Persistence of Free-Living Protozoan Communities across Rearing Cycles in Commercial Poultry Houses[▽]

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The introduction and survival of zoonotic bacterial pathogens in poultry farming have been linked to bacterial association with free-living protozoa. To date, however, no information is available on the persistence of protozoan communities in these environments across consecutive rearing cycles and how it is affected by farm- and habitat-specific characteristics and management strategies. We therefore investigated the spatial and temporal dynamics of free-living protozoa in three habitats (pipeline, water, and miscellaneous samples) in three commercial poultry houses across three rearing cycles by using the molecular fingerprinting technique denaturing gradient gel electrophoresis (DGGE). Our study provides strong evidence for the long-term (ca. 6-month) persistence of protozoa in broiler houses across consecutive rearing cycles. Various free-living protozoa (flagellates, ciliates, and amoebae), including known vectors of bacterial pathogens, were observed during the down periods in between rearing cycles. In addition, multivariate analysis and variation partitioning showed that the protozoan community structure in the broiler houses showed almost no change across rearing cycles and remained highly habitat and farm specific. Unlike in natural environments, protozoan communities inside broiler houses are therefore not seasonal. Our results imply that currently used biosecurity measures (cleaning and disinfection) applied during the down periods are not effective against many protozoans and therefore cannot prevent potential cross-contamination of bacterial pathogens via free-living protozoa between rearing cycles.

Free-living bacterivorous protozoa are increasingly implicated in the survival and transmission of bacterial pathogens (31). Food-borne pathogens like *Campylobacter* and *Salmonella*, important agents of (gastro)enteritis often related to the consumption of contaminated chicken meat, may survive, multiply, and be transported in the environment through association with various protozoan organisms (5, 6, 8, 10, 15, 20, 23, 30, 33, 40, 41). Some bacteria resist digestion by protozoans through adaptation of the intraprotozoan environment by alternation of the maturation pathway of food vacuoles (11, 22, 25), while others may survive or grow saprophytically in the extraprotozoan environment upon materials released from protozoan cells (2). To date, knowledge of poultry colonization by zoonotic pathogens and their survival in poultry environments is still limited (26, 44), and the presence and persistence of potential vectors such as free-living protozoa in poultry environments have been largely unexplored. In a recent study, we described free-living protozoan diversity and spatial distribution in commercial poultry houses during a single rearing cycle (7). In the present study, the dynamics of protozoan communities across and within three consecutive rearing cycles (down and rearing periods), in relation to habitat- and farm-specific management strategies, were examined. Information on the persistence of protozoan communities across rearing cycles is crucial to evaluate their po-

tential as a transmission route for human bacterial pathogens, like *Campylobacter* and *Salmonella*, between consecutive poultry flocks.

MATERIALS AND METHODS

Sampling strategy and collection. Three commercial poultry farms in Belgium (farms A, B, and C) were sampled on nine occasions between August 2006 and January 2007 (Fig. 1), encompassing the following three consecutive rearing cycles: I (sampling occasions 1 to 3), II (sampling occasions 4 to 6), and III (sampling occasions 7 to 9). Per sampling occasion, two broiler houses per farm were sampled. During each rearing cycle, samples were taken 1 to 4 days before the chickens arrived but after decontamination and disinfection (i.e., the down period; sampling occasions 1-4-7), 2 to 7 days after introduction of the chickens (sampling occasions 2-5-8), and between days 38 and 41 of the rearing period before slaughter of the chickens (sampling occasions 3-6-9). These will be referred to as the three different phases of each rearing cycle (Fig. 1). Air temperature inside the broiler houses varied between and within rearing cycles, being equal to the outside temperature during the down periods (from 21°C in the summer to 3°C in the winter) and maintained between 28.6 and 34.4°C and between 20.6 and 25.5°C when broilers were present during the second and third phases, respectively (Fig. 1).

All samples were taken with sterile equipment and collected in sterile flasks. Three different habitats were sampled inside each broiler house, as follows: pipeline, water, and miscellaneous habitats. The "pipeline" habitat consisted of 2- to 4-liter water samples from pipelines in the anterooms of the broiler houses before the water entered the rearing area of the chickens. The water at farms A and B came directly from a local well (60-m depth), while farm C used (chlorinated) main supply water. The "water" habitat represented 50-ml water samples (delivered via the pipelines) taken from drinking nipples (farm A) or cups (farms B and C) inside the rearing areas of the broiler houses. As the drinker systems were not yet connected to the water supply system during the down periods, this habitat could not be sampled during this phase. The "miscellaneous" habitat consisted of the following samples obtained from the rearing areas of the broiler houses: dust, condensation water, films on the exterior surface of the water lines, and damp areas on walls and in litter. Farms A and B used commercial wood shavings, whereas farm C applied untreated straw as

