

Diversity and occurrence of free-living protozoa on food and in food-related environments

Natascha Chavatte

Dissertation submitted in fulfilment of the requirements for
the degree of Doctor in Veterinary Sciences (PhD)

2016

Promotors:

Prof. Dr. Kurt Houf

Department of Veterinary Public Health and Food Safety
Faculty of Veterinary Medicine
Ghent University

Prof. Dr. Koen Sabbe

Department of Biology
Faculty of Sciences
Ghent University

Members of the examination committee

Chairman

Prof. Dr. Annemie Decostere

Dr. Julie Baré

Prof. Dr. Edwin Claerebout

Dr. Lieve Herman

Prof. Dr. Ir. Mieke Uyttendaele

Dr. Jeroen Van Wichelen

Prof. Dr. Anne Willems

To cite this thesis

Chavatte N. (2016). Diversity and occurrence of free-living protozoa on food and in food-related environments. Thesis submitted in fulfilment of the requirements for the degree of Doctor in Veterinary Sciences (PhD), Faculty of Veterinary Medicine, Ghent University.

The author and promoters give the permission to consult and to copy parts of this work for personal use only. Any other use is subject to the Laws of Copyright. Permission to reproduce any material contained in this work should be obtained from the author.

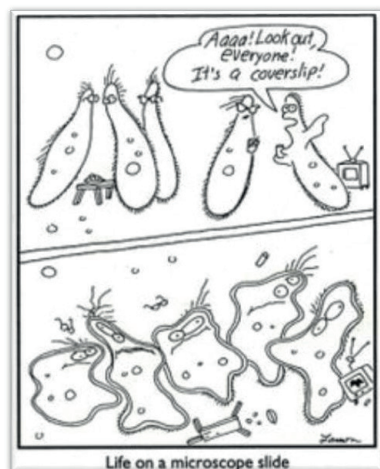


Table of contents

Table of contents	5
List of abbreviations	13
Preface	21
General introduction	25
1 Free-living protozoa	27
1.1 <i>Morphology</i>	27
1.2 <i>Motility and feeding</i>	29
1.3 <i>Life cycle and reproduction</i>	31
1.4 <i>Classification and phylogenetic relationships</i>	31
1.5 <i>Diversity</i>	35
1.6 <i>Ecology</i>	37
2 Methodology	43
2.1 <i>Recovery and cultivation</i>	43
2.2 <i>Identification and quantification</i>	45
3 Impact of free-living protozoa on food safety and public health	49
3.1 <i>Free-living protozoa in food-related environments</i>	49
3.2 <i>Free-living protozoa on food products and in drinking water</i>	51
3.3 <i>Free-living protozoa in animals and humans</i>	53
3.4 <i>Free-living protozoa in biofilms in the food environment</i>	55
3.5 <i>Pathogenic and opportunistic free-living protozoa</i>	57
3.6 <i>Interactions of free-living protozoa with foodborne pathogenic bacteria</i>	59
Scientific aims	69
Chapter 1. Co-occurrence of free-living protozoa and foodborne pathogens on dishcloths: implications for food safety	73
Abstract	75

1	Introduction.....	77
2	Material and methods	79
2.1	<i>Development and evaluation of two protocols for the recovery and quantification of FLP from dishcloths</i> .	79
2.1.1	Cultivation of FLP	79
2.1.2	Evaluation of the protocols	79
2.2	<i>Occurrence, enumeration and diversity of FLP on used dishcloths.....</i>	81
2.3	<i>Bacteriological analysis of used dishcloths.....</i>	83
2.4	<i>Data analysis</i>	85
3	Results	87
3.1	<i>Development and evaluation of two protocols for the recovery and quantification of FLP from dishcloths</i> .	87
3.2	<i>Occurrence, enumeration and diversity of FLP on used dishcloths.....</i>	89
3.3	<i>Bacteriological analysis of used dishcloths.....</i>	91
3.4	<i>Evaluation of dishcloth usage and kitchen practices.....</i>	91
4	Discussion	93
	Acknowledgements	99
	Chapter 2. Abundance, diversity and community composition of free-living protozoa on vegetable sprouts.....	107
	Abstract.....	109
1	Introduction.....	111
2	Material and methods	113
2.1	<i>Occurrence, abundance and diversity of FLP</i>	113
2.2	<i>Total aerobic bacteria and Escherichia coli</i>	115
2.3	<i>Data analysis</i>	115
3	Results	117
3.1	<i>Occurrence and abundance of FLP</i>	117
3.1.1	Within-lot variation	117
3.1.2	Between-lot variation.....	119
3.2	<i>Diversity of free-living protozoa</i>	119
3.3	<i>Bacterial load on vegetable sprouts</i>	125

4 Discussion	125
Acknowledgements	131
Chapter 3. Free-living protozoa in the gastrointestinal tract and feces of pigs: Exploration of an unknown world and towards a protocol for the recovery of free-living protozoa	137
Abstract	139
1 Introduction	141
2 Material and methods	143
2.1 <i>Development and testing of protocols for the recovery of FLP from the porcine gastrointestinal tract and feces</i>	143
2.1.1 Filtration	145
2.1.2 Sedimentation	145
2.1.3 Ludox and magnesium sulphate density gradient centrifugation	145
2.1.4 Formalin-ethyl acetate sedimentation concentration	147
2.2 <i>Exploration of FLP in the porcine gastrointestinal tract and feces</i>	147
2.2.1 Recovery of FLP from pig feces.....	147
2.2.2 Recovery of FLP from the pig gastrointestinal tract	147
2.2.3 Morphological identification of FLP	149
2.2.4 Molecular biological identification of FLP	149
3 Results	151
3.1 <i>Development and testing of protocols for the recovery of FLP from pig feces</i>	151
3.2 <i>Exploration of FLP in the porcine gastrointestinal tract and feces</i>	153
3.2.1 FLP in pig feces	153
3.2.2 FLP in the pig gastrointestinal tract.....	153
4 Discussion	155
Acknowledgements	161
General discussion	169
1 Methodological remarks	169
<i>Recovery</i>	169
<i>Cultivation</i>	173

<i>Purification</i>	175
<i>Quantification</i>	175
<i>Identification</i>	177
2 Diversity, occurrence and abundance of free-living protozoa on dishcloths, vegetable sprouts and in the porcine gastrointestinal tract and feces	181
<i>Diversity</i>	181
<i>Occurrence</i>	187
<i>Abundance</i>	191
3 Direct or indirect impact on food safety and public health	191
4 Future perspectives	197
References	203
Summary	233
Samenvatting	241
Curriculum vitae	251
Dankjewel	257

List of abbreviations

18S rRNA	small subunit (18 Svedberg) ribosomal ribonucleic acid
ALOA	Agar Listeria according to Ottaviani & Agosti
AM_MPN	amoeba numbers
am_rich	amoeba richness
API	analytical profile index
ARB	amoeba resistant bacteria
ATCC	American Type Culture Collection
BPA	Baird Parker agar
BPW	buffered peptone water
CCA	canonical correspondence analysis
CCAP	Culture Collection of Algae and Protozoa
CFA	Campy food agar
CFU	colony forming units
CIL_MPN	ciliate numbers
cil_rich	ciliate richness
CIN	cefsulodin-irgasan-novobiocin
cox1	cytochrome c oxidase
DC	direct counting
DCA	detrended correspondence analysis
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DOM	dissolved organic matter
EHEC	enterohaemorrhagic <i>E. coli</i>
EMA	ethidium monoazide
ERIC-PCR	enterobacterial repetitive intergenic consensus polymerase chain reaction
FISH	Fluorescent <i>in situ</i> Hybridisation
FLA	free-living amoeba
FLA_MPN	flagellate numbers
FLP	free-living protozoa
FLP_MPN	total FLP numbers
FLP_rich	total FLP richness
GIT	gastrointestinal tract
HUS	hemolytic uremic syndrome
IMS	immunomagnetic separation
ITS	Internal Transcribed Spacer
LOD	limit of detection
LOQ	limit of quantification
mCCDA	modified charcoal-cefoperazone-deoxycholate agar
MCPT	Monte Carlo Permutation Test
MOI	multiplicity of infection
MPN	most probable number
MSRV	Modified semi-solid Rappaport-Vassiliadis agar
NGS	Next-Generation Sequencing

NNA	Non-Nutrient Agar
OTU	Operational Taxonomic Unit
PAS	Page's amoeba saline
PCA	plate count agar
PCA	Principal Component Analysis
PCR	polymerase chain reaction
PPY	proteose peptone yeast extract medium
PSB	peptone-sorbitol-bile
PYG	proteose peptone yeast extract glucose
qPCR	real-time quantitative PCR
RDA	redundancy analysis
SEM	scanning electron microscopy
SG	specific gravity
STEC	Shiga toxin-producing <i>Escherichia coli</i>
TAB	total aerobic bacteria
TBX	tryptone bile X-glucuronide
TEM	transmission electron microscopy
T-RFLP	terminal restriction fragment length polymorphism
TSB	tryptic soy broth
VNBC	viable but non-culturable
VTEC	verocytotoxin-producing <i>E. coli</i>
XLD	xylose lysine desoxycholate

*Passion is about doing what you love
Dedication is perseverance and hard work
Add them together = fuel for success!*

Brooke Griffin

Preface

Preface

Free-living protozoa (*i.e.* unicellular heterotrophic eukaryotes) are important bacterial consumers controlling bacterial biomass and forming an important trophic link in aquatic and terrestrial food webs. They are common in both natural and anthropogenic environments.

