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# Risk factors for detection, survival, and growth of antibiotic-resistant and pathogenic *Escherichia coli* in household soils in rural Bangladesh

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Running Head: Soils as reservoirs for *E. coli* in Bangladesh

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## ABSTRACT

Soils in household environments in low- and middle-income countries may play an important role in the persistence, proliferation, and transmission of *Escherichia coli*. Our goal was to investigate the risk factors for detection, survival, and growth of *E. coli* in soils collected from household plots. *E. coli* was enumerated in soil and fecal samples from human, chicken, and cattle from 52 households in rural Bangladesh. Associations between *E. coli* concentrations in soil, household-level factors, and soil physicochemical characteristics were investigated. Susceptibility to 16 antibiotics and the presence of intestinal pathotypes were evaluated for 175 *E. coli* isolates. The growth and survival of *E. coli* in microcosms using soil collected from the households were also assessed. *E. coli* was isolated from 44.2% of the soil samples with an average of 1.95 $\log_{10}$ CFU/g dry soil. Soil moisture and clay content were associated with *E. coli* concentration in soil, whereas no household factor was significantly correlated. Antibiotic resistance and pathogenicity were common among *E. coli* isolates, with 42.3% resistant to at least one antibiotic, 12.6% multidrug-resistant (≥3 classes), and 10% potentially pathogenic. Soil microcosms demonstrate growth and/or survival of *E. coli*, including an enteropathogenic, ESBL-producing isolate, in some, but not all, of the household soils tested. In rural Bangladesh, defined soil physicochemical characteristics appear more influential for *E. coli* detection in soils than household-level risk factors. Soils may act as reservoirs in the transmission of antibiotic-resistant and potentially pathogenic *E. coli*, and therefore may impact effectiveness of water, sanitation, and hygiene interventions.


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43 **IMPORTANCE**

44 Soil may represent a direct source or act as intermediary for transmission of antibiotic-resistant
45 and pathogenic *Escherichia coli*, particularly in low-income and rural settings. Thus, determining
46 risk factors associated with detection, growth, and long term survival of *E. coli* in soil
47 environments is important for public health. Here we demonstrated that household soils in rural
48 Bangladesh are reservoirs for antibiotic-resistant and potentially pathogenic *E. coli*, can support
49 *E. coli* growth and survival, and defined soil physicochemical characteristics are drivers of *E.*
50 *coli* survival in this environment. In contrast, we found no evidence that household-level factors,
51 including water, sanitation, and hygiene indicators, were associated with *E. coli* contamination of
52 household soils.

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53 **INTRODUCTION**

54 The relative importance of different routes of enteric disease transmission is not well understood
55 (1), even for the model organism, traditional indicator of fecal contamination, and frequent
56 pathogen *Escherichia coli* (2). *E. coli* transmission is traditionally considered to occur via the
57 fecal-oral route (2) or through interactions with environmental compartments contaminated with
58 feces (i.e., water, hands and soils) (3, 4). Interactions of infected, colonized, and susceptible
59 hosts (human and animal) with environmental compartments plays an important role in enteric
60 disease transmission, and *E. coli* specifically (3). *E. coli* pathotypes infect multiple host species
61 (i.e., humans, ruminants, and chickens) that are often in close contact and share space, especially
62 in low and middle income countries (LMICs) (1). Understanding transmission of *E. coli*
63 pathotypes is important in developing effective water, sanitation, and hygiene (WASH)
64 interventions (3).
65
66 Research and WASH interventions have primarily focused on improving microbial quality in
67 water and food. Recent evidence suggests that other reservoirs (i.e., hands, soil), also act as
68 intermediaries of transmission either directly (i.e., hand-to-mouth contacts, soil ingestion) or
69 indirectly (through interactions with other environmental matrices) (5–9). Effective interventions
70 may need to limit transmission through microbial control of these additional reservoirs. Indeed,
71 of three recent randomized controlled trials of WASH investments in Bangladesh, Kenya (the
72 WASH Benefits Trials), and Zimbabwe (the SHINE trial), only the WASH Benefits Trial in
73 Bangladesh showed reductions in child diarrheal disease (10–13). The failure of WASH
74 investments on improvement of health may be partially attributed to the failure of the

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interventions to adequately reduce enteric pathogens and fecal contamination in environmental compartments, including soils (14).

Pathogen transmission via soil is particularly relevant for children given the high rates of observed soil ingestion in LMICs (5, 6, 15). For example, one study in rural Zimbabwe estimated that a one year-old child may ingest more than 20 g of soil per day as a result of both active soil ingestion and mouthing episodes with soil contaminated hands (8). Furthermore, the soil in households in LMICs is frequently found to contain high concentrations of *E. coli* (6, 16, 17). The detected *E. coli* include multiple intestinal pathotypes, as evidenced by a study in Tanzania (17), indicating that soils may be contributing to pathogenic *E. coli* transmission in these settings. Similarly, soil may play a role in transmission of antibiotic-resistant *E. coli*, which have also been detected in soils (18). Consumption of fresh produce grown in soils contaminated with *E. coli* also represent a health risk (19), as it has been shown that even after washing concentration of bacteria can remain high (20).

The high concentrations of *E. coli* in soil may be linked to soil-associated growth and/or survival. Growth dynamics of *E. coli* have been studied in soils, sand, and sediments to demonstrate potential limitations of *E. coli* as an indicator of fecal contamination (21–23). For example, Ishii et al. hypothesized that *E. coli* are naturalized to the soil environment as stable members of the soil microflora based on isolation of the same *E. coli* genotypes at the same location repeatedly over one year (21). In addition, the phylogenomic analysis of five *Escherichia* clades (isolated primarily from environmental compartments), which are phenotypically indistinguishable but genetically distinct from *E. coli* (24, 25), has strengthened

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the view that there are environmentally-adapted lineages. This was previously suggested by Byappanahalli et al. after observing distinct DNA fingerprints of *E. coli* strains from soils as compared to strains from animal sources (26). The existence of environmentally-adapted lineages suggests the possibility of strain-specific adaptation for survival and/or growth in soil. However, it is also clear that survival and/or growth is influenced by environmental factors including temperature, water content, nutrient availability, soil texture, pH, solar radiation, and the presence of soil indigenous microflora (27–31).

In recent years, more attention has been given to the role that environmental matrices play in pathogenic *E. coli* transmission. However, fundamental questions remain about the importance of *E. coli* adaptability, survival and growth in the environment. In this study we evaluate *E. coli* ecology in soils collected from Mirzapur, Bangladesh, with the focus of soil as a reservoir for *E. coli* transmission. Specifically, we investigated risk factors associated with the detection and concentration of *E. coli* in household soils in rural Bangladesh. We also assessed survival and growth dynamics of antibiotic-resistant and potentially pathogenic *E. coli* in soil microcosms to further highlight mechanisms by which soil intrinsic properties influence *E. coli* detection, survival, and/or growth.