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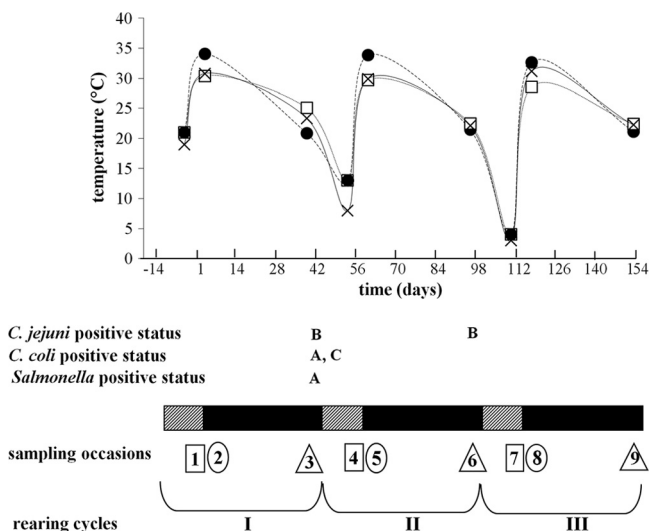
FIG. 1. Temperature and bacterial status of the broiler farms. Striped and black boxes represent the down periods (approximately 2 weeks) and rearing periods (6 weeks), respectively. Squares, circles, and triangles represent phases 1 (down period), 2 (beginning of rearing period), and 3 (end of rearing period), respectively; the numbers inside the symbols represent the sampling occasions (1 to 9). Letters represent the farms positive for *C. jejuni*, *C. coli*, or *Salmonella* species at the end of each rearing period. Temperature is expressed as the mean from two broiler houses per farm (●, farm A; □, farm B; ×, farm C).

bedding material. Farm A stored the shavings in a shed, while farm B applied new packages when required. Upon entry in the laboratory (within 1 h after sampling), miscellaneous samples were diluted in 10 ml sterile demineralized water. All samples (pipeline, water, and miscellaneous) were then collected on 0.22-μm white GSWP filters (Millipore) using sterile equipment and stored at −20°C. Prior to molecular analyses, filters were pooled per habitat and per broiler house for each farm and sampling occasion, which resulted in a total of 140 pooled samples. Cecal droppings collected during the last phase of each rearing cycle were investigated for the presence of *Campylobacter* and *Salmonella*, according to the method of Rasschaert et al. (28). More detailed information on farm characteristics and management strategies can be found in the work of Baré et al. (7).

Molecular community profiling using denaturing gradient gel electrophoresis (DGGE). DNA of the pooled samples was extracted using the bead-beating method, with phenol extraction and ethanol precipitation (36). Extracted DNA was purified on a Wizard column (Promega Corporation, Madison, WI), according to the manufacturer's recommendations. Given the polyphyletic nature of protozoa, the design of a universal set of protozoan-specific primers is impossible (35). Therefore, a universal set of eukaryote-specific 18S rRNA gene primers was used in order to capture as much eukaryotic (including protozoan) diversity as possible.

PCR amplification was carried out according to the study by van Hannen et al. (36), with the exception of performing 30 amplification cycles instead of 25. PCR amplification procedures were performed with a Bioman thermocycler (Westburg, Leusden, Netherlands). Each PCR mixture contained 1 to 4 μl of template DNA, 2 μl primers (12.5 pmol), 5 μl deoxynucleoside triphosphate (dNTP) (200 μM each; GeneAmp, Applied Biosystems Inc., CA), 2 μl bovine serum albumin (BSA) (400 ng; Roche Diagnostics GmbH, Germany), 5 μl of buffer I (Applied Biosystems), and 2.5 μl *Taq* DNA polymerase (2.5 U AmpliTaq DNA polymerase; Applied Biosystems) and was adjusted to a final volume of 50 μl with sterile water (Merck & Co., NJ). The presence of PCR products was determined by analyzing 5 μl of product on 1.66% agarose gels, staining it with ethidium bromide (Merck & Co.), and comparison with a molecular weight marker (SmartLadder; Eurogentec, Seraing, Belgium).

