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Day-to-Day Dynamics of Commensal *Escherichia coli* in Zimbabwean Cows Evidence Temporal Fluctuations within a Host-Specific Population Structure

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ABSTRACT To get insights into the temporal pattern of commensal *Escherichia coli* populations, we sampled the feces of four healthy cows from the same herd in the Hwange District of Zimbabwe daily over 25 days. The cows had not received antibiotic treatment during the previous 3 months. We performed viable *E. coli* counts and characterized the 326 isolates originating from the 98 stool samples at a clonal level, screened them for *stx* and *eae* genes, and tested them for their antibiotic susceptibilities. We observed that *E. coli* counts and dominant clones were different among cows, and very few clones were shared. No clone was shared by three or four cows. Clone richness and evenness were not different between cows. Within each host, the variability in the *E. coli* count was evidenced between days, and no clone was found to be dominant during the entire sampling period, suggesting the existence of clonal interference. Dominant clones tended to persist longer than subdominant ones and were mainly from phylogenetic groups A and B1. Five *E. coli* clones were found to contain both the stx_1 and stx_2 genes, representing 6.3% of the studied isolates. All cows harbored at least one Shiga toxin-producing *E. coli* (STEC) strain. Resistance to tetracycline, penicillins, trimethoprim, and sulfonamides was rare and observed in three clones that were shed at low levels in two cows. This study highlights the fact that the commensal *E. coli* population, including the STEC population, is host specific, is highly dynamic over a short time frame, and rarely carries antibiotic resistance determinants in the absence of antibiotic treatment.

IMPORTANCE The literature about the dynamics of commensal *Escherichia coli* populations is very scarce. Over 25 days, we followed the total *E. coli* counts daily and characterized the sampled clones in the feces of four cows from the same herd living in the Hwange District of Zimbabwe. This study deals with the day-to-day dynamics of both quantitative and qualitative aspects of *E. coli* commensal populations, with a focus on both Shiga toxin-producing *E. coli* and antibiotic-resistant *E. coli* strains. We show that the structure of these commensal populations was highly specific to the host, even though the cows ate and roamed together, and was highly dynamic between days. Such data are of importance to understand the ecological forces that drive the dynamics of the emergence of *E. coli* clones of particular interest within the gastrointestinal tract and their transmission between hosts.

KEYWORDS *Escherichia coli*, antibiotic resistance, cattle, commensal, Shiga toxin-producing *Escherichia coli*

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The bacterium *Escherichia coli* has the noteworthy characteristic of being both a gut commensal of a broad range of vertebrates, making its habitat very large (1), and a versatile and ubiquitous pathogen (2). Whether it is a pathogen (3) or not (4), *E. coli* passes from one host to another and settles in a new host via the fecal-oral route, either directly or indirectly via the environment. Although pathogenic strains have been extensively investigated and tracked to prevent health issues, key features of the gut commensal populations from which they emerge remain to be investigated.

As an example, Shiga toxin-producing *E. coli* (STEC) isolates are enteric pathogens in humans and are responsible for bloody diarrhea and hemolytic-uremic syndrome, which is the most frequent cause of acute renal failure in children (5). STEC strains are primarily found in the gastrointestinal tract of ruminant animals, where they mainly live as commensals. Cattle feces are regarded as the main source of infection through the contamination of water or meat during the food chain process (6, 7). However, the high degree of variability in the prevalence, duration, and level of shedding among cattle herds prevents us from defining a proper risk of human exposure to STEC isolates (8, 9).

It has also been shown that gut commensal populations are implicated in the emergence and persistence of antibiotic-resistant pathogenic clones (10). When the gut microbiota is exposed to antibiotic molecules, resistant *E. coli* clones are selected (11). The rise in the incidence of carriage of these specific clones increases the resistance gene pool that may be transmitted to opportunistic pathogenic clones present in the gut.

Moreover, knowledge about commensal *E. coli* populations would be of particular interest to understand the epidemiology of pathogens that use the same routes as *E. coli*. It would help us to predict which pathways of transmission between host populations that these pathogens are most likely to use (12–14). Commensal *E. coli* strains are exchanged between hosts of the same or different species, and their exchange has already been regarded to be a proxy for pathogen dissemination (15, 16). The use of an indicator of pathogen transmission would be of great interest in some complex ecosystems where the high degree of ecological overlap between humans, domestic animals, and wild animals leads to a greater risk of interspecies pathogen spillover (17–19).

Nonetheless, the population structure of commensal *E. coli* isolates is driven by multiple factors, leading to different patterns among individuals between and within species (20). Furthermore, most of the few studies that have aimed to obtain an understanding of the temporal dynamics of *E. coli* populations show that a high degree of clonal diversity exists over years (21), months (22, 23), or weeks (24, 25). Combined with the already known intermittent nature of the shedding of STEC in cattle (9, 26), these studies highlight the fact that a precise description of the dynamic pattern at the clonal level requires shorter sampling intervals. Individual-level sources of heterogeneity need to be untangled in upstream studies of the shedding and transmission patterns of clones of interest, such as STEC or antibiotic-resistant clones. The purpose of this study was to provide an in-depth baseline depiction of the temporal fluctuations in the gastrointestinal populations of *E. coli* in domestic cattle living at a well-studied wildlife/livestock interface. We describe the daily variations, over a period of 25 days, in the total *E. coli* counts, the clonal diversity, and the population structure, with a focus on STEC and antibiotic-resistant *E. coli* strains, in cows living in a region where the antibiotic pressure is controlled and low.

RESULTS

The between-individual variability in *E. coli* counts was higher than the within-individual variability. We performed viable *E. coli* counts on the 98 fecal samples from 4 cows over 25 consecutive days (25 samples each from two cows, labeled cows C1 and C2, and 24 samples each from the two other cows, labeled cows C3 and C4). All four cows shed *E. coli* in their feces, and clones were detected on each day of the sampling period, except in the feces from cow C4 on day 17. The total mean *E. coli* count was 3.7