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## RESULTS AND DISCUSSION

### Household characteristics, animal ownership, and feces management

Survey data on household characteristics, including animal ownership and feces management, allowed comparison of the study site to previous studies, and provided insight into the importance of household-level factors that may contribute to increased *E. coli* in the soil. We found enrolled households were generally more affluent, with respect to durable assets and animal ownership, than typical rural households in Bangladesh, as described by the 2014 Demographic Health Survey (32) (Table 1, Table S1). For example, households reported higher ownership of electricity, televisions, mobile phones, refrigerators, wardrobes, fans, cows/bulls, and chickens/ducks (Table S1). To assign households to wealth quartiles based on durable assets, animal ownership, and household characteristics, composite wealth indices were constructed using principal components analysis. Indices ranged from -0.39 to 4.52, and correlated moderately with self-reported monthly expenditures (Spearman's $\rho = 0.53$, $p < 0.001$). Wealth quartiles were defined using k-means clustering, with 33%, 21%, 29%, and 17% of households categorized in the poorest, second, third, and wealthiest quartiles, respectively (Table 1). Wealth quartiles represent variation in wealth only among households enrolled in study.

Among enrolled households, sanitation was generally improved relative to the status reported in the 2014 Demographic Health Survey for rural Bangladesh (32). For example, 71.2% of households in this study had improved latrines with basic sanitation services as compared to 43.6% in rural Bangladesh (Table 1, Table S1). Nevertheless, visible feces were observed in 57.7% of the toilets/latrines, while only 11.5% had soap. Toilet/latrines were shared among 1 to 5 people in 51.9% of the households while 48.1% were shared among 6 to 19 people (Table 1).



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Among the 18 households with children under five years (Table 1), 55.6% reported the child uses the toilet and none reported the use of diapers. The most common way (44.4%) to manage the child feces was disposal into the garbage. All the households had domestic animals (Table 1, Table S1) and all reported that the animals defecate on the ground inside the household plot. Diarrhea (defined as 3 or more episodes of loose/watery stool per day) or respiratory symptoms (runny nose and cough) seven days prior to the interview date was reported in at least one member of the household in 11.5% and 67.3% of the instances, respectively (Table 1).

**_E. coli_ concentrations in household soils**

Presumptive *E. coli* was isolated from 44.2% (n=23/52) of the soil samples collected in the household plots with an average ± standard deviation of 1.95 ± 0.88 $\log_{10}$ *E. coli* CFU/g dry soil and a maximum count of 3.86 $\log_{10}$ *E. coli* CFU/g dry soil. The mean and maximum *E. coli* concentrations observed in this study were similar to other studies in Tanzania and Zimbabwe (17, 33), but lower compared to a previous study in rural Bangladesh (16). Species identification using the API-20E system confirmed *E. coli* in the 23 soil samples (100%). The majority of isolates (21/23) were identified with a confidence level > 95%, while only 2/23 showed lower discrimination confidence. The API-20E results also indicated high phenotypic diversity among the isolates, as indicated by 10 unique biochemical profiles. Random amplified polymorphic DNA (RAPD) confirmed high degree of genetic diversity among the soil isolates. All isolates showed unique fingerprint patterns and only nine isolates clustered together in three RAPD types with similarity greater than 80% (RAPD types G, I, and K; Fig. S1). PCR detection of the *E. coli* gene *uidA* from DNA extracted directly from the soil samples increased, albeit not substantially, *E. coli* detection from 44.2% to 57.7 % (n=30/52). This result indicates that the culture method

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used for isolation was able to recover *E. coli* in the majority of soil samples where *E. coli* DNA was detected.

**Associations between soil characteristics and *E. coli* concentrations in soil**

We evaluated *E. coli* concentration associations with different soil characteristics measured as these varied across households. Soil water content was significantly correlated with the concentration of *E. coli* in soils (Spearman's $\rho = 0.48$, $p = 0.0003$; Table S2), consistent with previous studies (16, 28, 33). Water content in the 52 soil samples varied between 9.8% and 38.4% with a mean ± standard deviation of 20.8 ± 7% (Table S2). The only other soil physicochemical parameter that was found associated with *E. coli* concentrations was the percentage of clay, with an inverse correlation (Spearman's $\rho = -0.47$, $p = 0.0095$; Table S2). The mechanism explaining the inverse relationship between *E. coli* concentration and clay is unclear. In agreement with our findings, Lang et al. reported higher background concentration of *E. coli* in a sandy loam soil (73% sand, 19% silt, and 8% clay) than in a silty clay soil (11% sand, 53% silt, and 36% clay) (34). In contrast, previous studies have observed a higher proportion of bacteria (35) and preferential attachment (36) in the clay fraction of soil compared to the other fractions. *E. coli* O157 was also observed to survive longer in loam and clay soils compared to a sandy soil (37). In addition, Brennan et al. showed that the addition of different clay minerals (clay mineral composition varies among soils) influenced other physicochemical soil properties and differentially affected survival of enteropathogens (38). The contrasting results may also be due to differences in the methods for bacterial recovery, suggesting further evaluation of the methodology for *E. coli* enumeration in soils may be warranted. Correlations with other soil

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properties (field capacity; permanganate oxidizable active organic carbon; active organic carbon; total nitrogen; percentage of clay, silt, and sand in soil) were not significant (Table S2).

**Associations between household characteristics and *E. coli* concentrations in soil**

Differences in household characteristics, WASH indicators, diarrhea/respiratory symptoms, and animal ownership could not account for differences in *E. coli* concentration in soils (Table 1). This study was designed to include the same number of households with ruminants and without ruminants in order to determine if ruminants significantly increase *E. coli* contamination in the household soil environment. Our results indicate that the presence of ruminants in the vicinity of the household plot was not associated with *E. coli* concentration (Wilcoxon $p = 0.31$) (Table 1) or presence/absence of culturable *E. coli* in soils (Fisher's exact $p = 0.58$). In previous studies, the presence of roaming animals and animals in general has been associated with higher level of *E. coli* in soils, although the difference in concentration was low (0.22 and 0.54 $\log_{10}$ CFU/g dry soil, respectively) (16, 33). Our study was likely underpowered to observe significance between WASH indicators and concentration of *E. coli* in soils at the previously observed effect size. For example, all the households included in our study had chickens and other domestic animals that defecate inside the household plot and could potentially contribute to contamination of soils by *E. coli*. Indeed, not only a ruminant-associated molecular source tracking (MST) marker (BacR) but also an avian-associated MST marker (avian-GFD) have been detected in soil samples in rural Bangladeshi households (39). Nevertheless, the absence of a clear relationship between *E. coli* contamination in soils and household-level factors stands in contrast to the relationships observed with soil properties (moisture content, clay percentage).