DGGE was performed with the D-Code system from Bio-Rad Laboratories (Hercules, CA), mainly as described by van Hannen et al. (36). Equal amounts of DNA (650 ng μl⁻¹) were applied to a 28 to 57% gradient polyacrylamide gel (acrylamide/bisacrylamide ratio, 37.5:1; 100% denaturant corresponded to 7 M urea and 40% deionized formamide). Electrophoresis was performed for 990

min at 70 V; the temperature was set at 60°C. DGGE gels were stained with SYBR gold dye (Invitrogen, Paisley, United Kingdom), photographed, and processed by Quantity One 1-D analysis software (Bio-Rad Laboratories). Eukaryotic DNA obtained from previous studies performed in the laboratory was utilized to generate DGGE standards, for which three lanes were included per gel. The positions of the standards were applied to align the digitized DGGE images using BioNumerics 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Sequence information on the bands (see below) was used to check the grouping of bands into band classes or phylotypes. The number of different phylotypes within a (group of) sample(s) was defined as phylotype diversity. All data were combined in a matrix based on relative band intensities (i.e., the relative contribution of each band to the total band signal in the lane), which was then used for the data analyses.

Sequence information was obtained by sequencing DNA amplicons from purified excised DGGE bands. Sequencing was performed with the ABI Prism kit (PE Biosystems) using the 1427F primer (no GC clamp) (36) and an Applied Biosystems ABI 3130XL genetic analyzer. Bands were identified by screening the partial 18S rRNA gene sequence against GenBank sequences using BLAST (July 2009) (3). Protozoan phylotypes were classified by functional group (i.e., ciliate, flagellate, or amoeba) (13) and taxonomic group according to the recent eukaryotic classification of Adl et al. (1).

Data analysis. Principal components analysis (PCA; implemented using the program CANOCO 4.5 for Windows) (32), based on the covariance-variance matrix and with scaling focused on phylotype correlations, was used on log(x + 1)-transformed relative band intensities to assess spatial and temporal variations in phylotype composition of the samples. Four samples did not yield any protozoan DGGE bands and were removed from the analyses. The final data set thus consisted of 136 samples and 17 identified protozoan phylotypes. Variation in the relative abundances of the phylotypes was partitioned into spatially (farm and habitat) and temporally (rearing cycle and sampling phase within rearing cycles) structured components by performing partial regression analyses using redundancy analyses (RDA) in the CANOCO 4.5 for Windows program (9, 18, 39). This variation partitioning (VP) approach allowed separation of the pure effects of each component and their joint effects (39). The environmental matrix used in these analyses consisted of dummy variables for all four components (21). The forward selection procedure was used to select only those variables (per component) that contributed significantly to explaining the variation in the phylotype data using Monte Carlo permutation tests (4,999 permutations) (39). Both PCA and the VP analysis were first performed on the complete data set. As habitat appeared to be the dominant component structuring the protozoan communities (see Results), separate analyses of the data of each habitat were subsequently performed.

RESULTS

Bacterial status of the broiler flocks. All farms were *Campylobacter* positive for at least one rearing period. *Campylobacter jejuni* was isolated from cecal droppings in farm B (broiler houses X1 and X2) on sampling occasions 3 and 6; *Campylobacter coli* was isolated from farm A (broiler houses X1 and X2) and farm C (broiler house X1) during the first rearing period. *Salmonella*-positive birds were found only in farm A (broiler house X1) on sampling occasion 3 (Fig. 1).

Free-living protozoan phylotype diversity. A total of 62 eukaryotic phylotypes were distinguished. Most excised bands showed high similarities (>97%) to known sequences. In total, 38 unique sequences were recovered, belonging to the Amoebozoa (2 sequences), Chromalveolata (13), Excavata (2), Opisthokonta—fungi (9), Opisthokonta—Metazoa (1), Opisthokonta—others (1), Archaeplastida-Plantae (5), and Rhizaria (1) and unknown affiliations (4). Seventeen sequences were affiliated with free-living protozoa (6 ciliates, 8 flagellates, and 3 amoebae), with most identified free-living protozoa (64.7%) belonging to the Chromalveolata (Table 1). The most common protozoan phylotypes (present in more than half of the samples) had affinities with the *Spumella*-like flagellate JBM/S11, *Colpoda* spp., a *Glissomonad* sp., and

TABLE 1. BLAST matches of free-living protozoan sequences obtained from DGGE bands

