarce, leaving in question baseline food safety hazards associated with poultry in informal production contexts.

Exposure to animal feces is often ignored in exposure assessments,[31–33] which may help explain why recent large-scale, rigorous trials of community-based environmental control measures have resulted in minimal improvements in child health outcomes.[31–34] Annual global production of feces from animals (primarily cattle, chickens, and sheep) is estimated at 29.7x109 kg, approximately four times the amount of human fecal biomass, and ratios of animal feces to human feces are increasing.[35] Improvements to domestic water quality, environmental sanitation, and personal hygiene may mitigate exposure to both human and animal-sourced fecal pathogens.[10,36] However, water, sanitation, and hygiene (WASH) interventions focused on human fecal contamination may be insufficient without an explicit focus on the management of animal fecal waste.[37] Low-income populations bear the greatest burden of possible onsite fecal exposures, from animals in or near the home.[35] Considering pathways of enteropathogen transmission along animal value chains is critical to understanding animal contributions to the burden of diarrheal disease in children.

In this study, we investigated food safety in an LMIC urban context, to understand the nexus between food safety and WASH. We quantified food safety hazards along the chicken value chain in Maputo,

Mozambique to identify settings that could potentially pose a health risk to children and updated value chains (Chapter 1) to reflect microbial hazards at key settings. We compared the carriage of *Campylobacter* and *Salmonella* between broilers (raised for meat), layers (raised for eggs), and indigenous chickens (free-range and raised for meat and eggs) and between key settings. The purpose of this work is to provide actionable data to inform the development of interventions to mitigate zoonotic (directly from chickens to children) and foodborne (indirectly via contaminated chicken meat and/or eggs) transmission of enteropathogens in low-income settings. Our approach utilized microbial measures to assess key hazards across the chicken value chain.

Materials and Methods

Study setting. Maputo, the capital city of Mozambique, is growing rapidly and its population experiences widespread food insecurity.[38] Maputo has a growing poultry sector and is a national leader in poultry production.[39] The Food and Agriculture Organization of the United Nations (FAO) has classified poultry production in Maputo into three systems: a) village or backyard, b) commercial, and c) industrial and integrated.[39] Village or backyard systems have minimal levels of biosecurity and local consumption and trade of birds.[39] Commercial systems have low to high levels of biosecurity and commercial marketing of birds.[39] Industrial and integrated systems have high levels of biosecurity with standard operating procedures to improve food safety and only commercial marketing of birds.[39] Agriculture makes up 24% of Mozambique's gross domestic product (GDP)[40], and 55% of poultry producers are small-scale farmers (household or family-owned farms raising 100-2000 birds).[39] Mozambique has a target to double the productivity and income of small food producers and family farmers by 2030 as part of its own Sustainable Development Goal- related targets.[41] It is common for households to own domestic animals and poultry, and chicken presence has been associated with fecal indicator bacteria in soil in Maputo.[42] Government agencies lack the capacity to monitor biosecurity measures and enforce animal welfare

regulations in households or on small farms, and existing regulations are followed primarily by commercial poultry producers (Chapter 1).

Diarrhea accounted for 6% of deaths in children under 5 in Mozambique in 2019.[43] In a recent study in Maputo, *Salmonella*, *Campylobacter*, and *Cryptosporidium* were detected in 21%, 10%, and 4.4% of children's stools, respectively, in households located in informal settlements.[34,44] Our team previously reported that sanitary hazards are pervasive throughout the chicken value chain, particularly in the unregulated, informal sector, and children can come into contact with enteropathogens from microbial hazards in chicken meat or fecal droppings at households and small-scale farms (Chapter 1).

Study design and selection of participants. We conducted a cross-sectional study of contamination of chicken carcasses and chicken feces in six districts and across six settings (defined below) in Maputo, Mozambique, including small-scale farms, informal markets, grocery stores, corner stores, households, and depots. In each setting, we sampled chicken carcasses and/or feces. Setting types were identified during formative work as key locations that are highly likely for children to come into direct and/ or indirect contact with enteropathogens from chicken feces through contaminated chicken meat and chicken products (Chapter 1).

Small- scale farms. Small-scale farms were defined as individual farms that raised broilers and/ or layers for sale. These farmers do not return their flock to commercial companies for processing once chickens reach market size, and are responsible for marketing and selling their own chickens. No formal registries of small-scale farms were available at the district level. To have representation of farmers within the six districts, 14 neighborhood secretaries (local officials who oversee community operations and have knowledge of local farming practices) were consulted to identify farmers raising broilers and layers for sale. Neighborhood officials accompanied field staff to guide them to the farms. Fecal samples were collected from broilers and/or layers from 26 small-scale farms (one to six farms per neighborhood). No

carcasses were collected at these locations, as it was more common to process chickens at informal markets than at farms.

Informal markets. Open-air or wet informal markets were defined as locations where live chickens are sold and/or butchered. Local field staff generated a list of 18 markets in Maputo, markets not selling chickens were removed, and then markets were randomized for selection (*N=15*). At each market, vendors were selected for participation in the order in which they were situated in the market, starting with the vendor nearest to the market opening. In total, 63 fecal and 43 carcass samples were collected from broilers, layers, and indigenous chickens from one to four vendors at 10 markets (*N = 20*).

Grocery stores. Grocery stores were defined as enclosed markets selling goods, including frozen whole chickens and chicken parts. A Google Maps search was conducted to generate a census of 24 grocery stores, representing both smaller stores and major retail chains, which were then randomized for selection. Three to four frozen broiler carcasses were collected from six grocery stores (*N* = 19).

Corner stores. Corner stores were defined as small convenience shops selling a limited range of food items. Corner stores, which were included after learning from local staff that they are a less expensive alternative to grocery stores, were purposively-sampled in districts having few or no grocery stores. Two to four frozen broiler carcasses were collected from four corner stores (*N* = 13).

Households. Households were defined as a group of people sharing common living quarters. Households were eligible for inclusion in our study if they had at least one child less than five years old and owned indigenous chickens that were not raised for sale. Neighborhood secretaries were consulted to identify households keeping indigenous chickens, but not raising them for sale. Indigenous chicken fecal samples were collected from 19 households across seven neighborhoods. Households differed from farms in that farms raised their chickens for sale and did not necessarily have a child < 5 years old.

Veterinary depots. Veterinary depots were commercially-owned storefronts selling broiler chicks and farming supplies, including feeders, medications, vaccines, and feed. Fecal samples (11 at Depot A, six at Depot B, and three at Depot C) were collected from three veterinary depots that represented the main poultry suppliers in Maputo ($N = 20$).

We collected a total of 75 chicken carcasses for rinses and 116 pooled fecal samples (described below) from broilers, layers, and indigenous chickens at the aforementioned locations from June 2019 to September 2019. Pooled fecal samples from 20 broiler chicks were collected in July 2021 **(Table 1)**. Sample numbers reflect the prevalence of each of the chicken types in the Maputo, with broilers ($N = 136$) being the most common chicken type, followed by indigenous chickens ($N = 53$), and then layers ($N = 22$).

Table 1. Summary of number of fecal samples and carcass rinses collected across each setting and type of chicken. Note that not all sample and chicken types were available for all locations; see text for details.

Type of chicken	Pooled Feces				Carcass Rinses			Total
	Depot	Small-scale Farm	Household	Market	Grocery Store	Corner Store	Market	
Broiler	20	25	-	30	19	13	29	136
Layer	-	8	-	10	-	-	4	22
Indigenous	-	-	20	23	-	-	10	53
Total	20	33	20	63	19	13	43	211

Pooled fecal sample collection and processing. Sample collection methods varied by site: at small-scale broiler and layer farms, fecal droppings were taken from distributed locations throughout each broiler and/or layer house; at markets, fecal droppings were collected from chicken holding cages; at households, fecal droppings were collected either from the household yard or holding cage; at depots, fecal droppings were collected from four different boxes of broiler chicks that arrive in boxes of 80-100 chicks. For all settings, fecal droppings were collected using sterile spatulas (Grainger Supply, Norcross, GA), inserted into sterile 2 mL conical tubes (DOT Scientific, Burton, MI), transported to the laboratory in coolers on ice, and processed the same day. Samples were pooled in the laboratory to get a more representative sample of enteropathogen contamination at each site. Four fecal samples from the same setting, chicken type, and participant were pooled by equal weights[45] to approximately 1g and transferred into PowerBead DNA extraction tubes (Qiagen, Louisville, KY). Due to the small mass and loose consistency of broiler chick feces, fecal samples from depot broiler chicks were weighed to approximately 0.25 g.

Carcass rinse sample collection and processing. Two to four whole frozen broiler chickens were purchased at each grocery and corner store, with special attention paid to sampling across a variety of brands. Frozen, pre-wrapped broiler carcasses were kept in their original packaging. One to four raw carcasses were purchased per vendor at markets, depending on the availability of each type of chicken. All carcasses were placed in Nasco poultry rinse bags (VWR, Bridgeport, NJ) and then placed in coolers on ice for transport to the laboratory and processed the same day.

Carcasses were rinsed individually in 400 mL of 0.1% buffered peptone water (PBS; Quality Biological, Inc., Gaithersburg, MD) and hand shaken, moving the bag in an arc motion, for one minute, following established whole carcass rinse protocols.[46] Frozen carcasses were first thawed in a cold-water bath and removed from their packaging. After shaking, 100 mL of the carcass rinse solution was aliquoted into two 50 mL conical tubes. Rinse aliquots were centrifuged for ten minutes at 400 RPM. Pellets were

resuspended in 1 mL of 1X PBS, and 250 µL of the resuspended pellet solution was transferred into PowerBead DNA extraction tubes (Qiagen, Louisville, KY).

DNEasy PowerSoil Kit (Qiagen, Louisville, KY) extraction buffer "Solution C1" was added to each fecal and carcass rinse sample to fix all microbes in the sample. Six freeze-thaw cycles were preformed to break open *Cryptosporidium spp*. oocysts.[47,48] For each freeze-thaw cycle, extraction tubes were placed in the -80°C freezer for ten minutes and then flash thawed in a dry heat block (Thermomixer Comfort, Eppendorf, Germany) at 98°C.

DNA extraction for chicken fecal samples and carcass rinses. DNA was extracted from approximately 1 g of pooled fecal samples (0.25 g for depot broiler chick feces) and 250 µL of the carcass rinse solution using the DNEasy PowerSoil Kit (Qiagen, Louisville, KY) according to the manufacturer's protocol. A tissue lyser (Tissue Lyser LT, Qiagen, Hilden, Germany) was used at speed three for three minutes to optimize cellular lysis prior to extraction. Purified DNA was eluted with 80 µL of 10 mM tris buffer (Qiagen, Louisville, KY) at pH 8 and immediately stored at -80°C prior to downstream analyses. A negative extraction control (NEC), diethylpyrocarbonate (DEPC) treated water (Life Technologies Corporation, Carlsbad, CA) was included in each set of extractions.

qPCR analysis. Quantitative PCR (qPCR) assays were used to quantify *Salmonella spp*.,[49] *Campylobacter* spp.,[50] *Campylobacter jejuni and Campylobacter coli*[51] and *Cryptosporidium* spp.[47] Prior to qPCR analyses, each sample was spiked with $2.5x10^6$ copies of an artificially designed inhibition control gene target (Integrated DNA Technologies, Coralville, IA).[52] Samples were analyzed in duplicate on a Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA). All qPCR plates included duplicate reactions with nuclease free water as a no-template control (NTC) and the NEC. Each qPCR reaction had a final volume of 20µL and contained 10µL 2X qPCR Perfecta qPCR ToughMix Low ROX mastermix (Quanta BioSciences, Inc, Gaithersburg, MD), 10 µM each forward and reverse primer, 1 µM probe, and

4 µL of DNA template. All probes used the FAM reporter dye and BHQ-1 quenchers (Biosearch Technologies Inc., Petaluma, CA). Cycling conditions for all assays were as follows: 95°C for three min, 95°C for 15 s, and 45 cycles of 53-63°C for 30 s. Primers and annealing temperatures for assays used are summarized in **Table 2.**

Seven-point standard curves were prepared using known quantities of gblock gene fragments (Integrated DNA Technologies, Coralville, IA) that included the template reference sequence at ten-fold dilutions ranging from 10^6 to 10^1 gene copies per reaction. A standard curve was included on each plate across all assays. Gene target abundance was estimated from Ct values by interpolation to a standard curve as the mean concentration of duplicate reactions and reported as gene copies per gram of feces or gene copies per carcass rinse. Standard curves were averaged for each assay, and averaged curves were used for data analysis.

Table 2. Summary of qPCR primers and annealing temperatures

Pathogen	Gene target	Primer sequence (5' to 3')	Annealing temp	Reference
Campylobacter spp.	16S rRNA-F 16S rRNA-R	CACGTGCTACAATGGCATAT GGCTTCATGCTCTCGAGTT	60°C	Lund et al. 2014[50]
C. jejuni/ coli	cadF-F cadF-R	CTGCTAAACCATAGAAATAAAATTTCTCAC CTTTGAAGGTAATTTAGATATGGATAATCG	55°C	Platts-Mills et al. 2014[51]
Salmonella spp.	*invA*-F *invA*-R	GCTGCTTTCTCTACTTAAC GTAATGGAATGACGAACAT	55°C	Heymans et al. 2018[49]
Cryptosporidium spp.	Actin locus-F Actin locus-R	ATCGTGAAAGAATGACWCAAATTATGTT ACCTTCATAAATTGGAACGGTGTG	53°C	Yang et al. 2014[47]
Internal amplification control	IAC-F IAC-R	CTAACCTTCGTGATGAGCAATCG GATCAGCTACGTGAGGTCCTAC	63°C	Deer et al. 2010[52]

Limits of detection and quantification. Standard curves were analyzed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[53] Detection and quantification limits were determined according to methods previously described.[54] The limit of detection (LOD) was determined based on the standard curve as the lowest amount of template for which there was amplification for 90% of the runs. The limit of quantification (LoQ) was defined as the lowest concentration that was accurately quantified with an acceptable level of uncertainty and was calculated for each assay as follows: $Ct_{LoQ} = Ct_{LoD} - 2(\sigma_{LoD})$. If zero or one well amplified, results were deemed non-detectable (ND) and assigned a value half of the LoD. If both duplicates amplified but were beyond the lower limit of the dynamic range or LoQ, results were deemed detected but not quantifiable (DNQ) and assigned the value of the LoD. If both duplicates amplified but were beyond the upper limit of the dynamic range, results were assigned the highest quantifiable concentration. For calculating prevalence of gene target, if both wells amplified, the sample was treated as positive. Average assay efficiency was 99% for the inhibition control target, 99% for *Salmonella spp*., 100% for *Campylobacter spp*., and 99% for *C. jejuni/ coli*. Mean slope, y-intercept, R^2, and efficiency for each assay is listed in **Table S1 (Supplementary Material).**

Quantifying C. jejuni/coli *in child stool samples*

To establish a plausible zoonotic link to chicken exposure, we examined the potential for child infection in our study region with *C. jejuni*/*coli*, the human pathogenic *Campylobacter* species known to be carried by chickens. Our decision was based on a recent study in Ethiopia, where *Campylobacter* spp. was detected in 51% of children stool samples (n = 100) by conventional PCR.[55] The second most commonly detected *Campylobacter* species in children's stool by meta-total RNA sequencing (MeTRS) was C. *hyointestinalis*, which is not associated with chickens.[55] Therefore, We carried out assays on children's stool samples collected during the Maputo Sanitation (MapSan) study.[56] MapSan stool samples were previously analyzed for 15 enteric pathogens, including *Campylobacter spp.* and *Salmonella spp*. using the Luminex MagPix xTAG Gastrointestinal Pathogen Panel (GPP, Luminex Corp, Austin, TX). Full

methods used in the MapSan study are described elsewhere.[44] We examined child stool samples (*N*=64) that had previously tested positive for *Campylobacter* spp. via Luminex using our *C. jejuni*/*coli* assays. Half of the MapSan child stool samples tested for *C. jejuni/coli* were collected in compounds where poultry were observed at the time of sample collection (*N*=32), and half were collected in compounds with no observed poultry (*N*=32).[34,44] We also tested for associations between *C. jejuni/coli* and *Salmonella* spp. in stool and observed poultry in the compound at the time of specimen collection.

DNA was extracted from child fecal samples using the QIAcube automated extraction system (Qiagen, Hilden, Germany). A pretreatment step was performed to mechanically disrupt tissue and ensure maximum extraction efficiency. Approximately 100 mg of each sample and 1 mL of ASL lysis buffer (Qiagen, Germantown, MD) were added to SK38 bead tubes (Bertin Corporation, Rockville, MD). Three NECs were included. Samples were vortexed for five minutes, incubated for ten minutes at room temperature, and then centrifuged for two minutes at 14000 RPM. DNA was extracted from 200 µL of supernatant using the. Extracted DNA was immediately stored at -80°C prior to downstream analyses. qPCR analyses were performed as stated above to detect *C. jejuni/coli*.

Statistical analysis. Separate statistical analyses were conducted for *Salmonella* spp. and *C. jejuni/coli* using R 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Shapiro-Wilks tests were run to determine data normality. Means of $\log_{10}$ transformed concentrations were calculated for each pathogen and reported as average gene copies per gram of feces or average gene copies per carcass rinse. Kruskal-Wallis tests were performed to compare median pathogen concentrations in chicken feces and carcass rinses by setting and to determine if children in the MapSan study with observed exposure to poultry had different median concentrations of *C. jejuni/coli* in stool than children without observed exposure to poultry. Dunn's tests were performed as a follow up to significant Kruskal-Wallis comparisons to identify which groups were different. Odds ratios (ORs) and 95% confidence internals (CIs) were calculated using Fisher's exact tests that compared pathogen prevalence between chicken type and setting and to compare

pathogen prevalence between poultry exposure groups at the time of MapSan child stool sample collection. Results with p-value < 0.05 were considered statistically significant.

Ethics statement. The Institutional Review Board at Emory University (IRB00108546) and the Research Council to the Veterinary Faculty at Eduardo Mondlane University determined that this research was exempt from human subjects review, and the Municipality of Maputo (Reference number 754/SG/426/GP/2019) authorized this research. Prior to each interview, the study's purpose and participant rights were explained in Portuguese, and participants provided verbal informed consent.

