

## Microscopic and Molecular Studies of the Diversity of Free-Living Protozoa in Meat-Cutting Plants<sup>∇</sup>

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The diversity of free-living protozoa in five meat-cutting plants was determined. Light microscopy after enrichment culturing was combined with sequencing of PCR-amplified, denaturing gradient gel electrophoresis (DGGE)-separated 18S rRNA gene fragments, which was used as a fast screening method. The general results of the survey showed that a protozoan community of amoebae, ciliates, and flagellates was present in all of the plants. Protozoa were detected mainly in floor drains, in standing water on the floor, on soiled bars of cutting tables, on plastic pallets, and in out-of-use hot water knife sanitizers, but they were also detected on surfaces which come into direct contact with meat, such as conveyor belts, working surfaces of cutting tables, and needles of a meat tenderizer. After 7 days of incubation at refrigerator temperature, protozoa were detected in about one-half of the enrichment cultures. Based on microscopic observations, 61 morphospecies were found, and *Bodo saltans*, *Bodo* spp., *Epistylis* spp., *Glaucoma scintillans*, *Petalomonas* spp., *Prodiscophrya collini*, and *Vannella* sp. were the most frequently encountered identified organisms. Sequencing of DGGE bands resulted in identification of a total of 49 phylotypes, including representatives of the Amoebozoa, Chromalveolata, Excavata, Opisthokonta, and Rhizaria. Sequences of small heterotrophic flagellates were affiliated mainly with the Alveolata (Apicomplexa), Stramenopiles (Chrysophyceae), and Rhizaria (Cercozoa). This survey showed that there is high protozoan species richness in meat-cutting plants and that the species included species related to known hosts of food-borne pathogens.

Protozoa are unicellular eukaryotic microorganisms which are ubiquitous in nature and anthropogenic environments. Protozoa feed on bacteria, microalgae, and particulate or dissolved matter. In turn, they serve as food for other protozoa and metazoa. Besides the prey-predator relationship, there is a particular association of bacteria with protozoa, namely, survival and/or replication of a bacterium within a protozoan. First, some bacteria commonly described as obligate endosymbionts live intracellularly in the cytoplasm or the macronucleus (29, 30, 32, 34) and often cannot be cultivated outside the protozoan host. Second, in the last two decades, more attention has been paid to bacteria which were not expected to have an intracellular protozoan life cycle. Internalization of human pathogens (*Helicobacter pylori*, *Mycobacterium bovis*) (60, 66) and food-borne pathogens (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*) (6, 7, 14, 28, 35, 42, 59, 61, 68) in protozoa such as *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, *Acanthamoeba rhyodes*, and *Tetrahymena pyriformis* has been demonstrated. The mechanism of the bacterium-protozoan interaction has been intensively studied for *Legionella pneumophila* (1, 26). The facultative intracellular lifestyle of bacterial (food-borne) pathogens in protozoa is of special concern for several reasons: (i) some protozoa, such as *A. castellanii*, *A. polyphaga*, *Glaucoma* sp., and *Tetrahymena* spp., produce small vesicles

which can contain living bacteria (10, 13, 14, 31), and these vesicles might be inhaled or can contaminate the environment; (ii) bacteria surviving within protozoa or protozoan cysts resist unfavorable conditions, such as desiccation and exposure to disinfectants (2, 27, 36, 37); and (iii) an increase in antimicrobial resistance and virulence of bacterial pathogens after passage through protozoa has been demonstrated (8, 17). The association of food-borne pathogens with free-living protozoa is of particular interest because it might explain how, besides well-known strategies such as biofilm formation, some food-borne pathogens persist in food-processing areas despite daily cleaning and disinfection.

Identification of protozoa is traditionally accomplished by using morphology (microscopy and ultrastructure analysis) and locomotion, although PCR-based techniques have increasingly been found to be valid. Morphological identification is often hampered by the small size of protozoa, limited or difficult diagnostic criteria for some taxa, and the time-consuming process necessary to obtain identification expertise. More recently, culture-independent techniques, such as denaturing gradient gel electrophoresis (DGGE), thermal gradient gel electrophoresis, single-strand conformation polymorphism, and terminal restriction fragment polymorphism, have been used to study protozoan communities in various ecosystems (19, 41, 43, 47). Molecular identification is obtained after sequencing of structural or functional genes. Moreover, several studies based on 18S rRNA genes revealed high phylogenetic diversity of uncultivable organisms (46, 48, 49), indicating that the protozoan biodiversity was much higher than that determined by traditional methods. In a few studies, traditional and molecular methods have been combined (4, 57). Compilation of the re-

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TABLE 1. Overview of protozoan status of sample points, as determined by microscopic observation and cumulative data from molecular sequence analyses