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**Antibiotic resistance pattern and presence of extended-spectrum beta-lactamase (ESBL) genes**

The level of susceptibility to a panel of 16 antibiotics was evaluated among the 175 *E. coli* isolated from soil (n=23) and fecal samples from human (n=50), chicken (n=51), and cattle (n=51). Overall, 42.3% of the isolates were resistant to at least one antibiotic category and 12.6% were resistant to 3 or more antibiotic categories, thus classified as multidrug resistant (MDR) (Table 2). Resistance to tetracycline (27.4%) and ampicillin (20.6%) were predominant, followed by resistance to nalidixic acid (12.6%) and trimethoprim-sulfamethoxazole (10.3%). Resistance to other antibiotics were less prevalent (1.1 - 5.7%), while no resistance to piperacillin-tazobactam, meropenem, imipenem, and amikacin was observed (Table S3).

Resistance was more commonly observed in *E. coli* isolated from chickens (56.9%) and humans (54.0%) than in *E. coli* from ruminants (15.7%). The proportion of *E. coli* isolates from soil resistant to at least one antibiotic category (43.5 %) was closer to the proportional resistance among *E. coli* from chicken and human isolates than in isolates from ruminants. Notably, 13.0% of soil isolates were MDR (Table 2). The similarity in prevalence and resistance patterns observed among *E. coli* isolates from soils, human feces, and chicken feces, aligns with prior work identifying similar genotypic and phenotypic characteristics among isolates from soil, human feces, and chicken feces (40). The data support the potential for human and/or chicken feces to be a source of soil *E. coli*. Although antibiotic resistance data of *E. coli* from household soils is scarce, as the majority of prior studies focused on resistance in agricultural soils, the prevalence observed here is concerning, especially considering that we did not use antibiotic-selective media for isolation. Whether the *E. coli* isolated are a result of direct fecal input or if

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they represent environmental populations that are genetically different from the fecal sources is currently unknown and represents an interesting research topic for further investigation.

Interestingly, resistance to third-generation cephalosporins was detected with a frequency slightly higher in *E. coli* isolated from soils than in *E. coli* isolated from fecal sources (Table S3). Third-generation cephalosporins are an important family of antibiotics widely used for treatment of infections with Gram-negative bacteria. Soils are regarded as selective environments due to the presence of many antibiotic compounds produced by soil bacteria (41). Furthermore, anthropogenic release of antibiotics and antibiotic derivatives into soils may contribute to the proliferation of antibiotic-resistant bacteria (18). For example, most cephalosporins administered parenterally to humans and animals, are eliminated rapidly through urine (42). Therefore, selection of antibiotic-resistant bacteria not only occurs in the individual or animal taking the antibiotic but may also occur in the environmental compartment receiving the residues (18). Nonetheless, it is important to consider that soil resistomes are complex and antibiotic resistance genes have been documented in high abundance in soils regardless of recent anthropogenic influence (43, 44).

We found ten isolates resistant to third-generation cephalosporins, from which seven (two isolated from soils) were confirmed as ESBL producers by the double-disk synergy test (DDST) and carried the beta-lactamase gene $bla_{\text{CTX-M-group-1}}$. In addition, two isolates co-harbored another ESBL gene ($bla_{\text{TEM}}$ or $bla_{\text{OXA-1-like}}$). The presence of *E. coli* resistant to third-generation cephalosporins (including ESBL producers) in domestic soils in Bangladesh, suggests that this environmental compartment may play a role in child exposures to antimicrobial-resistant



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bacteria. Children (3 to 18 months) in a similar setting were observed to frequently ingest soil and mouth hands and objects after touching soil (8, 15). Exposure to ESBL-producing organisms through soil contact is concerning, as septicemia caused by ESBL-producing organisms has an elevated risk for fatality relative to septicemia caused by antibiotic susceptible infections (45).

**Distribution of intestinal pathotypes among *E. coli* from soil and fecal sources**

Overall 10.3% of the 175 *E. coli* isolates possessed at least one of ten intestinal virulence-associated genes tested. Enteropathogenic *E. coli* (EPEC) was the most prevalent pathotype encountered (4.6%) with seven of the eight EPEC isolates classified as atypical EPEC (only carrying the *eae* gene) and the other as typical EPEC (carrying both *eae* and *bfp* genes). EPEC was more frequently found in *E. coli* isolated from chickens feces (7.8%) than other sources, and it was the only pathotype detected in chicken feces. In contrast, Shiga toxin-producing *E. coli* (STEC) marked by the presence of *stx1* or *stx2*, was only detected in cattle feces. Of the 51 cattle isolates tested, 11.8% were classified as STEC. Human fecal isolates showed higher diversity of virulence-associated genes (*eae*, *bfp*, *aaiC* and *lt*), as three different pathotypes (EPEC, enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC)) were detected in human fecal isolates. From soil samples, one isolate was found to carry *aat* and *aaiC*, indicative of EAEC, while another isolate carried *eae*, indicative of atypical EPEC (Table 3). Detection of *eae* gene in DNA extracted directly from soils revealed presence of EPEC in an additional soil sample. Enteroinvasive *E. coli* (EIEC) was not detected in any of the studied isolates (Table 3).

Notably, the proportion of potentially pathogenic *E. coli* reported in this study is not directly comparable to other studies where enrichment for pathotypes or pooled DNA extraction followed

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by molecular methods have been performed (17, 46). In our study *E. coli* was isolated in tryptone bile x-glucuronide (TBX) agar, which is a selective agar for *E. coli* detection irrespective of pathogenicity, thus the *E. coli* isolated in this media represent the total culturable *E. coli* present in the samples. The presence of virulence genes in 8.7% of the randomly selected *E. coli* colonies recovered from soil samples (one per sample) suggests that within this study site, a surprisingly high proportion of *E. coli* in soil are potentially pathogenic.

**Survival and growth of EPEC in domestic soil microcosms**

Four EPEC isolates, including both antibiotic sensitive and resistant strains (Table S4), readily grew in the autoclaved natural standard soil, a commercially available sandy loam soil described further in the methods section. Specifically, substantial growth was observed from day 0 (seeded at a concentration of ~$10^3$ CFU/g dry soil) to day 3, when all isolates were detected at concentrations of $10^8$ CFU/g dry soil (Fig. 1a). Beyond day 3, the concentration decreased, but remained higher than the concentrations observed immediately after spiking (Fig. 1a). The kinetics of growth and persistence were similar for all four isolates (Fig. 1a, Fig. S2a). In contrast, in non-autoclaved soil, there was a sharp decrease in the concentration at day 7 post-seeding (Fig. S2b). By day 14, all four isolates were no longer detectable. These results support previous findings that soil microflora reduce survival of *E. coli* in soil environments (28, 29). Soil microflora impacts *E. coli* survival and/or growth through competition for available nutrients and/or direct antagonistic relationships, such as predation by protozoa (47–49). Additionally, autoclaving the soil may promote *E. coli* growth through release of nutrients, as for example ammonium-N (50). In addition, the availability of organic compounds is important for *E. coli* growth in soil environments (23, 27). Interestingly, adapting the EPEC isolates in