Results

A total of 211 samples were analyzed by qPCR: 116 fecal and 75 carcass samples from fully grown chickens and 20 fecal samples from broiler chicks.

No *Cryptosporidium* spp. were detected in this study. No *C. jejuni/coli* or *Salmonella* spp. were detected in broiler chick feces. The results below summarize pathogen data for chicken carcass and fecal samples from fully grown chickens.

Prevalence of Campylobacter and Salmonella in chicken samples

Campylobacter spp. was detected in 92 (83%) fecal samples and 65 (87%) carcass rinses. Five of the 116 fecal samples were excluded from analysis due to having contaminated field blanks. Samples that initially tested positive for *Campylobacter* spp. were subsequently analyzed for *C. jejuni/coli*. From here forward, we report only on *C. jejuni/coli* results, as these are the *Campylobacter* species that are found in both chickens and humans; 91% and 80% of fecal and carcass rinse samples, respectively, that tested positive for *Campylobacter* spp. also tested positive for *C. jejuni/coli*. *Campylobacter* spp. results can be found in the supplemental material **(Tables S2-S3, Figure S1)**. A summary of prevalence and average *C. jejuni/coli* and *Salmonella* spp. concentrations by sample and chicken type and setting are shown in **Tables 3 and 4**.

Table 3. Chicken Fecal Sample Results: Prevalence and log10 mean copies (SD) per gram of chicken feces for *C. jejuni/coli* and *Salmonella* spp.

	Statistic	Broiler		Indigenous		Layer	
		Farm	Market	Household	Market	Farm	Market
C. jejuni/coli[a]	Prevalence	14/23 (61%)[b]	30/30 (100%)	11/18 (61%)[b]	18/23 (78%)	3/7 (43%)[b]	8/10 (80%)
	Mean	$7.7x10^5$ ($1.5x10^6$)	$3.6x10^6$ ($7.5x10^6$)	$1.2x10^5$ ($3.7x10^5$)	$1.0x10^5$ ($2.3x10^5$)	$2.2x10^5$ ($4.2x10^5$)	$2.1x10^6$ ($5.7x10^6$)
***Salmonella* spp.**	Prevalence	0/25 (0%)	7/30 (23%)	3/20 (15%)	2/23 (9%)	0/8 (0%)	1/10 (10%)
	Mean	ND	$1.5x10^3$ ($6.4x10^3$)	$2.4x10^2$ ($8.9x10^1$)	$2.2x10^2$ ($5.8x10^1$)	ND	$2.2x10^2$ ($6.4x10^1$)

[a]Only samples that were both positive for *Campylobacter* spp. and had sufficient amounts of extracted DNA were also analyzed for *C. jejuni/coli*.
[b]Denominator is less than what is presented in Table 1 due to blanks being contaminated with *Campylobacter* spp.
Non-detectable fecal samples were assigned half the value of the limit of detection (16.2 copies for *Salmonella* spp.).

Table 4. Chicken Carcass Rinse Results: Prevalence and log10 mean copies (SD) per carcass rinse for *C. jejuni/coli* and *Salmonella* spp.

	Statistic	Broiler			Indigenous	Layer
		Corner Store	Grocery Store	Market	Market	Market
C. jejuni/coli[a]	Prevalence	3/12 (25%)	6/7 (86%)	29/29 (100%)	10/10 (100%)	4/4 (100%)
	Mean	$1.2x10^1$ ($4.0x10^1$)	$8.3x10^1$ ($4.3x10^1$)	$1.4x10^3$ ($2.6x10^3$)	$2.6x10^3$ ($2.9x10^3$)	$5.3x10^2$ ($2.4x10^2$)
***Salmonella* spp.**	Prevalence	2/13 (15%)	5/19 (26%)	5/29 (17%)	3/10 (30%)	1/4 (25%)
	Mean	$1.9x10^1$ (6.1)	$9.4x10^3$ ($2.8x10^4$)	$4.6x10^1$ ($9.3x10^1$)	$2.1x10^1$ (7.8)	$2.0x10^1$ (8.3)

[a]Only samples that were both positive for *Campylobacter* spp. and had sufficient amounts of extracted DNA were also analyzed for *C. jejuni/coli.*

C. jejuni/coli was detected in 84 (76%) fecal samples and 52 (84%) carcass rinses. 13 carcass rinse samples that tested positive for *Campylobacter* spp. were not subsequently analyzed for *C. jejuni/coli* due to low sample volume. *C. jejuni/coli* was detected in fecal and carcass rinse samples in all settings and chicken types. The odds of purchasing a whole chicken contaminated with *C. jejuni/coli* were higher at markets compared to corner stores (OR=92.3, 95% CI= 9.7, 4688.1), and chickens at markets were more likely to shed *C. jejuni/coli* in feces than chickens at farms (OR=6.0, 95% CI= 1.9, 20.8) and households (OR=5.0, 95% CI= 1.2, 20.6). Crude ORs and 95% CIs between settings and between chicken types are shown in **Table S4**. In fecal samples, prevalence of *C. jejuni/coli* was highest at markets (89%) compared to farms (57%) and households (61%) and highest in broilers (83%) compared to indigenous (71%) and layers (65%). In carcass rinses, detection of *C. jejuni/coli* was highest at markets (100%) compared to grocery stores (86%) and corner stores (25%). Indigenous and layer carcass samples were only collected at markets, where there was a 100% prevalence.

Salmonella spp. were detected in 13 (11%) fecal samples and 16 (21%) carcass rinses. Prevalence of *Salmonella* spp. in fecal samples was highest at markets (16%) and highest in broilers (13%). No *Salmonella* spp. was detected in broiler or layer feces from small-scale farms. Prevalence of *Salmonella* spp. in carcass rinses was highest at grocery stores (26%) and highest in layers (25%). However, comparisons of differences in *Salmonella* spp. prevalence between settings and chicken types were not significant.

Quantification of Campylobacter and Salmonella in chicken samples

C. jejuni/coli was present at concentrations ranging from $9.1x10^1$ to $2.9x10^7$ mean copies and 7.3 to $1.0x10^4$ mean copies in feces and carcasses, respectively. Median concentrations of *C. jejuni/coli* in feces and carcasses were $4.3x10^4$ and $3.9x10^2$, respectively. *C. jejuni/coli* concentrations in fecal samples were highest at markets ($7.5x10^6$ mean copies). Generally, we detected high levels of *C. Jejuni/coli* in chicken feces among broilers ($2.7x10^6$ mean copies) and in carcass rinses among indigenous chickens ($2.6x10^3$

mean copies). We found statistically significant differences in C. *jejuni/coli* concentrations in carcass rinses between all settings and in feces between markets and households (**Table S5**).

Salmonella spp. was present at concentrations ranging from $2.0x10^2$ to $3.5x10^4$ mean copies and $1.6x10^1$ to $1.0x10^5$ mean copies in feces and carcasses, respectively. Median concentrations of *Salmonella* spp. in feces and carcasses were $2.0x10^2$ and $1.6x10^1$, respectively. Overall mean copy numbers were lower than for *C. jejuni/coli*, with a small number of samples driving the upper range. In particular, just two samples (3%) from carcass rinses at grocery stores had high concentrations. At markets, *Salmonella* concentrations in both fecal samples and carcass rinses were elevated, although this difference was only statistically significant when comparing fecal samples between markets and farms. **Figure 1** summarizes pathogen concentrations in feces and carcass rinses by setting and sample type.

Prevalence and quantification of C. jejuni/coli in child stool samples

Concentrations of *C. jejuni/coli* in child stool ranged from $9.1x10^1$ mean copies to $1.1x10^9$ mean copies with a median concentration of $8.4x10^5$ mean copies. Prevalence (97%) and median concentration of *C. jejuni/coli* ($1.4x10^6$ mean copies) were slightly higher in child stool samples where poultry were observed at the time of sample collection. However, we did not detect statistical differences in median concentration ($P = 0.2$) and prevalence ($P = 0.4$) of *C. jejuni/coli* in MapSan child stool samples from compounds where poultry were present versus those where poultry were not observed **(Table 5)**.

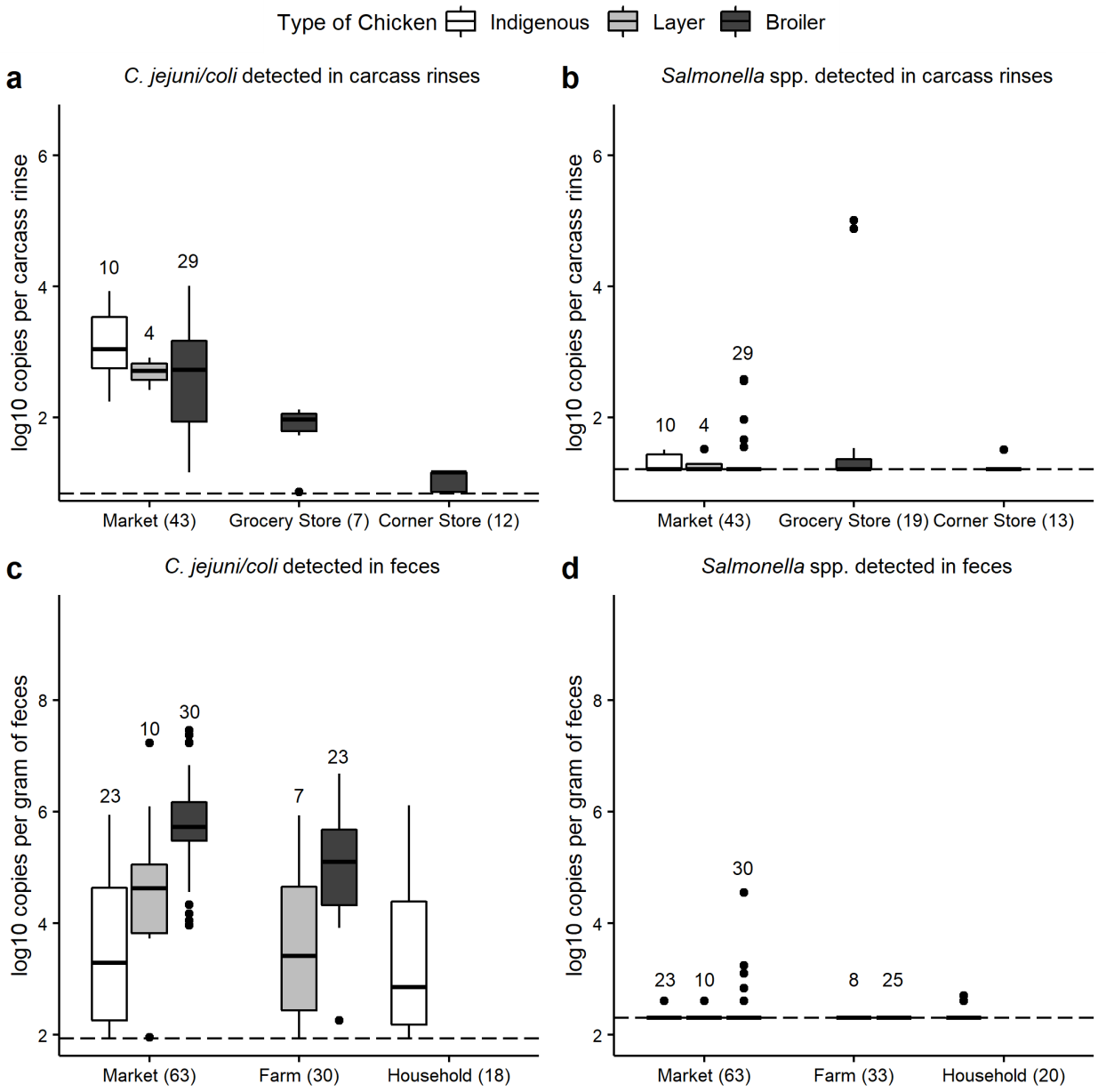

Figure 1. Box plots comparing concentrations of pathogens detected in fecal and carcass rinse samples by qPCR. X-axis labels represent settings (total number of samples). Sample numbers are denoted above box plots for the market and farm settings, where samples were collected from multiple chicken types. All non-detectable samples are included; dashed lines indicate thresholds for non-detects at half the assay LOD. Not all chicken types were available at every location; see text for details.

Table 5. Prevalence, mean (SD), and median copies of *C. jejuni/coli* per gram of feces in child stool samples

	Prevalence	Mean Copies	Median Copies
All stool samples	59/64 (92%)	$2.4x10^7$ ($1.4x10^8$)	$8.4x10^5$
Poultry Present	31/32 (97%)	$1.0x10^7$ ($2.6x10^7$)	$1.4x10^6$
Poultry Not Present	28/32 (88%)	$3.8x10^7$ ($2.0x10^8$)	$3.2x10^5$

Updated ChickFlows

To better understand microbial hazards along the value chains and highlight high-risk settings for child exposures to enteropathogens carried by chickens, we updated ChickFlows diagrams (Chapter 1) to include microbial load and prevalence data at different settings. Updated broiler, layer, and indigenous value chains are presented in **Figures 2-4** and display higher contamination levels as chickens move along the value chains. At the start of the broiler value chain, *Salmonella* spp. and *C. jejuni* were not detected at depots, but at farms *C. jejuni* were detected, and once broilers were sold at grocery stores, corner stores, and informal markets, both pathogens were detected. Similarly, for the layer value chain, no *Salmonella* spp. were detected at farms, but both pathogens were detected at informal markets. Though the indigenous chicken value chain is less formalized and primarily restricted to household/community and informal market settings, *C. jejuni/coli* were highest in indigenous feces once chickens were sold at informal markets.

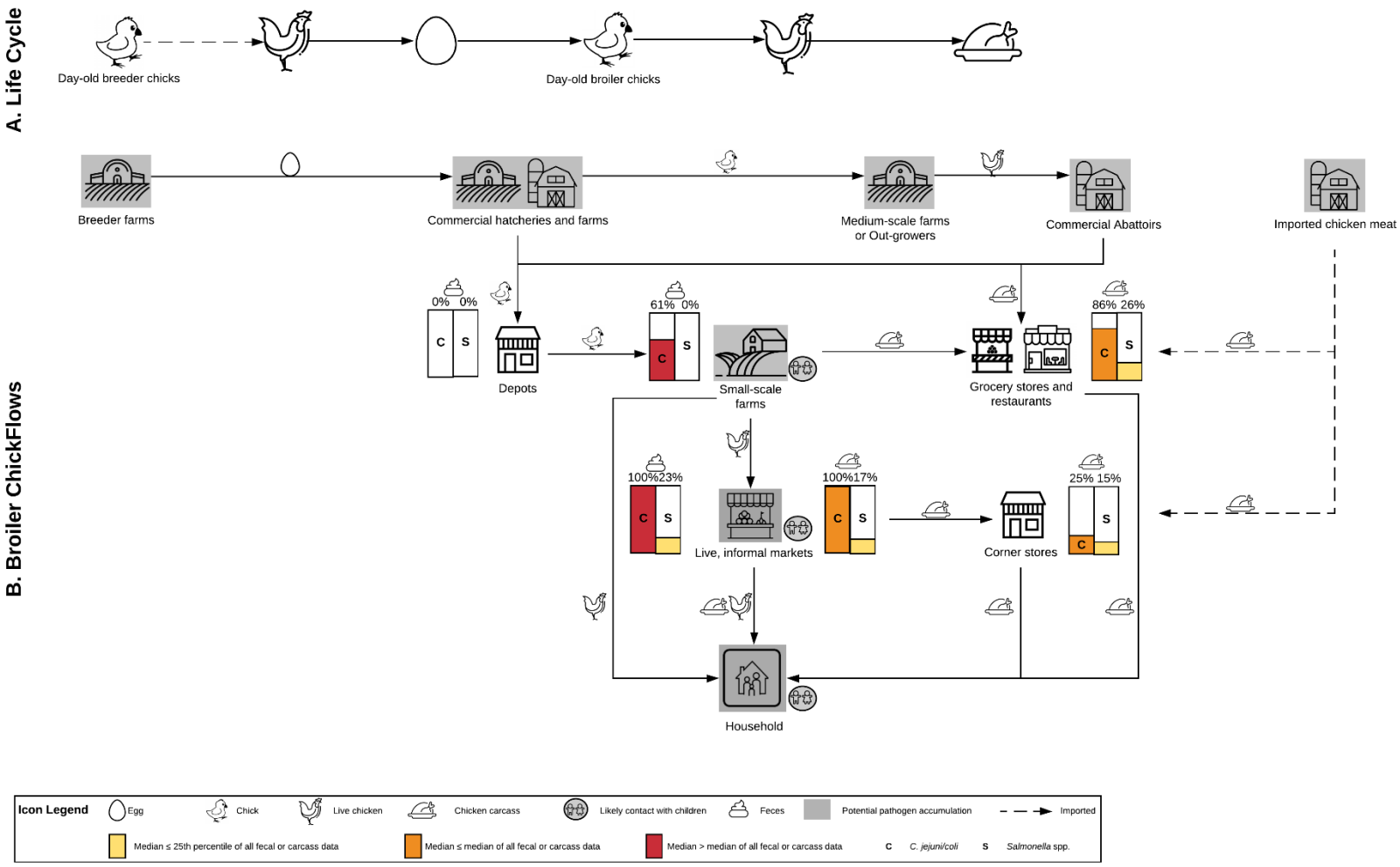

Figure 2. Broiler ChickFlows value chain. This updated map outlines the broiler value chain and incorporates pathogen data for *C. jejuni/coli* and *Salmonella* spp. The yellow-orange-red scale compares median pathogen concentrations for the specific sample type and location to the overall median pathogen concentration for that sample type. Bar scales correspond to pathogen prevalence.