There is increasing evidence that FLP play an important role in the epidemiology and ecology of bacterial pathogens. Associations with FLP have been implicated in the transmission and persistence of (foodborne) pathogenic bacteria, which still is an ongoing problem. Food and food-related environments create an ideal niche and meeting place for FLP and foodborne pathogens, enhancing the close association between both microbial groups. In order to fully understand pathogen epidemiology there is a pressing need to improve the knowledge of the ecology and the associations between both microbial groups in these environments.

Information about the occurrence and diversity of FLP on and in food and in food-related environments is generally lacking as they are often ignored components in food microbiological research.

This thesis forms part of a large research effort aimed at understanding the ecological role of FLP in the persistence and transmission of pathogenic bacteria in food and food-related environments. It builds on an ongoing expertise of both research groups with complementary skills and complements previous work carried out in the labs of the promoters which documented the presence and diversity of FLP in broiler houses, meat-cutting plants, domestic refrigerators and on lettuce.

The choice of the examined matrices during this thesis was based on the fact that both microbial groups (FLP and pathogenic bacteria) can co-occur and as diverse as possible by means of at consumer level the domestic environment (dishcloths), ready-to-eat fresh produce (vegetable sprouts) and livestock animals (gastrointestinal tract of pigs). The base of this doctoral thesis is, from an ecological point of view, providing new insights about the presence and diversity of FLP on and in food and in food-related environments. Describing FLP communities from above matrices will contribute to unravel their ecological significance and function in these environments and in the persistence of (foodborne) pathogenic bacteria.

The earth is a microbial planet, on which macro-organisms are recent additions highly interesting and extremely complex in ways that most microbes aren't, but in the final analysis relatively unimportant in a global context

Mark Wheelis

General introduction

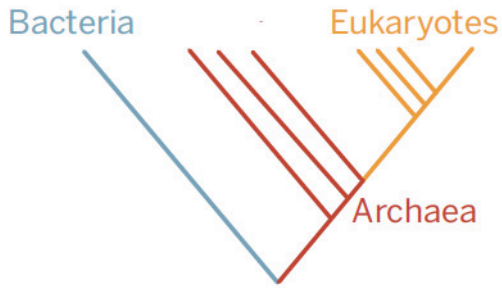


Figure 1. Three domains of the tree of life. Figure taken from Leslie (2015).



Figure 2. Variety in cell size and shape of free-living protozoa, drawn to scale next to a pinhead. Figure taken from Finlay (2002).

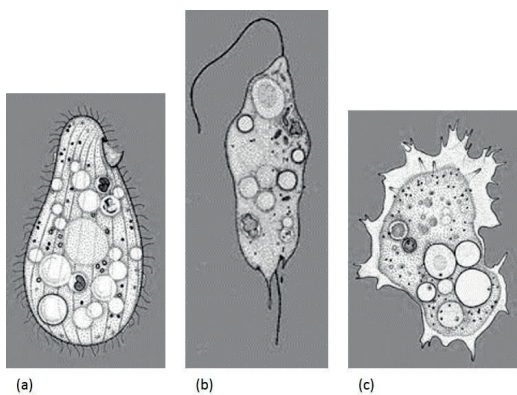


Figure 3. Examples of FLP species, representing each morphogroup: (a) ciliate *Tetrahymena*, (b) flagellate *Cercomonas* and (c) amoeba *Acanthamoeba*. Figures taken from <http://pinkava.asu.edu/starcentral/microscope/>

1 Free-living protozoa

Within the **tree of life** three main domains are distinguished: Bacteria, Archaea and Eukaryota (Fig. 1). All **eukaryotes** have a cytoskeleton, an internal skeleton that gives the cell its shape and ability to move. Through this skeleton all organelles are kept in their proper place or are able to move from one side of the cell to the other (Alberts et al., 1994). Another feature of eukaryotic cells is the possession of a nucleus, an endoplasmic reticulum, Golgi-apparatus and mitochondria.

Within the domain of Eukaryota, the group of **Protists**, which are unicellular organisms and from an evolutionary point of view a very diverse group (see 1.6.), are situated. **Protozoa** are defined as heterotrophic (metabolic energy derived from the consumption of organic matter) protists and likewise form a diverse and heterogeneous group of microorganisms (Hausmann et al., 2003). Besides the above eukaryotic features, protozoa can be very complex. The cell can contain specialized structures like photoreceptors, sensory bristles, leg-like appendages, 'mouth' parts and muscle-like contractile bundles. Protozoan cell size ranges between 2 μm and 2 mm and the variety in cell shape is infinite (Fig. 2). In this thesis focus will be on **free-living protozoa (FLP)**, which do not have an obligate parasitic or symbiotic life cycle.

1.1 Morphology

Based on **morphology** and locomotion FLP are divided into **three morphogroups**: ciliates, flagellates and amoebae (Fig. 3).

Ciliates (Fig. 3a) are characterized by cilia, small thread-like structures which occur in high numbers over the cell body and have their function in motility and feeding (Sigeo, 2005). Somatic cilia (cilia on the cell body) are used for locomotion, while buccal cilia (cilia near the oral apparatus) are used for feeding. The waving movement of the buccal cilia creates water currents by which food particles are collected near the cytostome (oral apparatus). Some ciliates, like the hypotrichs, have cilia arranged in groups (cirri) which function as walking appendages. Another feature of ciliates is the presence of a polyploid macronucleus, which controls somatic functions, and a diploid micronucleus, used during sexual reproduction. Many ciliates move by swimming, have a cell size range between 20 – 200 μm and are common in aquatic and soil habitats.

Flagellates (Fig. 3b) are characterized by one or more flagella, implanted at the anterior side of the cell. Flagella are similar in structure to the cilia and have their function in movement and feeding, but also for attachment to substrata. Most flagellates have a small cell body size (<20 μm), resulting in the uptake of only small food particles like bacteria or dissolved organic matter (DOM). Heterotrophic flagellates include microflagellates (≥ 15 –200 μm), nanoflagellates (2-15 μm) and picoflagellates (<2 μm) (Boenigk and Arndt, 2002). Most flagellates move by gliding, but swimming and (temporarily) attached organisms also exist. Some groups harbour a lorica (shell-like house) or are characterized by a collar.

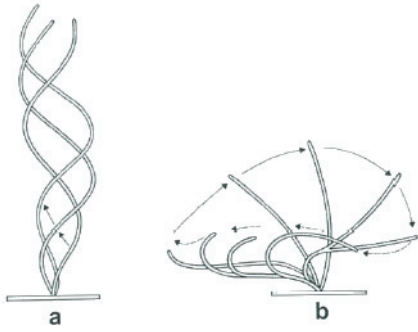


Figure 4. Illustration of the flagellar (a) and ciliary (b) movements. Figure taken from Hausmann et al. (2003).

Amoebae (Fig. 3c) are protozoa which lack flagella or cilia. However some members of the Heterolobosea (see section 1.5.) have a flagellated stage in their life cycle. A major taxonomic feature of amoebae are the pseudopodia or cytoplasmic extrusions, used for phagocytose and locomotion. There are testate amoebae, featured by a thick test or lorica, and naked amoebae (Sigg, 2005). The division in morphogroups does not reflect the phylogenetic position and relationships (see section 1.5.) of these organisms, except in the case of the ciliates which form a monophyletic group within the Alveolata.

1.2 Motility and feeding

Most FLP are **motile** and swim, crawl or glide in a medium or on a substrate. Other protozoan species are **sessile** and typically feature a (contractile) stalk which is often involved in feeding. Most protozoa are **solitary** organisms although colony forming species like peritrich ciliates (e.g. *Epistylis*), also occur.

There is a subtle difference between locomotion and motility. **Locomotion** implies progression of an organism through the medium in which the organism changes its place and position. **Motility** includes locomotion, but includes also the creation of water currents and intracellular movements (Hausmann et al., 2003).

Motility by means of **beating cilia or flagella** (Fig. 4) is the most common form. It results in a smooth movement of the cell (e.g. swimming) and is frequently seen in ciliates and flagellates. Flagellates swim or glide while the flagellum can be held stiffly or can have a waving movement or is used to migrate over the substratum. Most common in amoebal species is **amoeboid movement** by means of the extension of pseudopodia. During pseudopodial extension cytoplasmic streams results in cell shape change or cell movement.

FLP are **heterotrophic**: for energy and nutrients they need to convert organic molecules.

As a general mechanism nutrient uptake can be done by **pinocytosis**, a non-specific uptake of dissolved nutrients through membrane invagination, e.g. some euglenoid flagellates capture nutrients near the flagellar pocket via pinocytosis. The other and probably most common mechanism is **phagocytosis**, *i.e.* uptake of particulate matter through membrane invagination which require a specific receptor dependent process.

Within protozoan communities there are **bacterivores** (feeding on bacteria), which is the most common feeding mode, **herbivores** (feeding on photosynthetic organisms, mainly microalgae), **detritivores** (feeding on dead particulate organic material), **omnivores** (feeding on different kinds of prey or trophic levels) and **carnivores** (feeding on other protozoa or metazoa)(Pernthaler, 2005). Many protozoa can be allocated to a variety of feeding modes and the classification into a single specific feeding type is often incorrect.