Sample	Light microscopy					Sequence analysis	
	Total no. positive/total no. of sample points	No. positive for:			Total no. of morphospecies	No. of sample points positive for protozoa <sup>a</sup>	No. of phylotypes
		Amoebae	Flagellates	Ciliates			
<b>No contact with meat</b>							
Air conditioning in cold storage	0/2	0	0	0	0	— <sup>b</sup>	—
Board tenderizer (underside)	1/1	1	1	0	2	0	0
Ceiling	0/1	0	0	0	0	—	—
Conveyer belt (underside)	2/2	2	2	0	8	2	4
Cutting table (underside)	1/3	1	1	0	4	0	0
Door	0/1	0	0	0	0	—	—
Floor	3/6	1	3	0	3	1	1
Floor drain	13/18	6	11	4	19	11	13
Hot water knife sanitizer (out of use)	2/2	2	2	2	18	2	13
Plastic pallet	2/3	2	2	0	11	2	8
Plastic strip	2/4	0	2	2	7	2	4
Rail	2/7	1	2	2	8	2	7
Soiled bar cutting table	6/6	3	6	3	28	4	7
Standing water on the floor	5/6	5	4	3	17	4	12
Wall	3/10	1	3	1	7	3	6
Wall truck (near air conditioning)	0/2	0	0	0	0	1	1
<b>Direct contact with meat</b>							
Tenderizer							
Needles	1/3	1	1	0	2	1	3
Board (top)	1/3	1	1	0	3	1	3
Conveyer belt (top)	2/6	0	2	0	5	1	7
Cutting table (working surface)	2/10	2	2	0	5	1	2
<b>Others</b>							
Meat residues	5/5	2	5	3	18	5	13
Water supply	0/4	0	0	0	0	0	0

<sup>a</sup> A positive score was assigned when at least one band in the profile was affiliated with protozoan sequences (the results obtained with both primer sets for  $t_0$  and  $t_7$  are grouped together). The number of sample points analyzed was lower than the number analyzed for enrichment cultures (see Results).

<sup>b</sup> —, sample point which was not analyzed or whose profile contained a prominent band(s) which was affiliated with eukaryotic sequences other than protozoan sequences.

sults obtained by two approaches leads to better species description for a particular environment.

In order to investigate the role of environmental free-living protozoa in the contamination of food by food-borne pathogens, an inventory of these unicellular eukaryotes in food-processing environments is a necessary first step. Although the bacterial flora of meat-processing environments has been described, to our knowledge, no studies of the environmental protozoan communities in these environments have been performed previously. The present study was designed to determine the diversity of free-living protozoa in meat-cutting plants using enrichment cultures and sequencing of PCR-amplified, DGGE-separated 18S rRNA gene fragments.

#### MATERIALS AND METHODS

**Sample preparation and processing.** Five meat-cutting plants (one plant processing beef [plant A], two plants processing pork [plants B and C], and two plants processing beef, pork, and poultry [plants D and E; plant D also treated game]) were visited during the period from February to May 2007. Samples were taken after a waiting period of at least 2 h after cleaning and disinfection. A total of 105 samples were collected (Table 1). Liquid samples ( $n = 22$ ) were taken with a sterile syringe or sterile pipette and transferred to sterile plastic tubes. Sterile cotton wool moistened with sterile demineralized water and dry cotton were used to sample dry and wet surfaces, respectively. Samples were collected from a surface area of 126 cm<sup>2</sup> marked with a sterile template which was randomly

placed on the sample point. Cotton wool samples ( $n = 71$ ) were placed in numbered plastic bags, which were tightly closed to avoid drying of the cotton. Upon arrival in the lab (at most 2 h after sampling), 6 ml of sterile demineralized water was added to each cotton wool sample, which was subsequently gently massaged. The cotton wool was manually squeezed, and 5 ml of liquid was transferred to a sterile tube for further processing. For needles of tenderizers samples were obtained with sterile cotton swabs ( $n = 3$ ), and the samples were further processed as described above. Meat residues ( $n = 5$ ) which were found in corners or on the floor were transferred to sterile plastic tubes, and 5 ml of sterile demineralized water was added to each sample and vigorously shaken (vortexed). The supernatant was used for enrichment cultures. Tap water ( $n = 4$ ) was collected in a sterile bottle.

**Determination of microbiological hygiene status.** The general hygienic status of the plants was determined using the agar contact plate method as described in Commission Decision 2001/471/EC (21). Surface samples were collected from control points (conveyors, cutting tables, knives, mincing and packaging machines, saws, etc.) as described in the hazard analysis and critical control points plans of the meat processors. Plate count agar (Oxoid) was used as the cultivation medium, and the plates were aerobically incubated at  $37 \pm 1^\circ\text{C}$  for 24 h.

**Cultivation of protozoa and morphological identification.** One milliliter of an initial dilution or original liquid sample was transferred to a petri dish containing sterile Page's amoeba saline solution (Culture Collection of Algae and Protozoa recipe [www.ccap.ac.uk]) and heat-sterilized rice grain. All petri dishes were incubated for 7 days at refrigerator temperature ( $5 \pm 1^\circ\text{C}$ ) in the dark and were examined microscopically (Olympus CKX41 inverted microscope) within 1 week for the presence of protozoa. Sample points were considered positive for protozoa when at least one representative of amoebae, ciliates, or flagellates was observed. Protozoa were identified morphologically using standard taxonomic

sources for protozoan identification (22–25, 51, 52) and were classified as described by Adl et al. (3).

