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Molecular Detection and Antimicrobial Susceptibility of *Salmonella enterica* and *Escherichia coli* Isolated From Honeybee Gut in Holeta Town, Western Shewa, Ethiopia

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Abstract

A cross-sectional study conducted in Ethiopia from December 2021 to June 2022 investigated the prevalence of *Escherichia coli* and *Salmonella enterica* in honeybee colonies in Holeta Town, West Shewa. Out of 200 honeybee samples analyzed using bacteriologic culture, biochemical, and PCR tests, 15 (7.5%) were positive for *E. coli* and 11 (5.5%) for *S. enterica*. Additionally, PCR targeting the *invA* gene detected *S. enterica* in 10 samples (5%). Notably, traditional hives showed higher rates of pathogen occurrence compared to modern colonies. None of the *E. coli* isolates exhibited virulence genes. The study found significant associations ($P<0.05$) between *Salmonella* isolation and factors such as feed supplement, water type, and colony collapse. Antimicrobial susceptibility testing revealed that all *E. coli* and 63.6% of *S. enterica* isolates were resistant to *Ampicillin, Clindamycin, and Penicillin.* Conversely, all *E. coli* isolates were susceptible to *Streptomycin,* while only *Streptomycin* (100%) and *Trimethoprim* (63.6%) showed effectiveness against *S. enterica.* The findings suggest that management practices play a crucial role in honeybee health and pathogen contamination. Implementing modern hives and adopting good management practices including inspection, feeding, sanitation, and disease control are recommended to mitigate the impact of pathogens on honeybee colonies in the study area.

Keywords: *E. coli*; Honeybee Colony; I*nva* Gene; PCR; *Salmonella*

Abbreviations: VP: Voges-Proskauer; XLD: Xylose-Lysine Desoxycholate; HE: Heckton Enteric; BHI: Brain Heart Infusion; TSB: Tryptone Soya Broth.

Introduction

Beekeeping is the art of rearing honeybee colonies for economic benefit to exploit its products: honey, pollen, grain, propolis, and brood, and in practice for a long time [1]. The act of honeybee colonies was in cave art in Spain and Egypt in about 7000 B.C., for the first time [2]. It is one of the major agricultural activities that generate several opportunities for employment through its products [3]. Ethiopia has long been one of the leading producer countries of honey and beeswax [4]. Because of its excellent agro-climatic conditions and biodiversity, which have supported the establishment of a diverse honeybee flora and a large number of bee colonies, the country generates 98% of its honey and beeswax from traditional hives [5].

The health of honeybees has been one of the most critical areas of apiculture study in recent years. Globally, it deals with the mass of honeybee colony losses [6]. Colonies of honeybees are now declining in many regions of the world, which may be related to the negative impact of several pathogens that affect honeybees. In addition, one of the biggest challenges to managing bee colonies is the spread of parasites and diseases [7]. The exact cause of colony collapse disorder (the phenomenon that occurs when the majorities of worker bees in a colony disappears and leave behind a queen, plenty of food, and a few nurse bees to care for the remaining immature bees and the queen) is poorly understood. However, it commonly occurs because of several harmful circumstances [8]. Many pathogens can cause the collapse of a honeybee colony, including viruses, bacteria, and parasites [9]. In particular, several parasites attack honeybee colonies, resulting in significant harm. These pests include varroa mites, wax moths, small hive beetles, vespa hornets, and parasitic flies [10].

The gastrointestinal tracts of honeybees are home to diverse microorganisms, including bacteria [11]. As a result, numerous recent researches thus focus on the microbiota of their digestive tract [12,13]. The virulence, adult host mortality, and transmission of honeybee disease were difficult to understand and poorly documented. Polluted water is a reservoir of pathogenic bacteria [6,14], and access to it affects the health of insects, especially honeybees [15]. The use of reclaimed water sugar solution as drinking water has negative impacts on the deaths of honeybee colonies and can alter the shape of the midgut of honeybees [16].

The high incidence of bacteria present in the gut of honeybees is a public health risk, as the synanthropic behavior of bees may be conducive to dissemination through a wide variety of routes [17]. Worker honeybees collect food from sugar that is prepared, processed, and stored, and thus it may increase the likelihood of the risk of bacterial transmission. Beekeepers usually feed sugar solution during the lack of sufficient food, but the quality and diversity of sugar sources can affect the number of bees [18].