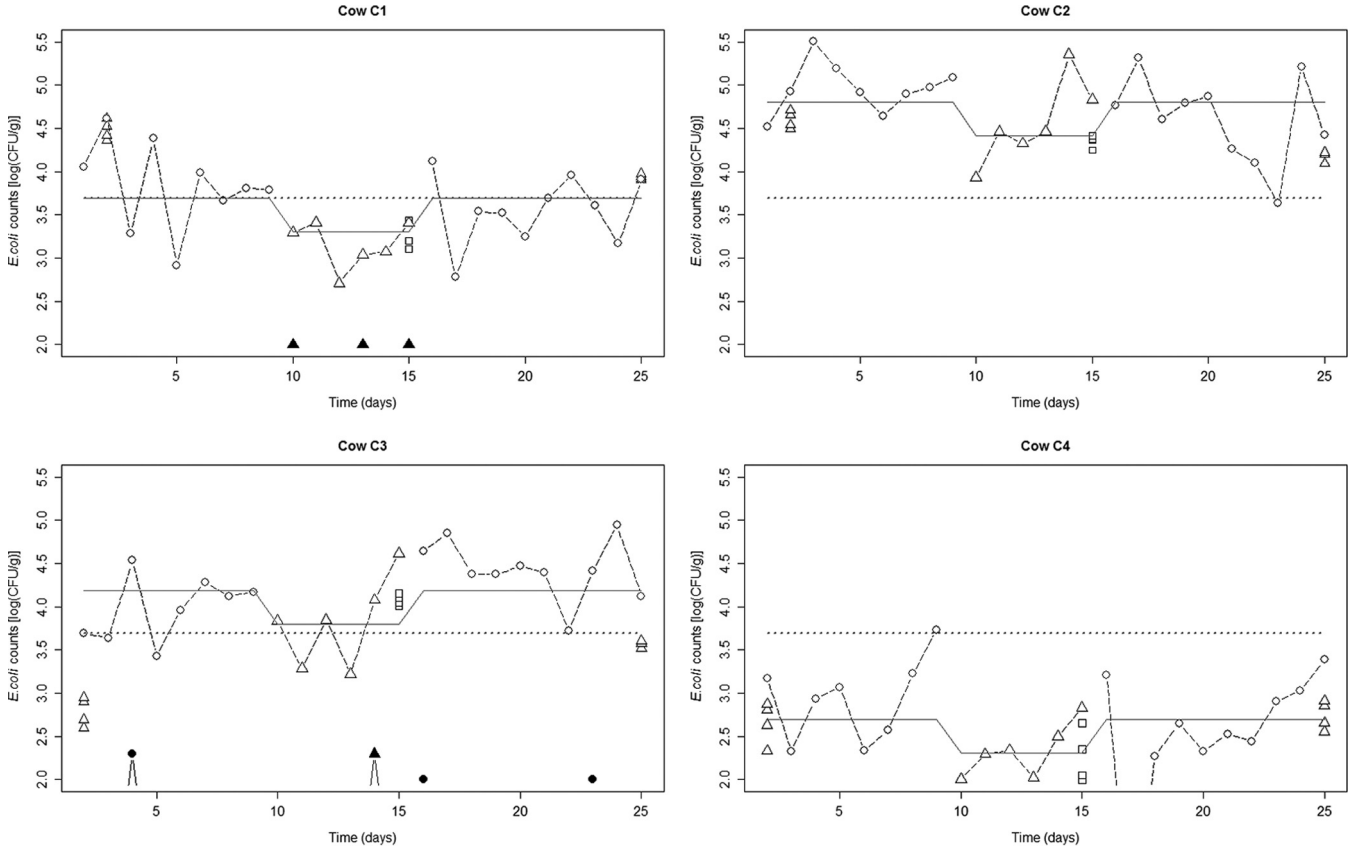

FIG 1 Plots of observed counts of commensal *E. coli* isolates in the four cows over a 25-day period, cow-specific predicted profiles, and counts of antibiotic-resistant *E. coli* clones. Each symbol represents the observed *E. coli* counts. Circles, triangles, and squares, one, two, and three freeze-thaw cycles, respectively. For each cow, the three dashed lines joining the symbols represent the three sets of observed viable *E. coli* counts made after the different freeze-thaw cycles. The drop in the counts between days 10 and 15 compared to that on the other days is due to the additional freeze-thaw cycle. White symbols, counts of total viable *E. coli* isolates; black symbols, counts of *E. coli* isolates resistant to tetracycline or penicillins; dotted lines, the overall mean of the total viable *E. coli* counts; continuous curves, cow-specific predicted profiles of shedding.

log CFU/g of feces. The mean *E. coli* counts for cows C4, C1, C3, and C2 were 2.6 log CFU/g, 3.7 log CFU/g, 3.9 log CFU/g, and 4.6 log CFU/g, respectively. The range of the *E. coli* counts ran from 2 log CFU/g to 5.5 log CFU/g (Fig. 1). Within the cows, the range was narrower. Indeed, for each of the four cows, the *E. coli* counts comprised a range of 2 log units.

Using a linear mixed-effects model, we assessed whether the *E. coli* counts changed over time and how they differed between hosts. No time trend was found during the sampling period. The number of freeze-thaw cycles that a sample underwent had a significant effect on *E. coli* counts (Table 1). *E. coli* counts were reduced by 0.39 log CFU/g (95% confidence interval [CI], −0.51, −0.27) (number of CFU per gram divided by 2.5; 95% CI, 1.9, 3.2) after 2 freeze-thaw cycles (samples from days 10 to 15 and restreaked samples from days 2 and 25). They were reduced by 0.78 log CFU/g (95% CI, −0.99, −0.56) (number of CFU per gram divided by 6.0; 95% CI, 3.6, 9.8) after 3 freeze-thaw cycles (restreaked samples from day 15).

On the basis of our model (Table 1), the between-individual and within-individual variability contributed to 69% and 28% of the total variability, respectively, with the residual variability being 3%. Therefore, there was more variation in *E. coli* counts between samples taken from different cows than among samples taken from the same animal. Thus, the cow host contributes more to the diversity of the *E. coli* counts than the day-to-day fluctuations within cows.

Commensal *E. coli* clones were specific to their host and fluctuated over the course of several days and weeks. (i) Characteristics of the clones detected. We randomly picked one *E. coli* isolate from all plated fecal samples and five isolates per

TABLE 1 Fixed and random estimated parameter values for the final linear mixed-effects model

Parameter	Estimate	95% CI[a]		*P* value
		Lower limit	Upper limit	
Fixed effects				
Intercept (no. of log CFU/g)	3.84	3.06	4.62	
Two freeze-thaw cycles (no. of log CFU/g)	−0.39	−0.51	−0.27	$<10^{-16}$
Three freeze-thaw cycles (no. of log CFU/g)	−0.78	−0.99	−0.56	$<10^{-16}$
SD of random effects (no. of log CFU/g)				
Cow level	0.78	0.38	1.58	
Time level	0.50	0.42	0.58	
Residual	0.17	0.14	0.21	

[a]Normal approximation to the distribution of the maximum likelihood estimators.

plated fecal sample from days 2, 15, and 25 that had been restreaked four times. Of the 319 *E. coli* isolates, a total of 32 distinct clones were detected using a combination of quadruplex PCR phylogrouping, determination of the *trpA* allele, random amplified polymorphic DNA (RAPD) PCR, and detection of the *stx* and *eae* genes by PCR (see Table S1 in the supplemental material). Over the sampling period, 11, 8, 7, and 9 clones were detected in cow C1, cow C2, cow C3, and cow C4, respectively.