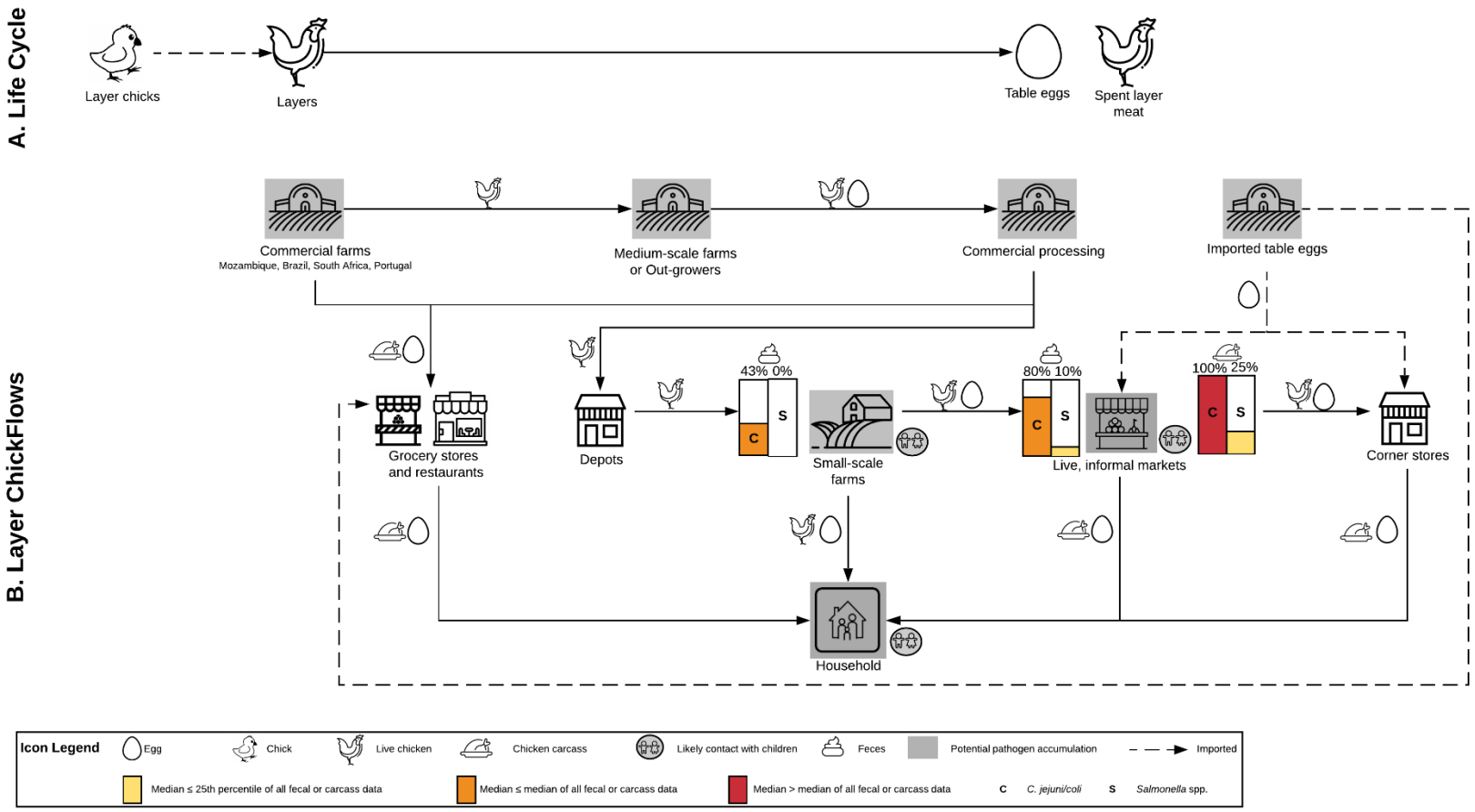

Figure 3. Layer ChickFlows value chain. This updated map outlines the layer value chain and incorporates pathogen data for C. jejuni/coli and Salmonella spp. The yellow-orange-red scale compares median pathogen concentrations for the specific sample type and location to the overall median pathogen concentration for that sample type. Bar scales correspond to pathogen prevalence.

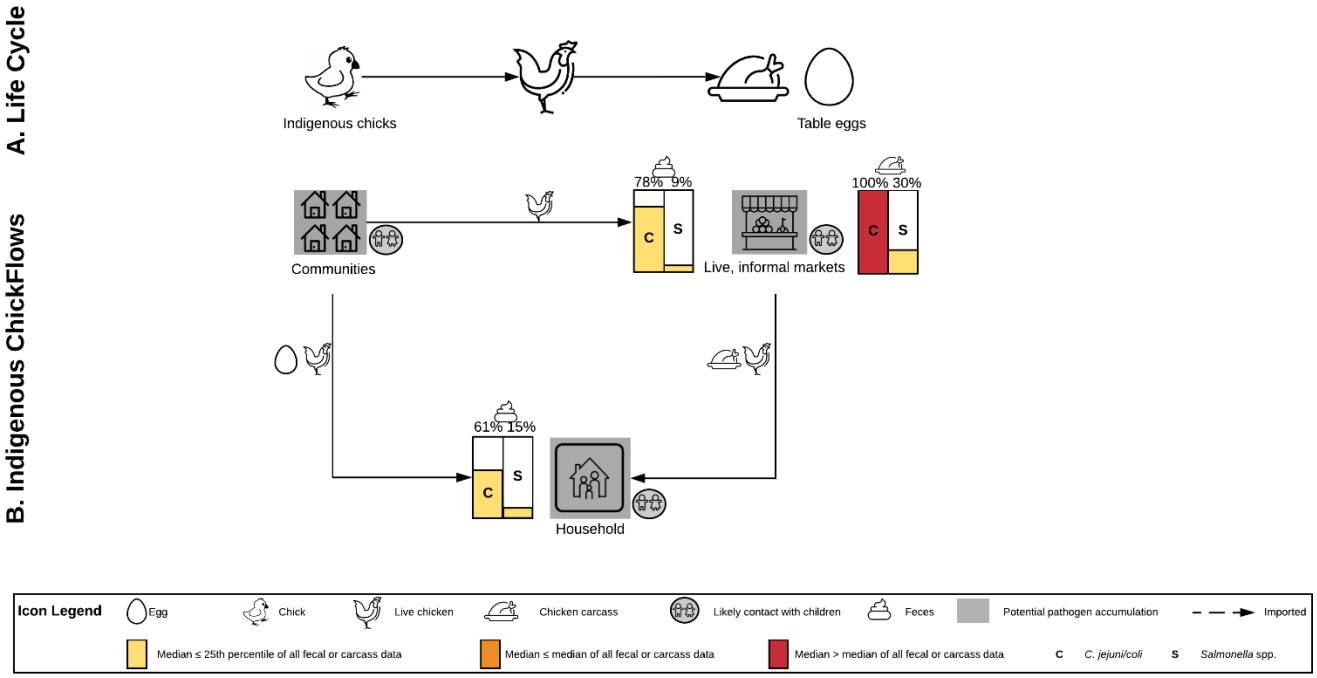

Figure 4. Indigenous ChickFlows value chain. This updated map outlines the indigenous chicken value chain and incorporates pathogen data for *C. jejuni/coli* and *Salmonella* spp. The yellow-orange-red scale compares median pathogen concentrations for the specific sample type and location to the overall median pathogen concentration for that sample type. Bar scales correspond to pathogen prevalence.

Discussion

We evaluated the prevalence and concentrations of enteropathogens carried and zoonotically transmitted by chickens (*C. jejuni/coli* and *Salmonella* spp.) along chicken value chains in Maputo, Mozambique, i.e., in broiler, layer, and indigenous chicken feces and carcass rinses at high exposure risk settings. The value chain frameworks guided identification of sampling locations, from production through marketing, to determine potential human exposure risks and to ultimately inform the development of intervention strategies to mitigate microbial hazards and associated exposure risks. We also confirmed that children <5 years old in Maputo are infected with *Campylobacter* spp. carried by chickens, thus linking child exposure to chicken feces and contaminated chicken meat to potential for infection. Water and sanitation interventions to reduce childhood exposures to enteropathogens have focused primarily on containing human fecal contamination, and on household-level interventions, but have been met with limited success.[31,32,57,58] Refocusing efforts to include interventions in public spaces (i.e. hygiene interventions at informal markets) and to improve food safety, especially in the production of animal-sourced foods, could potentially have a greater impact in reducing enteropathogen infections in children. Our study highlights the need for safe animal fecal waste management along the chicken value chain and for food safety interventions at informal markets.

This study makes three important contributions to the growing body of work investigating childhood risks of enteropathogen exposure from poultry in LMICs. To our knowledge, this study employed a risk-based sampling approach[19] to quantify C. *jejuni/coli* and *Salmonella* spp. along broiler, layer, and indigenous chicken value chains in Maputo, Mozambique. Second, we have both confirmed childhood infections with *C. jejuni/coli* and identified settings along the chicken production system where children can have direct and/or indirect contact with poultry feces. Third, our study complements previous research in sub-Saharan African countries investigating contamination of chicken meat and carriage in live chickens[19,59,60] and suggests several strategies to mitigate childhood exposures to animal feces.

At the start of the value chain (production), layers and broilers were not colonized with *C. jejuni/coli* or *Salmonella* spp., but by the time chickens reached the end of the value chain (consumers), all chickens types were contaminated with and shedding *Salmonella* spp. and C. *jejuni/coli*. No *Salmonella* spp. were detected in farm samples. Though we did not longitudinally follow the same lot of chickens, our results suggest that broilers and layers become colonized with *Salmonella* spp. at some point between transport from farms to being at the market for sale and during processing and distribution to grocery stores and corner stores. Overall, concentrations of *Salmonella* spp. at grocery stores were low, but two carcass samples were highly contaminated. Previous studies have reported higher recovery of *Salmonella* from grocery store chicken meat samples than from wet markets.[61–63] Grocery stores may have more sanitary conditions than wet markets, but there still exists a risk of chickens becoming contaminated with *Salmonella* during commercial processing. The highly contaminated samples may also have resulted from longer storage as compared to freshly processed chickens.[64] This structured approach allowed us to target data collection at pre-identified, high-risk settings (Chapter 1) along each value chain (broilers, layers, and indigenous chickens). The value chain approach provides a useful tool for a targeted assessment of human enteropathogens along both formal and informal animal food production systems.

Our findings highlight the need for food hygiene interventions focused on chicken processing at informal markets. Contamination exists in both the formal and informal chicken production systems, but in Maputo, 90% of households purchase foods from informal markets,[65] thus justifying a closer look at food safety conditions. Intervention studies aimed at improving meat safety at informal markets that focused on training and providing the equipment necessary to safely butcher meat found that the butchers recalled essential hygiene practices taught during the intervention, but the benefits were short-term with no implementation of these measures or additional institutional support.[66] Thus, there is a need for interventions developed in consultation with local governments to improve food safety and hygiene in informal settings.

We found evidence of *C. jejuni/coli* in child feces (92%), confirming that children in Maputo are infected with *Campylobacter* species carried by poultry. Prevalence and median concentration of *C. jejuni/coli* were slightly higher in child stool samples where poultry were observed at the time of sample collection (though not significantly). Our work supports previous research in Africa demonstrating that Ethiopian children <5 years old who were exposed to domestic poultry (hens, pigeons) were 2.9 times more likely to have *Campylobacter* infections than children without exposure.[67] This points to the likelihood of children being at an increased risk of acquiring *C. jejuni/coli* infections from exposure to domestic indigenous chickens, which we found shed high levels of *C. jejuni/coli*. Though children may be exposed to indigenous chicken feces at home, there are additional exposure pathways outside of the ones that are considered by conventional WASH studies, such as contaminated foods coming home.[10] Interventions for limiting household exposures to poultry feces have shown mixed results,[68–70] and while sanitation interventions focus on the household or compound, enteropathogens may be entering the home from outside sources. Therefore, it may be more efficient to control microbial hazards at sources of contamination along the value chain rather than solely at the end user.

Concentrations and prevalence of *C. jejuni/coli* were higher overall than *Salmonella* spp. across all chicken types and settings along the value chain, with up to 100% prevalence at farms and at markets. In particular, there is a high risk of purchasing chickens contaminated with and/or shedding C. *jejuni/ coli* at informal markets. We found that chicken carcasses collected at markets had the highest prevalence and concentration of *Campylobacter* spp. and *C. jejuni/coli*, followed by grocery stores and corner stores. *C. jejuni/coli* was detected in 100% of broiler, layer, and indigenous carcasses and broiler feces sampled at markets. While farm management can reduce *Campylobacter* in flocks, even flocks having a lower prevalence of *Campylobacter* colonization can result in up to 100% of contaminated carcasses after being fully processed.[71–73] Intestinal content from positive flocks can cross-contaminate chicken meat and the surrounding processing environment.[71–73] The processing step is key to mitigating the potential for fecal contamination and intestinal contents on chicken meat.

Carriage and prevalence of *Salmonella* spp. in market samples were lower than C. *jejuni/coli*. In addition to fecal cross-contamination, *Salmonella* spp. could have been introduced via contaminated transport crates and containers or exposure to *Salmonella*- positive birds.[74] Chickens at the market may have been more susceptible to *Salmonella* infection from heat stress and food deprivation prior to processing.[75]

As both *Salmonella* and *Campylobacter* are widespread in food animals in Africa, particularly in poultry,[60] strategies to mitigate enteropathogen exposures in children should include a focus on animal food production systems. Intervention strategies targeting animal feces management along the chicken value chain could reduce microbial hazards on foods at point of sale. Informal markets could establish monitoring programs with frequent microbial testing to determine HACCP for targeted hazard reduction interventions, but this would be costly to implement. Market vendors could also be trained in strategies for infection control in live birds, managing fecal waste, and general food hygiene associated with poultry processing.

This research was subject to several limitations. First, there is potential selection bias arising from the selection of sampling locations. Sampling was performed according to the identification of small-scale farms and households keeping indigenous chickens by neighborhood officials. The census of grocery stores relied on the identification of major retailers via Google Maps, followed by updates from local field staff. Second, comparisons between settings and chicken types did not account for variability within samples collected from the same locations, due to a relatively small sample size. Not all comparisons between chicken type-location combinations were possible because not all locations had all chicken types present. Third, the freeze-thaw cycles for detecting *Cryptosporidium* spp. may have been insufficient at breaking open the oocysts to release DNA, resulting in no detection of *Cryptosporidium* spp. in any samples. The prevalence and abundance of *Cryptosporidium* in chicken feces and carcasses along the value chain in Maputo warrants continued investigation.

Conclusion

Management of feces from chickens and other animals remains inadequate in LMICs and poses a risk to child health. Mitigation strategies should combine an understanding of production systems and food safety with an evaluation of exposure risks. WASH researchers and veterinary scientists have the opportunity to engage each other to develop strategies to raise and market poultry in a way that is safe for children. Our approach of quantifying microbial hazards along the chicken value chain could be applied to livestock systems associated with other animals and/or in other LMICs to highlight where to target future interventions aimed at improving child exposures and health outcomes. Our findings have highlighted the need for monitoring microbial hazards in live chickens and chicken meat at point of sale. Future work could provide longitudinal assessments of the impact of poultry feces management strategies on child health.

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Declarations

The authors declare no competing interests.

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Supplementary Material

Table S1. Summary of qPCR assay performance

Target (gene)	Slope	R2	y-intercept	Efficiency
Campylobacter spp. (16S rRNA)	-3.4	100%	40.0	96.3
C. jejuni/coli (cadF)	-3.4	99%	38.8	97.8
Salmonella spp. (*inv*A)	-3.3	99%	40.2	99.0
Internal amplification control	-3.3	99%	40.7	99.3

PCR conditions, including master mix, primer concentrations, and platform were identical for the samples run in Mozambique and at Emory University.

Table S2. Chicken Fecal Sample Results: Prevalence and mean copies (SD) per gram of chicken feces for *Campylobacter* spp.

	Broiler		Indigenous		Layer	
	Farm	Market	Household	Market	Farm	Market
Prevalence	14/23 (61%)*	30/30 (100%)	14/18 (78%)*	21/23 (91%)	4/7 (57%)*	9/10 (90%)
Mean	$1.2x10^6$ ($3.3x10^6$)	$5.9x10^6$ ($6.5x10^6$)	$4.0x10^5$ ($1.5x10^6$)	$1.7x10^5$ ($3.4x10^5$)	$2.8x10^5$ ($7.3x10^5$)	$1.9x10^6$ ($3.4x10^6$)

*Two farm broiler, two household indigenous, and one farm layer samples had positive field blanks and were discarded from analysis.

Table S3. Chicken Carcass Rinse Results: Prevalence and mean copies (SD) per carcass rinse for *Campylobacter* spp.

	Broiler			Indigenous	Layer
	Corner Store	Grocery Store	Market	Market	Market
Prevalence	5/13 (39%)	17/19 (90%)	29/29 (100%)	10/10 (100%)	4/4 (100%)
Mean	$1.7x10^1$ ($1.5x10^1$)	$1.0x10^3$ ($6.4x10^2$)	$6.1x10^3$ ($1.1x10^4$)	$9.3x10^3$ ($1.1x10^4$)	$2.5x10^3$ ($1.8x10^3$)

Table S4. Crude odds ratios and 95% confidence intervals of associations between settings and chicken types and pathogen prevalence

Comparison Groups	Fisher's Exact Test Comparisons	*C. jejuni/coli* in Feces	*C. jejuni/coli* in Carcass Rinses	*Salmonella* spp. in Feces	*Salmonella* spp. in Carcass Rinses
Settings	Markets to farms	***6.0 (1.9, 20.8)***	NA	6.8 (0.9, 306.3)*	NA
	Markets to households	***5.0 (1.2, 20.6)***	NA	0.9 (0.2, 4.5)*	NA
	Farms to households	0.8 (0.2, 3.2)	NA	0.1 (0.0, 1.5)*	NA
	Markets to grocery stores	NA	11.6 (0.5, 753.1)*	NA	0.7 (0.2, 3.4)
	Markets to corner stores	NA	***92.3 (9.7, 4688.1)****	NA	1.4 (0.2, 15.8)
	Grocery store to corner store	NA	7.8 (0.9, 110.2)*	NA	1.9 (0.3, 23.9)
Chicken Types	Broilers to indigenous	1.9 (0.7, 5.6)*	0.3 (0.0, 2.7)	1.1 (0.3, 4.8)	0.6 (0.1, 4.0)
	Broilers to layers	2.6 (0.7, 9.6)*	0.7 (0.0, 7.4)	2.5 (0.3, 118.1)	0.7 (0.1, 41.8)
	Indigenous to layers	1.3 (0.4, 4.8)*	2.1 (0.0, 187.8)	2.2 (0.2, 111.9)	1.3 (0.1, 89.6)

*2x2 table cell contained a zero value. A value of 1 was added to each 2x2 cell for comparison.
Bold italicized associations have a statistical significance of $p<0.05$.