Phylotype no.	Abbreviation	Closest relative(s)	GenBank accession no.	% similarity (no. of matching bp/total no. of bp)	Group	
					Taxonomic	Functional
16	UROGLEN	*Uroglena* sp. CCMP2768	EF165132	95.9 (140/146)	Chromalveolata	Flagellate
19	LOXOPH1	*Loxophyllum* sp. GD-070419	EU242511	97.1 (136/140)	Chromalveolata	Ciliate
20	LOXOPH2	*Loxophyllum* spp.[a]		97.6 (122/125)	Chromalveolata	Ciliate
21	VAN	*Vannella* sp. CAZ6/1	AY929914	90.8 (157/173)	Amoebozoa	Amoeba
22	PLATY	*Platyamoeba placida*	AY294150	92.8 (141/152)	Amoebozoa	Amoeba
23	ENCHELYS	*Enchelys polynucleata*	DQ411861	99.3 (141/142)	Chromalveolata	Ciliate
25	COLP	*Colpoda* spp.[a]		98.1 (158/161)	Chromalveolata	Ciliate
26	PARAPHYS	*Paraphysomonas vestita*	Z28335	86.8 (132/152)	Chromalveolata	Flagellate
27	GLISSOM	*Glissomonad* sp. Panama 103	EU709272	98.1 (152/155)	Rhizaria	Flagellate
29	SPUMEL	*Spumella*-like flagellate JBM/S11	EF043285	100 (146/146)	Chromalveolata	Flagellate
30	VORTICEL	*Vorticella* spp.[a]		98.6 (145/147)	Chromalveolata	Ciliate
32	SPUM/PAR	*Spumella*-like flagellate JBAS37	AY651094	99.3 (149/150)	Chromalveolata	Flagellate
		Paraphysomonas vestita	AF109325	99.3 (149/150)		
34	CYCL GL	*Cyclidium glaucoma*	EU032356	98.7 (155/157)	Chromalveolata	Ciliate
35	NAEGLE	*Naegleria* spp.[a]		99.4 (159/160)	Excavata	Amoeba
37	SPHAERO	*Sphaeroeca* WS 3-Uni05	AJ867611	87.2 (136/156)	Opisthokonta	Flagellate
48	STRAMEN	Stramenopile E2-85	EU528042	97.3 (142/146)	Chromalveolata	Flagellate
58	RHYN NAS	*Rhynchomonas nasuta* HFCC348	DQ46552	100 (166/166)	Excavata	Flagellate

[a] Several species within one genus all gave the same similarity results.

Naegleria spp. Almost one-fourth of the protozoan phylotypes (23.5%) represented protozoan genera known to host pathogens, like *Vannella*, *Platyamoeba*, *Cyclidium*, and *Naegleria* (23, 29, 34).

Samples possessed an average protozoan phylotype diversity of 5.4 ± 2.6 (minimum, 0; maximum, 12). The average phylotype diversity (across all farms) was lower in miscellaneous samples than in those from the other habitats, as follows: 4.7 ± 2.4 versus 5.8 ± 3.0 (pipeline samples) and 5.7 ± 2.2 (water samples). There also were differences between habitats and farms: the average phylotype diversity was highest in the pipeline samples from farm A (6.3 ± 1.0) and in the water (10.0 ± 3.9) and miscellaneous (6.1 ± 1.8) samples from farm C. The average phylotype diversity was lowest in the pipeline samples from farm C (3.7 ± 1.9) and in the water (5.2 ± 1.7) and miscellaneous (4.1 ± 2.8) samples from farm B.

Spatial distribution and temporal patterns in free-living protozoan communities. On the basis of preliminary ordinations, five outlier samples were identified and removed from the complete data set prior to PCA and variation partitioning (VP) of the data set. VP of the complete data set (131 samples and 17 protozoan phylotypes) showed that only 11% of the variation in the protozoan data can be significantly explained by the introduced dummy variables for habitat (ca. 6%), farm (3.5%), and sampling phase (1.6%) (see Fig. 3). No overlap in the variation explained by any of the four environmental components was found. Variation in the species data could not be significantly related to dummy variables for rearing cycles, indicating that no significant change in the protozoan community composition occurred from one rearing cycle to another.

Because of the important effect of habitat on the variation in free-living protozoan communities and in order to assess whether significant changes in composition occurred between rearing cycles within the different habitat types, separate PCAs (Fig. 2) and VP analyses (Fig. 3) of the three habitat types were performed. The VP analyses showed that 18.6%, 6.7%, and 14.8% of the variation in the protozoan data obtained from the