**DNA extraction.** Three milliliters was removed from the original liquid sample or initial dilution ( $t_0$ ), and 2 ml was randomly withdrawn from each enrichment culture on day 7 ( $t_7$ ). The subsamples were centrifuged for 20 min at  $20,800 \times g$  at 4°C. The upper part was carefully withdrawn, and the pellet was suspended in the remaining 500  $\mu$ l (final volume) of supernatant, which was subjected to DNA extraction. DNA extraction was performed with a ChargeSwitch genomic DNA micro tissue kit (Invitrogen) using a final eluent volume of 75  $\mu$ l. The DNA extracts were stored at -20°C until analysis.

**PCR amplification.** PCR amplification was performed with a PE Applied Biosystems 9700 temperature cycler. 18S rRNA gene primer sets Euk1A–Euk516r-GC (19) and F1427GC-R1616 (63) were used with the following modifications: 800 ng bovine serum albumin (Roche) was added to the PCR mixture, and 35 cycles of denaturation were used for both primer sets.

**DGGE analysis.** Protozoan communities from  $t_0$  and  $t_7$  samples were analyzed by DGGE.  $t_0$  and  $t_7$  PCR products for the same sample points were placed on a gel next to each other. A molecular marker was not included on the gels. Approximately 42  $\mu$ l of PCR product and 10  $\mu$ l of loading dye (30% [vol/vol] glycerol, 0.125% bromophenol, 20 mM Tris-HCl) were applied to individual wells. A sample of bovine or porcine DNA was included on some gels which contained samples probably positive for the presence of this DNA (e.g., samples from conveyer belts and cutting tables). All the DGGE analyses were performed using the DCode universal mutation detection system (Bio-Rad). The polyacrylamide gels consisted of polyacrylamide in 1 $\times$  Tris-acetate-EDTA (TAE) buffer diluted from a 50 $\times$  TAE solution (2 M Tris base, 0.95 M glacial acetic acid, 50 mM EDTA). Two gels were used: a 30 to 50% gradient in a 6% (vol/vol) polyacrylamide gel and a 30 to 55% gradient in a 8% (vol/vol) polyacrylamide for primer sets Euk1A–Euk516r-GC and F1427GC-R1616, respectively (acrylamide/bisacrylamide ratio, 37.5:1; 100% denaturing polyacrylamide solution contained 7 M urea and 40% deionized formamide). The 24-ml gradient gels were cast using a gradient former and a pump set at a constant speed of 5 ml/min. The gels were run for 960 min at 50 V in 1 $\times$  TAE buffer at 60°C and stained for 1 h in 1 $\times$  TAE buffer containing 0.9 mg/liter ethidium bromide.

**Recovery of DNA from DGGE gels.** The most prominent bands were sequenced, except for those whose positions were similar to the positions of bands for cows or pigs. The middle portions of selected DGGE bands were excised and transferred to 1.5-ml Eppendorf tubes containing 30  $\mu$ l of 1 $\times$  TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The tubes were incubated overnight at 4°C. Four microliters was used for the next PCR, which was followed by DGGE to check the band position and purity. After the purity was ascertained, PCR was performed with the same primer set without a GC clamp.

**Sequencing and analysis of excised gel bands.** Sequencing was performed using an Applied Biosystems ABI3130XL genetic analyzer. PCR products were purified for sequencing using shrimp alkaline phosphatase (1 U/ $\mu$ l; Amersham) and exonuclease I (20 U/ $\mu$ l; Epicentre Technologies) for 15 min at 37°C, followed by 15 min at 80°C. This material was subsequently used for cycle sequencing without any further purification using an ABI Prism BigDye V 3.1 terminator cycle sequencing kit (Applied Biosystems). Sequences were exported from BioEdit (33) as FASTA files and compared with the NCBI GenBank database using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) (5). All BLAST searches were performed in December 2007.

## RESULTS

**General hygiene status of the plants.** Only plant E fulfilled the legal requirement of a viable bacterial count of 0 to 10 CFU/cm<sup>2</sup> for all surface samples tested ( $n = 16$ ). For plants A to C, the limit was exceeded in only a minority of the samples (plant A, 2/10 samples; plant B, 1/20 samples; plant C, 2/20 samples). In plant D, seven of 10 samples were unacceptable, including samples from the balance, board meat tenderizer, cutting tables, and saws, which were heavily contaminated.

**Recovery of eukaryotic organisms after enrichment culturing.** A total of 50.5% of 105 samples examined were positive for protozoa after enrichment culturing ( $t_7$ ) (Table 1), and 43.7% of 71 cotton samples resulted in positive cultures. About one-third of the protozoan-positive enrichment cultures were obtained from liquid samples (both samples from out-of-use

hot water knife sanitizers, 13 of 18 samples from floor drains, and two samples from standing water on the floor). All samples from meat residues were positive after enrichment. In samples taken from air-conditioning systems, ceiling, doors, truck walls, and water supplies no protozoa were detected after 1 week. Flagellates, amoebae, and ciliates were observed in 94.3, 58.5, and 37.7% of the 53 positive enrichment cultures, respectively. Amoebae and/or flagellates were detected in cultures obtained from places which come in direct contact with meat, such as the working surfaces of two cutting tables, two top surfaces of conveyer belts, and the board and needles of one tenderizer. Metazoa, such as nematodes (present in five enrichment cultures) and rotifers (present in three enrichment cultures), were observed in two of the five plants but were not identified further. Fungi (mainly yeasts), either as pure cultures or in the presence of protozoa, were found in approximately one-quarter of the enrichment cultures.