Much like the human gut microbiota, many bee gut bacteria are specific to the bee gut and can be directly transmitted between individuals through social interaction [19]. The main risk factor of pathogen transmission is water: apart from honeybee friendliness, water is highly contaminated with human pathogenic bacteria [20]. The usage of antibiotics for controlling infections affects other microbes, including the beneficial bacteria present in healthy hosts. The selection forces imposed by an antibiotic can result in an accumulation of resistance determinants, those often encoded on mobile genetic elements and readily transferred among community members [21]. Tetracycline and fumagillin are the two common antibiotics used to treat honeybee diseases nowadays [22]. Furthermore, disrupted gut microbiota due to antibiotic use, pesticide exposure, or dietary changes has been linked to higher pathogen loads and host mortality [23].

The frequent prevalence of bacteria found in the bee's gut can result in a concern for public health, as the synanthropic behavior of bees may be conducive to dissemination through a broad range of routes [17]. The lack of sufficient food is partially a management issue in apiculture practices. Beekeepers usually feed sugar solution during starvation, but the quality and diversity of sugar sources can affect several bees [18]. The commonly isolated species of *Enterobacteriaceae* from honeybees include *Enterobacter*, *Klebsiella*, and *Serratia* [24]. Strains isolated from hives can cause mortality when administered to workers in the laboratory orally [25]. Potentially, these *Enterobacteriaceae* pathogens are under recognized as causes of mortality since infected worker honeybees usually leave the hive to die. *S. enterica* and *E. coli* is the usual pathogenic intestinal microflora of adult honeybees from *Enterobacteriaceae* [11,24].

Low productivity and poor quality of honeybee products are the main economic impediments for honeybee apiculture and rural beekeepers. Most research on microbiomes in the intestine of honeybees has emphasized the lactic acid bacteria, which are known to have antimicrobial activity [26]. In Ethiopia, there are limited studies on honeybee health. Even if available, their main focus was on predators and pests visible to the human eye [27]. As the synanthropic behavior of bees may be conducive to dissemination through a wide variety of routes, the high incidence of bacteria present in the bee's gut is a risk to public health [17]. There is a lack of studies on the pathogenic intestinal gut of microbial honeybees in Ethiopia. This study aims to isolate and identify *E. coli* and *S. enterica* from the honeybee gut, determine the antimicrobial susceptibility of the isolates, and identify potential risk factors associated with honeybee disease management in the study area.

Materials and Methods

Description of Study Area: The study was conducted from December 2021 to June 2022 in Holeta town in the central highlands of Ethiopia. Holeta, the capital of the Welmera district, is located in the Oromia special zone surrounding Finfine, 44 kilometers west of Addis Ababa on the highway to Ambo. The city is found at the latitude of 38° 30'E and 9° 3'N, as seen in Figure 1. It is about 2400 meters above sea level. With a bimodal distribution, the region receives a mean annual rainfall of 1100 mm, with 70% falling during the rainy season from June to September and the remaining 20% falling during the minor wet season from February to April. The annual

temperature is 11 to 22°C with a relative humidity of 50.4%. The town has eight kebeles (the smallest administrative unit). Based on data from Holeta town administration, the total human population is about 36,705 [28].

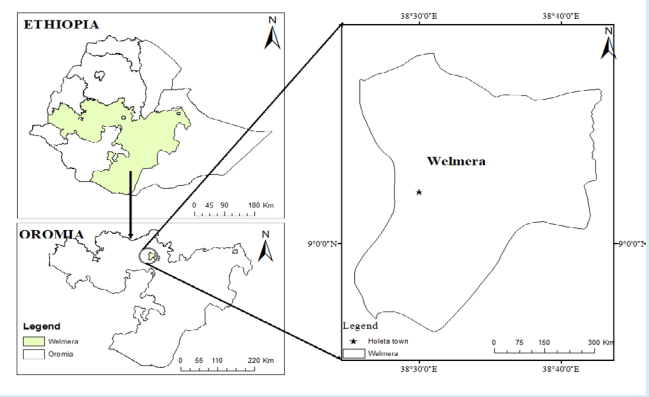

Figure 1: Map of study area. Source: (ArcMap desktop software, 2022).

In this study area, there are three types of beekeeping practices: traditional, transitional, and modern hives. The apiculture practice is profitable in this area, and the Holeta Bee Research Center encourages the community to participate in beekeeping activities and give bee colonies to the local community. Generally, Welmera district has 10876 traditional, 4512 transitional and 1856 modern hives: whereas, Holeta town has 42 Traditional, 40 Transitional, and 58 modern bees hives [29]. *Study population:* The study populations were adult worker honeybees managed in traditional and modern hives in Holeta town, Ethiopia. *Study Design:* A cross-sectional study was conducted to isolate and identify *E. coli* and *S. enterica* from honeybee guts and to determine the isolates' antibiotic resistance profile.