pipeline, water, and miscellaneous habitats, respectively, can be explained by the introduced variables. The farm from which samples were collected contributed importantly in all three habitat types; significant differences in community composition between the farms persisted throughout the whole sampling period. The sampling phase contributed to almost half of the explained variation in the miscellaneous habitat, suggesting that significant change in community composition occurred within the rearing cycles in this habitat but not in the other habitats (note, however, that the down period in the water habitat could never be sampled). In the pipeline habitat only, a slight (3.3%) but significant change between the rearing cycles was observed. The PCAs confirmed that pronounced differences existed between the farms, as shown for the pipeline and miscellaneous habitats in Fig. 2. The protozoan communities in the pipelines obtained from farms A (spread out along the positive side of the first axis in Fig. 2A) were different from those in the pipelines obtained from farms B and C (which both cluster more tightly on the negative side of the first axis in Fig. 2A). The communities obtained from farm A were characterized mainly by the presence of chromalveolate flagellates (*Spumella*-like and *Paraphysomonas* flagellates) and the ciliate *Loxophyllum* (LOXOPH2), and those obtained from farms B and C were characterized mainly by an unidentified chromalveolate flagellate (STRAMEN), the ciliates *Vorticella* (VORTICEL) and *Colpoda* (COLP), and the amoeba *Vannella* (VAN) (Fig. 2A). Variation in protozoan species composition in the miscellaneous habitat (Fig. 2B) was most pronounced in farm C and, to a lesser degree, in farm A, while the samples obtained from farm B were more similar to one another and clustered closely together on the negative side of the first axis. Samples on this side of the first axis were negatively characterized mainly by the absence of chromalveolate and rhizarian flagellates (except a *Uroglena* sp.) and the ciliates *Loxophyllum* (LOXOPH1 and -2) and *Colpoda* (COLP). All miscellaneous samples taken during sampling phase 3 in all three farms (Fig. 2B) clustered strongly together and are also
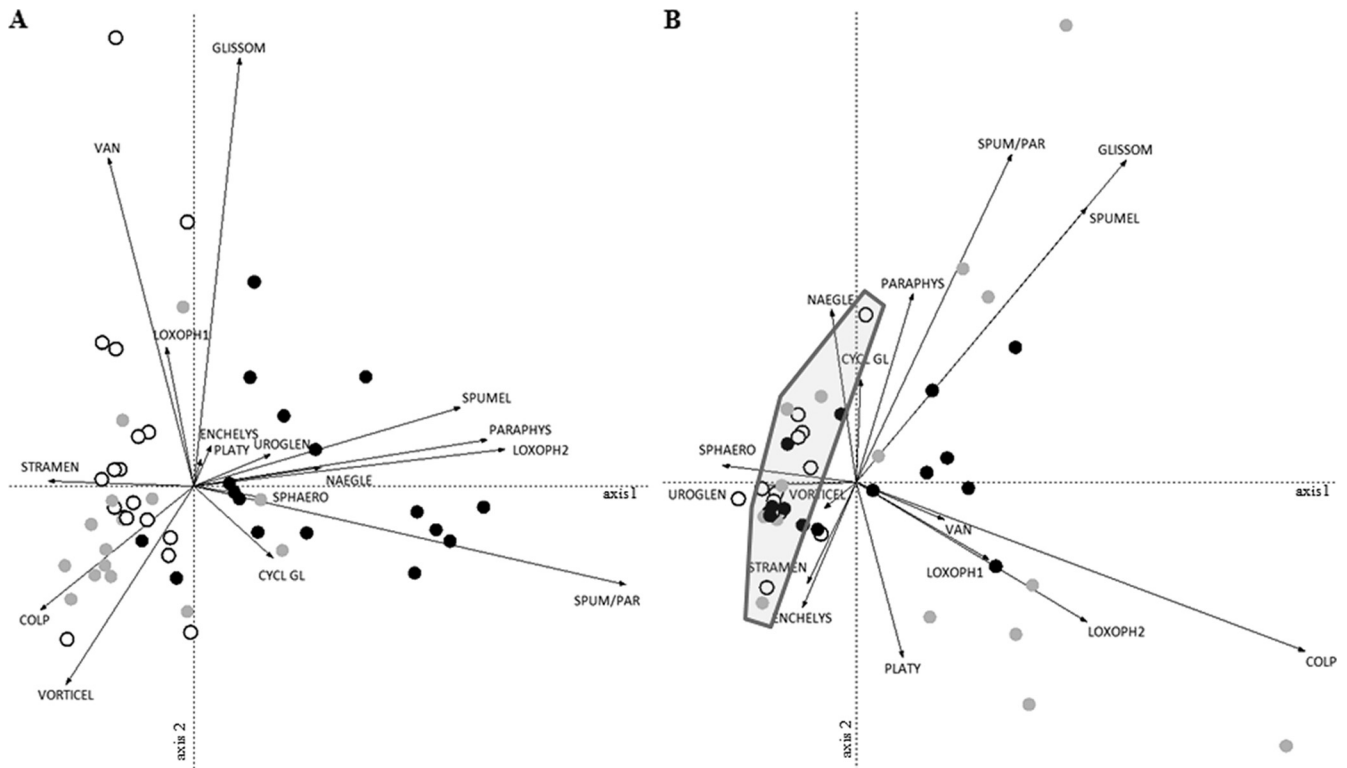
FIG. 2. PCA correlation biplots (axes 1 and 2) showing samples (symbols) and free-living protozoans (arrows) (labels are shown in Table 1) from analyses of the pipeline (A) and miscellaneous (B) samples. Farms A, B, and C are represented by black, white, and gray circles, respectively. (B) The gray polygon encircles the samples taken during sampling phase 3 from the three farms.