The sample processing efficiency was checked by addition of 6 ml of sterile Page's amoeba saline and heat-sterilized rice grain to the squeezed cotton wool, followed by incubation for 7 days at refrigerator temperature and microscopic determination of the cultured protozoa after 1 week. The population obtained did not substantially differ from the  $t_7$  enrichment culture, although there were some species which were not found in the corresponding  $t_7$  cultures.

**Morphological diversity of protozoa.** Representatives of amoebae, ciliates, and flagellates were found in each plant. Higher numbers of morphospecies were observed for cutting plants C (37 morphospecies, including 16 identified morphospecies), A (22 morphospecies, including 14 identified morphospecies), and B (22 morphospecies, including 9 identified morphospecies) than for plants D (18 morphospecies, including 7 identified morphospecies) and E (13 morphospecies, including 10 identified morphospecies) (Table 2). Enrichment cultures from six soiled table bars resulted in a total of 28 different morphospecies (Table 1). Other sample points showing high species diversity were floor drains, out-of-use hot water knife sanitizers, meat residues, and standing water on the floor. From a total of 61 different observed morphospecies (27 flagellates, 26 ciliates, and 8 amoebae), 27 protozoa were identified to the genus or species level (Table 2). The Chromalveolata comprised 15 identified ciliates. *Epistylis* sp., *Glaucoma scintillans*, and *Prodiscophyra collini* were ciliate species which were found in three of the five plants. Although flagellates were abundant in 94.3% of the positive enrichment cultures, the majority of these organisms could not be identified by light microscopy. Identified flagellates belonged to the Cercozoa and Euglenozoa. *Bodo* species 1 and *Petalomonas* species 2 were found in all meat-cutting plants. Amoebozoa were represented by Tubulinea and Flabellinea. *Thecamoeba* sp. was found in four of the five plants. Irregular, small ( $\leq 10 \mu$ m), dark amoebae were observed in some enrichment cultures, but their taxonomic positions could not be determined.

**DGGE profiles.** A total of 410 PCR products obtained from 100  $t_0$  extracts (excluding four meat residue samples and one needle tenderizer sample) and 105  $t_7$  extracts (each amplified with both primer sets) were subjected to DGGE. In 143 lanes, no bands were detected. The absence of profiles can be explained by an absence of eukaryotic DNA, breakdown of DNA due to chlorine residues originating from disinfection products,

TABLE 2. Morphologically identified protozoan taxa observed in enrichment cultures

Supergroup	First rank	Second rank	Species	Meat-cutting plant <sup>a</sup>					
				A	B	C	D	E	
Amoebozoa	Flabellinea	<i>Cochliopodium</i>	<i>Cochliopodium actinophorum</i>			+			
		Thecamoebida	<i>Thecamoeba</i> sp.	+	+	+	+		
	Tubulinea	Vannellida	<i>Vannella</i> sp.	+		+			
		Tubulinida	<i>Hartmannella</i> sp. <i>Saccamoeba</i> sp.		+	+	+		
Chromalveolata	Alveolata	Ciliophora	<i>Colpoda steinii</i>				+		
			<i>Colpidium colpoda</i>	+					
			<i>Dexiostoma campylum</i>	+				+	
			<i>Glaucoma scintillans</i>	+	+	+			
			<i>Glaucoma</i> sp.		+				
			<i>Tetrahymena pyriformis</i> complex	+				+	
			<i>Paramecium aurelia</i> complex	+					
			<i>Epistylis coronata</i>	+					
			<i>Epistylis</i> sp.			+	+	+	
			<i>Vorticella</i> sp.	+					
			<i>Cinetochilum margaritaceum</i>				+		
			<i>Cyclidium glaucoma</i>					+	+
			<i>Acineria incurvata</i>						+
			<i>Chilodonella uncinata</i>	+					
<i>Prodiscophrya collini</i>				+	+	+			
Excavata	Euglenozoa	Euglenida	<i>Petalomonas</i> species 1	+		+		+	
			<i>Petalomonas</i> species 2	+	+	+	+	+	
			Kinetoplastea	<i>Bodo saltans</i>	+		+	+	+
		Heterolobosea	Vahlkampfiidae	<i>Bodo</i> species 1	+	+	+	+	+
				<i>Bodo</i> species 2			+		+
				<i>Vahlkampfia</i> sp.					+
									+
Rhizaria	Cercozoa	Cercomonadida	<i>Allantion tachyploon</i>			+			

<sup>a</sup> +, present. The total numbers of identified morphospecies for plants A, B, C, D, and E were 14, 9, 16, 7, and 10, respectively.