We detected all the main *E. coli* phylogenetic groups during the sampling, except for phylogenetic groups C and F. The phylogenetic groups most often recovered from among the isolates were phylogenetic groups A and B1 (48.9% and 37.6% of the isolates, respectively), followed by E (7.5%), B2 (4.4%), and D (1.6%). Of the 32 detected clones, 14 were assigned to phylogenetic group B1, 6 belonged to phylogenetic group A, 8 belonged to phylogenetic group E, 3 belonged to phylogenetic group B2, and 1 belonged to phylogenetic group D. In cow C1, 5 clones (45.5% of the clones) from 78 isolates belonged to phylogenetic group A and 4 clones (36.4%) from 5 isolates belonged to phylogenetic group B1. For cows C2, C3, and C4, the clones mainly belonged to phylogenetic groups B1 and E (50.0% and 25.0% of the clones for cow C2, respectively; 28.6% and 42.9% of the clones for cow C3, respectively; and 55.6% and 22.2% of the clones for cow C4, respectively). However, the only clones belonging to phylogenetic group A that were isolated from cows C3 and C4 were highly prevalent, as these clones were most often isolated from cow C3 (62.7% of the isolates) and were the second most often isolated from cow C4 (39.4% of the isolates) (Fig. S1).

(ii) The clonal compositions of the *E. coli* populations varied among cows. The dominant clone on days 2, 15, and 25 was different between cows. In cow C1, two clones belonging to phylogenetic group A were successively dominant on days 2 and 15 (90.0% and 65.0% of the isolates for the dominant clone on day 2 and the dominant clone on day 15 in this cow, respectively; Fig. S1). In cow C2, a clone belonging to phylogenetic group E and one belonging to phylogenetic group B1 were codominant on day 2 (45.0% and 30.0% of the isolates on day 2 in this cow, respectively). Then, the phylogenetic group B1 clone was found to be dominant on day 15 (60.0% of the isolates on day 15). On day 25, it was replaced by two other B1 clones that were codominant (35.0% and 30.0% of the isolates on day 25; Fig. S1). In cows C3 and C4, dominant B1 clones on day 2 (42.0% and 37.0% of the isolates for the codominant B1 clones in cow C3 and 56.0% of the isolates for the dominant clone in cow C4) were replaced by clones belonging to phylogenetic group A between days 2 and 15 (95.0% and 86.0% of the isolates on day 15 in cows C3 and C4, respectively).

We detected only three clones shared between cows. Cow C1 shared a clone belonging to the B2 phylogenetic group with cow C3 (clone B2-122-1) and a clone belonging to the B1 phylogenetic group with cow C2 (clone B1-1-1-*stx*) (Table S1 and Fig. S1). Clone A-185-1, which was found to be dominant in cow C4 at days 15 and 25, was also found in cow C1, but it was subdominant (4.7% of the isolates in cow C1) (Table S1 and Fig. S1). No clone was shared by three or four cows.

TABLE 2 Diversity measurements of *E. coli* clones within cows on days 2, 15, and 25

	Shannon's index (*H*′)				Piélou's evenness (*J*′)			
Cow	Day 2	Day 15	Day 25	Mean (SD)	Day 2	Day 15	Day 25	Mean (SD)
C1	0.39	0.86	1.16	0.80 (0.39)	0.36	0.78	0.65	0.60 (0.22)
C2	1.40	1.06	1.57	1.34 (0.26)	0.78	0.77	0.88	0.81 (0.06)
C3	1.18	0.20	0.71	0.70 (0.49)	0.85	0.29	0.51	0.55 (0.28)
C4	1.12	0.41	0.84	0.79 (0.36)	0.81	0.59	0.76	0.72 (0.12)

We calculated Shannon's index (*H*′) and Piélou's evenness (*J*′) for each sample on days 2, 15, and 25 to compare the clone richness and their distribution between cows (Table 2). Neither Shannon's index nor Piélou's evenness was found to be significantly different between cows ($P = 0.5$ when both Shannon's index and Piélou's evenness were corrected using the Benjamini and Hochberg method [27]). The clones were specific to their hosts, but clone richness and evenness were not significantly different between cows.

(iii) The *E. coli* populations were highly dynamic over a few days. Of the 25 clones detected on days 2, 15, and 25, 15 were persistent (Table S1). We found three persistent clones in cows C1, C3, and C4. Cow C2 had six persistent clones (Fig. 2). Among these 15 persistent clones, 10 were dominant on at least 1 day and belonged to phylogenetic groups A and B1. The five remaining clones were subdominant and belonged to phylogenetic groups A, B1, B2, D, and E. The mean prevalence was not high for all persistent clones. The maximum mean prevalence was 59.3% of the isolates on days 2, 15, and 25 for clone A-108-1 in cow C3, which was absent on day 2 but