characterized by the absence of many protozoans, indicating that during each rearing cycle, a significant change in community composition took place in this habitat between the down period and the other sampling phases. *Spumella*-like organisms and a *Glissomonad* sp. were found mainly in miscellaneous samples during the down periods (sampling phase 1) and sampling phase 2 (Fig. 2B).

Persistence of free-living protozoa. Protozoan phylotypes were defined as persistent if they persisted in the broiler house environment from one rearing cycle to another (Fig. 4). A small number of protozoans was detected on all consecutive sampling occasions in some farms and/or habitats. *Loxophyl-*
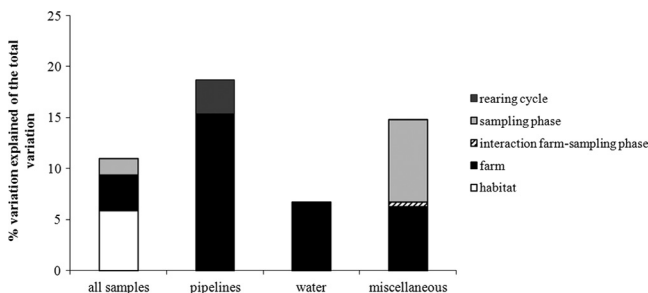

FIG. 3. Contribution of spatially (habitat, farm) and temporally (rearing cycle, sampling phase) structured variation to the total variation in free-living protozoan community composition for the whole data set (all samples) and for each habitat separately. Only significant variables were included in the analysis (see the text for more details).

lum spp., *Paraphysomonas vestita*, and *Spumella*-like flagellate JBAS37/*Paraphysomonas vestita* were found in all pipeline samples from farm A, while *Colpoda* was present in all pipeline samples from farm B. *Spumella*-like flagellate JBM/S11, *Naegleria* spp., and *Colpoda* spp. could be detected in all water samples from farms A, B, and C, respectively (Fig. 4). No phylotypes persisted during the whole sampling campaign in the miscellaneous habitat. Other protozoans persisted between at least two consecutive rearing cycles, i.e., they were detected in sampling phase 3 of one rearing cycle, during the down period (sampling phase 1), and in sampling phase 2 of the next cycle (note, however, that because the down period for the water habitat could never be sampled, protozoan persistence in this habitat pertains to a presence in sampling phase 3 of one cycle and sampling phase 2 of the next) (Fig. 4). Examples of these include a *Glissomonad* sp., *Paraphysomonas vestita*, and *Naegleria* spp. (all habitats); *Spumella*-like flagellate JBAS37/ *Paraphysomonas vestita* and *Colpoda* spp. (pipeline and water); *Loxophyllum* spp. (pipeline); and *Cyclidium glaucoma* (water) (Fig. 4).