failure of the extraction method, or inhibition of the PCR. A subset of 109 profiles was not analyzed further for reasons such as weak bands, failure to amplify the excised bands, or dominance of fungi in the enrichment cultures. For the remaining 158 profiles, preliminary sequence analysis of prominent bands revealed that 68 profiles were affiliated with fungal and/or metazoan (Mammalia, Nematoda) sequences. However, not all bands of these 68 profiles were excised, and the possibility that some weak bands matched protozoan sequences cannot be excluded. Ninety profiles contained at least one band which was affiliated with protozoan DNA sequences. Also, not all bands could be excised, and visual selection was made. A number of DGGE profiles for  $t_0$  samples and the corresponding  $t_7$  samples revealed distinct changes in the presence of bands and/or the relative band intensities, suggesting that enrichment culturing changed the relative composition of the protozoan community (Fig. 1). Although not all sample points were included in sequence analyses (see above), the data confirmed the finding obtained by microscopy that out-of-use hot water knife sanitizers, floor drains, standing water on the floor, and meat residues showed the highest species diversity (Table 1).

**Molecular affiliation of protozoan DNA sequences.** All sequences were affiliated with eukaryotic organisms, which confirmed the specificity of the two eukaryotic primer sets and is in agreement with the original studies (19, 64). A total of 310 bands were sequenced, and 174, 102, and 17 of the sequences were affiliated with protozoa, fungi, and metazoa, respectively.

Seventeen sequences were excluded due to sequence errors or ambiguous matches. Although the sequences with the highest BLAST scores often matched uncultured protozoan sequences, the most closely related organisms are shown in Table 3. Higher numbers of phylotypes were found for plants C ( $n = 21$ ) and E ( $n = 20$ ) than for plants A ( $n = 15$ ), D ( $n = 13$ ), and B ( $n = 12$ ). The Chromalveolata was the largest group (25 phylotypes), followed by the Rhizaria (10 phylotypes), Amoebozoa (five phylotypes), Excavata (three phylotypes), Opisthokonta (three phylotypes), and Archaeplastida (two phylotypes). Within the Chromalveolata group, Chrysophyceae and Colpodellida were the flagellate groups which were encountered most often (Table 3). *Spumella*-like flagellate JBM/S11, which clusters with *Spumella elongata* (12), *Colpodella tetrahymenae*, and *Telotrochidium matiense* were found in four plants. The Rhizaria were mainly represented by *Cercozoa* spp. and the Heteromitidae. *Bodomorpha* (3, 50), *Heteromita globosa*, the "Costa Rica" flagellate (16), and soil flagellates AND21 and AND24 (39) belong to the latter group. *Bodomorpha* sp. and *Lecythium* sp. were the cercozoan phylotypes which were most frequently encountered in meat-cutting plants. Archaeplastida (Chlorophyta) were found in plants A and C. Representatives of the Excavata (Euglenozoa) and Opisthokonta (Choanomonada) were rarely found, and, with the exception of *Parabodo caudatus*, their BLAST scores were low (<92%). Remarkably, *Petalomonas* spp. were frequently observed in enrichment cultures, but they were detected only twice by the molecular tool. Amoebal DNA was seldom re-

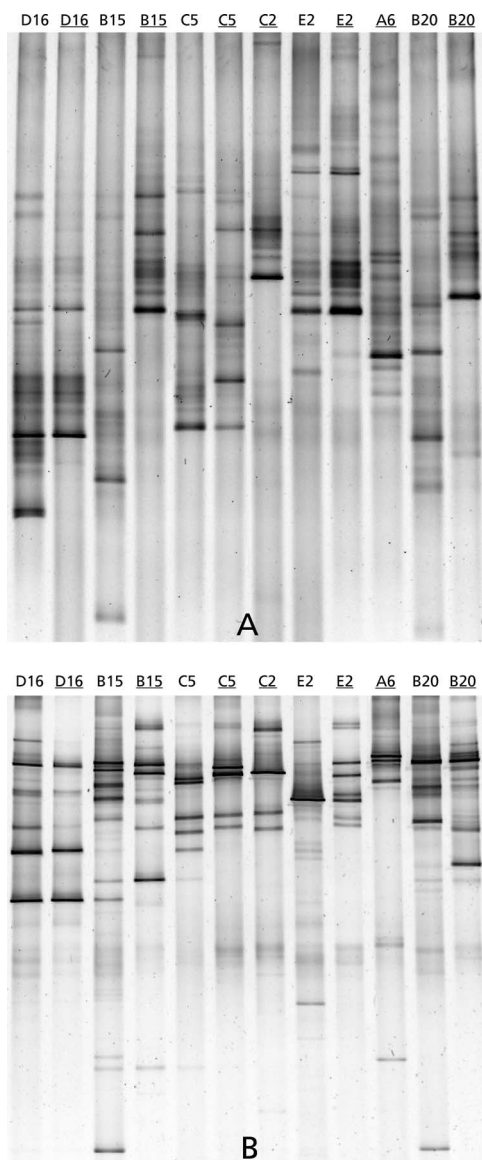


FIG. 1. DGGE patterns obtained using primer set F1427GC-R1616 (A) and primer set Euk1A-Euk516r-GC (B) for sample points in five meat-cutting plants (plants A to E). Sample points: D16, needles of a meat tenderizer; B15, top of conveyer belt; C5, out-of-use hot water knife sanitizer; C2, floor drain; E2, standing water on the floor; A6, soiled table bar; B20, plastic pallet. Underlining indicates corresponding  $t_7$  enrichment cultures.

tried; only five species were found, and they belonged to the Flabellinea ( $n = 2$ ), Tubulinea ( $n = 2$ ), and incertae sedis Amoebozoa Spongomonadida ( $n = 1$ ).