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297 autoclaved soil before facing non-autoclaved soil substantially extended the survival time (Fig.
298 S2c). The adaptation experiment here is analogous to *E. coli* entering the environment via feces.
299
300 We next followed the fate of the four EPEC isolates in three other soils collected from the
301 households (soils HH-15, HH-29, and HH-34; Table S5). While no significant growth or survival
302 differences were seen among the four isolates, we observed growth varied by soil source (Fig.
303 1b). While the concentrations of the isolates increased in one soil (soil HH-29), mirroring what
304 was observed in the natural standard soil, concentrations of all isolates fell below the detection
305 limit as early as day 3 post-seeding in the other two soils (soils HH-15 and HH-34) (Fig. 1b).
306 This striking differences in EPEC growth and survival among different soils collected from the
307 households led us to study more Bangladeshi soils. In total, we selected 10 soils, five of which
308 had detectable *E. coli* and five of which did not at the time of sampling in the households (Table
309 S5). Growth and survival kinetics of one *E. coli* strain (26-H: isolated from human feces,
310 classified as typical EPEC, resistant to third-generation cephalosporins, ESBL producer, and
311 carrier of the CTX-M beta-lactamase; Table S4) was observed in half of the soils (Fig. 2).
312 Specifically, in four of the five soils where *E. coli* was detected at the time of collection, isolate
313 26-H was able to persist for 14 days after spiking the non-autoclaved soil fraction. In the other
314 soil (soil HH-25), isolate 26-H did not grow or persist (Fig. 2). In contrast, in four of the five
315 soils with no previous *E. coli* detection, isolate 26-H was not detected after spiking the non-
316 autoclaved soil. One soil (soil HH-11) with no previous *E. coli* detection was permissive of *E.*
317 *coli* survival (Fig. 2). No obvious soil characteristic related to growth was identified. For
318 example, the pH values of the ten soils tested were very similar and close to neutral values

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(Table S5). Furthermore, soil-derived supernatant did not directly inhibit *E. coli* growth on laboratory media, suggesting no *E. coli* growth inhibitor is present in the soils (data not shown).

*E. coli* growth is dependent on soil moisture content, as observed in soil microcosms. Specifically, *E. coli* 26-H rapidly decreased in number below the lower limit of detection in autoclaved soil 2.2 with adjusted moisture content of 5% (field capacity ~ 44.8%) (Fig. S3). In contrast, when the moisture content of soil 2.2 was adjusted to 10%, 15%, or 20%, the concentration of the isolate increased by 5 orders of magnitude (from ~$10^3$ to ~$10^8$ CFU/g-dry) within 7 days. The results align with the aforementioned observed correlation between soil moisture content and *E. coli* concentrations. The results also align with prior work identifying water content as a major driver of survival kinetics of bacteria in soils (27, 28, 51) especially at growth permissive temperatures. Notably, small differences in soil moisture content may also influence *E. coli* survival and/or growth, particularly in the presence of soil microflora, which contributes to a more competitive environment. Quantification using culture-based methods may also influence recovery, as they may be unable to recover stressed bacterial cells, as for example cells that have entered the viable but non-culturable (VBNC) stated, at low moisture content or under other environmental stressors (52, 53).

Overall, the findings of our study indicate that soil physicochemical properties influence the detectability, concentration, and growth potential of *E. coli* - including potentially pathogenic and antibiotic-resistant variants - in households in rural Bangladesh. In contrast, WASH indicators were not significantly associated with *E. coli* contamination of household soils in our study site. These findings suggest studies investigating transmission of *E. coli* in household



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environments should consider soil ecology as a moderating variable between household-level risk factors and *E. coli* detection. Soils may act as reservoirs in *E. coli* transmission by enabling growth of antibiotic-resistant and potentially pathogenic *E. coli* variants, as demonstrated by our microcosm studies. Risks from *E. coli* growth in soil are high, given the observed high rates of soil ingestion (both directly and indirectly) among children in Bangladesh and other LMICs. We also found that strain-specific adaptations to growth in soil may not be compulsory for persistence in soil, as no differences in growth and survival rates among the isolates were observed. Moreover, the presence and demonstrated growth of pathogenic and antimicrobial resistant *E. coli* in these household soils suggests that other pathogenic bacterial species with ecology similar to *E. coli* may have the potential to persist and/or grow in soil and therefore also pose a risk to human health.

Further studies are warranted to determine the importance of growth and persistence of *E. coli* and other pathogens in situ to complement our microcosm evidence. Elucidating the origin and fate of pathogenic bacteria in domestic soil environments is important in order for designing effective measures to control transmission. For example, programs to promote upgrading soil flooring in households may help to reduce pathogen transmission as shown by the 13% reduction in diarrheal disease observed in Mexico's Piso Firmo program (54).

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## MATERIALS AND METHODS

*Ethics statement and study site*

This study was performed following an approved protocol by the ethics committees of the Swiss Federal Institute of Technology Zurich (ETH Zurich, Switzerland) and the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b, Dhaka, Bangladesh). The study was conducted in 52 households with dirt/soil flooring located in rural villages of Mirzapur upazilla in Tangail district of Bangladesh (26 households with ruminants and 26 households without ruminants) during February to April of 2016. Researchers/enumerators from icddr,b, conducted household surveys, soil sampling, and fecal sampling. A questionnaire-based survey was conducted on households assets and infrastructure, gastrointestinal/respiratory illness among household members, and agricultural/livestock practices as well as spot-check observations for WASH infrastructure. Based on household assets, infrastructure, and livestock ownership, household wealth was indicated by constructing a composite wealth index using principal component analysis and k-means clustering. Environmental and fecal sampling included the collection of one soil sample, one human fecal sample, one chicken fecal sample, and one cattle (ruminant) fecal sample (if present) from each participating household, as later described.

*Soil and fecal sample collection*

Soils (n = 52), from the front yard of the households with no visible feces, food or trash, were collected. Approx. 150 g of soil were aseptically retrieved from an area of 60 $cm^2$ and < 2 cm depth, and stored on ice in a sterile Fisherbrand® sample bag (Fisher Scientific, Pennsylvania, USA). Human fecal samples were provided in a stool container by household members (18 to 64 years old). Fecal samples from chickens and cattle, with fresh and glossy appearance, preferably



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right after observing the animal deposit the feces, were aseptically collected by the enumerator. Samples were stored on ice and transported to the Enteric and Food Microbiology Laboratory at icddr,b, where they were stored at 4°C and processed within 24 hours of collection.

*Soil physicochemical analyses*

Soil physicochemical analyses were performed at the Department of Soil, Water and Environment of the University of Dhaka. For all the soil samples, soil dry gravimetric water content (GWC) was determined by drying 1 g of soil at 100°C for 16 h or until mass remained constant. Field capacity (55) and permanganate oxidizable active organic carbon (mg/kg) (56, 57) were also measured. In addition, for a subset of 30 soil samples, particle size (58), active organic carbon (%C) with the Walkley-Black chromic acid wet oxidation method (59), and total nitrogen (%N) by the Kjeldahl method (60) were determined. For 10 soils used for the microcosm studies, soil pH was determined in a 0.01 M calcium chloride solution at 1:1 soil:solution ratio (61).