Sample Size Determination and Sampling Strategy: Because honeybees are highly sociable insects, a colony is critical to their health. Unlike other animals, considering of a single honeybee is difficult. The proportion study, involving 40 honeybee colonies, was conducted using purposive sampling. Five honeybees were randomly selected from each colony, yielding 200 honeybees. The district's agricultural bureau suggested four kebeles based on their potential for beekeeping (Table 1). *Sample and Data Collection:* Live honeybees were collected using screw-capped jars, assigned separate codes indicating colony number and date, transported by icebox, and then dissected aseptically at the Holeta Agricultural Biotechnology Research Center Laboratory [30]. Ethanol (70%) was used to sterilize the surface of honeybees. The dissected area and material were fixed with 70% and 97% ethanol, respectively. Honeybees were rinsed thrice with sterile water before using sterilized forceps to detach the stinger midgut, with the complete gut attached from the honeybee's abdomen. Each gut was placed in a sterile petri dish. After carefully swabbing the stomach, each swab was placed in a test tube containing 9 mL sterilized buffered peptone water. The samples were incubated at 37C for 24 h under aerobic conditions. Finally, *E. coli* and *S. enterica* were detected in the samples.

Site	Number of Colonies			Sample Collected Per Colony	Overall Number of Samples
	Modern	Traditional	Total		
Walmera	4	1	5	5	25
Sadamo	3	2	5	5	25
Meda Gudina	2	3	5	5	25
Gelgal Kuyu	4	6	10	5	50
HBRC	15	0	15	5	75
Total	**28**	**12**	**40**		200

Table 1: Sample distribution among study kebeles/sites of Holeta town and hive type.

During the survey, information about honeybee disease management practices and potential risk factors were collected. Observational and questionnaire assessments were conducted to obtain information regarding the management conditions of beekeepers. The data collection format included details of professional training, feed and watering activities, and information on honeybee handling. *Isolation and Identification of E. coli:* The *E. coli* isolation was done according to the protocol of ISO-16654 2002 standard [31]. The pre-enriched gut swab samples were subsequently sub-cultured onto MacConkey agar for primary screening of *E. coli* and incubated at 37°C aerobically for 24 hours. Suspected colonies of *E. coli* (pinkish color appearance) were then subcultured on Eosin Methylene blue agar. The *E. coli* suspected colonies having a dark center and a greenish metallic sheen were sub-cultured onto nutrient agar for confirmation by biochemical tests such as triple sugar iron agar, catalase, indole, methyl red, Voges-Proskauer (VP), and citrate tests according to the standard procedure.

Isolation and Identification of Salmonella: Isolation and identification of *Salmonella* from the gut of honeybee swab samples were performed according to the procedure recommended by the International Organization for Isolation of *Salmonella* [32]. A loop full sample from the test tube containing 9 ml sterile buffered peptone water was transferred to 10 ml of Rappaport Vassiliadis-soy peptone broth (RV; Oxoid, UK) and incubated at 42 °C for 24 h. Since the international standard organization method specifies Xylose-Lysine Desoxycholate (XLD and Heckton Enteric agar (HE) agar are optional selective media for *Salmonella* species, plating onto agar media plates parallel on both XLD agar and HE agar were carried out after 24 h and 48 h of incubation. Typical *Salmonella* colonies were sub-cultured on nutrient agar at 42°C for 24 h and subjected to further biochemical confirmation [33].

Molecular detection of S. enterica (invA gene): To extract Salmonella genomic DNA, each isolate underwent culture on brain heart infusion (BHI) agar plates and was then incubated for 24 hours at 37°C, following the procedure detailed in the appendix. Subsequently, a pure colony was collected using a 10µl loop and suspended in nuclease-free water by gentle swirling in an Eppendorf tube, followed by vortexing for 30 seconds. The tube was heated in a thermal block at 95-100°C for 10 minutes, allowed to cool for 2 minutes at room temperature, and then centrifuged at the highest speed in a mini-centrifuge for 5 minutes. After centrifugation, 50µl of the resulting supernatant was carefully transferred to a new tube, avoiding the pellet. This supernatant served as template DNA and was stored at -20°C until further use. Subsequently, up to 5µl of the collected supernatant per 50µl PCR reaction or up to 2.5µl per 25µl PCR reaction was utilized as a template for PCR amplification. Finally, the quality and quantity of the extracted DNA were assessed using a gel electrophoresis system and NanoDrop (spectrophotometer), as described by Bedassa, et al. [33].