**Detection of eukaryotic DNA other than protozoan DNA.** A large proportion of the sequences (102/310 sequences) were affiliated with fungi, including the Ascomycota ( $n = 72$ ), Basidiomycota ( $n = 23$ ), Glomeromycota ( $n = 3$ ), Urediniomycetes ( $n = 3$ ), and Chytridiomycetes ( $n = 1$ ). The yeast *Yarrowia lipolytica* was the species that was encountered most frequently (32 of the 72 ascomycete sequences, and in some DGGE lanes for a given sample point up to three bands were found at different migration positions), and it was found in all

plants. Seven sequences matched sequences of Nematoda (genera *Aduncospiculum*, *Choriorhabditis*, *Panagrolaimus*, *Pristionchus*, and *Rhabditis*), one sequence matched an Arthropoda sequence, and one sequence matched an Oligochaeta sequence. Mammalian DNA was detected in eight cases, and the sequences were affiliated with cow, pig, or deer sequences.

## DISCUSSION

The aim of this study was to determine the biodiversity of free-living protozoa in meat-cutting plants. Light microscopy after enrichment culture was combined with sequencing of PCR-amplified, DGGE-separated 18S rRNA gene fragments, which was used as a fast screening method. Preliminary microscopic observation of a few  $t_0$  samples revealed a low number of protozoa, and there were difficulties in analyzing fat- or protein-rich samples, which justified the decision to incorporate enrichment culturing. Moreover, the development of cryptic species (i.e., species inactive at the time of sampling) under favorable conditions (i.e., enrichment culture) indicated that they were present in the  $t_0$  samples. The enrichment cultures were incubated at a low temperature since the ambient processing temperature in the meat-cutting plants may not be more than 12°C. Two universal eukaryotic primer sets, those of Díez et al. (19) and van Hannen et al. (63), were used to capture a higher fraction of the eukaryotic communities. Because it was not practical to sequence all bands and analyze a subset of DGGE profiles, both of which inevitably resulted in underestimation of the species composition, it was not possible to make a detailed comparison of the two primer sets and the results obtained for  $t_0$  and  $t_7$  samples. Therefore, the survey resulted in a general overview of the protozoan community composition obtained by microscopy and sequencing.

A total of 105 samples were taken, and 53 of them resulted in protozoan-positive enrichment cultures, as determined microscopically. In most samples which yielded a protozoan-positive enrichment culture, residual organic material and/or water was present. For example, samples taken from floor drains, knife sanitizers, meat residues, soiled table bars, and standing water on the floor resulted in the highest number of protozoan-positive cultures. In addition, locations which were inadequately cleaned and disinfected because of ignorance or inaccessibility (e.g., holes in plastic pallets, undersides of cutting boards and conveyer belts, and upper sides of rails) harbored protozoa. Protein and fat residues provide an excellent nutrient source for bacteria, which in turn is favorable for the growth of protozoa. Detritus is rapidly colonized by small heterotrophic flagellates, whose concentrations can reach up to  $10^5$  organisms/ml or more (15). Of particular interest were the surfaces which come into direct contact with meat. Protozoa were found in enrichment cultures obtained from conveyer belts in plant B, and protozoan DNA was detected on the surface of a cutting table in plant E. In plant D, amoebae and flagellates were found in enrichment cultures obtained from two cutting tables and the board and needles of the tenderizer. The presence of protozoan DNA on needles was also observed at  $t_0$ , and the DGGE pattern was similar to that at  $t_7$ , suggesting that an active protozoan community was present at the moment of sampling (Fig. 1). The general hygiene status of some sample points as determined by microbial analysis in

TABLE 3. Protist community compositions at the different meat-cutting plants as determined by BLAST analysis of 18S rRNA gene sequences