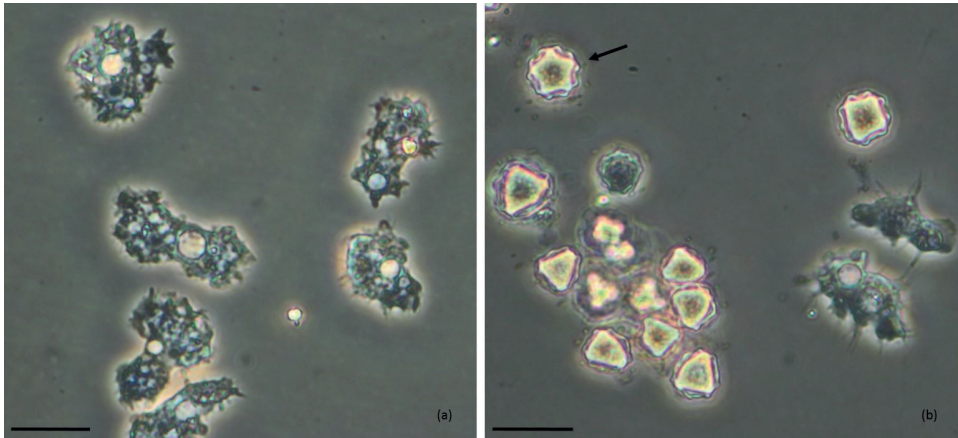


Figure 5. Trophozoites (a) and cysts (b, arrow) of *Acanthamoeba* sp. Scale bar = 20 μm ; inverted microscopy magnification x400.

1.3 Life cycle and reproduction

Within their **life cycle**, protozoa can change cell- morphology and physiology in response to internal or external signals (Hausmann et al., 2003). These changes can include encystment (formation of cysts or resting forms), excystment (reformation to trophozoites) and transformation from one cell type to another (e.g. amoeboid cell to flagellated cell). **Trophozoites** (Fig. 5) are the vegetative cell forms used for locomotion and multiplication; while **cyst** formation is triggered by external stimuli like nutrient depletion, desiccation or other unfavourable environmental conditions like changes in pH, temperature, salinity and oxygen levels (Corliss, 2001; Fouque et al., 2012; Khan, 2006). It must be highlighted that not all protozoan species are able to form cysts and some species, like aquatic ciliates or species kept in lab conditions, may have lost the ability to form cysts, due to the constant food supply (Finlay, 1990).

Free-living protozoa **reproduce** asexually and/or sexually. **Asexual** or vegetative reproduction is the main form of multiplication in protozoa and most common in flagellates and amoebae (Mulisch, 2003a). This process can be done by binary fission (mitosis resulting in two daughter cells), multiple fission (mitosis resulting in multiple daughter cells) or budding (separation of daughter cell(s) from a mother cell). **Sexual** reproduction involves the fusion of two haploid nuclei (after meiosis) to combine to a diploid zygote (Mulisch, 2003b).

A special type of sexual reproduction is seen in ciliates. This group (an example is *Paramecium caudatum*) reproduces sexually by **conjugation**, whereby two cells of complementary mating types conjugate or join, exchange genetic material and deliver progeny by fission or budding (Mulisch, 2003b).

Data about basic genetic information such as number of chromosomes of FLP and number of 18S rRNA copies are not mentioned in literature. For *Acanthamoeba castellanii* the number of genes is estimated ± 15450 (± 40 Mbp), encoding for ± 15000 proteins (Clarke et al., 2013).

1.4 Classification and phylogenetic relationships

The taxonomic classification of protists has been an issue of debate for a long time. Protozoa together with algae and slime moulds are often united in the **Protista** (Sapp, 2009), but this is a paraphyletic group, excluding the animals, plants and Fungi.

Eukaryotes were formerly classified in the kingdoms of Animalia, Plantae, Fungi and Protista (Margulis and Schwarz, 1988; Whittaker, 1969; Whittaker and Margulis, 1978), with the protist kingdom being perceived as being simple organisms, from which more complex multicellular species evolved. During the last 20 years, the classification of protists and the eukaryotic **tree of life** has repeatedly undergone thorough change and revision (Hampl et al., 2009; Katz, 2012). It is clear now that protists cover most of the evolutionary diversity of the eukaryotic tree, and that in the past they were often neglected because visually not dominant and hence often overlooked. In addition, their 'simple' morphology as observed by light microscopy also led to a serious underestimation of their diversity. Pioneer work in molecular phylogenies firmly nested the eukaryotic diversity into the crown of the tree at the same level of complexity as cells from multicellular life forms (Sogin, 1991; Sogin et al., 1989, 1986; Vossbrinck et al., 1987; Woese et al., 1990).

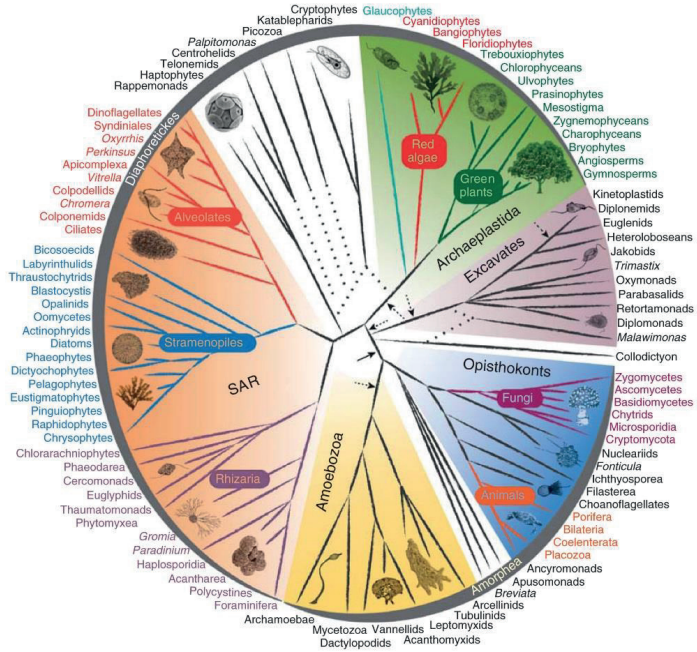


Figure 6. Eukaryotic tree of life, based on agreements between phylogenetic evidence and morphological characteristics, showing the evolutionary relationships between the main eukaryotic groups. Cartoons illustrate the diversity of the largest groups (coloured boxes). Dotted lines represent as yet uncertain relationships. Figure taken from Burki (2014).

The most recent **revision** of the **classification of eukaryotes** (based on ultrastructural, biochemical and molecular phylogenetic data) was provided by Adl et al. (2012). Eukaryotes are divided into five major assemblages or **supergroups**. Recently, Burki (2014) and Katz (2012) provided a review of eukaryote phylogeny based on most recent molecular genomic insights (Fig. 6).

The following supergroups are distinguished:

Opisthokonta: these mainly comprise the animals and Fungi.

Amoebozoa: form a monophyletic group; they include many free-living amoebae e.g. *Acanthamoeba*, *Vannella*. The Amoebozoa comprise the naked and testate lobose amoebae but also parasitic lineages of medical importance (*Entamoeba*), flagellated cells (*Phalansterium*) and mycetozoan slime molds.

The Opisthokonta and Amoebozoa are united in the larger group of Amorphea (Adl et al., 2012).

Excavata: a monophyletic group which comprises mainly heterotrophic protists, many of them anaerobes and parasites. Within the Excavata the Discoba are a group with many representatives of free-living protozoa, including the Heterolobosea (e.g. amoebae *Vahlkampfia*, *Naegleria*) and Euglenozoa (e.g. flagellates *Petalomonas*, *Notosolenus*).

Archaeplastida: these contain the main lineages of primary photosynthetic taxa: glaucophytes, rhodophytes or red algae and green organisms (green algae, land plants and non-photosynthetic parasitic taxa).

The SAR clade comprises the **Stramenopila**, **Alveolata** and **Rhizaria**.

Stramenopila are heterokonts, mostly biflagellated. They comprises a variety of organisms like the diatoms, the multicellular brown algae and oomycetes.

Alveolata are also a diverse lineage and include the group of Ciliophora, which includes all ciliate species.

Besides ciliates, also dinoflagellates and Apicomplexa (e.g. the malaria parasite *Plasmodium*) are part of the Alveolata.

Rhizaria include Cercozoa, Radiolaria and Foraminifera. A large diversity of free-living flagellates (e.g. cercozoan glissomonads) and amoeboflagellates belong to this supergroup.

	Endemicity	Ubiquity
Species diversity	High	Low
Rates of allopatric speciation ¹	Low, but see next	Low
Rates of non-allopatric speciation	High, ongoing	Low
Gene flow	Restricted	Constant
Dispersal rates	Low	High
Number of endemics	High, moderate	Low, none
Species abundancy	Only a few abundant; majority rare	Most species abundant
Rarity of species	Absolute (in space and time)	Relative (absence is due to unfavourable conditions)
Biosphere	Permanently rare	Temporarily rare
Species features	Phenotypic + genotypic ((semi)-cryptic species)	Phenotypic (morphospecies)

Table 1: Endemicity, according to Foissner (2008) vs ubiquity, according to Finlay (2004).