Table S5. P-values for Dunn's test comparisons of median pathogen concentrations between settings and chicken types

Comparison Groups	Fisher's Exact Test Comparisons	*C. jejuni/coli* in Feces	*C. jejuni/coli* in Carcass Rinses
Settings	Markets to farms	0.6	NA
	Markets to households	***0.0***	NA
	Farms to households	0.0*	NA
	Markets to grocery stores	NA	***0.0***
	Markets to corner stores	NA	***0.0***
	Grocery store to corner store	NA	***0.3***
Chicken Types	Broilers to indigenous	***0.0***	***0.0***
	Broilers to layers	***0.0***	0.4
	Indigenous to layers	0.0*	0.4

Dunn's test comparisons were performed for comparisons with significant Kruskal Wallis results.
Bold italicized associations have a statistical significance of $p<0.05$.
*No longer significant after adjustment for multiple testing

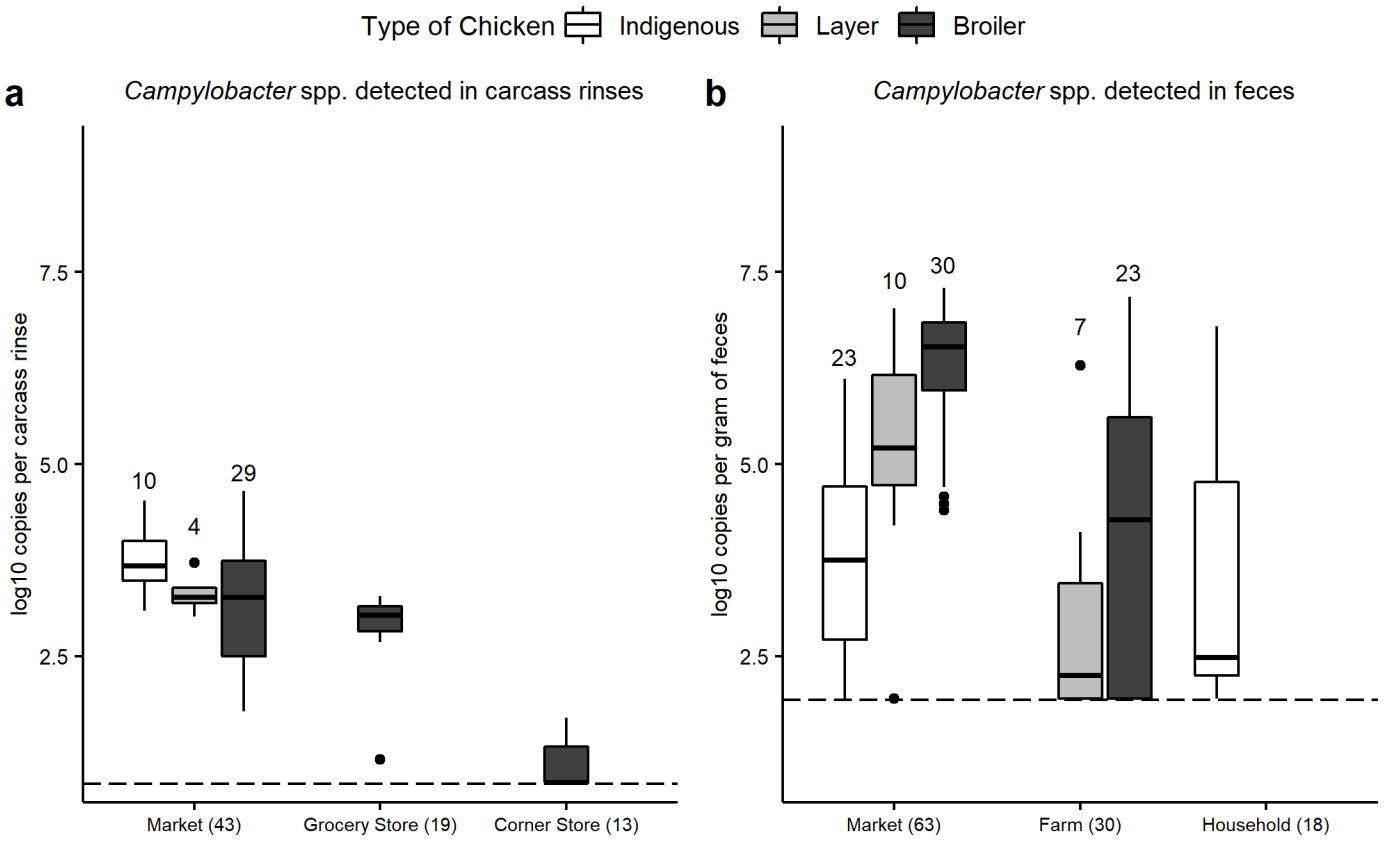

Figure S1. Box plots comparing concentrations of pathogens detected in fecal and carcass rinse samples by qPCR. X-axis labels represent settings (total number of samples). Sample numbers are denoted above box plots for the market and farm settings, where samples were collected from multiple chicken types. All non-detectable samples are included; dashed lines indicate thresholds for non-detects at half the assay LOD. Not all chicken types were available at every location; see text for details.

Chapter 3. Accumulation of microbial hazards associated with broiler chicken processing at wet markets: a time-series study in Maputo, Mozambique

Abstract

The burden of foodborne disease due to the consumption of animal source foods is substantial in low- and middle-income countries (LMICs). Wet markets provide access to fresh and affordable foods but are often poorly regulated and have the potential to spread both human and zoonotic diseases due to unhygienic practices. Contamination of chicken meat during slaughter and subsequent processing has been well documented at wet markets; yet, the accumulation of pathogens from the reuse of rinse water has not been investigated. This data would provide information on the role water plays in contributing to the cross- contamination of chicken carcasses. We conducted a time-series study at three wet markets in Maputo, Mozambique to assess the accumulation of *C. jejuni/coli*, *Salmonella* spp., and *E. coli* in paired rinse water and broiler carcass samples collected at 75-minute intervals. We collected 70 rinse water (including 10 baseline samples prior to any processing) and 60 paired broiler carcass rinse samples from 10 vendors (1 baseline and 6 paired samples). Chicken processing activity and associated hygiene practices were captured through direct observation, recorded using Open Data Kit (ODK) software, and reported for each vendor. All samples were analyzed for *E. coli* using the Colilert-18 test and for *C. jejuni/coli* and *Salmonella* spp. using qPCR. *C. jejuni/coli* and *E. coli* were detected in 30% and 88% of baseline samples, respectively. *Salmonella* spp. were not detected in any baseline samples. Starting with the first rinse, *C. jejuni/coli* and *E. coli* were detected in 100% of samples, and *Salmonella* spp. were detected in 42% of rinse water and 48% of carcass rinse samples. Mean concentrations (excluding baseline) were 4.4 log10 copies per 100 mL of rinse water and 3.6 log 10 copies per 100 mL of carcass rinse for *C. jejuni/coli*, 1.2 log10 copies per 100 mL of rinse water or carcass rinse for *Salmonella* spp., and 7.0 log10 MPN per 100 mL of rinse water and 6.6 log10 MPN per 100 mL of carcass rinse for *E. coli*. *C. jejuni/coli* showed an average 0.1 log10 copies (95% CI 0.0, 0.2) increase in rinse water and carcass rinse samples every 75 minutes. Our findings show that consumers are at a high risk of purchasing

chicken meat contaminated with human enteropathogens and may be at a greater risk if purchasing chickens later in the day due to pathogen accumulation. Contaminated chicken meat has the potential to contaminate the household environment and seed transmission. Low-cost and feasible interventions implemented during chicken processing may reduce microbial hazards on chicken meat before purchase.

Introduction

There is a high burden of foodborne disease associated with consuming animal source food (ASF), particularly in low- and middle- income countries.[1] The WHO identified foodborne transmission as the most important route for exposure to *Campylobacter* spp., non-typhoidal *Salmonella* (NTS), and Shiga toxin-producing *Escherichia coli* (STEC) across all subregions.[2] *Campylobacter* spp. and NTS, key pathogens carried by chickens, caused an estimated 80,527 combined foodborne deaths in 2010.[3,4] Urbanization increases risks of zoonotic diseases via poor sanitation and bringing livestock from urban to rural areas.[5] There is little in the way of interventions focused on food safety[6] and animal feces management[7] in resource-poor settings.

Live markets play a significant role in providing food security in LMICs, with access to fresh and affordable food being the main driver of consumers to these markets;[8,9] yet, food safety is a critical, but understudied component of food security.[10] Live markets, also referred to as informal or wet markets, sell a range of perishable goods, from fruits and vegetables to wild-caught animals, and often slaughter and process animals on site.[8,11] These settings are often fully or partially open-air, informal and have minimal enforcement of regulations.[11] Outbreaks have put the spotlight on wet markets and their high potential to spread zoonotic diseases,[8,12,13] but less attention has been paid to wet markets as a source of enteric pathogens that can seed households with infectious agents that may be passed within a household due to inadequate water, sanitation, and hygiene conditions. Slaughter and subsequent processing can contaminate chickens and the surrounding environment with *C. jejuni/coli*, *Salmonella* spp. and *E. coli*,[14–17] which are all present in the intestinal tracts of chickens.[18,19] In our recent work at informal markets in

Maputo, 100% of broiler carcasses sampled were contaminated with *C. jejuni/coli*, and 17% of carcasses were contaminated with *Salmonella* spp. (Chapter 2).

Despite potential microbial risks associated with purchasing foods from informal markets, populations value fresh foods from traditional markets[20] and depend on informal suppliers for sourcing food.[21] Thus, there is a need to monitor microbial food hazards at wet markets[13] to identify points where preventative measures can be implemented to improve food safety, and prevent cascading transmission downstream.

The Hazard Analysis Critical Control Point (HACCP) system provides a means to control food safety hazards[22] associated with poultry slaughter[23] but cannot be implemented without first identifying and assessing these hazards. Though widely adapted for poultry slaughter,[23] HACCP is difficult to implement in small or less developed businesses and low-income settings.[24] Prerequisites for implementing a successful HACCP program in the informal poultry sector include training, surveillance systems, and microbial data on the presence of *Salmonella*, *Campylobacter*, and other poultry-associated pathogens and have been cited as limitations in LMICs.[25]

Understanding the contamination of chicken meat from processing may be key to reducing and addressing the public health impact of exposures to these microbial hazards. Inadequate supply of fresh water may introduce opportunities for the cross-contamination of chicken meat.[26] In industrial production, an average of 26 L[27] of water is used per chicken. In the informal sector, individual vendors have limited and often intermittent access to water for food hygiene.[26,28] Past studies quantified and characterized contamination at live markets,[29–32] but have not focused on how pathogens accumulate in the informal slaughter and processing environment. Data on how pathogens accumulate in these environments would inform recommendations for hygienic processing of chickens at informal market settings. The objectives of this study were (i) to assess the accumulation of *C. jejuni/coli*, *Salmonella* spp., and *E. coli* on broiler

chicken carcasses and in rinse water as chickens were processed throughout the day and (ii) to examine chicken purchasing, processing activity and associated food hygiene practices.

Methods

Study setting

This study was conducted at three informal markets in Maputo, Mozambique. Maputo has a population of 1.1 million[33] and is comprised of seven municipal districts. The majority of Maputo households (71%) are designated as food insecure, as defined by four metrics of household insecurity that focus on food access, including the Household Food Insecurity Access Scale (HFIAS), the Household Food Insecurity Access Prevalence (HFIAP), the Household Dietary Diversity Score (HDDS), and the Months of Adequate Household Food Provisioning (MAHFP).[34,35] The informal food economy is the most important source of food,[34] and wet markets provide food to more than 90% of households.[35] Wet markets range from having infrastructure improvements by the municipality, such as toilets and drainage, to static and mobile vendors operating outside formal markets,[34] and access to water is an issue.[28]

Informal (non-industrialized) production of chicken meat is essential to Mozambique's poultry sector, contributing 70% of production capacity;[36] yet there is little capacity to enforce food standards and regulations (Chapter 1). Standards for good chicken production practices[37], eggs and egg products[38,39], chicken slaughter[40], and butchered chickens[41] exist but adherence to these standards is not monitored in or appropriate for the informal sector. Twelve percent of households in Maputo raise chickens for food.[35]

Study design

We conducted a time-series study at three informal markets (Markets A, B, and C) in Maputo, Mozambique between July 2021 and September 2021. The purpose was to determine how concentrations of *C. jejuni/coli* and *Salmonella* spp. (primary outcomes) and fecal indicator bacteria (secondary outcome) in processing water and on chicken carcasses varied throughout the day as broiler carcasses

were slaughtered and processed, and to characterize associated food hygiene practices. We visited ten live broiler vendors from three markets to collect freshly butchered broiler carcasses and rinse water samples and to observe chicken purchasing, butcher activity, and associated food hygiene practices.

Vendor Selection

We conducted a two-stage sampling process, first sampling markets, then sampling vendors within markets.

In the first stage of sampling, we purposively selected three markets from a list of 18 markets generated during previous sampling (Chapter 2). We were advised by our local agricultural consultant to sample these markets, because they serve low- and middle- income shoppers, are the largest and most frequented markets in the city, and have high chicken processing activity (as observed during previous sampling at these markets – Chapter 2).

In the second stage of sampling, we randomly selected vendors in each of the three markets. To create a sampling frame, markets were visited to map a layout of stands selling and processing broilers. Market maps included a layout of the immediate area surrounding chicken vendors, including the number of stands selling chickens, types of chickens and other goods being sold, locations of processing areas (if applicable), handwashing stations, sanitation facilities, drainage, and solid waste bins (**Supplementary Figures 1-3**).

Stands were eligible for inclusion if vendors sold and processed broiler chickens. At Markets B and C, vendors had a designated person for processing chickens, and this activity occurred in a set area away from the stand (the area belonging to an individual vendor for the sale and processing of chickens). Therefore, at Markets B and C, stands were only eligible for inclusion if the processing area was in close proximity to the stand to allow observation of both the stand and chicken processing. Stands were not eligible if vendors were observed processing layers, indigenous, or other types of poultry or were only

selling live chickens, but not processing. We previously observed differences in pathogen carriage based on the chicken type (Chapter 2), so vendors that only processed broilers were included to not introduce additional variables that may impact contamination levels. Stands were also ineligible if vendors did not pass COVID-19 screening questions (experienced symptoms or exposure within the past 2 weeks to someone with a suspected or confirmed illness). All eligible stands were randomly selected four vendors at Market A and three vendors each at Markets B and C using a random number generator in Excel. We asked vendors the approximate time they typically begin processing chickens and did not disclose why we asked for this information at the time to limit vendors from changing behaviors prior to data collection.

To guide enumerators to the stands in order of selection, we printed market sketches with numbers 1-8 above each pre-selected stand to correspond with the order in which they were randomly assigned. Among the selected vendors, we then sought informed consent on the day of sample collection. If any of the selected vendors did not consent, we went to the next randomly selected vendor.

Data Collection

We collected samples to reflect the microbial quality of chicken meat after chickens have been processed, and therefore used knowledge of chicken processing from previous sampling to determine our strategy for sampling rinse water and chickens. Specifically, in formative research, we observed the fresh slaughter and processing of chickens at informal markets in Maputo (Chapter 1). Processing is generally performed with minimal personal protective equipment and hygiene considerations. Live birds are kept in holding crates until slaughter, which begins with neck cutting and bleeding followed by scalding, defeathering, evisceration, harvesting of innards, and rinsing. Processed birds are typically kept at ambient temperature in bowls or plastic bags until purchased. Based on these direct observations of chicken processing, we determined that it was appropriate to sample carcasses and rinse water.

To understand water quality prior to any rinses, we sampled the rinse water (baseline) at each stand. To assess the relationship between time since initial butchering activity and pathogen concentration in water and on broiler carcasses, we sampled the first chicken that was processed and its rinse water at the same time. We repeated this paired sampling every 75 minutes (from approximately 7:30 to 15:30), which included a total of six paired broiler carcass and rinse water samples and ambient and rinse water temperature readings per vendor. **Figure 1** summarizes data collection activities.

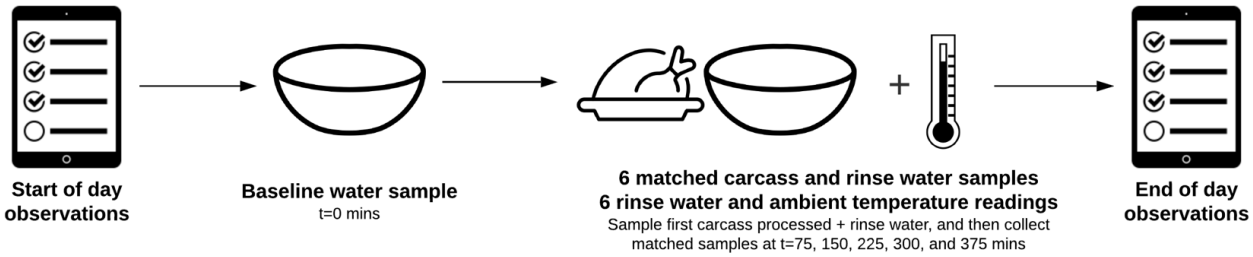

Figure 1. Summary of data collection activities. Data collection included start and end of day observations, collection of a baseline water sample prior to any chicken processing, six matched carcass and rinse water samples at 75-minute intervals, and rinse water and ambient temperature readings at the time of matched sample collection.

Observational Tool Development

We used a start and end of day observations checklist to understand processing activities and food hygiene practices at each stand, a start and end of day observations checklist was developed (**Supplementary Forms 1 and 2**). Checklists were informed by the results of our previous sampling at informal markets in Maputo (Chapter 2) and by a review of observational studies at markets (**Table 1**).[10,42–49]

The observation checklist was translated into Portuguese and reviewed by study enumerators for translation accuracy. To confirm correct coding and skip patterns before implementation, enumerators completed five practice forms, including one-on-one practice of the vendor questionnaire. Practice results were discussed to clarify discrepancies and improve the data collection tools.