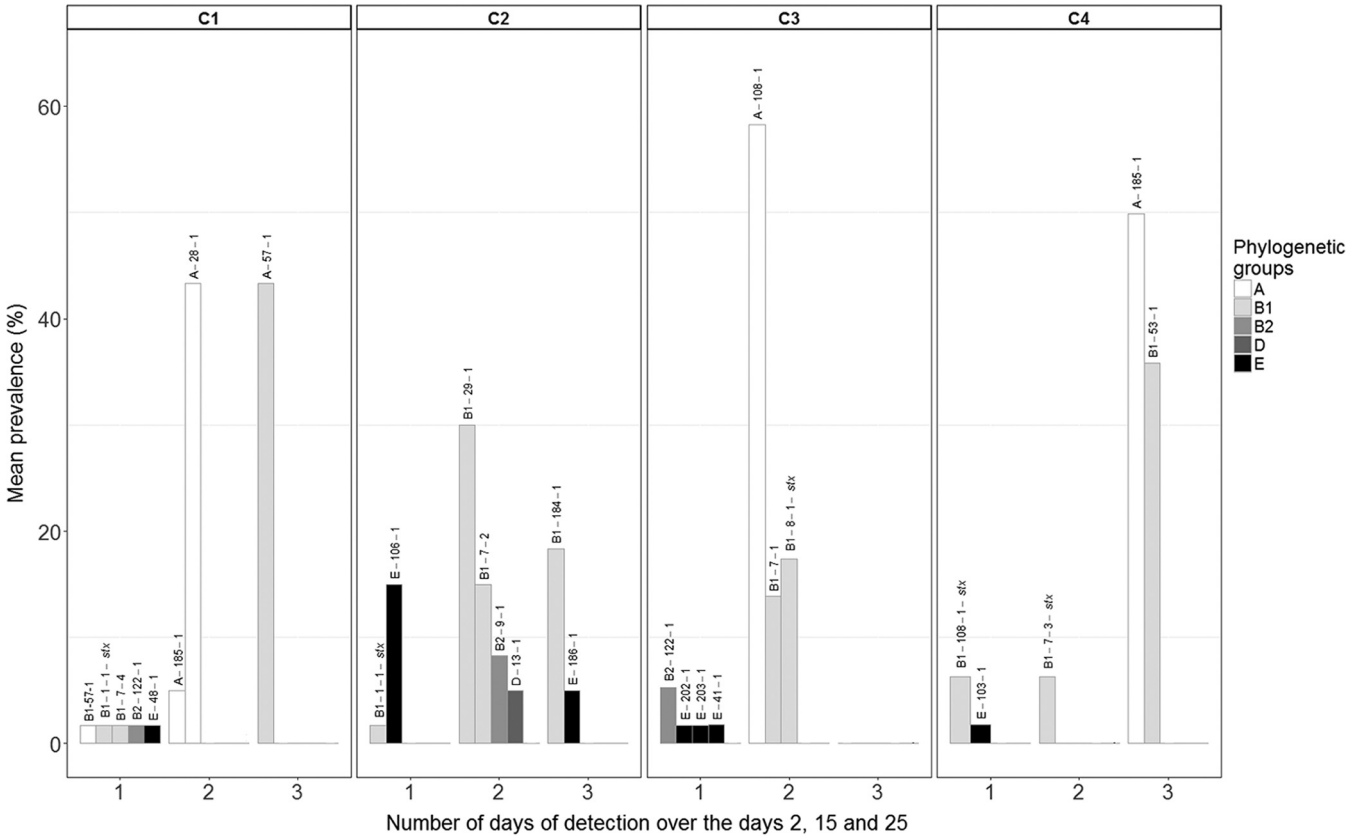

FIG 2 Mean prevalence of *E. coli* clones on sampling days 2, 15, and 25 according to the number of days of detection over these 3 days. Only the prevalences of clones that were detected at least once on these 3 days are represented. The clone nomenclature code was constructed as follow: XX-YYY-Z, where XX is the phylogenetic group, YYY is the *trpA* allele of the Pasteur Institute MLST database (67), and Z is the RAPD PCR pattern within each combination of phylogenetic group and *trpA* allele. When stx_1 and stx_2 genes were detected, the suffix *stx* was added to the clone nomenclature code. When the patterns differed by at least one genotyping technique, clones were considered to be different.

reached a prevalence of 95.0% and 80.0% of the isolates on days 15 and 25, respectively. The prevalence of the less abundant persistent clones was 5.0% of the isolates on days 2, 15, and 25 (clone A-185-1 in cow C1, clones D-13-1 and E-186-1 in cow C2; Fig. 2). This finding points out that the abundance of these clones undergoes fluctuations over intervals of several days. Indeed, no clone was found to be dominant within each cow during the entire study period. The prevalence of the most abundant clone on day 2 significantly changed over the resampling days in cows C1, C2, and C3 but not in cow C4 ($P = 4 \times 10^{-6}$, 7×10^{-5}, 1×10^{-3}, and 0.1, respectively, by Fisher's exact test with correction using the Benjamini and Hochberg method [27]; Fig. S1). For three of the four cows, the dominant clone on day 15 remained dominant on day 25 (clone A-28-1, clone A-108-1, and clone A-185-1 in cows C1, C3, and C4, respectively) but disappeared or was present at levels under the limit of detection for cow C2 (clone B1-29-1). Some of the subdominant clones were found to be persistent in cows C1 and C2 (one and three clones, respectively). We found transient clones in all cows (Fig. 2). Of the 13 transient clones, 12 were subdominant. They belonged to phylogenetic groups A, B1, B2, and E. Thus, the population of *E. coli* that persisted over a few days was multiclonal, was phylogenetically diverse, was dominated by clones belonging to phylogenetic groups A and B1, and changed substantially over several days.

We did not find any significant difference in Shannon's index and Piélou's evenness between days of sampling within cows ($P = 0.5$ and 0.8, respectively, when the values were corrected using the Benjamini and Hochberg method [27]). Thus, the clone richness and evenness of the commensal *E. coli* populations were not significantly different over a period of 25 days within a given host.

STEC strains were widespread but not dominant. Both the stx_1 and stx_2 genes were found in five *E. coli* clones, representing 6.3% of all isolates. Four clones belonged to the B1 phylogenetic group and one clone belonged to the A phylogenetic group (Table S1). All cows harbored at least one STEC isolate. Four clones were subdominant, whereas the B1-8-1-*stx* clone was codominant with a non-STEC clone on day 2 in cow C3. Cows C1 and C4 excreted two distinct STEC clones (clones A-1-2-*stx* and B1-1-1-*stx* in cow C1 and clones B1-7-3-*stx* and B1-108-1-*stx* in cow C4). Two of the STEC clones were found to be persistent, as the B1-8-1-*stx* clone was found on day 2 and day 25 in cow C3 and the B1-7-3-*stx* clone was found on day 2 and day 15 in cow C4 (Fig. 2). The B1-1-1-*stx* clone was isolated on day 25 in both cows C1 and C2 (one isolate in cow C1 and two isolates in cow C2). All STEC isolates were *eae* negative, and none exhibited the classical enterohemorrhagic *E. coli* (EHEC) O types.

Even though they were mainly rare and excreted at low levels, STEC isolates were present in all cows, and some were able to persist over a long period.