¹ Speciation occurring after populations become geographically isolated and diverge until reproductive barriers arise.

1.5 Diversity

The 'Everything is everywhere, the environment selects' statement was first postulated for microbial organisms by Beijerinck at the beginning of the 20th century (Beijerinck, 1913). Whether this is the case for free-living protists remains a controversial issue and different hypotheses exist (Table 1).

According to Foissner (2008) there are about 300 000 free-living protist species, many of them not yet described. Speciation is an ongoing process in many organisms. Protists have infinite possibilities to speciate due to their short generation times (enhancing mutations) and barriers to dispersal. Many protists persisted over geological time scales and survived great extinctions in habitat refugia.

In his '**moderate endemism model**', Foissner (2008, 1999a) stated a moderate rate of species extinction and a moderate (~30%) relative number of endemics, i.e. species with restricted distributions despite suitable habitats in other areas on earth.

Not all protist species have high abundances; only a few species are common, while the majority are rare with moderate, low or very low abundances. Rare species are rare in space and time, while abundant species are numerous, both spatially and temporally. Flagship species (i.e. conspicuous, easily recognizable and hard to miss species) with restricted distributions occur in a wide variety of protists and are the proof for protist endemism according to Foissner (2006). Human introduction of protist species is often neglected and may be a key factor in shaping the contemporary biogeographic ranges of protist species; an example concerns the introduction of diatoms to New Zealand (Vanormelingen et al., 2008).

In contrast, Fenchel and Finlay (2006) estimated that there are about 16600 free-living protists. According to **Finlay** (2002; 2004) microbial diversity is characterized by high abundance of organisms within populations, very short generation times and high dispersal rates, which results in low speciation rates (due to high gene flow). Due to these characteristics microorganisms are **ubiquitously dispersed** and have the ability to spread into virtually any habitat (by water circulation, groundwater networks, hurricanes, wind, transport via wet fur and feathers, etc.). Ubiquitous dispersal is 'the process of random dispersal across all spatial scales, up to the global scale.' Because of their astronomical population sizes, the probability that protist taxa will eventually cross physical barriers and are transported, via various environmental vectors or processes, to remote places is high. Absence of a taxon thus represents unfavourable local conditions, preventing establishment. Protist endemism does not exist and dispersal and extinction can rather be seen as neutral, statistical processes (related to abundance) rather than the result of species interactions.

Finlay believes most protist taxa are **cosmopolitan** which means 'thriving wherever its required habitat is realized'. For example, protozoan communities found in Arctic waters can also be isolated from Antarctic waters. Individual organisms are continuously dispersed worldwide, often as cysts. Consequently they end up in unsuitable habitats for population growth. These encysted, cryptic organisms remain invisible until the environmental conditions become optimal for growth. As such, sediments, soils, litter, etc. can be seen as seedbanks of diverse protist species (Pedrós-Alió, 2006).

1.6 Ecology

Ecology (Greek *oikos*, meaning home) is the scientific study of the distribution and abundance of **organisms** (i.e. *where* organisms occur, in what abundance, and *why*) and the **interactions** that determine distribution and abundance (Begon et al., 2006). The ecology of organisms is influenced by both **biotic** (other organisms) and **abiotic** (physical and chemical) factors.

In ecology a **community** is an assemblage of **populations** (i.e. a group of individuals of one species in a specific area) of potentially interacting species that co-occur in space and time.

Influence of environmental factors on protozoan communities

Abiotic factors are important determinants of protozoan ecology. Temperature, oxygen, pH, salinity, humidity and light are crucial environmental variables in the life cycle of protists and are conditions which can cause encystment in some FLP species (Greub and Raoult, 2004). An ecological characterization of ciliates species are summarized by Foissner and Berger (1996).

Temperature is one of the most important factors in the survival and distribution of protozoan species and their interactions with other microorganisms (Walochnik et al., 1998).

Most protozoan species are **mesophiles** which have a growth range between 15-45°C (optimum around 20-30°C) (Sigee, 2005).

The majority of protozoa depend on **aerobic energy metabolism** and most species have preferences for a specific **oxygen** tension (Fenchel, 2014). Most protozoa have O₂ preferences which correspond with those of their prey and some (mostly aerobic) protozoa show a chemosensory motile response to oxygen levels (Fenchel and Finlay, 2008). Based on their oxygen tolerance, protozoa can be divided into three groups: obligate anaerobes, microaerophiles and (fully) aerobes.

In general, free-living protozoa are **neutrophils**, which exist in **pH** between 5.5 – 8.5, but which have an optimum around neutrality. Pathogenic *Acanthamoeba* can grow at a pH ranging from 4 to 12 (Khan, 2006). Due to this broad pH range, FLP are able to withstand highly variable pH environments and colonize habitats with highly divergent pH.

Salinity is a major barrier for many single-celled eukaryotes. However, free-living protozoa may occur in environments ranging from **marine waters** (salinity 35 ‰) to **fresh water** (salinity <0.5 ‰).

Besides above abiotic factors, **humidity** and **light** will also have their influence on the occurrence, diversity and community composition of FLP in food and in food-related environments (i.e. environments where food is produced, processed or stored). Humidity is a determinant factor for many protozoan species. Primarily for their motility and as they are dependent on the available microscopic water (cf. soil habitats) to prevent them from desiccation (cf. cyst formation, section 1.3.).

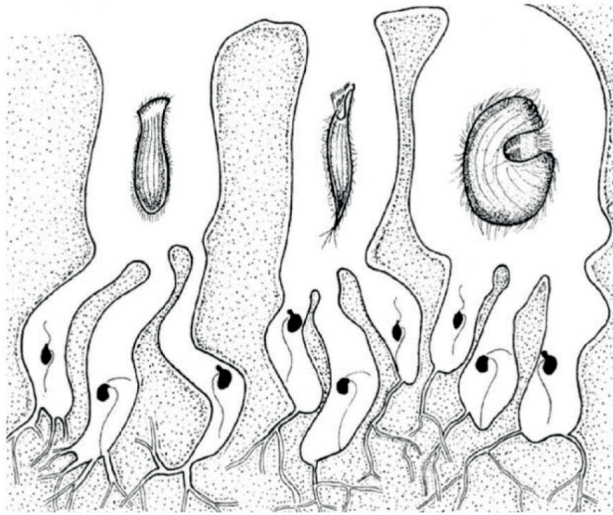


Figure 7. Schematic representation of protozoa in a soil environment. The larger protozoa on top all are ciliates, three species, each represented by one individual. The smaller protozoa below all are flagellates. Their habitat space is larger and they are more abundant than ciliates, but their habitat is qualitatively similar to that of ciliates, there are still only three species. The smallest spaces of the soil (i.e. the microscopic cracks and fissures) are too small to harbour protozoan species. Figure taken from Finlay and Fenchel (2001).

Biotic factors like food sources and interactions observed between free-living protozoa and other microorganisms like foodborne pathogens (see section 3.6.), play an important role in protozoan ecology. Other possible interactions with microorganisms are competition, predation, (ecto- or endo-) symbiosis, commensalism, mutualism and parasitism (Greub and Raoult, 2004).

In natural environments biotic factors are hardly measurable and very complex due to the presence of additional factors influencing the distribution and abundance of organisms. Moreover, anthropogenic factors (pollution, heavy metals, ...) also influence the ecology of protist communities.

Free-living protozoa in natural environments

Free-living protozoa are commonly found in **natural and anthropogenic environments**. In anthropogenic environments they can be found in swimming pools (Rivera et al., 1993), drinking water systems (Thomas and Ashbolt, 2011), air-conditioning units, hospitals (Thomas et al., 2006), health care facilities (Cateau et al., 2014), dental unit waterlines (Singh and Coogan, 2005), etc.

In natural environments FLP are common in soils and aquatic habitats.

Soil protozoa are totally dependent on the available microscopic water in soil cracks and pores (Esteban et al., 2006). Since soil is a highly variable habitat most soil protozoa are capable to form cysts to protect them against desiccation and most of the time remain in the encysted stage.

Foissner (1999b) stated that around 1600 soil FLP species were described, which is about 20-30% of the estimated FLP species number of soils. Their abundance is inversely correlated with their cell size, i.e. the smallest protozoa are the most abundant, while species richness remains constant (Fig. 7). In big pores, the biggest protozoa (i.e. ciliates) house, but mostly represented by only one individual; while in the small pores, the smallest protozoa (i.e. flagellates) house and are more abundant (20 times more abundant). The protozoan numbers increase with a factor 20 with decreasing cell size, while species richness remains constant. This is due to the fractal structure of soil, which results in qualitative similar 'micro' habitats.

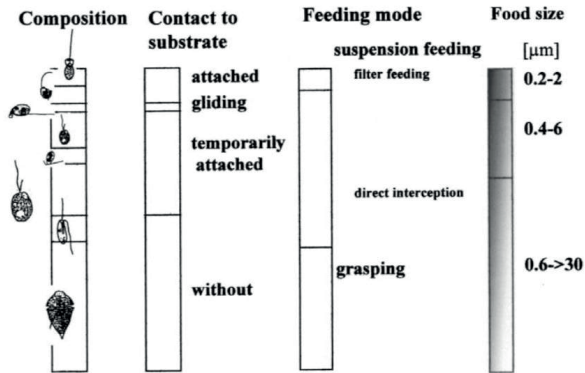
Within **aquatic** protozoa, a first differentiation must be made between pelagic and benthic organisms (Fig. 8).