DISCUSSION

Transmission of pathogenic bacteria via protozoan vectors from one broiler flock to the next requires persistence of these vectors in between rearing cycles. This could be inside the rearing area of the broiler house or in the protozoan pool in the immediate surroundings (such as in the water supply, litter,
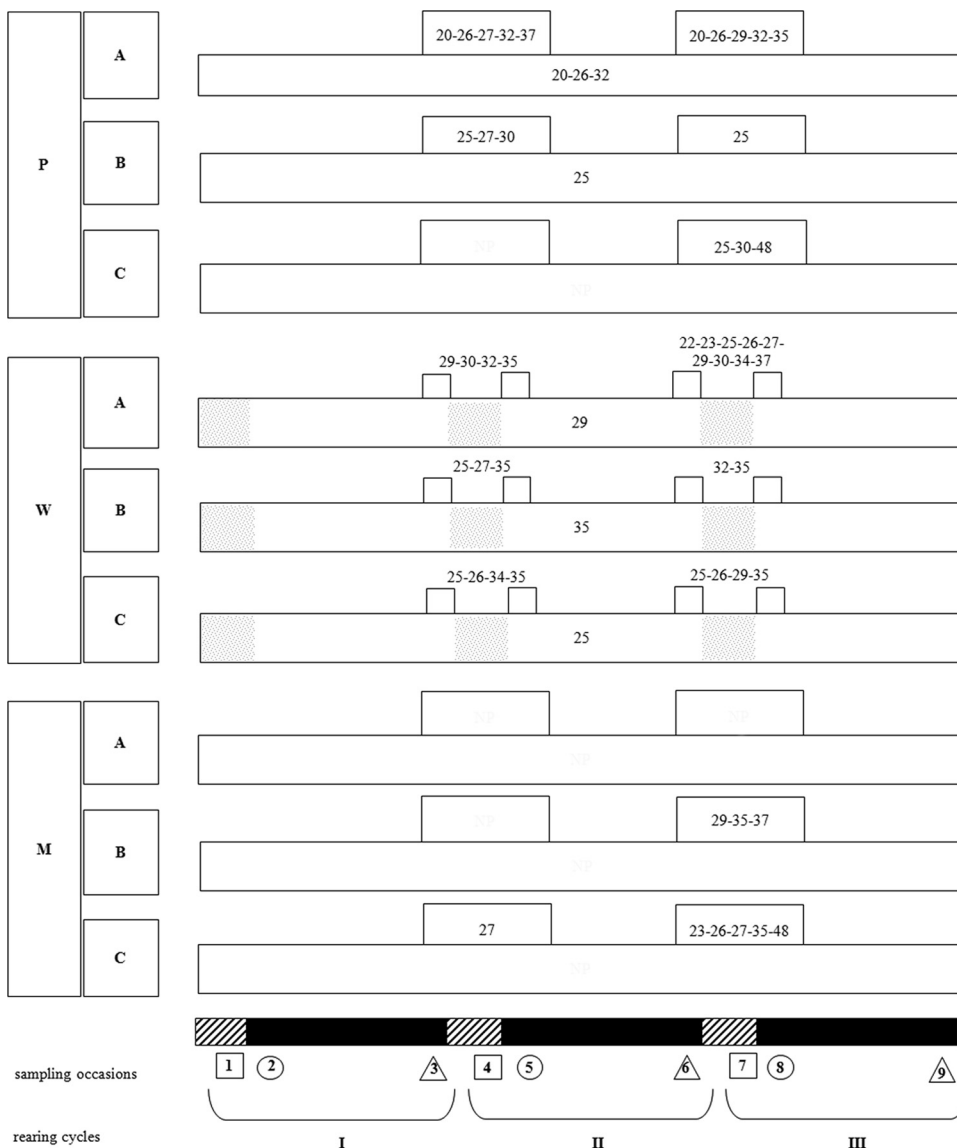
FIG. 4. Persistence of protozoan phylotypes during the whole sampling campaign and across down periods. Pipeline, water, and miscellaneous habitats are represented by P, W, and M, respectively; A, B, and C refer to the three farms. Gray boxes indicate that the water habitat could not be sampled during the down periods (see Materials and Methods). Free-living protozoa are represented by their phylotype numbers (Table 1). Long, horizontal boxes represent phylotypes detected on all consecutive sampling occasions, while short, horizontal boxes contain phylotypes which persisted between two consecutive rearing cycles.

or feed). Until today, no information about the occurrence, composition, and persistence of protozoan communities in various habitats (pipeline, water, and miscellaneous samples) across consecutive rearing cycles was available. Data obtained during the present study provide strong evidence for the long-term (ca. 6-month) persistence of free-living protozoa in broiler house environments across consecutive rearing cycles. Protozoan communities show almost no change across rearing cycles and remain highly habitat and farm specific.

Chromalveolata are the most frequently encountered protozoan group, which confirms published data (7). Most organisms belong to the Chrysophyceae (especially flagellates related to *Spumella*) or are ciliates (e.g., *Colpoda* and *Cyclidium*). Many small flagellates (such as *Spumella* and the cercozoan

Glissomonadida) are common bacterivores in soil and freshwater environments (12, 19, 42, 43). Through the formation of a gelatinous matrix that enhances bacterial growth (42), certain *Spumella* species are especially apt at colonizing environments where food is initially scarce. It is also worth noting that the amoeba *Naegleria* is one of the dominant organisms in all three habitats, as this genus comprises several human (*Naegleria fowleri*) and animal (e.g., *Naegleria australiensis* and *Naegleria italica*) pathogens which can cause central nervous system infections (37). Further, *Naegleria* is known to harbor bacterial pathogens (34). The diversity estimates in the present study are almost certainly an underestimate of true protozoan diversity in these environments, due to the inability to sequence all phylotypes and to potential DNA extraction and/or PCR biases (16, 38).