Table 1. Characteristics included in start and end of day observations checklists

Topic	Purpose of data collection	Timing of data collection
Market and vendor information	Collect basic information to identify the market and stand at which data was collected	Start and end of day
COVID-19 screening questions	To protect the enumerator and study participant	Start of day
Quantity and type of chickens	Assess quantity of chickens processed during sample collection period	Start and end of day
Presence of other live birds or animals	Assess if there is a potential risk of cross-contamination with other human enteropathogens	Start of day
Material of holding cages	Assess general hygiene associated with live chickens	Start of day
Water, sanitation, and hygiene (WASH)	Assess access to handwashing facilities and availability of soap and water for handwashing	Start of day
Presence of flies	Assess food hygiene waste storage	End of day
Observed and reported cleaning practices and waste storage	Assess food hygiene	End of day
Cold storage for processed chickens	Assess food hygiene	End of day
Location of chicken processing	Assess food hygiene	End of day

Start and end of day observations

We assessed vendor stand characteristics and food safety risks that could inform future intervention work. We conducted structured observations at each stand at the start and end of day. Following observations, vendors were asked questions related to WASH and food hygiene.

Sample collection

To assess the microbial quality of freshly processed chicken meat, a single, most recently processed broiler carcass was purchased (~ 4 USD) and placed individually into a Nasco poultry rinse bag (VWR, Bridgeport, NJ) at each time point. Participants were made aware that six broiler chickens would be purchased prior to sampling. Carcass samples were double bagged to prevent contamination and placed on ice for transport to the laboratory and processed same day.

We were interested in the cross-contamination of chicken meat during the final rinsing of chicken carcasses. Immediately following carcass sample collection, one rinse water sample, scooped into three sterile 50 mL conical tubes, was collected at each time point from the bowl or container used to perform a final rinse of chickens. All water samples from a single vendor were collected from the same bowl. The sides of each 50 mL conical were sanitized with 70% ethanol solution to prevent contamination during handling. A baseline water sample (100 mL) was also collected at the start of each day prior to butchering any chickens. Samples were placed on ice for transport to the laboratory and processed same day.

Field Data Management

Observations checklists were created in Excel and programed for data collection in Open Data Kit (ODK) (available from https://opendatakit.org/).[50] Observations data were collected electronically using password-protected Samsung Galaxy Android tablets (Samsung Electronics Co., Ltd., Suwon, South Korea) to improve the accuracy of data entry and enable immediate monitoring of results. Data were uploaded daily and stored securely using ODK.[50]

Laboratory Methods

Carcass Rinse Processing

Whole carcass rinses were performed in 400 mL 0.1% buffered peptone water (BPW) (VWR, United Kingdom) and hand shaken, moving the bag in an arc motion, for one minute.[51] After shaking, 200 mL of

the rinse solution were aliquoted into four 50 mL conical tubes. One 50 mL tube was set aside for quantification of *E. coli.* The remaining two 50 mL tubes were centrifuged for 10 minutes at 400 RPM. Pellets were resuspended in 1 mL of 1X PBS and kept at -20°C for DNA extraction within a week.

Rinse Water Processing

One 50 mL tube was set aside for quantification of *E. coli*. The remaining two 50 mL tubes were centrifuged for 10 minutes at 400 RPM. Pellets were resuspended in 1 mL of 1X PBS and kept at -20°C DNA extraction within a week.

DNA Extraction

Resuspended pellet samples were thawed for DNA extraction. 250 µL of the resuspended pellet solution was transferred into PowerBead DNA extraction tubes (Qiagen, Louisville, KY). DNA was extracted from 250 µL of rinse water and carcass rinse solution using the DNEasy PowerSoil Pro Kit (Qiagen, Louisville, KY) according to the manufacturer's protocol. Diethylpyrocarbonate (DEPC) treated water (Life Technologies Corporation, Carlsbad, CA) was included in each set of extractions as a negative control. A tissue lyser (Tissue Lyser LT, Qiagen, Hilden, Germany) was used at speed 3 for 3 minutes to optimize cellular lysis. Purified DNA was eluted with 80 µL of 10 mM tris buffer (Qiagen, Louisville, KY) at pH 8 and immediately stored at -80°C prior to downstream analyses.

qPCR Analysis

Quantitative PCR (qPCR) assays were used to quantify *Salmonella spp*.[52] and *C. jejuni/ coli*.[53] Each sample was spiked with $2.5x10^6$ copies of an artificially designed inhibition control gene target prior to analyses.[54] Primers and annealing temperatures for assays are summarized in **Table 2**. Seven-point standard curves were prepared, and limits of detection and quantification were calculated as previously described.[55,56]

Table 2. Summary of qPCR primers and annealing temperatures

Pathogen	Gene target	Primer sequence (5' to 3')	Annealing temp	Reference
C. jejuni/ coli	cadF-F	CTGCTAAACCATAGAAATAAAATTTCTCAC	55°C	Platts-Mills et al. 2014[53]
	cadF-R	CTTTGAAGGTAATTTAGATATGGATAATCG		
Salmonella spp.	*invA*-F	GCTGCTTTCTCTACTTAAC	55°C	Heymans et al. 2018[52]
	invA-R	GTAATGGAATGACGAACAT		
Internal amplification control	IAC-F	CTAACCTTCGTGATGAGCAATCG	63°C	Deer et al. 2010[54]
	IAC-R	GATCAGCTACGTGAGGTCCTAC		

Seven-point standard curves were prepared using known quantities of gblock gene fragments (Integrated DNA Technologies, Coralville, IA) that included the template reference sequence at ten-fold dilutions ranging from 10^6 to 10^1 gene copies per reaction. A standard curve was included on each plate across all assays. Gene target abundance was estimated from Ct values by interpolation to a standard curve as the mean concentration of duplicate reactions and reported as gene copies per gram of feces or gene copies per carcass rinse. Standard curves were averaged for each assay, and averaged curves were used for data analysis.

Limits of detection and quantification. Standard curves were analyzed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[55] Detection and quantification limits were determined according to methods previously described.[56] The limit of detection (LOD) was determined based on the standard curve as the lowest amount of template for which there was amplification for 90% of the runs. The limit of quantification (LoQ) was defined as the lowest concentration that was accurately quantified with an acceptable level of uncertainty and was calculated for each assay as follows: $CtLoQ = CtLoD - 2(\sigma LoD)$. If zero or one well amplified, results were deemed non-detectable (ND) and assigned a value half of the LoD. If both duplicates amplified but were beyond the lower limit of the dynamic range or LoQ, results were deemed detected but not quantifiable (DNQ) and assigned the value of the LoD. If both duplicates amplified but were beyond the upper limit of the dynamic range, results were assigned the highest quantifiable concentration. For calculating prevalence of gene target, if both wells amplified, the sample was treated as positive. Average assay efficiency was 98%

for the inhibition control target, 101% for *Salmonella* spp., and 102% for *C. jejuni/coli*. Mean slope, y-intercept, R2, and efficiency for each assay is listed in Table S1 (**Supplementary Table 1**).

IDEXX Colilert 18 Procedure and Analysis

Prior to starting sample collection, tests were performed to determine if BPW, used to perform carcass rinses, interferes with IDEXX Colilert 18 (IDEXX, Westbrook, ME) results. 100 mL of wastewater were collected from an open drain near the laboratory. Three, 99.9 mL aliquots of sterile distilled water and three, 99.9 mL aliquots of sterile 0.1% BPW were all spiked with 100 µL of wastewater. Samples were mixed with reagent, poured into a Quanti-Tray (IDEXX, Johannesburg, South Africa), sealed, and then placed in an incubator at 35°C for 18 hours. We found no difference in *E. coli* results between the groups, as confirmed by a t-test, and determined that BPW will not have a significant effect on carcass rinse results.

To quantify *E. coli* prevalence and concentration in carcass and rinse water samples, IDEXX Colilert-18 tests (IDEXX, Westbrook, ME) were performed same-day to the manufacturer's protocol. Rinse water and carcass rinse samples were diluted in sterile deionized water to an appropriate dilution for enumeration, which was determined after testing rinse water and carcass rinse samples from a market not included in this study. Generally, carcass rinse and rinse water samples were diluted 5-fold to 7-fold, and baseline water samples were analyzed undiluted, 1:10, and two-fold. Samples were processed immediately, and remaining samples were kept at 4°C. After 18 hours of incubation, if samples were determined to be unquantifiable (all cells were positive for *E. coli*), additional dilution series were performed immediately to ensure that all processing began within 24 hours. Due to an unexpected supply issue, Market A samples had slightly different quantification methods than Market B and C samples. For *Market A* samples, 100 mL of diluted sample were mixed with reagent, poured into a Quanti-Tray (IDEXX, Johannesburg, South Africa), sealed, and then placed in an incubator at 35°C for 18 hours. *Market B and C* samples were quantified using a Quanti-Tray/2000 (IDEXX, Johannesburg, South

Africa) and were incubated at 35°C for 18 hours. A Quanti-Tray/ 2000 comparator (IDEXX, Johannesburg, South Africa) was used to distinguish threshold positive results from negative results. Water quality was determined as most probable number (MPN) of *E. coli* per 100 mL.

Limits of detection and quantification. The lowest detectable concentration of *E. coli* for the Colilert-18 test was determined by the lowest dilution performed. Results below the detection limit were assigned a value equal to half the limit of detection. Results above the upper detection limit were assigned a value of 200.5 to standardize Quanti-Tray and Quanti-Tray/2000 results.

Statistical Analysis

Data were compiled, cleaned, and analyzed in R 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Separate statistical analyses were conducted for *C. jejuni/coli*, *Salmonella* spp., and *E. coli*. Pathogen prevalence data were calculated for baseline, rinse water, and carcass rinse samples. Shapiro-Wilks tests were run to determine data normality. Mean and median log10 transformed concentrations were calculated for each pathogen and reported as average and median log10 gene copies or MPN per 100 mL of carcass rinse or 100 mL of rinse water sample. To compare the distribution of pathogens in carcass rinse to rinse water samples, Wilcoxon Signed Rank Tests for paired data were performed.

To assess the accumulation of *C. jejuni/coli*, *Salmonella* spp., and *E. coli* on broiler chicken carcasses and in rinse water (Study Objective 1), we first plotted log10 averaged pathogen data and 95% confidence intervals (CIs) at each time point for a visual inspection of trends. The relationship between pathogen concentration and time since processing the first carcass was assessed with linear mixed effects models where the outcome of interest was pathogen concentration and the explanatory variable was time. Because paired samples were collected six times from the same vendor, vendor was included as a random intercept. For all statistical tests, results with p-value < 0.05 were considered statistically significant.

As we noticed during the visual inspection that the slope of the increase in log10 averaged pathogen concentration changed over time (details are reported in the Results section), we conducted change point analyses to statistically detect the timing and magnitude of changes in the slopes. Change point analysis is a method that detects changes in temporal trends in time series data.[57] To detect when the slope changed, we fit two lines to our data that have six time points (first sample, 75, 150, 225, 300, and 375minutes) by allowing the slope to change at each time point. For example, when the "change point" is at time point 2, we fit a line to the first two data points and the other line to the remaining data points. We repeated this process with all possible change points (time point 2, 3, 4, and 5) and selected the best-fit model based on the Akaike Information Criterion (AIC).[58] A difference in AIC values greater than 2 was considered significant.

To examine chicken purchasing, processing activity, and associated hygiene practices at markets (Study Objective 2), we summarized start and end of day observations data. Data were exported from ODK into Excel and imported into R 4.0.2 for analysis. To summarize processing activity and food hygiene practices, descriptive statistics (prevalence) were run. Data on the number of workers, processing activity, availability of handwashing facilities, and frequency of cleaning were reported for each vendor.

Ethics

The Institutional Review Board at Emory University (IRB00108546) and the Research Council to the Veterinary Faculty at Eduardo Mondlane University determined that this research is exempt from further human subjects review, and the Municipality of Maputo (Reference number 754/SG/426/GP/2019) authorized this research. Prior to data collection, the study's purpose and participant rights were explained in Portuguese, and participants provided verbal informed consent. Participants were made aware that enumerators would purchase broiler chickens for data collection. To take precautions against spreading COVID-19, enumerators completed daily screenings, including temperature screenings and self-

assessments of symptoms and exposure to any individual within the prior two weeks with a suspected or confirmed case of COVID-19. Vendors were also screened prior to recruitment.

Results

Eleven vendors were approached to consent to data collection. One vendor declined participation when asked for consent due to time limitations. Information on chicken processing activity, associated hygiene practices, and market characteristics were collected from ten vendors through observations at three wet markets. In total, 70 rinse water (including ten baseline) and 60 broiler carcass samples were collected and analyzed for *C. jejuni/coli*, *Salmonella* spp., and *E. coli*.

Market characteristics, vendor processing activity, and associated hygiene practices

Markets included in our study had variable access to handwashing facilities and overall low levels of hygiene: two of three markets had access to a handwashing facility, and of those with access, the sink or tap was away from the stand; only one stand had soap or detergent available at the sink or tap (**Table 3**). Across all markets, chicken selling and processing is located in a separate section of the market; however, this section borders stands selling vegetables at Market B and live goats at Market C. All vendors reported never cleaning broiler holding cages (all used porous material as bedding, such as cardboard boxes), and 90% of vendors reported never cleaning the general area around the stand. 90% of vendors processed chickens on a table or container elevated from the ground, and 10% of vendors processed chickens in a container on the ground. All vendors stored processing waste in open containers. 10% of vendors cooked chickens at the stand where chickens were processed. Flies were observed around chicken meat at each stand, and no cold storage was observed. 10% of vendors cleaned leafy greens at the stand. Ambient temperatures ranged from 14-30°C, and rinse water temperatures ranged from 15-28°C.

Vendors processed an average of 21 broilers during the observation period, ranging from having 15-80 broilers at the start of the observation period to 0-10 broilers at the end of the observation period. Only broilers were processed at each stand, and no other animals or poultry were observed.

Table 3. Summary of vendor processing activity and associated hygiene practices

Vendor	No. of workers	No. of broilers at start/ end of day	Cooks chickens	Processing location[a]	Handwashing Facility			Frequency of Cleaning		
					Location[b]	Water available	Soap or detergent available	Holding cages	Butcher materials	General area
Market A										
Vendor 1	3	20/0	No	Location 1	Location 4	Yes	Yes	Never	Daily	Never
Vendor 2	1	70/53	Yes	Location 1	Location 4	Yes	No	Never	Daily	Never
Vendor 3	1	40/10	Yes	Location 1	Location 4	Yes	No	Never	Daily	Never
Vendor 4	2	80/45	Yes	Location 2	Location 4	Yes	No	Never	Daily	2-6 times per week
Market B										
Vendor 5	1	60/42	No	Location 1	Location 4	Yes	No	Never	Daily	Never
Vendor 6	1	20/0	No	Location 1	Location 4	Yes	No	Never	Daily	Never
Vendor 7	1	30/14	No	Location 1	Location 4	Yes	No	Never	Daily	Never
Market C										
Vendor 8	1	15/0	No	Location 3	No facility	No	No	Never	Daily	Never
Vendor 9	1	25/0	No	Location 3	No facility	No	No	Never	Daily	Never
Vendor 10	1	30/12	No	Location 1	No facility	No	No	Never	Daily	Never

[a]Processing locations: Location 1- Table, elevated from the ground; Location 2- Container on the ground; Location 3- Container, elevated from the ground
[b]Handwashing facility locations: Location 4- Sink or tap away from the stand

Prevalence of C. jejuni/coli, Salmonella spp., and E. coli in carcass and rinse water samples

At baseline, detection of *C. jejuni/coli*, *Salmonella* spp., and *E. coli* were 30%, 0%, and 88%, respectively (**Table 4**). *C. jejuni/coli* were detected in 30%, 100%, and 100% of rinse water, and carcass rinse samples, respectively. *Salmonella* spp. were detected in 0%, 42%, and 48% of rinse water, and carcass rinse samples. *E. coli* were detected in 88%, 100%, ad 100% of rinse water, and carcass rinse samples. Samples (12 carcass rinse and 14 rinse water) were deleted from *E. coli* analyses due to detecting *E. coli* in two trip blanks and one field blank. One broiler rinse sample was unaccounted for during Colilert-18 analyses. Prevalence and log10 mean and median concentrations are summarized in **Table 3**. *Salmonella* spp. prevalence varied by market. At Market A, no *Salmonella* spp. were detected at two vendor stands, and one stand had one positive broiler sample.

Table 4. Prevalence and log10 mean concentration (SD) of *C. jejuni/coli, Salmonella* spp., and *E. coli* per 100 mL of carcass rinse or 100 mL of rinse water sample

	Statistic	Rinse Water, Baseline	Rinse Water, Sample 1	Rinse Water, Samples 1-6	Carcass Rinse, Samples 1-6
C. jejuni/coli	Prevalence	3/10 (30%)	10/10 (100%)	60/60 (100%)	60/60 (100%)
	Mean copies	1.3 (1.0)	3.8 (1.4)	4.4 (1.0)	3.6 (0.9)
	Median copies	0.8	3.8	4.4	3.6
Salmonella spp.	Prevalence	0/10 (0%)	4/10 (40%)	25/60 (42%)	29/60 (48%)
	Mean copies	ND	1.1 (0.5)	1.2 (0.6)	1.2 (0.6)
	Median copies	ND	0.8	0.8	0.8
E. coli	Prevalence	7/8 (88%)*	8/8 (100%)*	48/48 (100%)*	47/47 (100%)*
	Mean MPN	2.0 (1.9)	6.6 (1.1)	7.0 (0.8)	6.6 (0.8)
	Median MPN	1.9	6.9	7.0	6.8

*Samples with contaminated blanks were removed.
Non-detectable samples were assigned half the value of the limit of detection.

Quantification of C. jejuni/coli, Salmonella spp., and E. coli in carcass and rinse water samples

Low levels of *C. jejuni/coli* and *E. coli* were detected in baseline rinse water samples. Concentrations spiked after processing the first carcass and the following details exclude baseline samples. The first carcass contributed an average 3.8, 1.1, and 6.6 log10 mean copies of *C. jejuni/coli*, *Salmonella* spp., and

E. coli to the rinse water. Overall, we observed low levels of *Salmonella* spp. and high levels of *C. jejuni/coli* and *E. coli*. *C. jejuni/coli* were present in rinse water and carcass rinse samples at concentrations ranging from 1.7 to 6.4 log10 and 1.8 to 5.5 log10 copies per 100 mL of sample, respectively. *Salmonella* spp. were present in rinse water and carcass rinse samples at concentrations ranging from 0.8 to 3.1 log10 copies per 100 mL of sample for both sample types. *E. coli* were present in rinse water and carcass rinse samples at concentrations ranging from 3.9 to 8.6 log10 and 4.5 to 7.8 log10 MPN per 100 mL of sample, respectively.