*E. coli enumeration and isolation*

*E. coli* enumeration and isolation from soil and fecal samples was performed as previously described (33) with slight modifications. In brief, 5 ± 0.25 g of soil or 1 ± 0.25 g of feces were diluted in a sterile Fisherbrand® blender bag (Fisher Scientific, Pennsylvania, USA) and mixed by hand for 2 min in 30 ml of phosphate buffered saline (PBS). The mixture was let to settle for 15 ± 3 min to allow sedimentation of bigger particles and for reproducible ten-fold serial dilutions. For enumeration of *E. coli* from soils, 1 ml of each dilution were inoculated onto tryptone bile x-glucuronide (TBX) agar (Oxoid, Basingstoke, UK) by the pour plate technique.



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406 The lower limit of detection (LOD) for the soil samples was 0.99 $\log_{10}$ CFU/g of dry soil. For
407 isolation of *E. coli* from feces, 100 µl of each dilution were spread plated onto TBX agar. All
408 plates were incubated at 37°C for 18-24 h and one colony (for soil, human and chicken samples),
409 or two colonies (for cattle samples) were selected, based on blue-green color appearance on the
410 TBX media, for species confirmation using the API-20E system (bioMerieux, Marcy-L'Étoile,
411 France). The confirmed *E. coli* isolates were given a number corresponding to the household
412 where the sample was collected (1 to 52) follow by the sample type: "S" for soil, "H" for human
413 fecal, "CH" for chicken fecal and "C" for cattle fecal (i.e. 15-CH corresponds to the *E. coli*
414 isolate recovered from a chicken fecal sample collected from household 15). *E. coli* isolates were
415 stored at -80°C at icddr,b and sent to Eawag (Dübendorf, Switzerland) for further analyses.
416
417 *Random amplified polymorphic DNA (RAPD)*
418 RAPD fingerprinting was performed on *E. coli* isolated from soils, using primer "4" (5'-
419 AAGAGCCCGT-3') (discrimination index 0.983) and following a procedure described
420 previously (62). Results were analyzed with the software Bionumerics 4.5. Similarity was
421 determined using the Dice coefficient and clustering was performed by the unweighted pair
422 group method with arithmetic means (UPGMA). RAPD patterns with a Dice coefficient >80%
423 were considered as probably related and assigned to the same cluster or RAPD type.
424
425 *Molecular detection of E. coli in soils.*
426 Molecular detection targeting the conserved beta-glucuronidase gene *uidA* in DNA extracted
427 from soils was performed to establish whether or not the culture-based approach resulted in
428 false-negatives. For DNA isolation from soil, 0.25 g of soil were additionally collected from



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each household and added to a cryovial containing 1 ml of LifeGuard soil preservation solution (Qiagen, Hilden, Germany). Soil samples were stored at -20°C and processed before 30 days after collection. DNA was extracted using the PowerSoil DNA isolation kit (MoBio, California, US) following the manufacturer's instructions. Molecular detection of *E. coli* was performed by PCR, using primers targeting the beta-glucuronidase gene *uidA* (*uidA*_For 5'-GCGTCTGTTGACTGGCAGGTGGTGG-3' and *uidA*_Rev 5'-GTTGCCCGCTTCGAAACCAATGCCT-3'), a gene commonly found in *E. coli* (63) Reaction conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. DNA extracted from the *E. coli* strain ATCC 25922 was used as positive control, while DNAase free water was used as non-template control.

*Antibiotic susceptibility testing*

Antibiotic susceptibility of the 175 isolated *E. coli* was determined against 16 different antibiotic disks (Oxoid, Basingstoke, UK) by standard disk diffusion technique following the Clinical Laboratory Standards Institute (CLSI) guidelines and interpretation standards (64). The evaluated antibiotics included representatives of five different antibiotic categories: beta-lactams: ampicillin (AMP) and mecillinam (MEC) (penicillins); piperacillin-tazobactam (TZP) (beta-lactam-beta-lactams inhibitors); aztreonam (ATM) (monobactam); cefixime (CFM), ceftriaxone (CRO), cefotaxime (CTX) and ceftazidime (CAZ) (third-generation cephalosporins); meropenem (MEM) and imipenem (IPM) (carbapenems); aminoglycosides: amikacin (AMK); tetracyclines: tetracycline (TET); phenicols: chloramphenicol (CAM); quinolones: nalidixic acid (NAL) and ciprofloxacin (CIP); folate pathway: trimethoprim-sulfamethoxazole (SXT). Multidrug resistance



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was defined as non-susceptibility to at least one antibiotic in 3 or more categories as defined by Magiorakos et al. (65). Double-disk synergy test (DDST) was carried out on 10 *E. coli* isolates suspected to be ESBL producers (based on their resistance to third generation cephalosporins). The DDST was considered positive when expansion of the inhibition zone of CTX, CRO, and/or ATM disks towards a disk with clavulanic acid located 20 mm away was observed, as indicated in Jalier et al. (66) with some modifications (67).

*Detection of virulence-associated and extended-spectrum beta-lactamases-encoding genes by PCR*

Previously described PCR methods (68) were used for the detection of ten virulence-associated genes indicative of five different *E. coli* intestinal pathotypes in the 175 *E. coli* isolates: enteroaggregative *E. coli* (EAEC): *aaiC* (secreted protein) and *aat* (antiaggregation protein transporter gene); enteroinvasive *E. coli* (EIEC): *ial* (invasion associated locus) and *ipaH* (invasion plasmid antigen H); enteropathogenic *E. coli* (EPEC): *eae* (intimin) and *bfp* (bundle forming pilus); enterotoxigenic *E. coli* (ETEC): *lt* (heat labile enterotoxin) and *st* (heat stable enterotoxin) and Shiga toxin-producing *E. coli* (STEC): *stx1* and *stx2* (shiga toxins). Detection of *eae* was also directly performed on the DNA isolated from soils. In addition, detection of the beta-lactamase genes $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{OXA-1-like}}$ and $bla_{\text{CTX-M}}$, was performed on all ESBL-producing *E. coli* isolates by multiplex PCR with previously described primers (69). A bacterial strain known to carry the gene targeted by each primer pair was used as positive control. *E. coli* strain ATCC 25922 and water were used as negative and non-template controls, respectively.