For **pelagic** protozoa, i.e. free-swimming organisms, the position within the water column is related to the availability of nutrients, oxygen concentration and light. In fresh water environments nanoflagellates are major bacterial consumers (Pernthaler, 2005). Abundances of heterotrophic nanoflagellates (HNF) in pelagic habitats are between 20 -20000 HNF/ml (Boenigk and Arndt, 2002). Other protists dominating pelagic communities are heterotrophic flagellates, ciliates and dinoflagellates (Pfister et al., 2002; Sherr and Sherr, 1994).

Benthic environments like sediments and bottoms of rivers, lakes and seas, are covered with organic debris and detritus (Hausmann et al., 2003). Due to this high amount of organic matter, they contain large populations of sessile and motile protozoa like ciliates, flagellates and amoebae (Finlay and Esteban, 1998).

Seasonal and anthropogenic (pollution, wastewater plants) changes may influence the community composition of benthic and pelagic protozoa.

Pelagial



Benthic

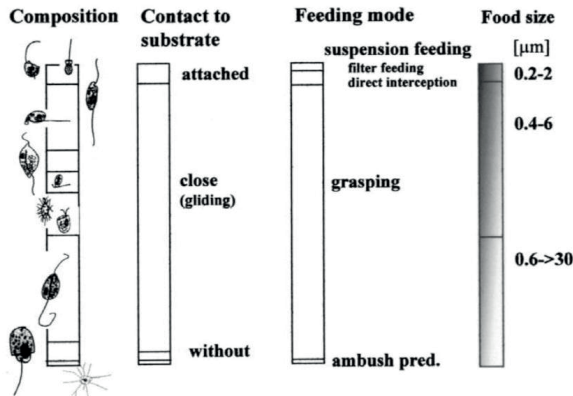


Figure 8. Comparison of benthic and pelagic flagellates on the basis of mean biomass. Right three bars give the percentage of total flagellate biomass that is formed by taxa characterized by their degree of contact to the substrate, their feeding mode or by their preferred spectrum of food size, respectively. Figure taken from Boenigk and Arndt (2002).

Functional role of free-living protozoa

Protozoan communities have **important functions** (grazing) in aquatic (pelagic and benthic) and terrestrial food webs (Sigeo, 2005). Due to these functions protozoa are important in the carbon turnover and nutrient recycling in the **food web** (Esteban et al., 2006).

Protists release nutrients (e.g. dissolved amino acids), not necessary for growth, into the environment. These nutrients stimulate growth of bacteria or other primary producers (Nagata and Kirchman, 1991). Bacterivorous grazing induces nutrient regeneration of nitrogen and phosphorus (Elser et al., 1990) and small bacterivorous protozoa close the microbial loop of the food chain, by forming a link between DOM consuming microorganisms (picoplankton, <1-2 μm) and (zoo)planktonic organisms that can only feed on cells with size >3-5 μm (Jürgens et al., 1996).

The occurrence of free-living protozoa on food and in food-related environments will be reviewed in section 3.

2 Methodology

2.1 Recovery and cultivation

In contrast to aquatic protistology, little information and few **protocols or methods** are available for **recovering FLP** from food-related environments or food matrices. Even less is known about how effective these methods are and which protocol is most appropriate to use for a specific matrix and morphogroup. Vaerewijck et al. (2010) confirmed cotton wool swabbing as an effective method to recover FLP from plastic surfaces like the interior walls and trays of refrigerators. To recover FLP from soft tissue surfaces, like lettuce leaves, washing, swabbing, excision and homogenization were applied and concluded to be valuable for the recovery of FLP. Washing lettuce leaves recovered the highest number of taxa (Vaerewijck et al., 2011). Depending on the morphogroup one wants to recover, a different approach should be used. The same rule can be applied for the matrix one wants to sample.

After the recovery of FLP from the target matrix, **cultivation** methods via **enrichment** are used to create suitable selective conditions that favour multiplication and growth of specific protist groups. Many selective media are available to cultivate FLP.

Commonly used is a solution containing inorganic salts like Page's Amoeba Saline (PAS) enriched with rice grains to stimulate bacterial growth (Page, 1988). Other possible media are enriched with plant infusions like cereal leaves. Organic-rich media like proteose peptone yeast extract medium (PPY) and proteose peptone yeast extract glucose (PYG) are more specific media and used to stimulate cultivation of *Tetrahymena* and *Acanthamoeba*, respectively. Agar-based media can also be used for FLP isolation. A standard method (Kalina and Page, 1992) to isolate and cultivate free-living amoebae from soil samples is to inoculate a drop of sample on non-nutrient agar (NNA) plates seeded with heat-killed bacteria (*E. coli*). Non-nutrient agar only supports limited growth of bacteria and results in multiplication of amoebae, who uses the heat-killed bacteria as food source.

Detailed guides on how to isolate and identify FLP can be found in literature (Berger and Foissner, 2003; Foissner, 1999b; Jeuck and Arndt, 2013; Smirnov and Brown, 2004).

Inadequate extraction and enrichment procedures result in undersampling of protist species, which is inevitable (Finlay et al., 2004; Foissner, 2008). Undersampling in space and time and sampling only a limited volume creates a problem for capturing rare species. However, Fenchel et al. (1997) observed that selective enrichment techniques may reveal rare and cryptic protist species.

Another obstacle is that some species remain viable but non-culturable (VBNC) under lab conditions. Due to competition of fast growing or highly abundant species, growth of other protozoan species may be suppressed.

It must be noted that lab culture conditions not always reflect environmental conditions and non-detection of an organism does not mean the absence of this organism. Until today no gold standard for protist species detection and identification exists (Boenigk et al., 2012).

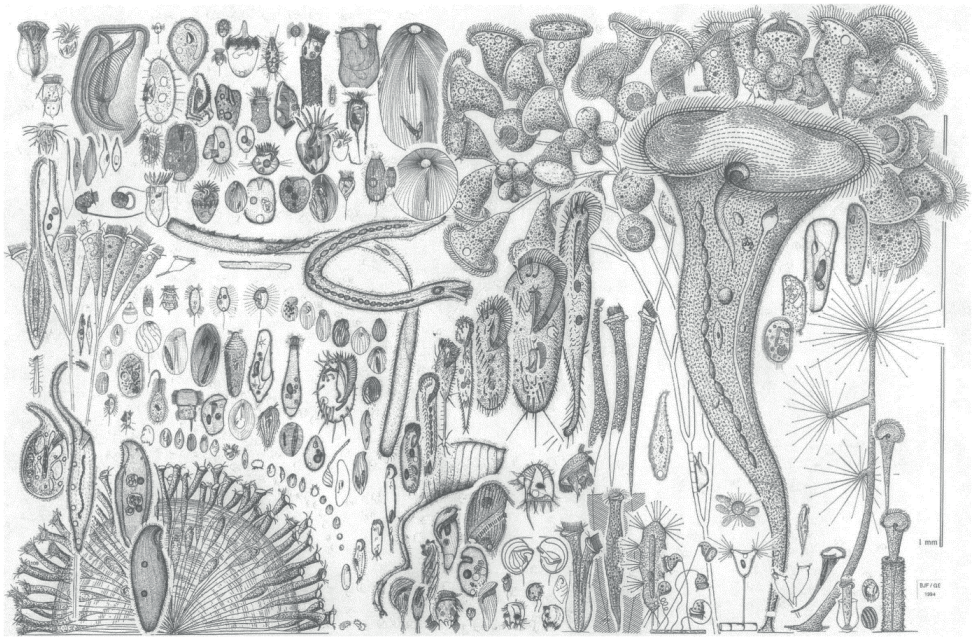


Figure 9. An example of the variety of free-living ciliated morphotypes. Figure taken from Finlay et al. (1996).

2.2 Identification and quantification

Identification

Identification of FLP is basically done using microscopy (**phenotypic** features) and molecular biological techniques (**genotypic** features).

The concept of 'morphotypes' (Fig. 9) refers to taxa that can be differentiated by means of their morphology alone when only analysed by light **microscopy** (Finlay et al. (1996). Identification is best performed on living FLP using (inverted) light microscopy. Some ciliate species require silver impregnation for more accurate identification (Foissner, 1999b). Movement is an important taxonomic characteristic to identify FLP species. To visualize more ultrastructural characteristics of the cell, electron microscopy (transmission electron microscopy, TEM; and scanning electron microscopy, SEM) are advised.

Identification based on morphology does not take 'cryptic species' into account. This concept was introduced to describe species that are phenotypically the same but genotypically different.

Since the 1990's, amplicon sequencing analyses (cultivation-independent - and cultivation-dependent methods) are increasingly used. These molecular methods can serve as complementary tools to morphological identification of FLP. **DNA-based methods** start with DNA extraction from environmental samples or cultures, followed by the amplification of target sequences by PCR. The PCR products can be cloned or sequenced or can be used for genetic profiling (see below).

Another technique which can be applied to identify specific FLP organisms is **Fluorescent *in situ* Hybridisation (FISH)**. A fluorescent labelled probe is designed that specifically binds to a complementary sequence of the target RNA. Due to the fluorescent labelling the complementary parts are visualized and individual microorganisms can be identified and quantified by fluorescent or confocal microscopy.