The PCR reaction setup included the following components: Promega GoTaq Green Master Mix (12 µl), Primer Salm 3 (0.81 µl), Primer Salm 4 (0.87µl), Nuclease-free Water (8.82 µl), and 2.5 µl of DNA template (each). The DNA extracted from Salmonella isolates served as the template for amplifying the highly conserved region of the invA gene using primers Salm3 (5´-GCTG CGCG CGAA CGGC GAAG-3') and Salm4 (5´-TCCC GGCA GAGT TCCC ATT-3´), which target a 389 base pair fragment of the conserved invA gene sequence specific to S. enterica [34].

Amplification was conducted in a thermocycler (BIO-RAD T100TM, Singapore) using the following cycling conditions: an initial incubation at 95°C for 5 min, followed by 35 cycles of amplification (denaturation at 95°C for 90 s, annealing at 60°C for 60 s, and elongation at 72°C for 90 s), ending with a final extension at 72°C for 7 min. *Molecular detection of E. coli (virulence genes (stx1, stx2, and eaea):* The DNA of *E. coli* was extracted using the boiling technique. Before DNA extraction, the isolates were cultured in LB broth at 37 °C for 18 h. Bacteria were pelleted from 1.5 ml LB broth, suspended in 200 µl of sterile deionized water, and incubated at 100 °C for 10 min. After centrifuging, the supernatant was used as template DNA and stored at −20 °C [35]. After extraction, DNA was subjected to PCR for the presence of virulence genes *stx1*, *stx2*, and *eaea*. The PCR reaction was set up in a 25µl mixture containing nuclease-free water (8 µl), both forward and reverse primers (2 µl), Gotaq master mix (Promega, USA) (12µl), and template (3µl). The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 950C; 35cycles, each consisting 40 s at 95°C, 40 s at 55°C, 30 s at 72°C; and a final extension of 1cycle for 8 min. at 72°C [36].

The amplified DNA products from Salmonella-specific PCR and virulence genes of *E. coli* were examined by electrophoresis on 1.2% agarose w/v gels (1.5 g of agarose was combined with 100 ml of 1 TAE buffer in a glass flask), stained with red gel, and visualized under UV light. Each gel received a current of 120 V. The PCR product was loaded onto an agarose gel (eight microliters along with 3 liters of the loading dye (B7025S, New England). A 100 bp DNA ladder was used as a marker for the PCR results [33]. *Antimicrobial Susceptibility Test:* Antimicrobial susceptibility tests were performed following the standard agar disk diffusion method, according to the Clinical Laboratory Standards Institute [37], using antimicrobial disks (Oxoid Basingstoke, England). The antimicrobials used in this study for both *S. enterica* and *E. coli* isolates were Ampicillin (AMP, 10µg), Chloramphenicol (CMP, 30µg), Ceftriaxone (CRO, 30µg), Streptomycin (S, 10µg), Tetracycline (TE, 30 µg), Oxy-tetracycline (OT, 30µg), Penicillin (P, 10µg), Clindamycin (CLN, 10µg) and Trimethoprim (TR, 5µg) (HIMEDIA, India). The isolates grown on nutrient agar were transferred to a test tube containing 5 ml tryptone soya broth (TSB) (Oxoid, England), and the broth culture was incubated at 37 °C for 24 h. The turbidity was then diluted with sterile normal saline for spectrophotometry at 620nM absorption between 0.08-0.1ABS. A sterile cotton swab was dipped into the suspension and then swabbed uniformly in three directions over the surface of a Muller Hinton agar plate (Oxiod, England) and held at room temperature for 30 min to avoid excess moisture. Antibiotic disks were then placed on inoculated plates using sterile forceps. Antibiotic disks were gently pressed onto the agar to ensure firm contact and incubated at 37 °C for 24 h. After incubation for 24 h, the diameters of the zones of inhibition were measured and compared with the zone size interpretative guidelines for the family Enterobacteriaceae (Table 2) and determined to be sensitive, intermediate, and resistant [37].

Data Management and Analysis: The data collected through the questionnaire survey and laboratory results were entered into Microsoft Excel and analyzed using SPSS (SPSS version-20) statistical computer software. Descriptive statistics were used to describe the frequency and percentage of *S. enterica* and *E. coli* occurrences. Chi-square was used to see the association of bacterial occurrence with different risk factors. A p-value < 0.05 was considered indicative of a statistically significant association.