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*Soil microcosm studies*

Growth and survival in soil was evaluated for four EPEC isolates (15-CH, 24-H, 26-H, and 29-CH), including one isolate sensitive to all antibiotics while the other three isolates showed different resistance profiles (Table S4). Some experiments were conducted only with the EPEC isolate 26-H (resistant to third-generation cephalosporins, ESBL producer, and carrier of the CTX-M beta-lactamase). Experiments were performed with a natural standard soil (soil type No. 2.2) from LUFA Speyer Germany (http://www.lufa-speyer.de/index.php) and 13 soils collected from the households. The natural standard soil No. 2.2 is a commercially available sandy loam soil with known physicochemical properties (Table S5), has not received pesticides, biocidal fertilizers, or organic manure for at least 5 years, and so was used here as a control soil. Soils were sieved through a 2.36 mm mesh followed by sterilization by 3 consecutive rounds of autoclaving. Soil GWC was determined with 0.5 g of soil following the procedure mentioned earlier. Before starting the experiments the soil GWC was adjusted to 15% ± 1% GWC with sterile double distilled water ($ddH_2O$). For one experiment the soil GWC was adjusted only at the start of the experiment (Fig. 1) while for the others (Fig. 2, Fig. S2, and Fig. S3) the GWC was adjusted if necessary after each time point measured. As autoclaving the soil impacts the indigenous soil microbiota and likely affect some physicochemical soil properties (70, 71), we compared the survival dynamics of the four EPEC isolates in autoclaved vs. non-autoclaved standard soil. To find a scenario that likely resemble a more realistic condition that *E. coli* encounters in domestic soil, a mix of sterilized and unsterilized soil in a ratio of 1:19 or 1:1 was used. In this case, the sterile autoclaved soil fraction was seeded with *E. coli* and incubated for seven days before spiking the non-autoclaved soil fraction with the seeded autoclaved soil. The GWC adjusted soils (4-5 g) were placed into 50 ml tubes and maintained at room temperature



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until used. Triplicate soil samples were prepared for each condition and for each *E. coli* isolate evaluated. For inoculation into the soils, *E. coli* cells were prepared as previously described with modifications (72). In brief, each *E. coli* isolate from overnight cultures in LB broth were diluted into the same medium in triplicates to a staring $OD_{600}$ of 0.05 and grown to mid-logarithmic phase ($OD_{600}$=0.6) at 37°C and 220 rpm. Cells were harvested at 6500 g for 5 min, washed twice with 1X PBS to avoid media carry over and resuspended in 1X PBS to an estimated $10^8$ CFU/ml. The cell suspension was diluted and soils were inoculated to a concentration of $10^1$ - $10^4$ CFU/g of dry soil. As non-inoculated and water content controls, sterile dd$H_2O$ was added instead of the bacterial suspension. Soil-bacteria microcosms were mixed by inversion for 1 min, followed by vortex at maximum speed for 1 min. Right after mixing (day 0) the CFU/g of dry soil were measured by withdrawing and suspending approx. 0.5 g of the inoculated soil (exact weights were recorded for each sample) into 1X PBS, followed by 1 min vortex at maximum speed and centrifugation at low speed (200 g for 2 min) to sediment soil particles. The resulting supernatant was subjected to ten-fold serial dilutions and 25 µl volume from each dilution was drop-plated in duplicates in TBX agar (73). The number of CFU was counted after overnight incubation at 37°C. The microcosms were incubated at 30°C, which is within the range of average temperature in the study area. Furthermore, Islam et al., reported significant linearity between atmospheric temperature and soil temperature at 5 cm depth in Bangladesh (74). Bacterial counts were determined at different time points over a period of up to 84 days, as described for day 0. The lower LOD for each microcosm experiment is indicated in the corresponding graphs.

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*Inhibition assay*

Inhibitory effect of soil on growth of *E. coli* was investigated with six Bangladeshi soils, three that were positive for *E. coli* isolation (HH-25, HH-46, HH-50) and three negative for *E. coli* isolation (HH-04, HH-09, HH-10). For this, a 1:1 soil:PBS solution was prepared, vortexed at maximum speed for 1 min and centrifuged at 200 g for 2 min. Ten µl of the supernatant from each soil:PBS solution were applied to the center of a Mueller Hinton agar plate previously inoculated with the *E. coli* strain ATCC 25922. Zones of inhibition were measured after overnight incubation at 37°C.

*Statistical analyses*

Data was analyzed using GraphPad Prism, version 7.0a (GraphPad Software, Inc. La Jolla, CA) and R version 3.4.3. All concentrations are expressed as $\log_{10}$ *E. coli* CFU per gram of dry soil, as the soil GWC was determined. When the CFU counts were below the lower LOD, half the lower LOD was assumed for all subsequent quantitative analyses . Wilcoxon signed rank test and Kruskal-Wallis test by ranks were used to compare mean ranks of *E. coli* concentration in soil among groups obtained from the survey data. To evaluate if the presence of ruminants is associated with presence and concentration of *E. coli* in soils, Fisher's exact and Wilcoxon signed rank test were used, respectively. Association between $\log_{10}$ *E. coli* CFU/g of dry soil and monthly expenditures, toilet age, or the soil physicochemical parameters was evaluated using Spearman's rank correlation analysis. Differences in the proportion of resistant isolates among sources were evaluated using Fisher's exact. For the soil microcosm results, significant differences in the geometric mean of $\log_{10}$ *E. coli* CFU/g of dry soil were evaluated using one-

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539 way ANOVA with post-hoc analysis (Tukey's multiple comparisons test) or independent
540 Student's *t*-test.
541
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765

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766 **Table 1.** Characteristics of the 52 households in Mirzapur, Bangladesh enrolled in this study
767 along with *E. coli* counts in soil.

| Characteristics | n | Percent (%) | *E. coli* $\log_{10}$ CFU/g-dry soil | | Sig. | Test |
|---|---|---|---|---|---|---|
| | | | Mean | SD | | |
| **Wealth Quartile** | | | | | p = 0.96 | *Kruskal-Wallis test by ranks* |
| First (Poorest) | 17 | 33 | 1.27 | 0.80 | | |
| Second | 11 | 21 | 1.25 | 1.05 | | |
| Third | 15 | 29 | 1.11 | 0.69 | | |
| Fourth (Wealthiest) | 9 | 17 | 1.41 | 1.08 | | |
| **Monthly Expenditures** | | | | | p = 0.86 | *Spearman's rank correlation ($\rho$ = -0.03)* |
| **Toilet/Latrine** | | | | | p = 0.29 | *Kruskal-Wallis test by ranks* |
| Improved - Basic | 37 | 71.2 | 1.14 | 0.79 | | |
| Improved - Limited | 13 | 25 | 1.46 | 1.00 | | |
| Unimproved | 2 | 3.8 | 1.81 | 1.17 | | |
| **Toilet was Serviced/Pit Emptied** | | | | | p = 0.61 | *Kruskal-Wallis test by ranks* |
| In the last month | 3 | 5.8 | 1.17 | 0.83 | | |
| Between 1 month and 1 year | 18 | 34.7 | 1.10 | 0.91 | | |
| Between 1 year and 5 years | 4 | 7.7 | 1.54 | 1.00 | | |
| Never | 27 | 51.9 | 1.31 | 0.84 | | |
| **Toilet Age** | | | | | p = 0.15 | *Spearman's rank correlation($\rho$ = -0.21)* |
| **Visible feces observed around the toilet/latrine** | | | | | p = 0.13 | *Wilcoxon signed-rank test* |
| No | 22 | 42.3 | 1.59 | 1.08 | | |
| Yes | 30 | 57.7 | 1.00 | 0.55 | | |
| **Soap present in toilet/ latrine** | | | | | p = 0.76 | *Wilcoxon signed-rank test* |
| No | 46 | 88.5 | 1.25 | 0.86 | | |
| Yes | 6 | 11.5 | 1.26 | 0.91 | | |
| **Number of Users** | | | | | p = 0.6 | *Kruskal-Wallis test by ranks* |
| 1 – 5 | 27 | 51.9 | 1.12 | 0.74 | | |
| 6 – 10 | 22 | 42.3 | 1.41 | 1.00 | | |
| > 11 | 3 | 5.8 | 1.15 | 0.81 | | |
| **Number of Users Under Five** | | | | | p = 0.43 | *Wilcoxon signed-rank test* |
| 0 | 34 | 65.4 | 1.21 | 0.81 | | |
| ≥ 1 | 18 | 34.6 | 1.31 | 0.96 | | |
| **Incidence of diarrhea** | | | | | p = 0.36 | *Kruskal-Wallis test by ranks* |
| In the last 7 days | 6 | 11.5 | 1.92 | 1.0 | | |
| Within last month | 8 | 15.4 | 1.00 | 0.53 | | |
| Within last 6 months | 7 | 13.5 | 1.12 | 0.59 | | |
| In more than 6 months | 31 | 59.6 | 1.21 | 0.92 | | |
| **Incidence of respiratory symptoms in the last 7 days** | | | | | p = 0.83 | *Wilcoxon signed-rank test* |
| No | 17 | 32.7 | 1.24 | 0.84 | | |
| Yes | 35 | 67.3 | 1.25 | 0.878 | | |
| **Chicken/Ducks** | | | | | p = 0.64 | *Wilcoxon signed-rank test* |
| <10 | 37 | 71.2 | 1.29 | 0.90 | | |
| ≥10 | 15 | 28.8 | 1.15 | 0.77 | | |
| **Cattle** | | | | | p = 0.31 | *Wilcoxon signed-rank test* |
| No | 26 | 50 | 1.40 | 0.96 | | |
| Yes | 26 | 50 | 1.10 | 0.74 | | |