Community profiling or fingerprinting techniques are molecular screening methods to study and compare microbial communities and are based on the analysis of total community DNA or RNA. These relatively simple profiling methods (Forney et al., 2004) are based on the amplification of specific sequences by PCR. The PCR products are separated on acrylamide gels, resulting in different bands, which can be isolated from the gel and subjected to sequencing. Examples of community profiling techniques are denaturing gradient gel electrophoresis (**DGGE**) (Muyzer et al., 1993), Temperature Gradient Gel Electrophoresis (**TGGE**) (Marie et al., 2006), terminal restriction fragment length polymorphism (**T-RFLP**) (Clement et al., 1998) and Single-Strand Conformational Polymorphism (**SSCP**) (Medlin et al., 2006).

Next-generation sequencing (NGS) is a high throughput and cost-effective technique and nowadays used to sequence (meta) genomes of microorganisms. It is applied to check for sequence variations within single individual genomes, but also for metagenomics. These techniques allow direct sequencing of microbial communities. The cloning steps are avoided, which makes this a less expensive approach (Nolte et al., 2010).

Quantification

No standard **enumeration** method exists to quantify FLP. Definitely not for FLP in food or food-related environments. Free-living protozoa can be quantified by direct counting and culture from serial dilutions.

Direct counting of cells is mostly done after fixation of the sample and by means of a counting chamber (Sedgewick-Rafter cell, or Fuchs-Rosenthal counting grid or Bürker counting grid) and a microscope. Direct counting is suitable for samples containing a high concentration of cells as the limit of detection (LOD) is high, e.g. for Fuchs-Rosenthal LOD is 10^3 cells/ml and for the Bürker counting grid it is 10^6 cells/ml.

Flow cytometry is an example of another technique applied to detect and count protists and other eukaryotic microorganisms (Marie et al., 2010; Rose et al., 2004). Fluorescence Activated Cell Sorting (**FACS**) is a specialized type of flow cytometer and based on the size and fluorescence characteristics, due to natural cell pigments or staining, of cells. With FACS the sorted cells can be subjected to DNA extraction and construction of clone libraries with universal eukaryotic primers of the 18S rRNA gene (Marie et al., 2010).

The **Most Probable Number** (MPN) method is a technique to estimate numbers of organisms present in a sample (de Man, 1975). After creation of serial dilutions with the appropriate media, the samples are incubated at the temperatures ideal for the target organisms. Dilutions must be chosen carefully and to such a degree that inocula in the tubes will sometimes but not always contain viable organisms. Serial dilutions of the samples are examined in multiple tubes (e.g. 3-tubes, 5-tubes, 8-tubes, 10-tubes). After incubation, the presence or absence of a target organism in a specific dilution is noted per tube (i.e. a positive tube). Each tube whose inoculum contains at least one viable organism will produce visible growth. The outcome, i.e. the number of tubes and the number of tubes with growth at each dilution, will imply an estimate (the most probable number) of the original, undiluted concentration of micro-organisms in the sample (per g or ml). The MPN can be computed by means of an equation, MPN tables (e.g. US Food, Drug and Administration manual and tables) (Blodgett, 2006) or an online MPN calculator (<http://www.freedomdownloadcenter.com/Business/MiscCalculators/MPNCalculator.html>).

Assumptions for the MPN method: the micro-organisms are distributed randomly within the sample and only viable organisms are enumerated.

More information on how to use the MPN tables (including examples and calculations) is provided in 'Microbiologie van voedingsmiddelen – Methoden, principes en criteria' (Dijk et al., 2012) and Bacteriological Analytical Manual - Appendix 2 - Most Probable Number from serial dilutions (Blodgett, 2006).

3 Impact of free-living protozoa on food safety and public health

Despite thorough cleaning and disinfection, pathogenic bacteria persist on food products, in drinking water and in food related environments. Some FLP are known as opportunistic pathogens (see 3.5). In addition, associations between foodborne pathogenic bacteria and FLP have been shown to play a role in the protection and persistence of these bacteria (Vaerewijck et al., 2014). These associations will be highlighted in section 3.6. Consequently, FLP can have both a direct and indirect impact on food safety and on public health. In this perspective, an overview on the current knowledge about free-living protozoa on food and food-related environments is given below.

3.1 Free-living protozoa in food-related environments

While knowledge about foodborne bacterial pathogens is rapidly increasing, knowledge about FLP in food-related environments is still rare.

Studies of protozoan communities in commercial **poultry houses** or in farms in general, is limited (Baré et al., 2011, 2009; Snelling et al., 2006a). In the study of Baré et al. (2009) protozoan communities in broiler houses were studied and appeared to be highly diverse with a higher protozoan diversity in water samples (drinking supplies, pipelines) compared to dry samples (litter, food). In total 91 morphotaxa (based on morphology) and 22 phylotypes (based on gene sequencing data on a subset of samples) were identified. Flagellates and ciliates were more typical for water and dry environments, respectively, while amoebae did not show any preference for a particular habitat. Almost half of the identified taxa were amoebae, about one quarter were ciliates and the remainder were flagellates. The protozoan communities were strongly determined by farm-and habitat-specific features and persisted across consecutive rearing cycles (Baré et al., 2011).

Other studies on protozoan communities in food-related environments focused on FLP in meat-cutting plants (Vaerewijck et al., 2008) and domestic refrigerators (Vaerewijck et al., 2010).

To assess protozoan occurrence and diversity in **meat-cutting plants**, Vaerewijck et al. (2008) combined light microscopy data with DGGE gene sequence data. Based on microscopy 61 morphospecies were observed and based on sequencing data 49 phylotypes were identified. Protozoan species were found in samples taken from floor drains, knife sanitizers, meat residues, standing water on the floor; but also from surfaces in direct contact with meat like conveyer belts and cutting tables. FLP taxa were more common in sampling sites with high humidity and organic material.

Vaerewijck et al. (2010) concluded that FLP are part of the microbial communities present in **domestic refrigerators**. Vegetable trays were mostly FLP positive and contamination by FLP probably occurred via cross contamination between vegetables and the plastic surfaces. The most common morphogroups were amoebae and flagellates, with ciliates typically occurring in vegetable trays.

In the above mentioned studies the most common FLP taxa belonged to the genera *Vannella*, *Hartmannella*, *Vahlkampfia* and Acanthamoebidae for amoeba; the ciliate genera *Colpoda*, *Chilodonella*, *Cyclidium*, *Epistylis* and *Glaucoma*, and heterotrophic nanoflagellate genera like *Bodo*, *Pleuromonas*, *Petalomonas* and the cercozoans. These FLP species are typically also inhabitants of both soil and aquatic environments.

3.2 Free-living protozoa on food products and in drinking water

The occurrence of FLP on food products has barely been investigated. Especially the occurrence of FLP communities on fresh produce, i.e. ready-to-eat vegetables and fruits, which are eaten raw, is of concern from a public health perspective.

Recent data about FLP on food is limited to few studies by Gourabathini et al. (2008) and Vaerewijck et al. (2011) who investigated the occurrence of FLP on **lettuce, spinach and butterhead lettuce**, respectively.

Flagellates were the most abundant (up to 10^5 cells/g) morphogroup recovered from lettuce (Gourabathini et al., 2008; Vaerewijck et al., 2011). Commonly found flagellates were *Bodo saltans* and *Spumella*-like species (Vaerewijck et al., 2011). *Vannella* and *Hartmannella*, both amoebae genera, were observed via light microscopy but not detected through sequence data. Ciliate species mainly belonged to the genera of *Colpoda* and *Cyclidium*.

The studies by Napolitano (1982), Napolitano and Colletti-Eggolt (1984), Rude et al. (1984) and Sharma et al. (2004) only focused on amoebae and recovered them from vegetables and mushrooms.

To our knowledge no studies were performed on the occurrence of FLP communities on **other fresh produce** like fruits, meat, fish or shellfish. Trypanosomatids (i.e. parasitic flagellates) were recovered from fruits and seeds (Catarino et al., 2001). Protozoan epibionts were found on shells and gills of crustaceans (Fernandez-Leborans, 2010). Fish-infecting amoebae were isolated from freshwater and marine fish and are agents of fish diseases (Dyková and Lom, 2004).

More studies were conducted about the occurrence and diversity of free-living amoeba in **drinking water** (Delafont et al., 2013) and drinking water systems (Loret and Greub, 2010; Thomas and Ashbolt, 2011). Notwithstanding disinfection, these habitats are often characterized by biofilm formation (see section 3.4) and remainders of organic matter, conditions which favour FLP growth.

In drinking water, *Hartmannella* was the most dominant genus. Other common genera were *Naegleria*, *Vannella*, and *Acanthamoeba*. These amoebae were also recovered from drinking water systems, but also *Vahlkampfia*, *Saccamoeba* and *Echinamoeba* were frequently encountered. Even though these environments are dominated by amoebae, ciliates (*Vorticella*) and flagellates (chrysozoans and kinetoplastids) were also recovered from drinking water supplies in the studies of Otterholt and Charnock (2011), Poitelon et al. (2009) and Valster et al. (2009).

Apparently, not only drinking water but also bottled mineral water can contain *Acanthamoeba* spp. (Maschio et al., 2015).