The pipeline, water, and miscellaneous habitats harbor more or less distinct protozoan communities. There is considerable overlap in community composition between the water samples and samples from the other habitat types, because water communities are affected by both the water supply from the anteroom and the aerial habitats inside the broiler houses. The present study shows that this overriding effect of habitat on the protozoan community composition is maintained across consecutive rearing cycles. In addition, the different farms are also characterized by specific communities, and those differences persist across rearing cycles as well. Variation between farms is most pronounced in the pipeline communities. This is probably due to the fact that different water sources are used, from the chlorinated main supply water in farm C to local groundwater in farms A and B. In the miscellaneous habitat, the high variability in the samples obtained from farm C may be related to the fact that untreated straw from the field was used as bedding material rather than commercial wood shavings, as used in farms A and B.

Within a specific habitat and farm, there are no pronounced changes in protozoan community structure with time. Significant temporal change observed in the miscellaneous habitat is uniquely related to change within and not between rearing cycles. Significant (albeit small) temporal variation between rearing cycles was observed only in the pipeline habitat, which may be related to external, possibly seasonal changes in the water supply communities. The within-rearing-cycle change in the miscellaneous habitat is due mainly to an overall decrease in diversity toward the end of each cycle (sampling phase 3). This appears to be counterintuitive, as one would expect higher diversity after early colonization stages. This decrease, however, may be due mainly to an increase in the diversity of nonprotozoan eukaryotes (especially Fungi) (data not shown). A high abundance of these organisms may interfere with the amplification of (rarer) protozoan DNA, resulting in lower apparent protozoan diversity.

The present study, for the first time, provides a strong indication that commercial poultry farms are inhabited by farm-specific (endemic) protozoan communities which persist in the broiler house environment half year round. This indication is based on the facts that (i) protozoa were recovered from samples taken during the down periods between rearing cycles and that (ii) unlike natural communities (4, 24), virtually no change in the protozoan community composition between consecutive rearing cycles is observed. If the protozoa in the broiler houses would only be newly recruited from the outside environment, a seasonal signal (and hence more variation between rearing cycles) in the data would be expected. Most of the protozoan phylotypes detected during the down periods (e.g., *Spumella*-like flagellate, *Paraphysomonas vestita*, a *Glissomonad* sp., *Vorticella* spp., *Naegleria* spp., *Platyamoeba placida*, and *Colpoda* spp.) belong to groups which are capable of forming cysts under unfavorable conditions (14, 19, 27, 42) and as such might survive the cleaning and disinfection procedures applied during the down periods.

Several persistent protozoa, like *Platyamoeba placida*, *Cyclidium* species, and *Naegleria* species, are known vectors or reservoirs for bacterial pathogens (23, 34), but for most protozoa detected during this study, this information is still lacking. *In vitro* experiments (under simulated broiler house con-

ditions) (8) recently revealed that *Campylobacter jejuni* was able to survive for up to 14 days (the advisable duration of a down period) (17) in association with *Acanthamoeba castellanii*, a genus previously observed in the broiler houses (7) but not without this organism. The presence of free-living protozoa can as such in theory enable the survival of pathogenic bacteria in between rearing periods. The logical next step will be to demonstrate the presence of *Campylobacter* or other pathogens inside persistent protozoans (and/or their cysts) in broiler house samples taken during the down periods. We tested several methods to detect the presence of *Campylobacter* in protozoa in samples, but these were as yet not successful. First, fixatives used for fluorescent *in situ* hybridization (FISH) analyses distorted the protozoa (including their internal organelles) (J. Baré, unpublished data). Second, the antibiotic gentamicin, which is utilized in studies of protozoa-bacteria associations (to make sure only internalized bacteria are detected) (20, 33), was not effective against the *Campylobacter* strains used but did affect the protozoon (*Acanthamoeba castellanii*) (8). In order to unequivocally establish internalization of *Campylobacter* in protozoa obtained from natural samples, an effective protocol for extracting the protozoa from the samples and killing free-living or externally associated bacteria should be designed.

The results obtained indicate that the biosecurity measures and cleaning and disinfection procedures used are not effective against (potential pathogenic) free-living protozoa, as organisms persisted during the down periods. If protozoa are involved in the transfer of pathogenic bacteria from one broiler flock to the next, the currently used approaches cannot prevent bacterial cross-contamination between rearing cycles via protozoa. Therefore, cleaning and disinfection procedures should be thoroughly evaluated for their effectiveness against protozoan trophozoites, cysts, and associated bacterial pathogens. The fact that even the main supply water used in farm C contained protozoa suggests that standard water chlorination procedures are not effective against all free-living protozoa present. Improvement of biosecurity measures, like for instance the avoidance of the use of natural straw as bedding material or the use of a hygiene barrier at the entrance of the broiler houses, should further reduce the entrance of organisms.

ACKNOWLEDGMENTS

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