We observed trends in pathogen concentration for both sample types. Concentrations in rinse water were consistently higher than in carcass rinse samples. *Salmonella* spp. in rinse water and carcass rinse samples followed the same continuous distribution ($p = 0.9$). This was not the case for *C. jejuni/coli* ($p = 2.0 \times 10^{-8}$) and *E. coli* ($p = 4.6 \times 10^{-4}$).

Influence of time since initial processing activity on C. jejuni/coli, Salmonella spp., and E. coli concentrations

Time was positively associated with a slight increase in *C. jejuni/coli* concentrations in rinse water and carcass rinses (**Table 5**). In both sample types, there was an average 0.1 log10 copies per 100 mL sample (95% CI 0.0, 0.2) increase in *C. jejuni/coli* concentration every 75 minutes. No statistical associations were observed between time and *Salmonella* spp. and *E. coli* concentrations.

Table 5. Summary of linear mixed effects regression results comparing associations between *C. jejuni/coli, Salmonella* spp., and *E. coli* and time, excluding baseline rinse water samples

Pathogen	Sample Type	Number of observations	Coefficient (10^{-3})	95% CI (10^{-3})	p- value
C. jejuni/coli	Rinse water	60	1.7	0.11, 3.2	***0.04***
	Carcass rinse	60	1.4	0.32, 2.5	***0.01***
Salmonella spp.	Rinse water	60	-0.075	-0.82, 0.67	0.84
	Carcass rinse	60	0.23	-0.42, 0.88	0.50
E. coli	Rinse water	48	-0.086	-1.6, 1.4	0.91
	Carcass rinse	47	-0.45	-1.7, 0.85	0.49

Bold italicized associations have a statistical significance of $p<0.05$.

Changes in slope were observed from a visual inspection of line plots with averaged pathogen data over time (**Figure 2**). Generally, change point analyses confirmed changes in slope with two exceptions (**Table 6**). No clear change points were detected for *C. jejuni/coli* in carcass rinses or for *Salmonella* spp. in rinse water samples. The time points at which slopes changed were not consistent across pathogens or sample types.

Table 6. Summary of change point analysis results

Pathogen	**Sample Type**	**Time point at which the slope changes**
C. jejuni/coli	Rinse water	225 mins*
	Carcass rinse	Changes were not detected
Salmonella spp.	Rinse water	Changes were not detected
	Carcass rinse	375 mins*
E. coli	Rinse water	150 mins*
	Carcass rinse	225 mins*

*The difference between the smallest AIC and the second smallest was >2

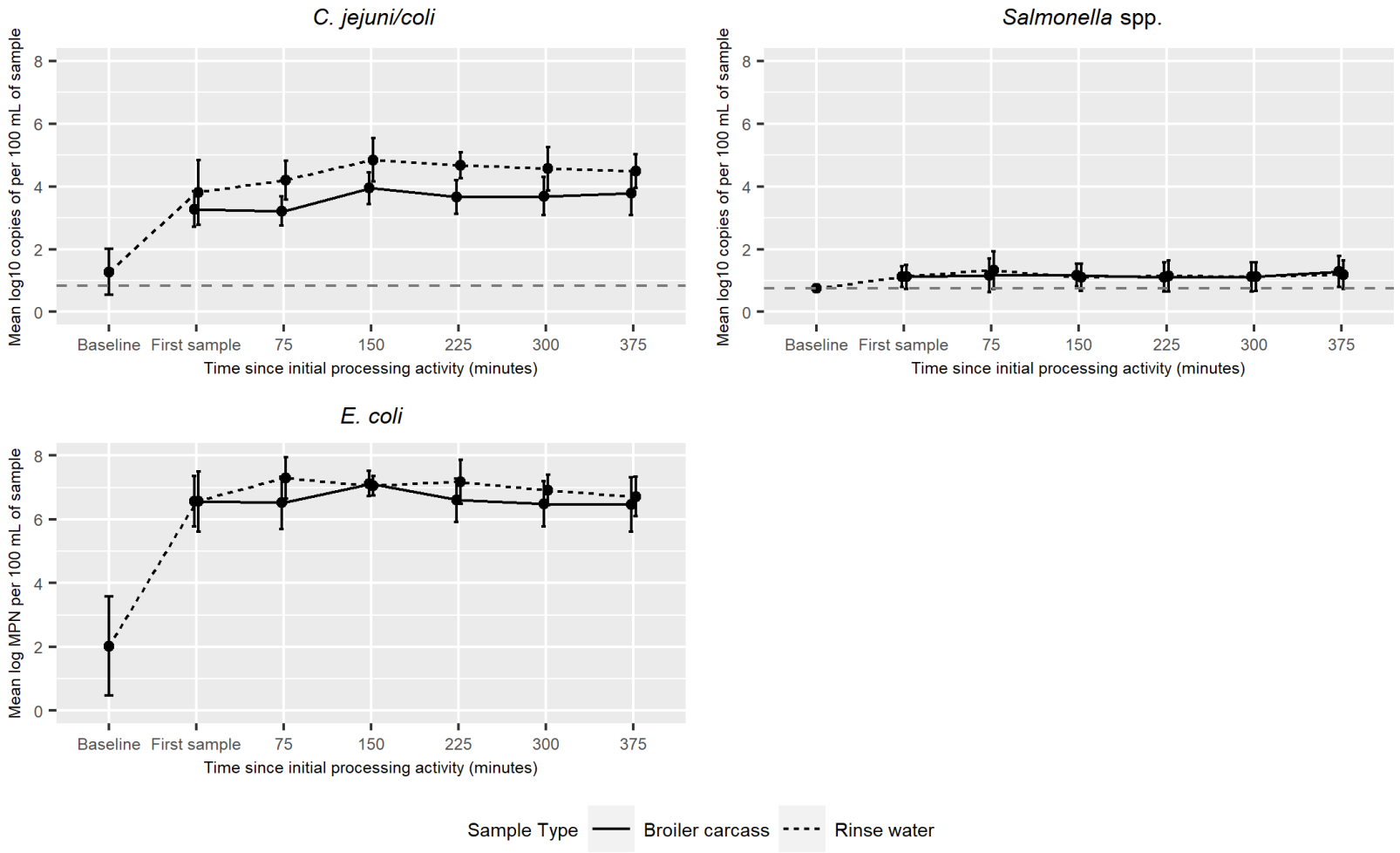

Figure 2. Line graphs comparing mean log10 concentrations of pathogens detected in rinse water and carcass rinse samples by qPCR and Colilert-18. 95% confidence intervals for average concentrations at each time point are plotted. All non-detectable samples are included. Gray, horizontal dashed lines indicate thresholds for non-detects at half the qPCR assay LOD. For E. coli, the LOD is <1 MPN/1,000,000, which corresponds to samples that were diluted 8-fold.

Discussion

We assessed the accumulation of enteropathogens (*C. jejuni/coli*, *Salmonella* spp., and *E. coli*) during broiler chicken processing at informal markets in Maputo, Mozambique, i.e., in broiler carcass and rinse water samples collected throughout the sampling period. We detected a slight increase in *C. jejuni/coli* concentrations as more broiler chickens were processed and observed poor food hygiene conditions. Every broiler carcass sampled was contaminated with *C. jejuni/coli* and *E. coli*, and *Salmonella* spp. was detected in 48% of samples. We observed unhygienic practices that may have contributed to the cross-contamination of chicken meat. Porous surfaces, lack of handwashing facilities, infrequent cleaning, and slaughter within the market are against the Food and Agriculture Organization of the United Nations' (FAO) recommended guidelines for minimal risks to health at live poultry markets.[59] These findings are consistent with prior studies at wet markets in Maputo (Chapter 2) and other LMIC settings.[9,14–16,60–62] While we did not sample the cooked chicken meat or produce we observed at vendor stands during sampling, there is a potential for the cross-contamination of ready-to-eat chicken meat and leafy greens with *Campylobacter* spp., *Salmonella* spp., and *E. coli*.[63–66]Chicken meat purchased from wet markets pose a high risk of introducing human enteropathogens into the household setting, as bacteria from chickens can transfer to kitchen surfaces[67] and other foods[68] and may ultimately seed household transmission.[68]

This study makes three important contributions. First, time-series sampling of rinse water and broiler carcasses allowed us to assess the accumulation of *C. jejuni/coli*, *Salmonella* spp., and *E. coli*. Wet market studies assessing microbial hazards in food have been largely cross-sectional and cannot make conclusions on how pathogen levels change throughout the span of a typical work day or longer periods of time. Second, we fill a data gap by providing quantitative data on poultry- associated pathogens, which has been cited as a first step to implementing successful HACCP programs along the informal poultry sector in Maputo.[25] Our two- stage sampling strategy allows us to generalize our findings to other frequently visited wet markets processing high volumes of poultry in Maputo. Third, our data highlights

food safety issues at wet markets and raises questions about how food security is assessed in these settings.

Our data displayed a spike in rinse water and carcass concentrations after processing the first carcass that remained elevated throughout the sampling period. We had hypothesized a continual increase in pathogen concentration. Rinse water likely became contaminated from the leakage of intestinal content during processing.[17] Contamination of the rinse water, at baseline, with *C. jejuni/coli* and *E. coli* could be due to the use of contaminated processing equipment[17] or a contaminated water source. Our data suggest that chicken meat may have taken on the contamination of the rinse water, suggesting that additional water provisions are needed but may not be an effective mitigation strategy without additional controls.

Differences in pathogen biology may explain why we observed increases in *C. jejuni/coli* concentrations, only, and different change points between the pathogens. Though *C. jejuni/coli* levels did increase linearly with time, changes were minimal after the first sample was processed. The increase in *C. jejuni/coli* over time may be due to an accumulation of bacteria from continued processing, and amplification of *C. jejuni/coli* is of concern, given the rinse water is a warm and concentrated bloody environment favorable for *C. jejuni/coli* growth.[69] We did not detect changes in *Salmonella* spp. concentration after the first carcass was processed, and overall levels were lower than for *C. jejuni/coli*. Generally, *Salmonella* spp. cells in poultry are low,[70] and at such low levels, large volumes of water are required for detection.[71,72] The 100 mL standard used for the detection of *E. coli*, which is the same volume processed for qPCR, may not have been sufficient to detect *Salmonella* spp. changes at low levels.[71] Change point analyses revealed different time points at which the slopes changed for each pathogen. The change point appears to correspond with pathogen concentrations. *Salmonella* was present at the lowest concentrations and the change point occurred after the longest period of time whereas *E. coli* was present at the highest concentrations and the change point occurred the earliest. The exact drivers of

differences in change point locations between the pathogens are not fully understood and warrant future investigation.

Our results suggest a need for a food hygiene intervention during chicken processing steps at informal markets. To our knowledge, interventions targeting the processing step have not been widely implemented in markets, but other strategies have been tested and found success in pathogen control. To control Avian Influenza, live poultry markets implemented periodic rest days and closures, depopulation, and disinfection and found significant reductions in virus amplification[73] and transmission within the market.[74] An intervention for improving meat safety at markets in Nigeria, which incorporated training and incentives, found that butchers had long-term retention of meat safety measures but rarely implemented these measures.[10] In commercial processing environments, chemical agents are routinely applied to broiler carcasses during carcass wash or spraying steps to reduce microbial loads.[18,75] However, pilot studies would need to test its effectiveness at reducing microbial loads in highly concentrated water with infrequent replacement as well as impact on taste, infrastructure needed to implement the intervention, and cost in an informal setting.

Washing carcasses with water only can reduce fecal indicator bacteria[76] and *Campylobacter* counts,[77] but this would require regular access to a clean water source, which is a challenge for vendors. While we did not measure the exact volume of water used for scalding and rinsing, the containers typically used for rinsing hold approximately 20 L of water. Scalding is performed in a large pot, similar to the size commonly found in a household kitchen (approximately 20 L). In comparison to commercial processing, where an average 26 L of water per chicken is used,[27] vendors may experience issues with water availability[28] and can process up to 20 chickens before changing the rinse water (Chapter 1).

Our findings highlight the microbial quality of foods sold at informal markets in Maputo, which raises questions about food security estimates and how they are assessed. Sustainable Development Goal Target

2.1 uses the Food Insecurity Experience Scale (FIES) to assess food insecurity;[78,79] however, access to safe food is not explicitly measured. Thus, food safety will likely be underprioritized as Mozambique works towards accomplishing its country-specific SDGs.[80] Higher levels of food insecurity in Mozambique may be observed if food security scales also included a measurement of safety.

This study has several limitations that should be considered when interpreting the results. Frist, we did not collect observational data on the frequency of changing rinse water, so we are uncertain if rinse water was replaced during the observation period and if so, any potential influence on pathogen concentrations. Based on the line graphs of averaged pathogen concentrations at each sampling point, we believe the rinse water was not replaced during the observation period. Second, the market is not a controlled environment where we could model the accumulation of enteropathogens on a single chicken over time. It is possible that the chickens we sampled had different levels of carriage prior to being processed, and we did not sample chicken feces to determine the impact of carriage on carcass rinse concentrations. Inactivation processes, such as sunlight, could have influenced pathogen concentrations[81] and explained the plateau we observed in concentration levels. Third, we used qPCR to detect *C. jejuni/coli* and *Salmonella* spp., so we cannot distinguish between the detection of live and dead bacterial cells.[82] Therefore, we may have overstated potential exposure risks. Future work could utilize culture methods to detect only viable bacteria, but the disadvantages, such as being time-consuming and requiring a high skill level,[83] should be weighed against the advantages that qPCR provides. Fourth, our data may not be best modeled by the linear mixed effects regression we performed. We saw from the change point analysis that our data can be explained by at least two slopes. However, the results should be interpreted with caution, as our change point analyses were based on only six time points. More frequent sampling may have removed random noise in the time series and revealed more robust underlying trends.

Conclusion

We found high levels of human enteropathogens in rinse water and on chicken meat that persisted throughout the day as more chickens were processed. Our results indicated that *C. jejuni/coli* accumulated during rinsing, which is of concern given this is the last "control" step before consumers purchase chicken meat. Improving the microbial quality of foods sold at wet markets is important to addressing the safety aspect of food security in low-resource settings. Further research could investigate challenges experiences by vendors processing chickens, vendors' perceptions of disease risk, and reasons for why certain food hygiene measures are not being implemented.

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Declarations

The authors declare no competing interests.

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Supplementary Material

Table S1. Summary of qPCR assay performance

Target (gene)	Slope	R2	y-intercept	Efficiency
C. jejuni/coli (cadF)	-3.3	99%	41.6	102.1
Salmonella spp. (*inv*A)	-3.3	99%	40.4	100.8
Internal amplification control	-3.4	99%	41.6	97.7

Figure S1. Market A Sketch

Figure S2. Market B Sketch

Figure S3. Market C Sketch

Form S1. Start of day vendor characteristics

Form S2. End of day vendor characteristics

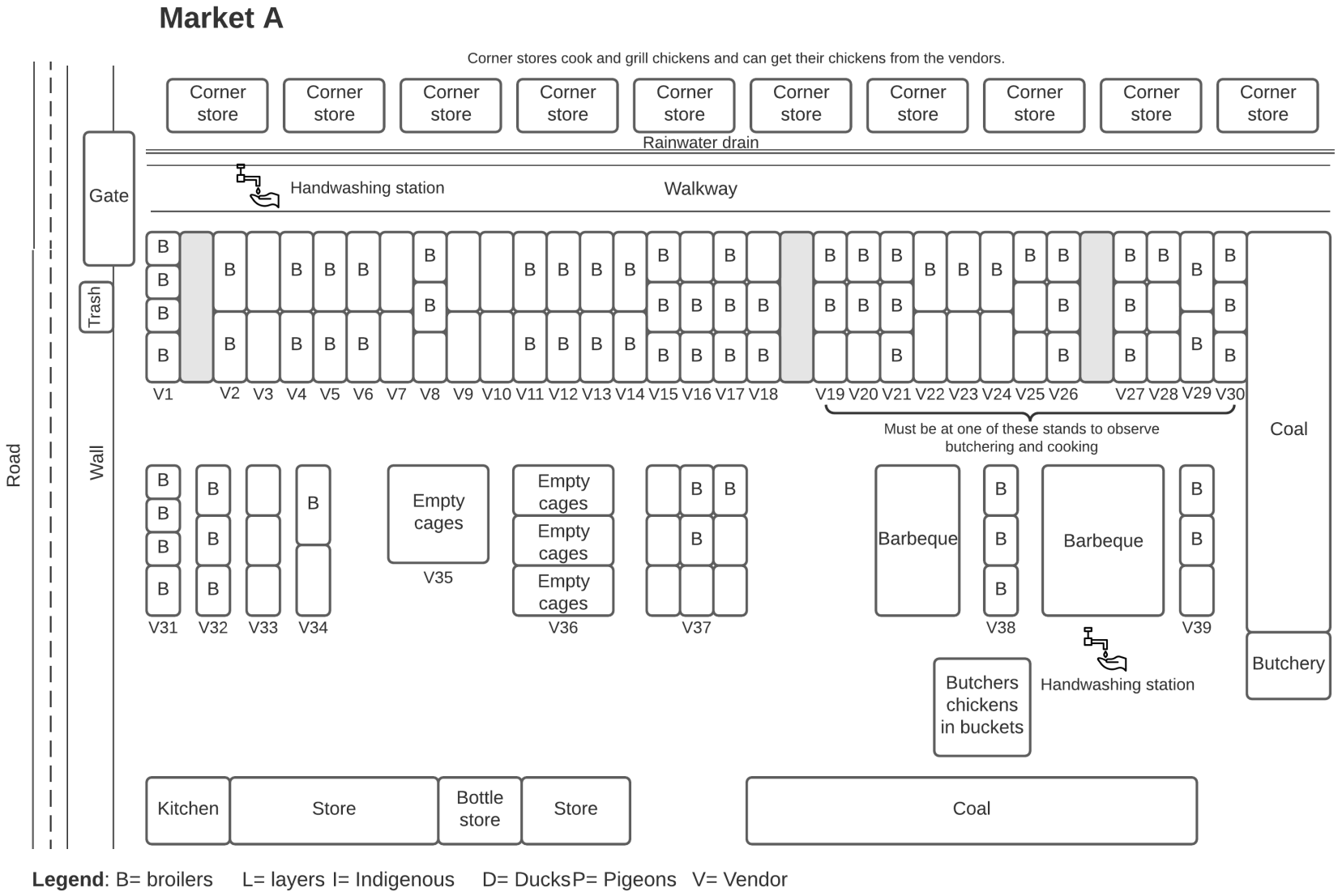

Figure S1. Market A Sketch. One to three vendors were observed at each stand. Market A sells live, processed, and cooked chickens. Vendors either process chickens at their stands or have a designated person to process chickens away from the stand. No restrooms were observed in the immediate chicken processing area.