Antimicrobial Agents	Concentration (µg/disc)	Susceptible (mm)	Resistant (mm)	Intermediate (mm)
AMP	10	≥17	≤13	14-16
CMP	30	≥18	≤ 12	13-17
CRO	30	≥21	≤13	14-20
CLN	10	≥21	≤14	15-20
S	10	≥15	≤11	14-Dec
TE	30	≥15	≤11	14-Dec
P	10 unit	≥17	≤14	15-6
TR	5	≥16	≤10	15-Nov
OT	30	≥15	≤11	14-Dec

Table 2: Zone diameter and Microbial inhibition concentration for *Enterobacteriaceae*.
Key: AMP=ampicillin, CMP=chloramphenicol, CRO=ceftriaxone, CLN=clindamycin, S=streptomycin, TE=tetracycline, P=penicillin G, TR=trimethoprim, OT=oxy-tetracycline.

Results

Among the bee samples examined, 5.5% and 7.5% were positive for *S. enterica* and *E. coli*, respectively, in culture. The highest number of isolates for *S. enterica* and *E. coli* were 2(8%) and 3(12%), respectively, from Meda Gudina kebele. From the total modern hive sample examined, 2.9% had positive for *S. enterica* and 5.7% positive for *E. coli*. From the traditional hive samples examined, 11.7% and 11.7% were positive for *S. enterica* and *E. coli,* respectively, as described in Tables 3 and 4.

Kebele	Hive Type	Number of Examined	Number of Positive	%	χ 2	P-value
Meda Gudina	Traditional	15	2	13.3		
	Modern	10	0	0		
	Total	**25**	**2**	**8**		
Sademo	Traditional	10	1	10		
	Modern	15	0	0		
	Total	**25**	**1**	**4**		
Welmara	Traditional	5	1	20		
	Modern	20	0	0	0.54	0.96
	Total	**25**	**1**	**4**		
Galgel Kuyu	Traditional	30	2	6.6		
	Modern	20	1	5		
	Total	**50**	**3**	**6**		
HBRC	Traditional	-	-			
	Modern	75	4	5.3		
	Total	**75**	**4**	**5.3**		
Total	Traditional	60	7	11.5	3.34	0.068
	Modern	140	4	2.9		
	Total	200	11	5.5		

Table 3: *S. enterica* isolated from the gut of a honeybee in HBRC and four kebeles of Holeta town.

Kebele	Hive Type	Number of Examined	Number of Positive	%	χ 2	P-value
Meda Gudina	Traditional	15	2	13.3		
	Modern	10	1	10		
	Total	**25**	**3**	**12**		
Sademo	Traditional	10	1	10		
	Modern	15	0	0		
	Total	**25**	**1**	**4**		
Welmara	Traditional	5	1	20		
	Modern	20	1	5	2.14	0.71
	Total	**25**	**2**	**8**		
Galgel Kuyu	Traditional	30	3	10		
	Modern	20	2	10		
	Total	**50**	**5**	**10**		
HBRC	Traditional	-				
	Modern	75	4	5.3		
	Total	**75**	**4**	**5.3**		
Total	Traditional	60	7	11.7		
	Modern	140	8	5.7	2.145	0.143
	Total	**200**	**15**	**7.5**		

Table 4: *E. coli* isolated from the gut of a honeybee in HBRC and four kebeles of Holeta town.

Occurrence of E. coli and S. enterica at Colony Level: In this finding, from a total of 40 colonies of honeybees, 37.5% and 27.5% were positive for *E. coli* and *S. enterica*, respectively. The highest *E. coli* and *S. enterica* prevalence was observed in Meda Gudina, at 60% and 40%, respectively (Table 5). According to the evaluation of the prevalence of *Salmonella* and *E. coli* for the type of bee hive (from which the colony was sampled), the contamination was higher in the transitional than in the modern ones. Table 6 shows 58.3% prevalence for both pathogens in the transitional period, 28.6% for *E. coli*, and 14.3% for *S. enterica* in modern hives.

Kebele		*E.coli*				*Salmonella*			
	N	n	%	χ^2	P	n	%	χ^2	P
M/Gudina	5	3	60	3.16	0.53	2	40	0.711	0.95
Sedamo	5	1	20			1	20		
Welmera	5	2	40			1	20		
G/Kuyu	10	5	50			3	30		
HBRC	15	4	26.7			4	26.7		
Total	40	15	37.5			11	27.5		

Table 5: *E. coli* and *S. enterica* isolates at colony level in Holeta town.