Antibiotic-resistant *E. coli* clones were detected at low levels. We detected very few *E. coli* clones resistant to tetracycline and penicillins in the 98 fecal samples plated on Drigalski agar containing tetracycline and ticarcillin, respectively. We found resistant clones in two cows, cows C1 and C3 (Fig. 1; Table S1). Only one clone resistant to tetracycline [due to the presence of the *tet*(A) gene] and penicillins (due to the presence of the bla_{TEM} gene) was found in the cow C1 on days 10, 13, and 15. It was a subdominant clone, as we found it to be present at a level that was at the limit of detection (2 log CFU/g) (Fig. 1), and it belonged to phylogenetic group E. This clone was also resistant to sulfonamides and trimethoprim (due to the presence of the *dfrA14* gene). We found two clones resistant to antibiotics in cow C3. One clone was resistant to tetracycline [due to the presence of the *tet*(B) gene], sulfonamides, and trimethoprim (due to the presence of the *dfrA17* gene) and was present at a level that was at the limit of detection. It belonged to phylogenetic group A (clone A-1-3-R, Table S1). We isolated this clone once, on day 23. The second clone was resistant to penicillins (due to the presence of the bla_{TEM} gene) and sulfonamides and was found in two isolates on both days 4 and 14 and in one isolate on day 16 (Fig. 1). It belonged to phylogenetic group D (clone D-11-1-R, Table S1; Fig. 1). All resistant clones were distinct from the other detected clones, which were susceptible to all the tested antibiotics.

Thus, despite the absence of antibiotic treatment in the herd in the previous 3 months, we detected resistant clones in two cows at a very low level. These clones were able to persist for at least a few days and harbored different molecular supports for resistance to tetracycline and trimethoprim (28).

DISCUSSION

Several studies have shown the existence of a temporal variability in the incidence of commensal *E. coli* populations at the year level (21), month level (23), or week level (25), mostly in humans but also in cattle and horses (29). This study followed on a daily basis and over a 25-day period the commensal *E. coli* populations of healthy adult cows in an extensive small-scale farming system with a focus on subpopulations of interest. This study (i) will allow the proposal of hypotheses for the basic mechanisms of *E. coli* adaptation as a commensal and its population dynamics between and within hosts and (ii) will serve as a basal reference of the normal temporal patterns and variability for later studies to interpret changes in clonal populations of interest, such as STEC and antibiotic-resistant clones or commensal *E. coli* populations, in the context of the identification of a transmission network.

The commensal *E. coli* population was host specific and highly dynamic. The most striking feature of our work was the important variability in the characteristics of the *E. coli* populations among cows. Despite the fact that we selected cows from the same herd and with high degrees of genetic and environmental similarity (e.g., the cows fed and drank from the same sources), the main source of variability for the *E. coli* counts was the between-individual variability, meaning that the *E. coli* population structure was host specific. For example, during the entire sampling period, cow C4 had very low *E. coli* counts, whereas cow C2 had almost twice as many *E. coli* organisms on a given day (Fig. 1). Moreover, very few clones were shared among the cows, with only 9% of the detected clones (3 of 32) being found to be shared, and none of the clones detected was found in all four cows.

However, we found several trends shared by the four cows: (i) the counts of *E. coli* were in the range of 10^2 to 10^6 CFU per g of feces, consistent with estimates of *E. coli* population sizes in cattle obtained by plating of fresh feces (30) or by PCR-based assays (31, 32); (ii) the phylogenetic groups recovered the most often were A and B1, which have been found to be associated with domestic cattle (28, 32, 33), and E, so the *E. coli* population pattern was close to that of the buffaloes living in the same area (28); (iii) the intestinal *E. coli* population of each cow was constituted of several persistent and transient clones, as previously described (34) (we also found that dominant clones persisted in the intestinal tract longer than subdominant clones [34]); and (iv) moreover, we observed switches of the dominant clones in all four cows during the sampling period. These shared trends suggest the existence of common mechanisms that control the ecological relationships among commensal *E. coli* populations.

Emergent dominant clones could be present in the gut at levels under the limit of detection for extended periods before they increase in abundance and are detected. Alternatively, they could be acquired exogenously and their number could rise quickly, as has been hypothesized in an experiment where healthy volunteers received ciprofloxacin for 14 days and quinolone-resistant *E. coli* emerged in their feces a few days after the end of the treatment (35). In agreement with our data (see Fig. S1 in the supplemental material), it has been shown in mice that the establishment of a dominant clone within the gut took a few days (36, 37), depending on its capacity to adapt its metabolism to the available carbon sources, which were mainly sugars (36, 38). The adaptation of a clone to its environment is driven by a combination of periodic selection and clonal interference, which is the competition of clones carrying different beneficial mutations at the population level (37, 39) and convergence and epistatic interactions at the genotype level (39, 40). Furthermore, a high level of polymorphism in the ability of the *E. coli* species to use carbon sources (41, 42) could explain the diversity of the observed clones. Other forces could be involved in the competition

between clones, such as the production of colicins and/or bacteriophages (42, 43) or resistance to grazing by protozoan predators, such as *Dictyostelium discoideum* (44).

Nonetheless, it should be remembered that the diversity and distribution of the *E. coli* genotypes are different among the different regions of the gastrointestinal tract, as environmental conditions are not the same in each of these regions (45–47). Thus, we cannot exclude the possibility that the clones detected were present in different parts of the gastrointestinal tract of their host, and their prevalence at days 2, 15, and 25 could have been the result of a bloom of a specific clone in a given fraction of the gastrointestinal tract.

STEC clones behave like other nondominant commensal clones. All cows shed STEC isolates but carried different clones and did not excrete them on the same days. These results highlight the intermittent and variable nature of STEC shedding and are in accordance with the findings in the literature dealing with STEC shedding in cattle (9, 26). The capacity of STEC strains to survive as subdominant clones in the intestinal environment could be the result of opposite pressures. Our results indicate that STEC clones do not have a better fitness than other clones for overgrowth of the gastrointestinal tract of cattle, as shown in mice (48). Nevertheless, STEC strains are able to occupy niches distinct from those of resident strains, which have a greater ability to metabolize glycolytic substrates, by exploiting other carbon sources, such as gluconeogenic substrates (48). Moreover, it has been shown that the presence of *stx* genes protects STEC clones against predation by grazing protozoa by enabling the survival of the clones in food vacuoles (49). Hence, the conjunction of these fitness advantages and disadvantages results in the presence of STEC clones at a low level but a high prevalence in cattle. Recently, Jones et al. characterized by multiple-locus variable-number tandem-repeat analysis and genome sequencing a set of O157:H7 isolates from a beef herd (50). They showed that four different lineages were present in the herd over a 9-month period and detected clonal replacement events over time and cross transmission between cows (50). Our results are in accordance with those of that study; however, no information on the prevalence of non-STEC clones was given.