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768 **Table 2.** Distribution of the 175 antibiotic-susceptible and -resistant *E. coli* isolates by source.

| Source | No. (%) of susceptible isolates | No. (%) of resistant isolates to one antibiotic in 1 to 3 or more antibiotic categories[a] | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 or more[b] |
| Soil | 13 (56.5) | 5 (21.7) | 2 (8.7) | 3 (13.0) |
| Human | 23 (46.0) | 10 (20.0) | 6 (12.0) | 11 (22.0) |
| Chicken | 22 (43.1) | 11 (21.7) | 10 (19.6) | 8 (15.7) |
| Cattle | 43 (84.3) | 7(13.7) | 1 (2.0) | 0 (0) |
| Total | 101 (57.7) | 33 (18.9) | 19 (10.9) | 22 (12.6) |

769 [a]Penicillins, monobactams, third generation cephalosporins, tetracyclines, phenicols and

770 quinolones.

771 [b]Resistance to 3 or more antibiotic categories were classified as multidrug resistant.

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772 **Table 3.** Distribution of intestinal pathotypes of *E. coli* isolated from soil and fecal samples.

| Source | No. *E. coli* | No. (%) of isolates positive for intestinal pathogenic virulence-associated genes | | | | | |
|---|---|---|---|---|---|---|---|
| | | EAEC[a] | EIEC[b] | EPEC[c] | ETEC[d] | STEC[e] | Any IPEC |
| Soil | 23 | 1 (4.4) | 0 (0) | 1 (4.4) | 0 (0) | 0 (0) | 2 (8.7) |
| Human | 50 | 2 (4.0) | 0 (0) | 2 (4.0) | 1 (2.0) | 0 (0) | 5 (10.0) |
| Chicken | 51 | 0 (0) | 0 (0) | 4 (7.8) | 0 (0) | 0 (0) | 4 (7.8) |
| Cattle | 51 | 0 (0) | 0 (0) | 1 (2.0) | 0 (0) | 6 (11.8) | 7 (13.7) |
| Total | 175 | 3 (1.7) | 0 (0) | 8 (4.6) | 1 (0.6) | 6 (3.4) | 18 (10.3) |

773 [a]EAEC: Indicated by the presence of *aat* or *aat* and *aaiC*

774 [b]EIEC: Genes *ial* and *ipaH* were not detected.

775 [c]EPEC: Indicated by the presence of *eae* or *eae* and *bfp.*

776 [d]ETEC: Indicated by the presence of *lt.*

777 [e]STEC: Indicated by the presence of *stx1* or *stx1* and *stx2.*

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778 **FIGURE LEGENDS.**

779 **Figure 1.** Survival dynamics of four EPEC isolates (15-CH, 24-H, 26-H, and 29-CH) in
780 autoclaved soils. **(a)** Geometric mean $\log_{10}$ CFU per gram of dry soil of four EPEC isolates
781 measured at day 0, 3, 7, 14, 28, 56, and 84 after spiking standard soil. Each symbol represents
782 the geometric mean $\log_{10}$ CFU per gram of dry soil and the error bar indicates the standard
783 deviation of three independent replicates per isolate. Lower limit of detection (LOD) is indicated
784 by the horizontal dotted line. Gravimetric water content (GWC) of the soil at each time point is
785 indicated by the dotted line and the right y-axis. **(b)** Aggregate of the concentration of four EPEC
786 isolates in the standard soil and soils collected from three households (HH-15, HH-29, and HH-
787 34). Each symbol represents the $\log_{10}$ CFU per gram of dry soil for each isolate and their
788 replicates (three independent replicates per isolate); the horizontal line is the geometric mean
789 $\log_{10}$ CFUs per gram of dry soil of all the isolates for each soil type (GWC is indicated) and on
790 each sampling day (day 0, 3, and 7); the dotted line indicates the lower LOD. When the CFU
791 counts were below the lower LOD, the value used to graph correspond to half the lower LOD.
792
793 **Figure 2.** Survival dynamics of *E. coli* 26-H (isolated from human feces, classified as typical
794 EPEC, resistant to third-generation cephalosporins, ESBL producer, and carrier of the CTX-M
795 beta-lactamase) in ten Bangladeshi household soils. Each symbol represents the geometric mean
796 $\log_{10}$ CFU per gram of dry soil and the error bar indicates the standard deviation of three
797 independent replicates per soil at day 0, 7, 14 and 28 (only for two soil). Day (-7) represents the
798 calculated CFU per gram used to seed the autoclaved fraction of the soils; day (0) is the CFUs
799 per gram of dry soil after spiking the non-autoclaved portion with the seeded autoclaved soil
800 (1:19 autoclaved: non-autoclaved ratio). The dotted line indicates the lower limit of detection

(LOD). When the CFU counts were below the lower LOD the value used to graph correspond to half the lower LOD.