Hive Type		*E. coli*				*S. enterica*			
	N	n	%	χ^2	P	n	%	χ^2	P
Traditional	12	7	58.3	3.17	0.075	7	58.3	1.726	0.89
Modern	28	8	28.6			4	14.28		
Total	**40**	**15**	**37.5**			**11**	**27.5**		

Table 6: *E. coli* and *S. enterica* isolates at colony level and type of hive.

Overall prevalence of S. enterica by invA PCR: Presumptive *S. enterica* isolates that were Salmonella-positive and passed through the selective medium were confirmed by *invA* PCR. As a result, 5% (10/200) of the total prevalence was *S. enterica* (Figure 2). *Occurrence of S. enterica* and E. coli based on Risk Factors: Among beekeepers in the study area, 82.5% replied that they provided supplementary feed for honey during periods of feed scarcity. The most common locally available feed types used as colony supplements are shiro, sugar, and honey. Honeybees collect water from 40% of streams, 35% of rivers, and 25% of tap water. The respondents were asked whether they had received training in beekeeping and management techniques. Accordingly, 62.5% of the interviewed beekeepers received beekeeping training from the HBRC. Among the variables assessed, the type of supplementary feed (p=0.03), water source (p=0.009), and occurrence of colony collapse (p=0.001) were statistically significant (Table 7).

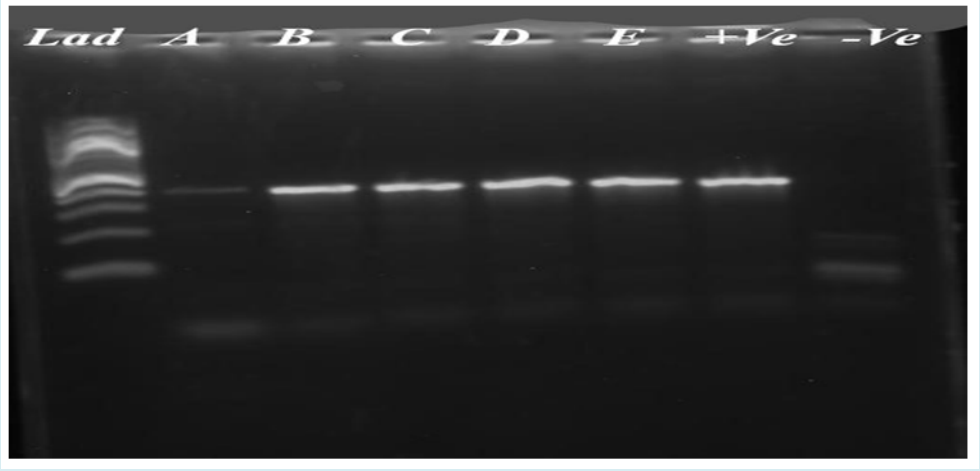

Figure 2: Representative gel pictures of *invA* PCR.
Key: (Lad=Ladder 100bp; A, B, C, D, E= S. enterica positive isolates, +ve=Positive control, and –ve= Negative control).

Management	Categories	N	%	*E. coli* Positive				*S. enterica* Positive			
				N	%	X^2	P	N	%	χ^2	P
Do you Provide supplement feed?	Yes	33	82.5	13	39.4	0.3	0.5	4	12.1	3.7	0.05
	No	7	17.5	2	28.6			7	100		
Type supplement	Sugar	9	22.5	5	55.6	3.7	0.3	2	22.2	3.8	0.03
	Shiro	13	32.5	6	46.2			3	23.1		
	Honey	11	27.5	2	18.2			2	18.2		
	Nothing	7	17.5	2	28.6			4	57.1		
Type of water	Stream	16	40	6	37.5	2.2	0.3	2	12.5	9.5	0.009
	River	14	35	7	50			8	57.1		
	Tap	10	25	2	20			1	10		
Frequency of cleaning equipment	Every week	15	37.5	4	26.7	1.3	0.3	3	20	1.8	0.41
	Every2 week	13	32.5	6	46.2			3	23.1		
	Monthly	12	30	5	41.7			5	41.7		
Do you get beekeeping training?	Yes	25	62.5	9	36	0.06	0.8	6	24	0.4	0.52
	No	15	37.5	6	40			5	33.3		
Have ever encountered colony collapse?	Yes	29	72.5	9	31	1.9	0.2	2	6.9	22.4	0.001
	No	11	27.5	6	54.6			9	81.9		
	Total	40	100	15	37.5			11	27.5		

Table 7: Cleaning, supplement feed, training, status of colony collapse, frequency of cleaning equipment of honeybee and *S. enterica* and *E. coli* positive risk factors.