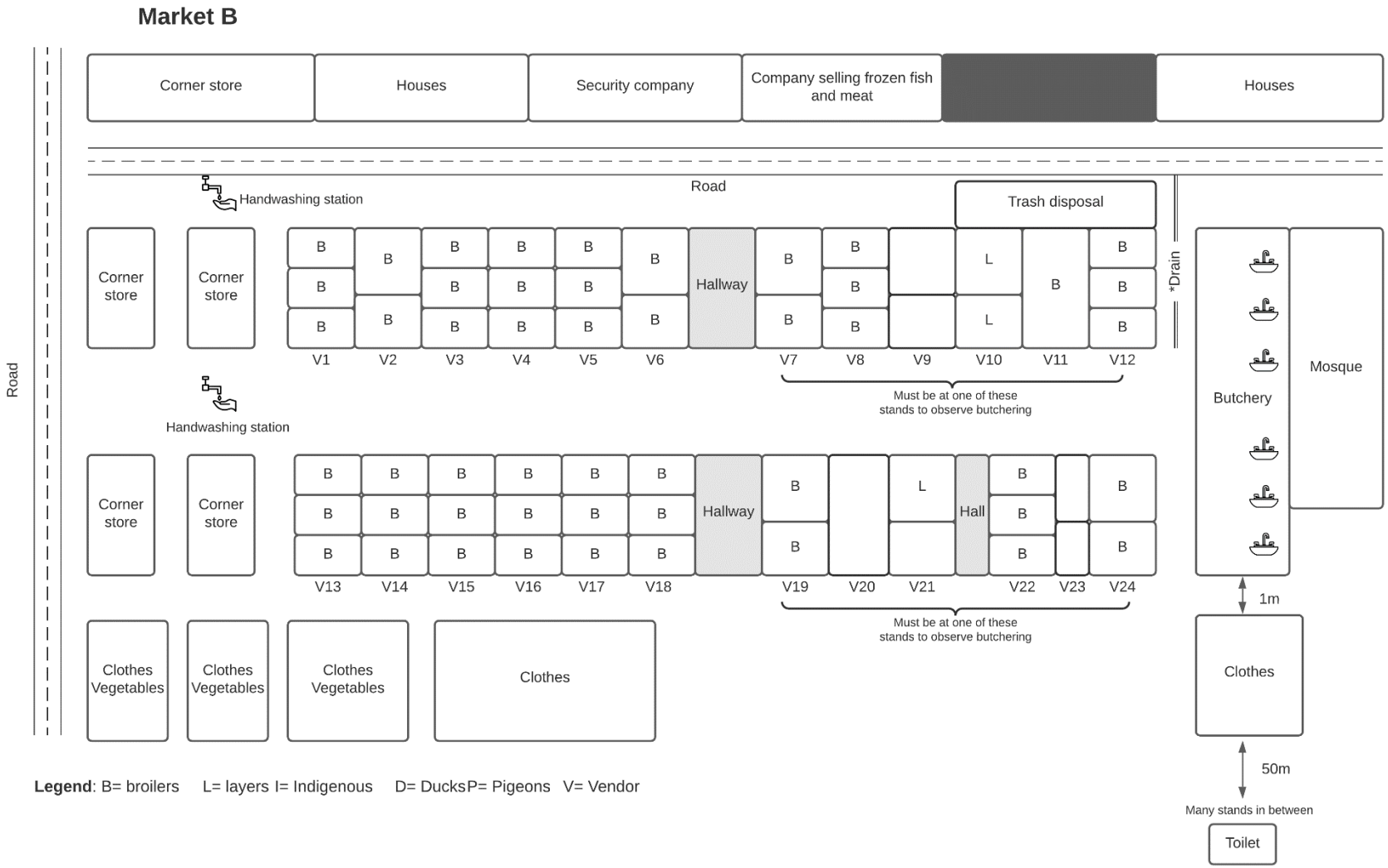

Figure S2. Market B Sketch. One vendor was observed at each stand. Market B sells live and processed chickens. Vendors have a designated person for processing chickens, and this occurs in a set area away from the stand.

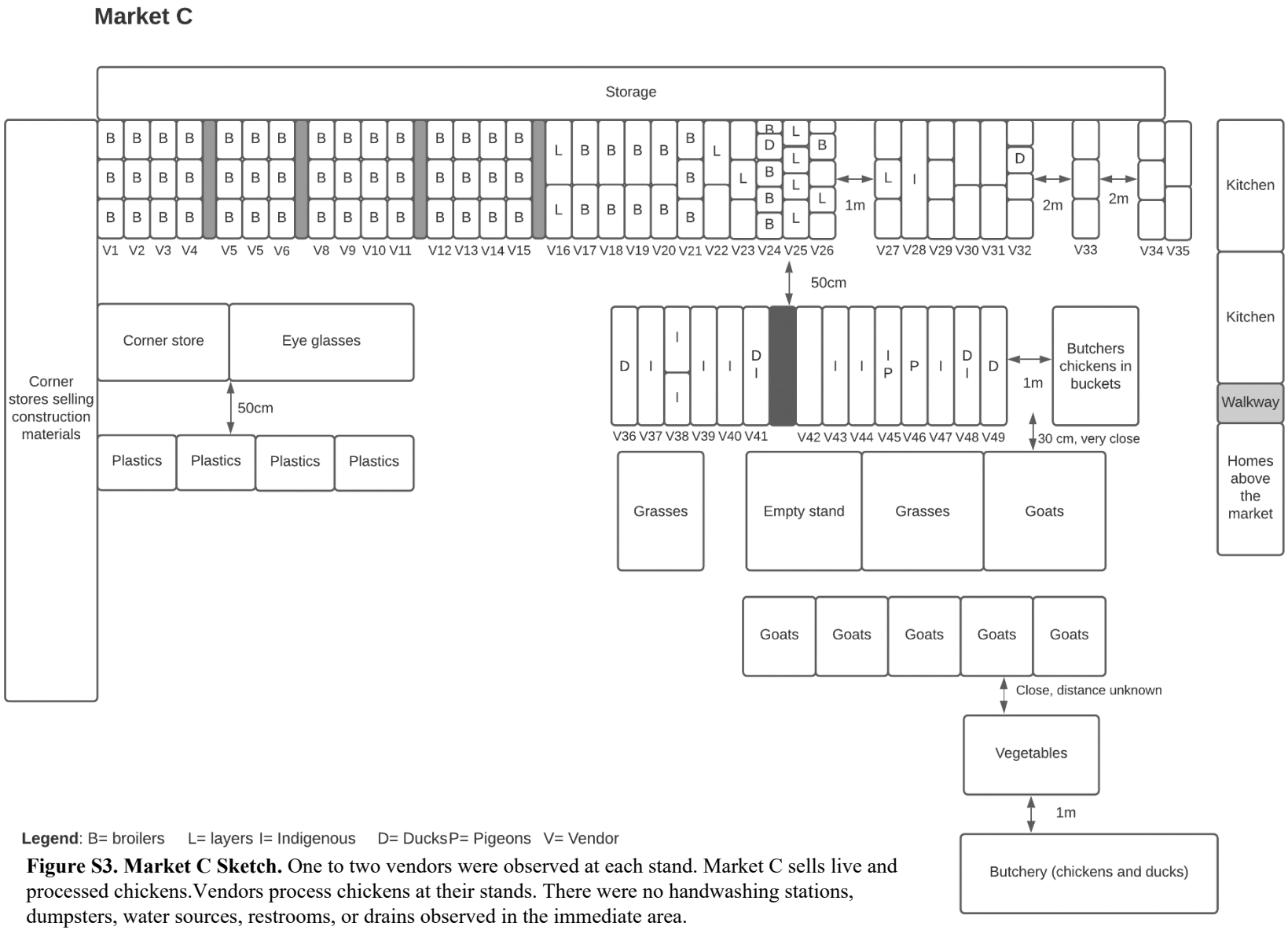

Figure S3. Market C Sketch. One to two vendors were observed at each stand. Market C sells live and processed chickens.Vendors process chickens at their stands. There were no handwashing stations, dumpsters, water sources, restrooms, or drains observed in the immediate area.

Form S1. Start of day vendor characteristics

Date of visit

State date and time of survey

IMEI (International Mobile Equipment Identity)

Preface: The purpose of our visit is to collect information on butchering and food hygiene practices and to also collect broiler carcasses and rinse water samples. We are asking vendors selling chickens in Maputo to participate in this study. Results will help us to develop food safety recommendations for butchering chickens.

Market location, consent, and COVID exposure

1. Enumerator name
    a. Enumerator 1
    b. Enumerator 2
    c. Enumerator 3
    d. Enumerator 4
2. Market name
    a. Market 1
    b. Market 2
    c. Market 3
3. Enter the vendor stand number.
4. Was consent given to at least one adult vendor to participate in the survey?
    a. No, ineligible
    b. No, refused
    c. Yes

Any YES response to the COVID responses below should be considered sufficient reason to postpone face-to-face visits if it cannot be explained by an underlying medical condition.

5. "1. Have you had any of the following symptoms in the last two weeks, which were not explained by a diagnosis with something other than COVID-19 (e.g., lung disease, heart failure, etc.), even if they were mild.
    ☐ Fever (greater than 37.3°C)
    ☐ Cough
    ☐ Shortness of breath or difficulty breathing
    ☐ Lost of sense of smell or taste
    ☐ Sore Throat
    ☐ Chills
    ☐ Muscle pain or body aches
    ☐ Nausea or vomiting
    ☐ Diarrhea
    ☐ Fatigue
    ☐ Headache
    ☐ Congestion, runny nose "

6. "2. In the last 14 days, have you lived, visited, cared for or been in a room for an extended period (<1 1/2 meters, face to face, >15 minutes) with someone who is under investigation or has been confirmed for COVID19/coronavirus infection?
   ☐ Yes
   ☐ No"
7. Did the participant pass the covid-19 screening?
   a. No
   b. Yes
8. (DON'T READ OUT LOUD. This is for documentation purposes only) Has the enumerator passed the covid-19 screening already today?
   a. No
   b. Yes
9. Informed consent

Module A: Live chickens

1. OBSERVE. Number of workers at the vendor stand where you are collecting samples
2. OBSERVE. This stand sells the following chicken products:
   a. Raw or butchered chicken
   b. Live chickens
   c. Cooked chickens
   d. Frozen chickens
   e. Eggs
3. OBSERVE. Number of live broilers at the start of the day
4. OBSERVE. Number of live layers at the start of the day
5. OBSERVE. Number of live indigenous chickens at the start of the day
6. OBSERVE. The maximum number of broilers, layers, or indigenous chicken in one cage.
7. OBSERVE. The minimum number of broilers, layers, or indigenous chicken in one cage.
8. OBSERVE. Presence of other live birds at the start of the day:
   a. Ducks
   b. Geese
   c. Turkey
   d. Wild birds
   e. Pigeons
   f. Other domesticated birds
   g. No other types of live birds present at the stand
9. OBSERVE. Broilers, layers, and/ or indigenous chickens are: a) kept in the same holding cages with other types of birds and/ or b) in cages touching the cages of other types of birds.
   a. No
   b. Yes
10. OBSERVE. The material of the holding cages:
    a. Wire or metal
    b. Plastic
    c. Porous material (ex: cardboard, paper, wood)
11. OBSERVE. The material of the bottom layer to catch feces in the holding cages:
    a. Wire or metal
    b. Plastic
    c. Porous material (ex: cardboard, paper, wood)

   d. Litter
   e. No bottom layer to catch feces; feces falls directly on the ground, floor, or cage below

Module B: WASH

1. Please show me where workers at your stand wash their hands.
   a. No handwashing place
   b. Fixed facility observed (sink/tap)
   c. Mobile object observed (bucket/jug/kettle)
   d. No permission to see
2. OBSERVE. Availability of water at the place for handwashing
   a. Water is available
   b. Water is not available
3. OBSERVE. Availability of soap or detergent at the place for handwashing
   a. Soap or detergent available
   b. Soap or detergent not available
4. OBSERVE. Was the handwashing station located at the stand?
   a. No
   b. Yes

Module C: Survey result

1. What was the result of the survey?
   a. Partially completed- respondent refused to complete
   b. Finished to completion
   c. Participant did not pass COVID screening
2. Additional comments

Take a picture of the consent form page with signature. Make sure the entire form is in frame and you are able to read its contents in the picture.

End date and time of the survey

Form S2. End of day vendor characteristics

Date of visit

State date and time of survey

IMEI (International Mobile Equipment Identity)

Preface: The purpose of this survey is to collect information on butchering and food hygiene practices.

Market location, consent, and COVID exposure

1. Enumerator name
    a. Enumerator 1
    b. Enumerator 2
    c. Enumerator 3
    d. Enumerator 4
2. Market name
    a. Market 1
    b. Market 2
    c. Market 3
3. Enter the vendor stand number.
4. Was consent given to at least one adult vendor to participate in the survey?
    a. No, ineligible
    b. No, refused
    c. Yes

Any YES response to the COVID responses below should be considered sufficient reason to postpone face-to-face visits if it cannot be explained by an underlying medical condition.

5. "1. Have you had any of the following symptoms in the last two weeks, which were not explained by a diagnosis with something other than COVID-19 (e.g., lung disease, heart failure, etc.), even if they were mild.
    ☐ Fever (greater than 37.3°C)
    ☐ Cough
    ☐ Shortness of breath or difficulty breathing
    ☐ Lost of sense of smell or taste
    ☐ Sore Throat
    ☐ Chills
    ☐ Muscle pain or body aches
    ☐ Nausea or vomiting
    ☐ Diarrhea
    ☐ Fatigue
    ☐ Headache
    ☐ Congestion, runny nose "
6. "2. In the last 14 days, have you lived, visited, cared for or been in a room for an extended period (<1 1/2 meters, face to face, >15 minutes) with someone who is under investigation or has been confirmed for COVID19/coronavirus infection?

☐ Yes

☐ No"

7. Did the participant pass the covid-19 screening?
    a. No
    b. Yes
8. (DON'T READ OUT LOUD. This is for documentation purposes only) Has the enumerator passed the covid-19 screening already today?
    a. No
    b. Yes
9. Informed consent

Module A: Live chickens

1. OBSERVE. Number of workers at the vendor stand where you are collecting samples
2. OBSERVE. Number of live broilers at the end of the day
3. OBSERVE. Number of live layers at the end of the day
4. OBSERVE. Number of live indigenous chickens at the end of the day
5. OBSERVE. Presence of other live birds at the end of the day:
    a. Ducks
    b. Geese
    c. Turkey
    d. Wild birds
    e. Pigeons
    f. Other domesticated birds
    g. No other types of live birds present at the stand
6. OBSERVE. Broilers, layers, and/ or indigenous chickens are: a) kept in the same holding cages with other types of birds and/ or b) in cages touching the cages of other types of birds.
    a. No
    b. Yes

Module B: WASH and food hygiene

READ: I will now ask some questions about cleaning at this stand, and then make some additional observations.

1. Does anyone ever clean any of the following?
    a. Holding cages
    b. Butcher surfaces and materials, such as knives and containers
    c. The general area surrounding your stand, including the floor/ ground
2. How often does anyone at the stand clean the holding cages?
    a. Never
    b. Monthly
    c. Weekly
    d. A few times a week (2-6 times)
    e. Daily
    f. Refused
    g. Don’t know
3. How often is the litter or bedding at the bottom of the cages changed?
    a. Never

   b. Monthly
   c. Weekly
   d. A few times a week (2-6 times)
   e. Daily
   f. Refused
   g. Don't know
4. How often does anyone at the stand clean butcher surfaces and materials, such as knives and containers?
   a. Never
   b. Monthly
   c. Weekly
   d. A few times a week (2-6 times)
   e. Daily
   f. Refused
   g. Don't know
5. How often does anyone at the stand clean the general area surrounding the stand, including the floor/ ground?
   a. Never
   b. Monthly
   c. Weekly
   d. A few times a week (2-6 times)
   e. Daily
   f. Refused
   g. Don't know
6. What materials are used for cleaning at the stand?
   a. Water only
   b. Water and soap (or detergent or industrial cleaning agent)
   c. Refused
   d. Don't know
7. OBSERVE. Did you ever see flies on or around chickens and chicken meat?
   a. No
   b. Yes
8. OBSERVE. Did you ever see leafy greens cleaned or prepared at the same stand where chickens are sold, cooked, and/ or butchered?
   a. No
   b. Yes
9. OBSERVE. Did you ever see animals other than poultry being sold at the same stand?
   a. No
   b. Yes
10. OBSERVE. Did you ever see cold storage (coolers, ice, etc.) for raw chickens?
   a. No
   b. Yes
11. OBSERVE. How was butcher waste stored at the stand?
   a. In a container or box with a lid or cover
   b. In a container or box without a lid or cover
   c. In a plastic bag
   d. No storage, waste is on the floor/ ground

12. OBSERVE. Where were chickens butchered?
    a. Directly on the floor/ ground
    b. On a surface on the floor/ ground
    c. In a container on the floor/ ground
    d. In a container, elevated from the floor/ ground
    e. On a table, elevated from the floor/ ground
13. OBSERVE. Did any of the workers bring children with him/ her while working at the stand?

Module C: Survey result

1. What was the result of the survey?
    a. Partially completed- respondent refused to complete
    b. Finished to completion
    c. Participant did not pass COVID screening
2. Additional comments

Take a picture of the consent form page with signature. Make sure the entire form is in frame and you are able to read its contents in the picture.

End date and time of the survey

Discussion

Smallholder livestock production may have financial and social benefits to households in LMICs,[1] but inadequate animal fecal waste management remains a challenge and poses risks to child health.[2,3] Water, sanitation, and hygiene (WASH) studies have largely focused on the containment of human waste with less attention on primary barriers specific to controlling exposures to animal feces.[4] While studies have measured child exposures to animal feces within households, it is difficult to develop appropriate mitigation strategies without an understanding of pathways beyond the domestic setting that contribute to direct and indirect exposures, such as via animal food systems. Therefore, the aim of this dissertation was to better understand the carriage of chicken-related enteropathogens and exposure risks along three chicken value chains.