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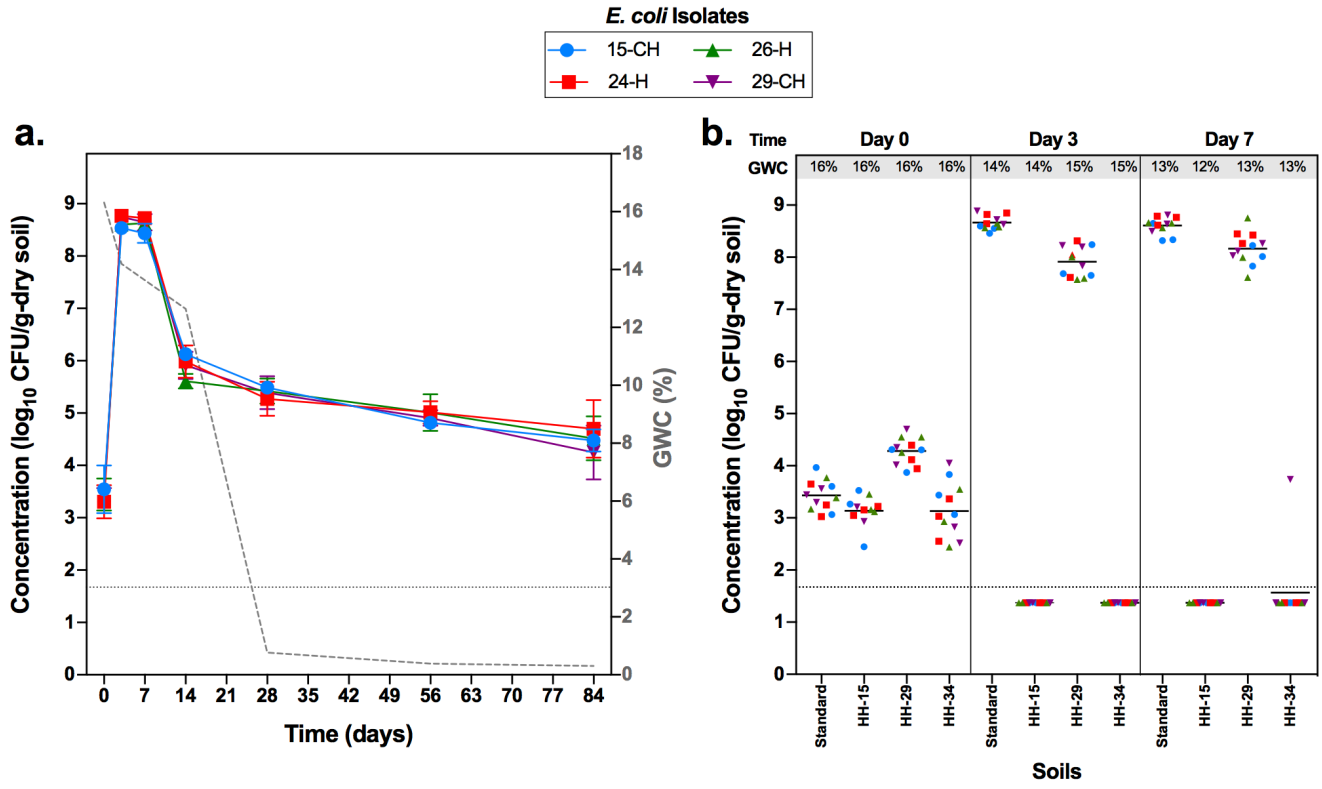
**Figure 1.**



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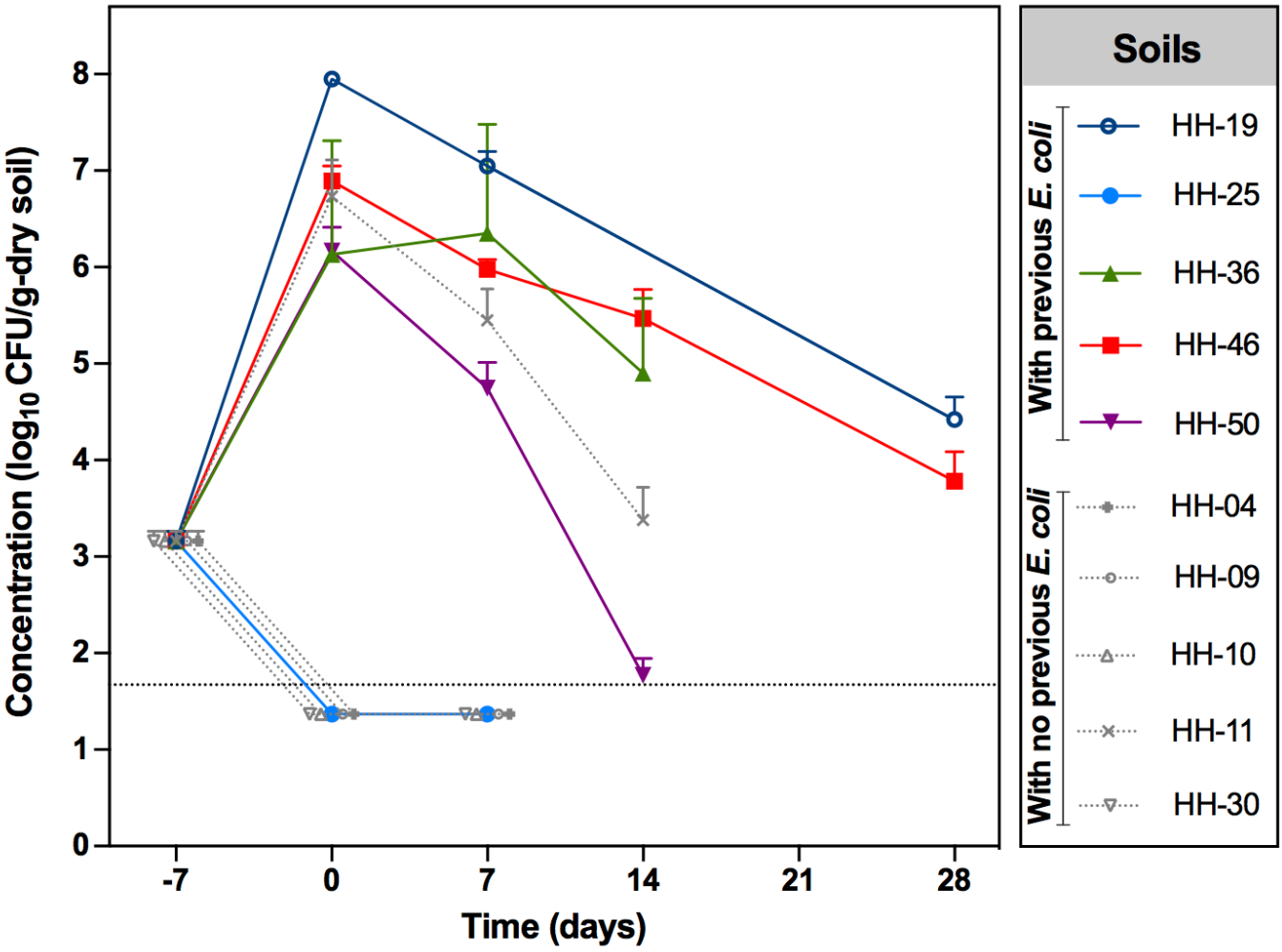
# Figure 2.

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