Supergroup	First rank	Second rank	Most closely related organism	Accession no.	Sequence similarity (%)	Meat-cutting plant <sup>a</sup>					
						A	B	C	D	E	
Amoebozoa	Flabellinea	Thecamoebida	<i>Sappinia diploidea</i>	DQ122380	97.2			+			
			Vannellida	<i>Platyamoeba placida</i>	AY294150	95.1			+		
	Tubulinea	Lyptomyxida	<i>Leptomyxa reticulata</i>	AF293898	78.4	+					
			Incertae sedis	<i>Echinamoeba exundans</i>	AF293895	98.9					+
	Incertae sedis	Spongomonadidae	<i>Spongomonas minima</i>	AF411280	97.6–100			+		+	
Archaeplastida	Chloroplastida	Chlorophyta	<i>Chloromonas</i> sp.	AF514406	99.2			+			
			<i>Polytoma uvella</i>	U22942	99.2–100	+					
Chromalveolata	Alveolata	Apicomplexa	<i>Ascogregarina culicis</i>	DQ462457	92.8					+	
			<i>Colpodella edax</i>	AY234843	90.1–90.9				+	+	
			<i>Colpodella tetrahymenae</i>	AF330214	88.2–98.8		+	+	+	+	
			<i>Colpodella</i> sp.	AY142075	95.8–96.9				+	+	
			<i>Cryptosporidium andersoni</i>	AY954885	80.2–81.0	+				+	
			<i>Colpidium campylum</i>	X56532	99.1	+					
			<i>Discophrya collini</i>	L26446	99.0				+		
			<i>Glaucomides bromelicola</i>	AJ810077	99.4	+					
			<i>Loxophyllum rostratum</i>	DQ190465	99.4					+	
			<i>Opisthonecta minima</i>	EF417834	97.6–97.8					+	
			<i>Prodiscophrya</i> sp.	AY331803	100					+	
			<i>Pseudocohnilembus persalinus</i>	AY551906	99.8					+	
			<i>Pseudoplatyophrya nana</i>	AF060452	99.5–99.6				+	+	
		<i>Sorogena stoianovitchae</i>	AF300287	95.7–97.6				+	+		
		<i>Telotrochidium matiense</i>	AY611065	96.2–100				+	+		
		<i>Vorticella microstoma</i>	DQ868347	97.9–99.6				+			
		<i>Glenodinium inaequale</i>	EF058237	80.9–85.0					+		
		Stramenopiles	Dinzoa	Bicosoecida	<i>Adriamonas peritocrescens</i>	AF243501	76.0				+
					<i>Ochromonas</i> sp.	EF165126	98.6				
					<i>Ochromonas</i> sp.	EF165144	99.4				
					<i>Paraphysomonas vestita</i>	Z28335	98.7	+			
					<i>Spumella</i> -like flagellate JBAS38	DQ388538	99.3	+			
					<i>Spumella</i> -like flagellate 1305	DQ388568	99.4–100	+			
<i>Spumella</i> -like flagellate JBM/S11	EF043285				99.4–100				+		
<i>Saprolegnia parasitica</i>	AB086898				99.8	+					
<i>Notosolenus ostium</i>	AF403159				75.1				+		
<i>Petalomonas cantuscygni</i>	AF386635				78.2				+		
Excavata	Euglenozoa	Euglenida	<i>Parabodo caudatus</i>	DQ207590	100			+			
Opisthokonta	Choanomonada	Acanthoecidae	<i>Diaphanoeca grandis</i>	DQ059033	91.3			+			
			<i>Stephanoeca diplocostata</i>	AF084235	91.3–91.9			+			
			<i>Monosiga ovata</i>	AF084230	82.4				+		
Rhizaria	Cercozoa	Cercomonadida	<i>Bodomorpha</i> sp.	DQ211596	95.3–100		+	+	+		
			<i>Cercomonas</i> sp.	U42448	98.8			+			
			<i>Cercomonas</i> sp.	DQ211598	99.4				+		
			<i>Cercomonas</i> sp.	AY884338	98.9				+		
			“Costa Rica” flagellate	AF411277	97.9				+		
			<i>Heteromita globosa</i>	U42447	97.2–100				+		
			<i>Proleptomonas faecicola</i>	AF411275	92.2	+			+		
			Soil flagellate AND21	AY965866	99.2–100				+		
		Soil flagellate AND24	AY965867	99.8–100				+			
		Incertae sedis	<i>Lecythium</i> sp.	AJ514867	89.7–98.0	+	+	+	+		
		Incertae sedis									
Eukaryotes			<i>Breviata anathema</i>	AF153206	96.7	+					

<sup>a</sup>+, present. The total numbers of phylotypes for plants A, B, C, D, and E were 15, 12, 21, 13, and 20, respectively.

plant D was unacceptable according to Commission Decision 2001/471/EC and might explain the presence of protozoa on surfaces expected to have a low germ content. However, our results suggest that a good hygiene score (plant E) does not necessarily correlate with an absence of protozoa in the food-processing environment.

The overall species compositions (i.e., the occurrence of amoebae, flagellates, and ciliates) were quite similar in all plants, and our data do not suggest that process-related activity (i.e., cutting of cow carcasses versus cutting of pig carcasses) resulted in a specific protozoan community. Plant C showed the highest species richness, as determined by both methods. Little agreement was found between the light microscopy results and the sequencing results, although the analysis of a subset of bands limited our ability to make a reliable comparison. By using light microscopy, 27 morphospecies were identified, compared to 49 phylotypes. Higher ciliate diversity was observed when light microscopy was used than when the molecular approach was used (26 and 11 taxa, respectively). The taxa which were retrieved by both methods were *P. collini* and *Vorticella* spp. One species was morphologically identified as *G. scintillans*, while sequence analysis of the sample point indicated an affiliation with *Glaucomides bromelicola*. Sequence analysis showed that *T. matiense* was the most frequently encountered ciliate species, although it was not microscopically observed, in contrast to *Epistylis* spp., which were frequently observed in enrichment cultures. *T. matiense* is more closely related to *Epistylis* than to any other peritrichous genus and probably evolved from Peritrichia with a noncontractile stalk (44). Small heterotrophic flagellates were abundant in enrichment cultures but were difficult to identify microscopically in a reliable way. This was compensated for by sequence analyses of excised DGGE bands. Flagellates were represented mainly by Alveolata (Apicomplexa), Stramenopiles (Chrysophyceae), and Rhizaria (Cercozoa). *Bodo* spp. and *Petalomonas* spp. were abundant in enrichment cultures but rarely detected by PCR. Amoeboid organisms were rarely identified by DGGE, although monopodial amoebae were frequently encountered in enrichment cultures. Our results agree with the finding that amoebae are rarely found in environmental molecular surveys (4, 11).