3.3 Free-living protozoa in animals and humans

FLP are present in various animals, often with specific functions. For instance, flagellates in the hindgut of termites play a role in the digestion of lignocellulose (Mikaelyan et al., 2014) and ciliated rumen protozoa are important in the digestion process in cattle and sheep (Belanche et al., 2014).

Information about the presence of FLP in animals and humans is limited, apart from studies dealing with intestinal parasitic protozoa. Only a few studies and reports are available on free-living amoebae (FLA) recovered from stool and feces. In **human stool**, *Acanthamoeba* spp. and other opportunistic pathogenic FLA species like *Hartmannella* sp., *Hyperamoeba* sp. and *Vahlkampfia* sp. have been recovered from both healthy and infected individuals (Bradbury and Forbes, 2013; de Moura et al., 1985; Mergeryan, 1991; Zaman et al., 1999a, 1999b).

Acanthamoeba strains belonging to the most pathogenic genotype T4 have been isolated from wild squirrel and cow **feces** (Lorenzo-Morales et al., 2007b; Niyiyati et al., 2009).

Most research is focused on the occurrence of *Acanthamoeba* spp. in **humans**, due to their medical importance. Most clinical isolates belong to genotype T4 which are the causative agent of a common eye infection (keratitis) often found in contact lens wearers. Other pathologies caused by *Acanthamoeba* are primary amoebic meningitis (PAM) and granulomatous amoebic encephalitis (GAE); the latter can occur in immunocompromised patients and is mostly lethal. More information about opportunistic FLA is provided in section 3.5.

Baré et al. (2009) reported the presence of FLP in caecal droppings and feces of broiler chickens. Though, generally the occurrence and diversity of FLP in livestock or domestic animals is largely unexplored.

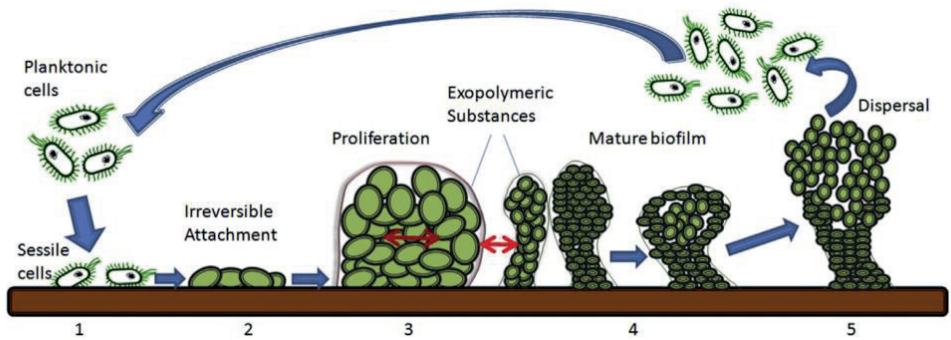


Figure 10. Schematic presentation of biofilm formation: (1) initial attachment; (2) irreversible attachment; (3) microcolony development by proliferation; (4) biofilm formation; and (5) dispersal. Figure taken from Rahaman Mizan et al. (2015).

3.4 Free-living protozoa in biofilms in the food environment

Biofilms consist of **microbial communities** embedded in an extracellular polymeric **matrix**, attached to a biotic or abiotic surface (Hall-Stoodley et al., 2004). Biofilm formation can be subdivided in five steps (Fig. 10): (1) initial attachment, (2) irreversible attachment, (3) microcolony development by proliferation, (4) biofilm formation/maturation and (5) dispersal.

Biofilms are heterogeneous layers of cells and the building blocks are **microcolonies** (Blaschek et al., 2007). They increase in thickness and complexity by the exchange of nutrients between the microcolony cells and surrounding environment. Biofilms can be single species or multispecies communities and are formed by redistribution of attached cells by surface motility; by binary fission of attached cells or by recruiting cells from the bulk fluid. Cells in biofilms have the ability to exchange genetic material and consequently may play a role in antibiotic resistance and increased virulence of pathogenic bacteria. By the formation of biofilms, microbial communities are **protected** against antimicrobial agents (Bridier et al., 2011). Cells embedded in the extracellular polymeric matrix express phenotypes that differ from the planktonic counterparts and show an increased resistance to biocides. Also due to this matrix, and the specific aggregation of microcolonies, the active molecules of biocides cannot penetrate and reach the internal layers of the biofilm.

Biofilm structural development and composition is nutrient dependent; e.g. *Escherichia coli* grows in biofilms when nutrients are available while other species grow in biofilms when there is nutrient deprivation. Besides nutrient availability, temperature, pH and oxygen are important factors in biofilm formation. The latter are also important in the ecology of free-living protozoa (see section 1.7.1). Due to the high amount of organic matter, biofilms provide ideal niches for free-living protozoa (Barbeau and Buhler, 2001). **Protozoan grazing** (Huws et al., 2005) is the most important factor in controlling biofilm community composition (Dopheide et al., 2008). These FLP species can act as reservoirs or hosts (see section 3.6) for waterborne pathogens like *Legionella pneumophila* (Thomas et al., 2006; Valster et al., 2010) and may as such reduce the efficacy of biocide treatment enhancing the creation of predation resistant species.

Knowledge about biofilms on food (Blaschek et al., 2007) and in food-related environments is still increasing. An overview of biofilms related with food or food-contact surfaces is provided by Rahaman Mizan et al. (2015). For example, microbial aggregates or biofilms on mung bean, alfalfa, clover and radish sprouts were confirmed by scanning laser microscopy (Fett and Cooke, 2005; Fransisca et al., 2011). Biofilms on food can be composed of a **consortium** of foodborne pathogens and spoilage microorganisms and may as such have a negative effect on food quality and food safety (Bridier et al., 2015).

3.5 Pathogenic and opportunistic free-living protozoa

Infectious and parasitic foodborne diseases are often caused by protozoan parasites like *Giardia duodenalis* (giardiasis), *Toxoplasma gondii* (toxoplasmosis), *Entamoeba histolytica* (amoebiasis), *Cryptosporidium parvum* (cryptosporidiosis), and *Cyclospora cayetanensis* (cyclosporiasis) (Dawson, 2005; Dorny et al., 2009).

Although the number of infections are low, e.g. the incidence rate of *Acanthamoeba* keratitis in wearers of contact lenses is 0.01-1.49 per 10000, (Khan, 2009), FLP may also cause **opportunistic infections** and as such have a direct impact on public health. Opportunistic infections mainly occur in immunocompromised patients (*Acanthamoeba* and *Balamuthia* encephalitis). Non-opportunistic infections are *Acanthamoeba* keratitis, *Naegleria* meningoencephalitis, and some cases of *Balamuthia* encephalitis, occurring in immunocompetent individuals (Schuster and Visvesvara, 2004a).

Besides *Acanthamoeba* spp., other (opportunistic) pathogenic free-living amoebae like *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia diploidea* are recognized as etiologic agents of encephalitis (Visvesvara et al., 2007). Opportunistic pathogenic amoebae don't depend on their hosts for transmission towards new hosts and no host to host transmissions of the specific diseases occur.

The first observation of potentially pathogenic free-living amoebae was realized by Culbertson (1958). Lab animals (monkeys) were used for poliovirus research. Culbertson observed that cytopathology in monkey kidney tissue, was caused by *Acanthamoeba* and not by the poliovirus, as expected. Culbertson isolated the amoebae, cultivated them and inoculated mice and monkeys with these *Acanthamoeba*. In the end all animals were killed by the amoebae.

The first case of **human** amoebic encephalitis was reported in Australia and was initially thought to be caused by *Acanthamoeba* but was later recognized as *Naegleria* (Fowler and Carter, 1965). The first human *Acanthamoeba* infections were observed in debilitated or chronically ill patients Martinez (Martinez, 1980).

Naegleria fowleri, an amoeboflagellate found in water and soil, is unable to survive in sea water. In general these amoebae are more sensitive to environmental conditions like desiccation and pH fluctuations. *N. fowleri* is the causative agent of primary amoebic meningitis (PAM), a sudden and severe disease, developing a few days after exposure to the water source. The early onset of infection (developing within several days upon exposure) and the difficult diagnosis results in death within 2 weeks after hospitalization (Schuster and Visvesvara, 2004a). Candidates that might serve as virulence factors are the release of the enzymes phospholipase or neuraminidase, and the creation of pores in target cell membranes that may have a lytic effect and aggressive phagocytotic activity.

Balamuthia mandrillaris was first isolated in the late 80's from the brain of a mandrill baboon, but was only described in 1993 by Visvesvara et al. (1993). *B. mandrillaris* is responsible for *Balamuthia* granulomatous amoebic encephalitis (BGE) and skin infections. These amoebae are present in soil, and once isolated they are slowly growing in cultures. BGE is a chronic disease lasting from a few weeks up to two years. Symptoms are hard to distinguish from viral or tuberculous meningitis, tuberculoma or neurocysticercosis. Typical early signs are fever, stiff neck, personality change, cerebellar ataxia, hemiparesis, aphasia and seizures (Martínez and Visvesvara, 2001).

One single reported human case of amoebic encephalitis was caused by *Sappinia diploidea* (Gelman et al., 2003). Symptoms were headache, vomiting, seizure, blurred vision and photophobia.