Antimicrobial Susceptibility Profiles of Isolates: The assessment of the antimicrobial susceptibility of all 15 *E. coli* and 11 *S. enterica* isolates from the honeybee samples collected from HBRC and four kebeles of Holeta town to the selected disks is shown in (Table 8). *S. enterica* isolates were highly susceptible to streptomycin (100%) and trimethoprim (63.6%), while 100% were resistant to Ampicillin, Clindamycin, and Penicillin. *E. coli* isolates were highly susceptible to Streptomycin (100%), Oxy-tetracycline, and Tetracycline (93.3% and Ceftriaxone (80%). All *E. coli* isolates were resistant to Penicillin and Clindamycin. *Honeybee Management Practices:* Of the 40 sampled owners and workers interviewed, about (80%) were male, and the rest (20%) were female. Workers of the most productive age, between 26-47 years, were actively participating in beekeeping. Among the households and workers interviewed, 22.5 %, 67.5%, and 10% were single, married, and divorced, respectively.

Antimicrobial Agent	(µg/disc)	*E. coli* (n=15)			*S. enterica* (n=11)		
		Susceptible N (%)	Resistant N (%)	Intermediate N (%)	Susceptible N (%)	Resistant N (%)	Intermediate N (%)
AMP	10	0 (0)	14 (93.3)	1 (6.6)	0 (0)	11 (100)	0 (0)
CMP	30	10 (66.6)	2 (13.3)	3 (20)	5 (45.4)	1 (6.6)	5 (45.4)
CRO	30	12 (80)	1 (6.6)	2 (13.3)	7 (63.6)	2 (13.3)	2 (13.3)
CLN	10	0 (0%)	15 (100)	0 (0)	0 (0)	11 (100)	0 (0)
S	10	15 (100)	0 (0)	0 (0)	11 (100)	0 (0)	0 (0)
TE	30	14 (93.3)	1 (6.6)	0 (0)	1 (9.09)	5 (45.4)	5 (45.4)
P	10 U	0 (0)	15 (100)	0 (0)	0 (0)	11 (100)	0 (0)
TR	5	11 (73.3)	2 (13.3)	2 (13.3)	7 (63.6)	2 (18.2)	2 (18.2)
OT	30	14 (93.3)	1 (6.6)	0 (0)	5 (45.4)	0 (0)	6 (54.5)

Table 8: Antimicrobial susceptibility profile of *E. coli* and *S. enterica* isolates.
Key: AMP=ampicillin, CMP=chloramphenicol, CRO=ceftriaxone, CLN=clindamycin, S= streptomycin, TE=tetracycline, OT=oxy tetracycline, TR=trimethoprim and P=penicillin.

Regarding educational status, (32.5%) of the respondents had no formal education, (17.5%) attended primary education (25%) of them went to secondary and preparatory school (15%) attended a diploma, and (10%) were a degree or higher, as shown in Table 9. Consequently, both groups (literate and informal education) were practicing beekeeping. However, these findings indicate that honeybee farmers in Holeta Town have low levels of education. The respondents were interviewed for the variable to describe the frequency of inspecting their apiary and honeybee colonies. Among these, 45% replied that they look externally into the hives every week, 35% every three days, and 7.5% every day (Table 9).

Demographic Variables	Categories	Frequency	Percentage (%)
Sex	Male	32	80
	Female	8	20
Education status	No formal education	13	32.5
	Primary education	7	17.5
	Secondary/preparatory	10	25
	Diploma	6	15
	Degree	4	10
Marital status	Single	9	22.5
	Married	27	67.5
	Divorced	4	10

Age	15-25 years	7	17.5
	26-36 years	14	35
	37-47	17	42.5
	Above 47	2	5
Are you aware of bacterial honeybee disease?	Yes	10	25
	No	30	75
Frequency of observation	Every day	3	7.5
	Every two day	5	12.5
	Every three day	14	35
	Every week	18	45

Table 9: The proportion of sex, age, educational, marital, frequency of observation, and awareness of bacterial honeybee disease of respondents.