Although both culture methods and PCR-based methods have their specific biases (45, 58, 65) and it was difficult to identify all observed species morphologically and impossible to sequence all bands from the gels, the data obtained show that the two-method approach resulted in identification of a higher number of species than either method separately. Our results confirm the results of other studies (4, 45, 57) which recommended that a combination of methods should be used to study protozoan communities. The use of two universal eukaryotic primer sets resulted in detection of many eukaryotic organisms other than protozoa, including a high number of fungi. Amplification of fungal or metazoan DNA has also been found in other studies (19, 43) and can be attributed to several factors. First, yeasts were abundant in the enrichment cultures, leading to a higher fungal DNA content in the extract. Second, a high copy number of rRNA genes is present in the genomes of some yeasts, such as *Y. lipolytica* (18), which increases the probability that the genes are amplified during PCR. Third, protozoa form a paraphyletic group, and the use of universal eukaryotic prim-

ers inevitably leads to amplification of nonprotozoan DNA. The use of specific primer sets instead of universal primers can eliminate this problem and is strongly recommended when particular groups need to be studied (9, 20, 40, 53).

It is still unclear how protozoa are initially introduced and transported in food-processing areas. Air, drinking water, and human activities are the most likely transmission routes. Air can contain (cysts of) protozoa, including amoebae, flagellates, and ciliates, such as *Colpoda steinii* (38, 54). Protozoa are known to be common inhabitants of drinking water. The possibility that protozoa are spread by means of droplets formed by the aerosolization of water that is sprayed or splashed during cleaning and disinfection processes cannot be excluded. Daily cleaning and disinfection of food-processing areas creates an environment with a relative high moisture content, which favors the development of protozoa. In a study of moisture-damaged buildings, the occurrence of flagellates and ciliates on different building materials was demonstrated, including the presence of amoebae, which was positively correlated with the water content of the sampled material (67). In another study, 47% (23/49) of the swabs taken from moist areas (floor tiles, drains of sinks, water taps) in a hospital were positive for amoebae (56). In this study, amoebae were detected in 29.5% of the enrichment cultures. The amoebae identified by Rohr et al. (56) mainly belonged to the genera *Hartmannella*, *Vahlkampfia*, and *Vannella*, which is in accordance with our data. *Acanthamoeba* spp. and *Naegleria* spp., however, were not found in the present survey. The protozoan community is subjected to different stress conditions, such as cold, desiccation, and cleaning and disinfection processes. Many, but not all, protozoa have the ability to encyst and can resist unfavorable conditions. Trophozoites and cysts have different susceptibilities to disinfectants, and cysts commonly show higher resistance (36). Our results suggest that protozoa in food-processing areas resist the daily cleaning and disinfection process, either due to excystment after the disinfection cycle, due to the inefficiency of disinfectants at the recommended user concentrations, due to inadequately cleaned surfaces, or due to uncharacterized cell response mechanisms. This suggestion is supported by the fact that protozoa have been found in other anthropogenic environments subjected to disinfection or having residual disinfectant concentrations, such as water systems and swimming pools (55, 62). However, susceptibility tests are necessary to determine the extent of resistance of free-living protozoa to disinfectants commonly applied in the food industry.

In conclusion, high protozoan species richness in meat-cutting plants was demonstrated. This diversity is preferably studied by a combination of microscopic observations and molecular techniques. As a recommendation for further studies, analysis of the composition of eukaryotic assemblages may be refined by designing specific primer sets to study particular groups of amoebae, ciliates, and flagellates. The ecological significance of free-living protozoa in food-processing areas remains unclear. Bacterivory by protozoa should influence the bacterial population in terms of number and species diversity. In this regard, protozoa might be considered "partners" in the control of bacterial levels. On the other hand, the diversity study showed that some of the protozoa are potential hosts for food-borne pathogens. Although an association of food-borne

pathogens with protozoa such as *T. pyriformis* has been shown, whether food-borne pathogens really interact with protozoa isolated from food-processing environments remains to be determined, since most previous studies were carried out with culture collection strains under laboratory conditions. A recent study, however, showed that free-living protozoa isolated from vegetables were able to internalize and release food-borne pathogens (31). The detection of food-borne pathogens in environmental protozoa, as demonstrated by fluorescence in situ hybridization, is a logical next step. Furthermore, the survival of protozoa (and their internalized bacteria) under stress conditions (desiccation, disinfectants, extreme pH values, and heat), the increase in virulence of food-borne pathogens after passage through environmental protozoa, and the effect of grazing activities of protozoa on microbial communities in food-processing areas are all open fields of research.

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