***E. coli* antibiotic-resistant clones are rare and subdominant.** We focused our study mainly on tetracycline and ticarcillin, because tetracycline is the most commonly used antibiotic in cattle and penicillins are the most commonly used antibiotics in human medicine in Zimbabwe and because very few cases of antibiotic resistance were previously found in cattle from the same cattle herd from which the cows that we studied were obtained (28). We detected only three *E. coli* clones that were resistant to antibiotics, which is consistent with the absence of antibiotic treatment of these cows in previous weeks. Two of these clones were able to persist at a low level for several days. Nevertheless, they did not seem to be present in their host during the entire sampling period, meaning that their clearance was fast, as it has been shown that the clearance of resistant clones was slower for clones with a high relative abundance (10). Moreover, we did not observe the spread of antibiotic resistance genes between clones. Therefore, under such ecological conditions, the fitness cost of resistance did not seem to be compensated for by others mechanisms, such as fitness-compensatory mutations and genetic coselection (51), and, consequently, did not allow the transmission of antibiotic resistance support among clones and among hosts.

Concluding remarks. Our data, summarized in Fig. 3, which is based on the phylogenetic reconstruction of the *trpA* gene of the studied clones over time, highlight (i) the important phylogenetic diversity of commensal *E. coli* isolates within a cow at a sampling point and between cows from the same herd, (ii) the shedding of different clones on a given day, (iii) the maintenance of a few clones throughout the course of the sampling period, and (iv) the intermittent shedding of STEC and antibiotic-resistant clones. The intestinal *E. coli* microbiota is host specific and may result from two types of pressure: common pressures affecting all cows caused by factors that shape ecological niches, such as body mass, diet, and gastrointestinal tract morphology (1, 33), and pressures specific to each individual, which could be due to interactions within its

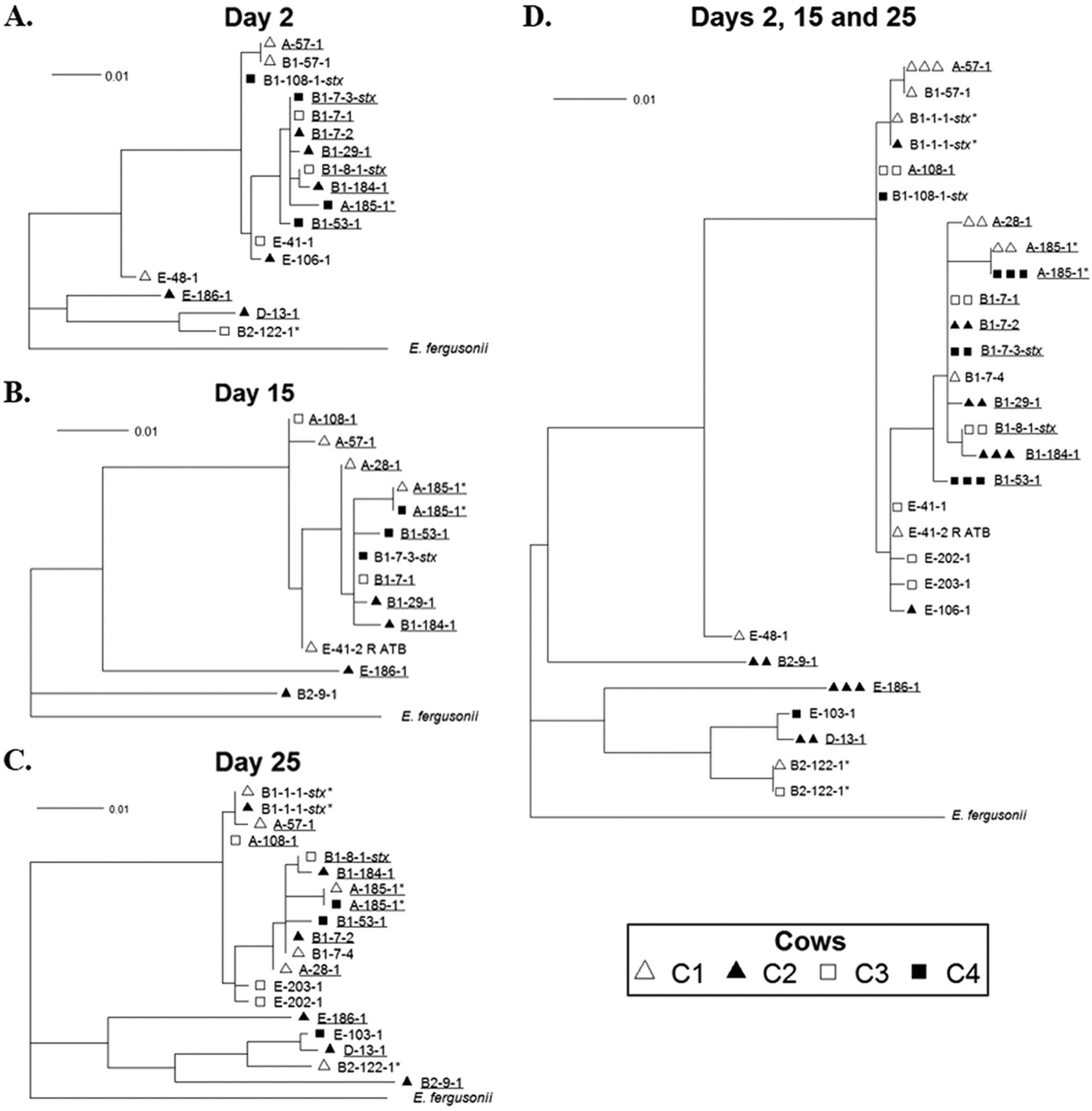

FIG 3 Phylogenetic trees of the *E. coli* clones isolated on days 2 (A), 15 (B), and 25 (C) and days 2, 15, and 25 (D) reconstructed from the sequences of the *trpA* gene by the PhyML program (73) and rooted on *Escherichia fergusonii*. For the clone nomenclature code, see the legend to Fig. 2. Persistent clones are underlined. The three shared clones A-185-1, B1-1-1-*stx*, and B2-122-1 are indicated with an asterisk. Antibiotic-resistant clone E-41-2, isolated on days 10, 13, and 15, is indicated on the phylogenetic trees in panels B and D by R ATB. On the tree in panel D, the number of symbols in front of each clone represents the number of times that it was isolated over the 3 days.

own microbiota (52) and with the host immune system (53), resulting in an *E. coli* population with a unique individual pattern at a given time point. The environment of the host may also have an impact on the observed fluctuations. Finally, STEC clones are submitted to the same ecological pressures as non-STEC clones. Such heterogeneity between individuals and over time should be taken into account in further studies that aim to use *E. coli* as a proxy for modeling the transmission network at a population level.