Discussion

Honeybee colony losses have become a significant global issue, primarily due to the emergence of new honeybee diseases. Controlling these diseases is vital to protect honeybee populations. Traditionally, antibiotics like Tetracycline have been used to manage honeybee diseases. However, the use of antibiotics in apiculture is banned in many European countries due to potential health risks to both humans and bees. In our study, we investigated the prevalence of *S. enterica* and *E. coli* in honeybee colonies, focusing on different management practices and hive types in various kebeles (local administrative areas) in Ethiopia. In our study, similar to the findings reported by Zafar, et al. [38] and Diriba, et al. [39], we observed the highest prevalence of *S. enterica* at 8% in Meda Gudina kebele. Additionally, the overall occurrence of *E. coli* in honeybee gut samples was 7.5%, with the highest isolation rate of 12% also recorded in Meda Gudina kebele. Traditional hives had a higher prevalence of both pathogens compared to modern hives, indicating that management practices play a crucial role in disease prevalence.

Modern hive beekeepers were more likely to follow better management practices, including proper feeding, watering, and hygienic practices, as advised by HBRC researchers. Traditional hives are more challenging to manage and are more susceptible to pests and diseases, leading to higher pathogen prevalence [40]. Poor management practices, such as inadequate feeding and improper water sources, were associated with higher prevalence of *S. enterica* and *E. coli*. Feed supplements, water sources, and colony collapse were significantly associated with the presence of these pathogens [41]. A significant number of beekeepers (62.5%) received training from HBRC and livestock experts, leading to better production management and disease control. Proper training helps beekeepers improve their practices, reducing the prevalence of diseases [42]. *S. enterica* isolates showed high resistance to common antibiotics like ampicillin, clindamycin, and penicillin, with resistance levels ranging from 6.4% to 100%, while *E. coli* isolates also exhibited high resistance to the same antibiotics. This resistance is likely due to the overuse of these antibiotics in livestock, posing a challenge for both human and animal health [43].

Implementing a holistic approach to improve honeybee health and reduce disease prevalence involves several key strategies. Transitioning to modern hive designs can greatly facilitate better management practices, providing easier access for inspection, feeding, and sanitation. Alongside this, expanding beekeeping training programs is crucial, focusing on practical aspects like disease identification, prevention strategies, and effective control methods. Regular hive inspections, both externally and internally, are essential for early disease detection and prompt intervention. Providing honeybees with adequate feed supplements and clean water sources during feed scarcity periods helps boost their immune systems and reduces pathogen prevalence. Maintaining sanitary conditions in the apiary through equipment cleaning and hygienic practices minimizes disease risks. Furthermore, conducting additional research to identify pathogen sources and understand their effects on honeybee and human health is vital for implementing targeted control measures. By integrating these recommendations, beekeepers can work towards maintaining healthy colonies and sustaining honeybee populations [44-47].

In this study, the overall Occurrence of *S. enterica* and *E. coli* in the gut of honeybees were 5.5% and 7.5%, respectively. In another way, the highest prevalence for these pathogens was Meda Gudina kebele. The occurrence of *E. coli* (20-60%) and *S. enterica* (20-40%) isolates among honeybee colonies in the study sites/kebeles signals the high distribution of *S. enterica* and *E. coli* present in the honeybee

environment. Moreover, this shows that the honeybee gut is an alternative habitat for human pathogenic bacteria. The type of hive and management practice was suggested to be possible risk factors for the prevalence of *S. enterica* and *E. coli* in honeybees. The statistical difference observed in *S. enterica* isolates was in the feed supplement, water source, and colony collapse ($P<0.05$). It was evidence of a low level of public awareness about bacterial honeybee disease and associated risk factors in the study area. In another way, there is evidence of antibiotic resistance isolates from bees in the study area. In conclusion, the honeybee gut is an alternate habitat for human pathogenic bacteria as a high load of these bacteria recovered from the alimentary canal of honeybees.

Our research underscores the significance of effective hive management techniques, hive design, and training in combating honeybee diseases. The adoption of modern hives and knowledgeable beekeepers plays a pivotal role in mitigating pathogen spread. Addressing antibiotic resistance and enhancing overall hive care are key factors in safeguarding honeybee populations and promoting the longevity of beekeeping practices. Further investigation is warranted to comprehensively grasp the implications of these pathogens and devise efficient disease control strategies.

Based on the above conclusion: the use of a modern hive instead of a traditional one is advisable for management practices like inspection, feeding, sanitation, and disease control easily; great emphasis should be given to training and extension programs for the community focusing on the practical aspects of general beekeeping, and more specifically on honeybee management; observation of bee colonies and cleaning feeding equipment are necessary for apiculture to improve honeybee health; further studies should be conducted to rule out the sources of targeted bacteria in the bee environment; the direct/indirect effect of the targeted bacteria to human and honeybee health should studied in detail.

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