We mapped value chains for broilers, layers, and indigenous chickens and characterized management and food hygiene practices that have the potential to expose children to chicken- related enteropathogens. We found that along the chicken value chain, children are most likely to encounter chicken-related enteropathogens during three scenarios: 1) direct contact with indigenous chickens and associated fecal waste at the household; 2) direct contact with fecal waste at households with small-scale farms; and 3) ingestion of contaminated chicken meat, eggs, and/ or produce from informal markets, grocery stores, and/ or small-scale farms. These settings can be targeted to mitigate risks. Our work highlights the intersection of formal and informal chicken production sectors and food safety issues that could undermine food security efforts. We also contribute the ChickFlows value chain framework[5] as a tool that can be implemented across contexts to understand risks along animal value chains for the purpose of informing targeted interventions.

Using the value chain framework from Chapter 1 to guide the identification of sampling locations, Chapter 2 examined the carriage and contamination of live chickens and chicken meat with *C. jejuni/coli* and *Salmonella* spp. We found higher contamination levels as chickens moved along each value chain. Informal markets were identified as high-risk settings for the purchase of live chickens and chicken meat

contaminated with *C. jejuni/coli*, calling into question how chicken processing contributes to the cross-contamination of chicken meat and suggests a need for microbial control strategies and food safety and hygiene education. Though prevalence and concentration of *Salmonella* spp. were low overall, highly contaminated grocery store chickens, relative to broiler chickens at markets and corner stores, show that risks are not limited to the informal production sector.

Chapter 2 also investigated childhood infections with *Campylobacter* spp. Our data confirmed that children in Maputo are infected with *C. jejuni/coli*, specific *Campylobacter* spp. carried by poultry, thus establishing a plausible zoonotic link to chicken exposure. The combined chicken fecal and child stool data suggest that interventions to control exposures to chicken fecal waste should be implemented across multiple settings along the value chain, as opposed to a sole focus on household-level interventions. However, we cannot rule out the possibility of these child infections being from exposures to other child feces or adult feces.

Chapter 3 built upon Chapter 2 market data to further investigate the contamination of chicken meat from processing water at informal markets. We detected *C. jejuni/coli*, *Salmonella* spp., and *E. coli* in chicken meat and rinse water samples, and *C. jejuni/coli* concentrations increased throughout the sampling period. The unhygienic practices observed are likely responsible for this contamination and could have contributed to background levels of *E. coli* and *C. jejuni/coli* in rinse water before any chickens were processed. Rinse water concentrations were consistently higher than carcass rinse concentrations, suggesting that carcasses may have taken on the contamination of the rinse water. Also considering consistent findings with Chapter 2, our data point to contamination as an ongoing challenge to food safety at markets processing live poultry. Our findings raise additional questions on the source and primary contributor of market contamination and demonstrate the need for food hygiene interventions to reduce microbial hazards on chicken meat before being purchased by the consumer.

Strengths and Limitations

Value chain framework and study design

We applied a value chain analysis to guide our investigation of microbiological hazards and associated exposure risks to children. Value chain analyses are "powerful frameworks" for studying food systems as they provide the foundational information needed to develop health interventions.[6] Our mapping of ChickFlows revealed a level of detail that would have been overlooked had we gone directly to the end user to characterize childhood exposures, including information on governance, challenges, relationships between key stakeholders, sanitary risks, and differences and similarities between value chains. By employing a triangulation convergence model mixed methods study design, we developed a comprehensive understanding of where risks of exposure to chicken-related enteropathogens exist and how these risks may vary along the value chains.

Our collection of time-series data to examine the accumulation of enteropathogens during processing strengthened our understanding of variability in microbial hazards throughout the day. Our data showed that *C. jejuni/coli* concentrations slightly increased throughout the sampling period, but *Salmonella* spp. and *E. coli* concentrations did not change linearly with time after processing the first carcass. Our study would be strengthened by collecting more frequent samples and sampling over a longer period of time, which is common in studies assessing chicken carcass contamination.[7,8] Also, our observations captured the volumes of chickens processed, which could help inform the selection of an appropriate carcass rinse intervention.

Chapter 1 and 2 data collection was cross- sectional and has limitations. Since we collected fecal and carcass data once at each sampling location, we were not able to determine the source or cause of contamination. Tracking the same lot of chickens, from production to the consumer, would not have been feasible given the difficulties associated with poultry value chains in emerging economies.[6] Chapter 2 results would be strengthened by longitudinal household assessments to determine dominant exposure

pathways to chicken feces and associated enteropathogens. Collecting repeated raw and cooked chicken meat, egg, and chicken fecal samples, child stool samples, observational data on contact with animals and their feces, and data on child consumption of chicken meat and eggs would provide conclusive evidence and link both environmental and foodborne exposures to enteropathogen infections in children. Collection of child stool samples were beyond the scope of the study, but we did verify that children are infected with *C. jejuni/coli* from a sub-analysis of the Maputo Sanitation Trial data. Though poultry carry *C. jejuni/coli*, cattle and humans also carry *C. jejuni/coli*.[9] We believe direct exposure to cattle feces is unlikely in our study area, as we did not observe any cattle during data collection.

Field methods

Chapter 3 results would be strengthened had we asked vendors the water sources they used to process chickens, tested the microbial quality of these water sources and collected observational data on water storage conditions and the number of chickens processed between water replacement.

Contamination was an issue in the field and during sample transport, highlighting a challenge associated with microbial data collection. We excluded *E. coli* results for 26 samples (20% of total samples) due to contaminated field and trip blanks. We did not exclude *C. jejuni/coli* and *Salmonella* spp. data for these samples, since the field and trip blanks did not indicate cross-contamination specific to these bacteria. Since *E. coli* is an indicator of fecal contamination and 100% of rinse water and carcass samples tested positive for *C. jejuni/coli*, it is likely that the discarded samples would have tested positive for *E. coli* regardless of potential cross-contamination. Virtually training study enumerators in aseptic techniques was challenging. For future studies, additional piloting prior to the start of sample collection could identify quality control issues and measures that can be improved upon to prevent the cross-contamination of samples.

Laboratory methods

Our microbial results contribute to baseline food safety data for *C. jejuni/coli*, *Salmonella* spp., and *E. coli* in chicken meat sold at Maputo markets, as these enteropathogens are not typically monitored. The Center for Environmental Hygiene under the Maputo City Directorate of Health tests hand swabs, utensils, and equipment used by vendors processing chickens and selling fresh chicken meat for total coliforms, fecal coliforms, *E. coli*, and Coagulase-positive staphylococci, but the frequency of testing is unknown. We also provide baseline data for the carriage of pathogens in chickens during production. Pooling fecal samples was an effective strategy for detecting the carriage of human enteropathogens in chicken feces. By pooling samples, we increased flock-sensitivity as compared to analyzing individual droppings.[10]

While qPCR is a quick, reliable, and sensitive method for detecting enteropathogens in various sample matrices, we cannot distinguish between live and dead bacteria. Culture methods would allow us to detect only viable bacteria, but require a high skill level, is time-consuming, and could have significantly higher false- negative results as compared to molecular methods.[11,12] Our analysis of samples via qPCR has implications for our understanding of risks.[11] When interpreting the results to characterize potential risks along each value chain, we are assuming that the concentrations we detected were of viable bacteria that present real hazards. For the purposes of this study, we wanted to quantify enteropathogen concentrations to understand potential exposure risks, and qPCR analyses were an appropriate method for answering our research questions.

We did not detect *Cryptosporidium* spp. in any chicken fecal or carcass rinse samples. Our follow up test of positive *Cryptosporidium* spp. samples from a separate study yielded negative results. The recovery of *Cryptosporidium* spp. from water samples is difficult if *Cryptosporidium* spp. is present in low numbers.[13] We centrifuged 100 mL of water sample, but detection in water can require a minimum of 10 L.[13] We applied mechanical pretreatment, via a bead beating step and six freeze thaw cycles, to break open the

thick- walled oocysts. These steps should have been sufficient to release DNA. Future studies should perform recovery efficiency tests to determine the quantity of *Cryptosporidium* spp. DNA extracted in comparison to the original amount in the sample, and if not sufficient, consider alternative methods for extracting samples.

Policy recommendations

Sustainable Development Goal Target 2.1 aims to end to hunger and ensure access to safe and sufficient food throughout the year by 2030.[14] Mozambique's 2020 review of progress on achieving SDGs reflected widespread food insecurity.[15] Though food safety is mentioned in Target 2.1, indicators of meeting this target include prevalence of undernourishment and of moderate or severe food insecurity. The Food Insecurity Experience Scale (FIES) does not measure access to safe food.[16] Thus, it seems likely that access to *safe* food will be underprioritized. This is evident in Mozambique's increase in poultry production without the same emphasis on ensuring poultry products are safe to eat, as observed in our study.

Standards do exist for good chicken production practices[17], eggs and egg products[18,19], chicken slaughter[20], and butchered chickens[21], but adherence to these standards is not monitored in or appropriate for the informal sector, and there are no incentives to comply. For example, INNOQ Standard 441[21] describes the appropriate management of poultry slaughter but refers to industrial equipment that informal vendors do not use, such as water and air chillers. Furthermore, our results exceed the permissible limits for fecal coliforms and *Salmonella* indicated in the national standards for slaughtered chickens[21], displaying a disconnect between policy and implementation of these policies. Currently, producers must pay a fee to access existing standards from the National Institute of Standards and Quality (Chapter 1), thus presenting a barrier for lower income suppliers.

To mitigate risks associated with the informal production and marketing of chickens and other animal source foods in Mozambique, specific actions to improve food safety should be considered. First, Mozambique could strengthen the surveillance of microbial hazards in foods by including more frequent testing of informally produced foods. These data would be useful in monitoring progress towards achieving food safety goals. Second, local leaders and informal producers could work together to develop effective food safety strategies. Informal dairy milk markets in Kenya, Tanzania, and India provide clear examples of how hygiene certifications and trainings can provide economic benefits and improvements in food quality and safety.[22] Joint programs between government and informal vendors can, in fact, be successful, and would require long-term funding from governments.[22] Mozambique could adopt similar programs and interventions but should target one behavior at a time.[23]

Future directions

This dissertation describes three chicken value chains in Maputo, Mozambique and provides evidence of microbial hazards and associated exposure risks. Our findings support the need for improvements in animal feces management along each chicken value chain. However, value chain complexity and lack of coordination between stakeholders introduce many opportunities for cross-contamination, even after a successful intervention. Therefore, our findings lead us to propose further research that would inform the development of interventions at the two settings that we believe could contribute to the greatest risk of child exposure to enteropathogens carried by chickens: informal markets and households.

Market- level interventions and future research

We detected very high levels of human enteropathogens in rinse water, carcass, and fecal samples from informal markets. Our results generate additional questions regarding the sources and primary contributor of the contamination we observed. We are unsure if the contamination we observed is due to cross-contamination in wash water or cross-contamination during initial processing steps prior to entering the wash water. If a carcass became contaminated from the spillage of its fecal contents, the source of

infection is still unknown. Future studies could investigate the carriage of human enteropathogens in chickens when they arrive at the market to determine if chickens are arriving colonized (either from the farm or during transport) and/or if chickens are primarily acquiring infections at the market while in the holding cages. Additionally, flies can serve as disease vectors, carrying and transferring *C. jejuni/coli* and *Salmonella* spp.,[24] and their role in contaminating chicken meat at informal markets is not well understood. The answers to these questions would help with the development of future interventions.

Food safety interventions at markets have shown limited effectiveness and have not been sustainable or scalable.[25] One explanation for this could be that interventions have focused primarily on food hygiene training, which involved multiple health messages, thus limiting intervention effectiveness.[23] Our data points to the carcass wash step as a critical control point to eliminate microbial hazards or reduce to an acceptable level. Future research could pilot interventions to reduce microbial hazards on chicken meat, such as a carcass wash intervention, and would require the use of behavior change methods. A recent quantitative risk assessment model was developed along the farm-to-fork pathway and found that chickens from a retail source have the highest contribution of *Salmonella* infections as compared to production settings and cross- contamination during serving and cooking.[26] A market-level intervention has the potential for great impact.

Our data provides a snapshot of current microbial hazards present at markets processing chickens and can be utilized as a baseline for setting food safety standards. The United States Department of Agriculture (USDA) utilizes performance standards tests to monitor the effectiveness of critical control steps.[27] The USDA has established a maximum acceptable percentage of 9.8% for *Salmonella* spp. and 15.7% for *Campylobacter* spp. on broiler carcasses in a moving 52-week period.[27,28] While these targets may not be realistically achieved in the short-term, Mozambique could use a similar approach but make adjustments so that it is appropriate in informal market settings. Before developing targets, we would need a better understanding of current sanitation and hygiene conditions at wet markets. Local entities could then

establish food safety standards specific to informal market settings and monitor incremental improvements until goals are achieved, and then implement measures to sustain improved food safety.

Household- level interventions and future research

Over 90% of households in Maputo purchase foods from markets[29] and 100% of carcasses and fecal samples from markets were contaminated with *C. jejuni/coli*. Future research could investigate the potential contamination of the household environment from the purchase of contaminated chicken meat and live chickens shedding enteropathogens. Cross-contamination and transfer of *Campylobacter* spp. and *Salmonella* spp. from chickens to the household environment is well-documented in high-income countries,[30–32] but these cross-contamination evens[33] are not widely understood in LMIC settings. Future work could also investigate household processing of chickens and any resulting cross- contamination. These data would inform the development of domestic food hygiene interventions. Though the primary focus of this dissertation work has been on potential exposures to children, we cannot overlook adults as carriers of *C. jejuni/coli* and their potential to seed household transmission.[9]

Our study showed that children may be at risk of exposure to *C. jejuni/coli* being shed in indigenous chicken feces. We observed indigenous chickens and their feces in the domestic environment and children in the same areas where chickens roamed. In addition, we learned that children may live at households that also have small-scale farms, thus putting children at risk of exposure to *C. jejuni/coli*. Household studies have measured children's direct ingestion of feces.[34] Previous interventions to reduce domestic exposures to chicken fecal waste, such as corralling poultry, providing animal feces scoops, improving flooring, and providing clean play spaces, have been limited to separating children from direct exposures to chicken fecal droppings.[35–38] Indirect exposures to enteropathogens via the consumption of contaminated foods remains unclear as studies investigating fecal-oral routes of enteropathogen transmission and enteric disease do not routinely sample food.[39,40] We recommend that future studies investigating child exposures to enteropathogens in the domestic environment include a food component.

This data would generate the evidence necessary to better estimate the burden of disease in children attributable to foodborne exposures and develop targeted mitigation strategies.

WASH implications

This study illuminates an opportunity for WASH, agricultural, and food safety researchers to collaborate and work towards reducing the burden of disease associated with exposures to zoonotic enteropathogens. The findings from this dissertation work suggest a need for WASH improvements along animal food value chains, as humans can become exposed to enteropathogens carried in animal feces via WASH-related pathways.[4]

WASH improvements along animal value chains have implications for accomplishing global SDGs to end food insecurity by 2030. Though food safety is not directly measured when assessing improvements in food security,[14] access to safe food implies having access to safe water for food preparation. SDG 6 aims to achieve universal access to safe drinking water by 2030,[14] but markets are not included. At markets, we observed lack of access to water for washing hands, cleaning, and processing and washing chickens. A call has been made to monitor WASH conditions at wet markets and recommended building wet market infrastructure, such as installing handwashing facilities and toilets and implementing cleaning protocols among other recommendations.[41] WASH research can contribute to the implementation and evaluation of these recommendations at markets.

WASH researchers have recognized that exposures to animal feces are understudied,[42] and as a result, studies are emerging that investigate exposures to animal feces and safe animal feces management strategies.[36,43,44] SDG 2.3 aims to double agricultural productivity of small-scale food producers, but this target does not address the agricultural waste and by-products that would result from increased production.[14] For example, at households keeping animals, household water sources used for cooking, cleaning, and handwashing may become contaminated with animal feces.[45] It is likely that more WASH studies will test interventions aimed at controlling animal feces and measure associated health outcomes.

Though WASH research is moving in the direction of measuring household exposures to animal feces, it is still largely focused on the domestic setting, and free- roaming animals justify a need for community level sanitation.

Our findings highlight a need for a greater focus on food safety, and WASH research could contribute to our understanding of the pathways of exposure to human enteropathogens along animal foods systems. Interventions are needed along animal food systems and should not put the burden of reducing fecal exposures solely on the end user, as often done in WASH studies in LMICs.[46] To develop interventions to mitigate exposure risks, more data are needed to:

- Understand key constraints (i.e. economic, infrastructural) that contribute to current food hygiene and WASH conditions at informal markets
- Understand risk perception of zoonotic disease transmission from processing chickens at wet markets
- Understand risk perception of zoonotic disease transmission from domestic animal husbandry practices
- Understand cultural considerations and preferences that impact how foods are sourced, prepared, and stored in LMIC settings
- Identify behaviors that could be targeted for interventions at settings along animal value chains

Prioritizing food safety may be key to truly achieving SDGs by 2030. Foodborne exposures to zoonotic enteropathogens have not been largely considered in WASH studies. Our study showed settings upstream of the end user where infected live chickens and contaminated chicken meat could be purchased, enter the domestic environment, and potentially seed household transmission. Therefore, WASH studies that do not consider foodborne exposures to enteropathogens carried in animal feces, when investigating enteric disease, could miss a key opportunity to better understand disease risks.

Conclusions

This dissertation aimed to characterize human enteropathogens in chicken meat and carried by chickens along three chicken value chains and associated opportunities for exposures to children. We took a food systems approach to first understand ChickFlows followed by an assessment of microbial hazards at key settings along each value chain. Our findings highlight the importance of understanding animal food systems when considering pathways of exposure to zoonotic enteropathogens and have applications for other urbanizing cities in Africa where livestock are being brought to the city center.[5] Our study provides further evidence of how a value chain approach can be used as a framework to identify risks to human health associated with animal food systems and can be implemented in other contexts. As Mozambique moves towards achieving SDGs country-wide, government entities must prioritize food safety as an important and necessary aspect of achieving food security. The results from each chapter have policy implications and inform the development of proposed next steps and intervention strategies.

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