MATERIALS AND METHODS

Study design and experimental setting. (i) Study site and sample collection. The study was conducted in the Hwange District of Zimbabwe, Africa. The Hwange National Park and its periphery are part of the Kavango-Zambezi Transfrontier Conservation Area (KAZA TFCA) (54). In this area, diverse wild African ungulates run alongside (without any physical separation) communal land, where small-scale farmers raise livestock (55) in a semiarid ecosystem (average rainfall, 600 mm per year). Approval by the Governmental Veterinary Services of Zimbabwe was obtained for sampling animals within the ANR SAVARID project ANR-11-CEPS-003. Sample collection ran from 25 March 2014 to 18 April 2014, at the end of the rainy season. A protocol of daily sampling of four Nguni cattle, named cows C1, C2, C3, and C4, for the detection of *E. coli* was implemented for 25 days. We chose females from the same herd, comprising approximately 20 heads, that were genetically closely related and that had the highest similarities, considering the field conditions. No animal from the herd had received antibiotic treatment in the 3 months preceding the sampling period. All cows had calved 2 months before. Three of the four cows were 4 years old. Cow C1 was 13 years old. Two of the cows were related, with cow C4 being the aunt of cow C2. All four cows roamed daily and returned to the kraal (i.e., an enclosure located close to the homestead) before sunset, where they stayed overnight. Moreover, we tried to limit the variations that may have been introduced by the sampling protocol by implementing it each day at the same hour, immediately after the return of the herd to the kraal. The feces were obtained either directly from the rectum or from the ground immediately after defecation and loaded in 1 ml of a solution of brain heart infusion containing 20% glycerol. Tubes containing fecal samples were labeled with a unique identifying code and transported from the field to a tank containing liquid nitrogen in less than 2 h in the research camp, where they were kept at −180°C until shipment by plane in dry ice to the IAME laboratory, INSERM, in Paris, France, at the end of the sampling period.

(ii) Bacterial counts and isolation of *E. coli* strains. Fecal samples were stored at −80°C upon their arrival at the IAME laboratory. A volume of 200 μl of stool-containing suspensions was cultured on Drigalski agar plates at 37°C for 24 h for the isolation of *Enterobacteriaceae*. Samples from days 1 to 9 and days 16 to 25 underwent one freeze-thaw cycle before plating, whereas samples from days 10 to 15 underwent two freeze-thaw cycles. All *Enterobacteriaceae* with different morphotypes that grew were identified to the species level by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry analysis (MALDI Biotyper; Microflex; Bruker). On the basis of the quantity of feces (1 mg) in each sample, we estimated the lower limit of detection of *E. coli* strains to be 10^2 CFU per gram of feces. The number of CFU per plate was determined, and a colony identified to be *E. coli* was randomly picked for further analyses. Then, all samples collected on days 2, 15, and 25 were restreaked on Drigalski agar plates four times, after an additional freeze-thaw cycle, to enhance the accuracy of bacterial counts. In order to study the clonal distribution, five *E. coli* isolates were randomly picked from each plate so that on each of the three days, 21 isolates were obtained from each cow. All *E. coli* isolates were stored at −80°C in a solution of 80% glycerol for further analyses. We assessed our ability to detect a minor clone, defined as a clone present at a frequency of 10% in the population of *E. coli* isolates (25) randomly selected on days 2, 15, and 25, by calculating the probability that at least one representative would be picked using the binomial formula $1 - (1 - p)^n$, where p is the frequency of the minor clone ($P = 0.10$) and n is the number of isolates picked (56). We estimated that the chance of detecting a minor clone on days 2, 15, and 25 was 88%.

(iii) Antibiotic resistance. Antibiotic resistance was studied both phenotypically and genotypically. First, global antibiotic resistance was analyzed by plating fecal samples on Drigalski agar plates containing 32 mg/liter ticarcillin, which was selective for penicillin-resistant isolates (57), and Drigalski agar plates containing 20 mg/liter tetracycline, which was selective for tetracycline-resistant isolates (58). The lower limit of detection was 10^2 CFU/g of feces. The species forming the colony was identified by MALDI-TOF mass spectrometry analysis and, when it was *E. coli*, was stored at −80°C for further analyses. Second, all the *E. coli* isolates were tested for susceptibility using the disk diffusion method according to the 2013 recommendations of the French Society for Microbiology (http://www.sfm-microbiologie.org/). Susceptibility to the following seven antimicrobial agents was tested: amoxicillin (25 μg), chloramphenicol (30 μg), kanamycin (30 IU), streptomycin (10 IU), trimethoprim (5 μg), sulfonamides (200 μg), and tetracycline (30 IU). Third, genetic determinants of tetracycline, penicillin, and trimethoprim resistance were determined by PCR assays. Detection of efflux pump-encoding genes [*tet*(A) to *tet*(E)] conferring tetracycline resistance was performed on the tetracycline-resistant *E. coli* isolates by using a multiplex PCR (59). Penicillin resistance was assessed by a PCR based-method (for the bla_{TEM} gene) on the penicillin-resistant *E. coli* isolates (60). Multiplex detection of the dihydrofolate reductase-encoding genes *dfrA1*, *dfrA5* and *dfrA14*, *dfrA7* and *dfrA17*, and *dfrA12* was performed on the trimethoprim-resistant *E. coli* isolates. Differentiation between the *dfrA5* and *dfrA14* genes, on the one hand, and the *dfrA7* and *dfrA17* genes, on the other hand, was performed by sequencing (28).

(iv) *E. coli* phylogenetic grouping, clone relatedness, and virulence gene and O typing. We attempted to determine the phylogeny between *E. coli* and *Escherichia* clade clones by combining three genotyping methods. First, all *E. coli* and *Escherichia* clade isolates were assigned to one of the seven main *E. coli* phylogenetic groups (A, B1, B2, C, D, E, and F) or to an *Escherichia* clade using the Clermont quadruplex PCR (61) and the PCR assay reported elsewhere (62). Second, we compared the nucleotide sequences of their *trpA* genes (807 bp). We chose this housekeeping gene because its phylogeny is known to be congruent with the phylogeny of *E. coli* species (63). Nucleotides sequences were obtained by Sanger sequencing and aligned using BioEdit software (64). The quality of sequencing was assessed by visual inspection of the chromatograms. We compared the sequences to those in the multilocus sequence typing (MLST) database of the Pasteur Institute (Paris, France) (65) and assigned the sequence

to the corresponding allele in the database. Third, we performed random amplified polymorphic DNA (RAPD) PCR using primer 1254 (5′-CCGCAGCCAA-3′) (35) and compared the RAPD PCR profiles of clones belonging to the same phylogenetic group and having the same *trpA* sequence. Two clones were considered to have distinct RAPD PCR profiles when the number of RAPD band differences was two or more (66). We constructed a clone nomenclature code to characterize all the detected genotypes as follows: XX-YYY-Z, where XX is the phylogenetic group, YYY is the *trpA* allele of the Pasteur Institute MLST database (67), and Z is the RAPD PCR profile within each combination of phylogenetic group and *trpA* allele. When the patterns differed by at least one genotyping technique, clones were considered to be different. To confirm that we correctly discriminated the clones with our combined genotyping techniques, we sequenced the housekeeping gene *gnd* (1,335 bp) for two representative clones of each pattern (or one clone, when we isolated only a single clone) and compared the nucleotide sequences as described above for the *trpA* gene. The *gnd* gene is known to be the target of frequent recombination events (63, 68), rendering it a good marker to check for genetic divergence between related clones. In all cases, the *gnd* sequences confirmed the delineation of the clones (data not shown).

The *stx* and *eae* genes were detected by PCR (69). The main O types associated with enterohemorrhagic *E. coli* (EHEC; O26, O103, O104, O111, O157) were detected using a PCR-based approach (69, 70). When one of these genes was detected, the name of the gene was added to the clone nomenclature code.

Statistical analyses. (i) Bacterial counts. A linear mixed-effects model was used to assess whether the *E. coli* counts changed over time. The response variable was the decimal log-transformed *E. coli* counts. We used the following explanatory variables for the fixed effects: a polynomial function of time for the temporal characterization of the *E. coli* counts and the number of freeze-thaw cycles that a sample underwent before plating. The last variable was included in the model in order to take into account the repetition of heat shocks caused by freezing and thawing, which could bias the effect of the other explanatory variables. Cow-specific random intercepts (b_i) were included in the model, allowing variations between the log-transformed *E. coli* counts at the cow level. The day of sampling was assessed as a cow-specific random effect. The equation of the full model is presented below:

$$y_{i,j} = \sum_{k=0}^{K} \alpha_k \times t_{i,j}^{k} + \beta_c \times c_{i,j} + b_i + \kappa_{i,j} + \varepsilon_{i,j}$$

where $y_{i,j}$ is the decimal log-transformed *E. coli* count for cow *i* on the *j*th day; α_k is the coefficient of the polynomial function of degree *k*; $t_{i,j}$ is the *j*th day for cow *i*; β_c is the coefficient associated with the fixed effect of the number of freeze-thaw cycles, with one freeze-thaw cycle being the reference; $c_{i,j}$ is the qualitative variable representing the number of freeze-thaw cycles of the sample from cow *i* on the *j*th day; b_i is the coefficient associated with the random effect of cow *i*; $\kappa_{i,j}$ is the coefficient associated with the random effect of time on the *j*th day for cow *i*; and $\varepsilon_{i,j}$ is the residual error.

We assumed that the cow-level random effect, the time-level random effect, and the residual errors were independent and had a normal distribution with a mean of 0 and variances of σ_b^2, σ_κ^2, and σ_ε^2, respectively. Explanatory variables were selected using a backward approach and the Akaike information criterion (AIC), by starting with all candidate variables and testing the deletion of each variable one by one. We started with a quartic function of time to detect nonlinear trends ($K = 4$). The suppression of covariates was stopped when no further decrease of the AIC was obtained. Then, we confirmed the significance of our model terms using the Wald test for single fixed effects.

The between-individual variability was defined as the difference in the *E. coli* counts between cows. The percentage of the total variability attributable to the between-individual variability was expressed as $\sigma_b^2/\sigma_{\text{tot}}^2$, where σ_{tot}^2 is the total estimated variation, which was expressed as $\sigma_{\text{tot}}^2 = \sigma_b^2 + \sigma_\kappa^2 + \sigma_\varepsilon^2$. The within-individual variability is defined as the change in the *E. coli* counts within each cow between measurements. The percentage of the total variability attributable to the within-individual variability was expressed as $\sigma_\kappa^2/\sigma_{\text{tot}}^2$.

(ii) Dominant *E. coli* clones and diversity indices. For a given sample collected on days 2, 15, and 25, we defined the dominant clone to be the one that was the most abundant with a proportion of greater than 50%, as adapted from a previous study (25). In the absence of a dominant clone, codominant clones were defined as the most abundant clones for which the sum of the proportions was greater than 50%. Subdominant clones were other clones detected in the sample. Within each cow, we considered the dominant clone on day 2 and compared its prevalence between days 2, 15, and 25 using Fisher's exact test. The four *P* values were adjusted using the Benjamini and Hochberg method (27), as we performed multiple comparisons. Significance was determined at a *P* value of <0.05. To assess the persistence of the clones within their host, we defined persistent clones, based on the samples from days 2, 15, and 25, as clones detected in two or three samples. A transient clone was defined as a clone that was detected on 1 day (either the 2nd, the 15th, or the 25th day). Mean prevalences within each cow over days 2, 15, and 25 were calculated for the clones detected on these days.

For each cow, we also estimated diversity indices to assess the structure of the *E. coli* population in samples from days 2, 15, and 25. Two different but related indices were calculated: (i) Shannon's index (H'), which was calculated as

$$H' = -\sum_{i=1}^{s} p_i \times \ln(p_i)$$

where p_i is the proportion of isolates found for the *i*th clone of a sample of *s* different clones (29, 71), and (ii) Piélou's evenness (J'), which was calculated as $J' = H'/\ln(s)$, where $\ln(s)$ is the maximum value of Shannon's index for the given community (72). It is interesting to use both of these indices, as these two

indices incorporate differently the richness and evenness of a community in one number. Shannon's index weighs heavily the clone richness of a sample by quantifying the uncertainty in predicting the clone identity of an individual that is taken at random from the sample, whereas Piélou's evenness measures the distribution of clones in a sample. Diversity indices were calculated separately for each cow on days 2, 15, and 25, and means for each cow were calculated by averaging the index for the 3 days. Shannon's indices were compared between cows by a one-way analysis of variance. One-way analysis of variance with repeated measures was used to compare Shannon's indices between days within cows. The same was done for Piélou's evenness. The four *P* values were adjusted using the Benjamini and Hochberg method (27).

All statistical analyses were performed using R software (R, version 3.0.2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00659-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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