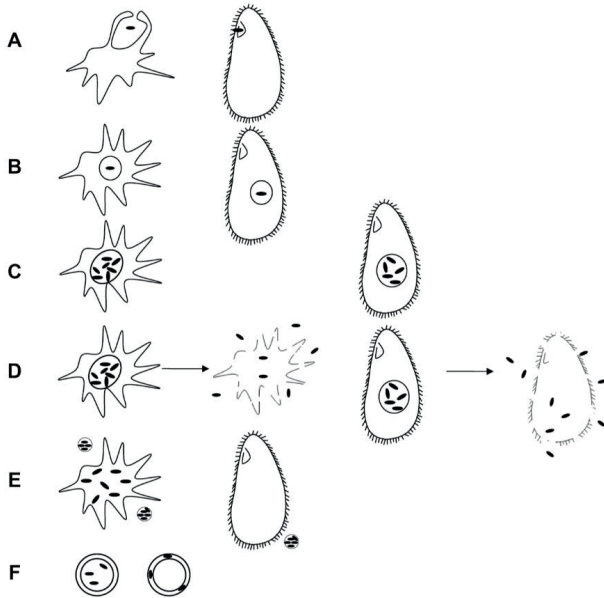


Figure 11. Overview of the different post-ingestional survival mechanisms of bacteria in free-living protozoa (left: amoeba, right: ciliate). (A) uptake of a bacterial cell; (B) survival of a bacterial cell in a protozoan; (C) survival and multiplication of bacterial cells in a protozoan; (D) multiplication and release of bacteria after lysis of the protozoan cell; (E) release of bacteria through expelled vesicles or fecal pellets; (F) survival of bacteria in cysts or cyst walls. Figure taken from Vaerewijck et al. (2014).

Other **emerging** pathogenic amoebae are *Hartmannella* and *Vahlkampfia* which are associated with amoebic keratitis (Abedkhozasteh et al., 2013; Niyiyati et al., 2010). Observations about pathogenic ciliates or flagellates are not so common. The pathogenic nature of scuticociliates like *Uronema marinum* in marine fishes like flounder and turbot was confirmed by several authors (Crosbie and Munday, 1999; Dragesco et al., 1995; Dykova and Figueras, 1994; Munday et al., 1997).

3.6 Interactions of free-living protozoa with foodborne pathogenic bacteria

In general, FLP and bacteria show a typical **predator-prey relationship**, by which the prey (bacteria) is detected, captured, ingested and digested by the predator (protozoan). However, other types of associations exist: neutral, mutualistic (or symbiosis, both have benefits of the association); commensalistic (one has benefits and the other one is not affected) or parasitic (one has benefits and the other one is harmed). Mutualistic interactions are the basis of the origin of mitochondria and plastids in eukaryotic organisms (Dyall et al., 2004) and some protozoa may use the symbionts' metabolites as a food source (Gast et al., 2009). Also ecto- or endosymbionts occur (Gast et al., 2009).

The term **amoeba resistant bacteria (ARB)**, was introduced by Greub and Raoult (2004), to define bacteria resistant to protozoan grazing and able to survive, grow and exit FLA after internalization. Other microorganisms like viruses and fungi can also be amoeba resistant. A distinction must be made between obligate endobionts (e.g. cyanobacteria, not discussed below) and bacteria with a transient stay inside the protozoan cell.

Potential defense strategies start with **pre-ingestional adaptations** by which bacteria avoid protozoan grazing (Matz and Kjelleberg, 2005). Pre-ingestion or extracellular defense mechanisms include changes in cell size and morphology (e.g. oversized bacteria) (Boenigk et al., 2004), toxin production, microcolony formation (Matz et al., 2004), modifications in bacterial cell surface molecules (Wildschutte et al., 2004) and increased bacterial motility (Matz and Jürgens, 2005).

Postingestional survival (Fig. 11) of bacteria in FLP can be categorized into three different groups: (1) bacteria which multiply and cause lysis of the protozoan cell; (2) bacteria which multiply without lysis of the protozoan cell and (3) bacteria which survive without multiplication inside the protozoan cell (Barker and Brown, 1994). Besides survival in trophozoites, survival of environmental bacteria inside cysts (*Legionella*) or cyst walls (*Mycobacteria*) has been reported (Adékambi et al., 2006; Kilvington and Price, 1990). A review of possible associations between free-living protozoa and their internalized pathogens is provided by Thomas et al. (2010).

Associations of free-living protozoa with four major foodborne pathogens *Campylobacter jejuni*, *Salmonella* spp., *E. coli* O157:H7 and *Listeria monocytogenes* were reviewed by Vaerewijck et al. (2014). These associations are dependent on external factors like food resources and temperature. But also biological factors like bacterial virulence and host susceptibility determine bacteria-FLP interactions.

Intraprotozoan growth and multiplication of *Campylobacter jejuni* was observed at 37°C but not at 25°C (Axelsson-Olsson et al., 2005). Baré et al. (2010) and Lambrecht et al. (2015a) observed prolonged survival of *C. jejuni* after co-cultivation with *Acanthamoeba castellanii*.

Foodborne pathogens and FLP share the same ecological niche and co-occur on food (Baré et al., 2011, 2009; Snelling et al., 2006a). Limited information about the co-occurrence of FLP and foodborne pathogens in food-related environments is available. Baré et al. (2011, 2009) confirmed the co-occurrence of FLP and *Campylobacter* in broiler houses. Vaerewijck et al. (2010) on the other hand did not detect (LOD <10 cfu/ml) *Listeria monocytogenes* in any of the examined refrigerators.

FLP can act as a **reservoir** for pathogenic bacteria, enhancing the persistence of these pathogens (Hadas et al., 2004). Baré et al. (2011, 2009) suggested that FLP (due to their ability to internalize campylobacters) play a role in the persistence of this foodborne pathogen in food-related habitats.

FLP can also act as a **protective niche** or shelter for bacteria against harsh environmental conditions (Barker and Brown, 1994; King et al., 1988). As mentioned previously, cysts but also trophozoites can protect internalized bacteria against physical and chemical unfavourable conditions like disinfectants and biocides (García et al., 2007; Kilvington and Price, 1990). *Acanthamoeba polyphaga* had a protective effect in the survival of *C. jejuni* when co-incubated at room temperature and at 4°C, in milk and orange juice (Olofsson et al., 2015). However this effect was not observed (neither in milk nor in orange juice) when heated to pasteurization temperatures.

Moreover, FLP are considered as **Trojan horses**, enabling internalized bacteria to pass the first line mammal immune system (Barker and Brown, 1994; Greub and Raoult, 2004).

Endocytobionts belonging to Parachlamydiaceae and Rickettsiales ruptured *Acanthamoeba* cells, which were isolated from a patient with keratitis (Scheid et al., 2008); suspecting that these bacteria may have a role in amoebal pathogenicity and use the amoebal cells as Trojan horses.

C. jejuni internalized in *A. castellanii* cells were fed to healthy broiler chickens in an experiment of Snelling et al. (2008). After one day of incubation, the chickens were colonized with *C. jejuni*.

FLP can serve as **vectors**, introducing pathogens into novel habitats, or as transmission routes towards hosts (Barker and Brown, 1994; Bouyer et al., 2007; Matz and Kjelleberg, 2005).

The ciliates *Tetrahymena* sp. and *Glaucoma* sp. are able to expel bacteria (like *Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*) containing vesicles (Brandl et al., 2005; Gourabathini et al., 2008).

Internalized bacteria can be released in the environment through induction of host cell lysis or through egestion of food vacuoles (vesicles) or its content (pellets). Bacteria inside these vesicles or pellets are more resistant to external stress factors (Anacarso et al., 2012). The role of *Acanthamoeba* in Legionnaires' disease was demonstrated by Rowbotham (1980) and Berk et al. (1998). Vesicles expelled by *Acanthamoeba* or the amoeba with internalized *Legionella* itself can be spread and cause the disease.

Additionally, protozoa are seen as **training grounds** and **evolutionary cribs** for foodborne pathogens (Molmeret et al., 2005), enhancing bacterial virulence and mediating bacterial gene transfer. A hyperinvasive strain of *Salmonella enterica* Typhimurium phage type DT104 was obtained after passage through rumen ciliated protozoa and *A. castellanii* (Brewer et al., 2011; Carlson et al., 2007; Xiong et al., 2010). Phage DT104 is a multiple antibiotic resistant strain (possession of SGI1, *Salmonella* genomic island 1) and is more virulent than related strains. Upon infection humans are two to three times more likely to be hospitalized compared to infection with other strains and infected calves are 13 times more likely to die than calves infected with antibiotic-sensitive *S. enterica* serovar Typhimurium. The study of Carlson et al. (2007) revealed that rumen protozoa are a mediator of DT104 hyperinvasion. After exposure to rumen protozoa there was an increased expression of SO13 (antibiotic-resistance gene, part of SGI1) and *hilaA* (a transcriptional regulator of *Salmonella* invasion). This upregulation resulted in hyperinvasiveness of DT104. However, this effect is a co-contribution both of the presence of rumen protozoa and SGI1.

Lateral gene transfer was observed between eukaryotic hosts, bacteria and viruses (Bertelli and Greub, 2012; Moliner et al., 2010). Amoeba-resistant bacteria are often specialized and consequently show a reduction in genome size compared to their ancestral relatives (Merhej et al., 2009). These intracellular bacteria may exchange genetic material. Amoebae represent a training place for these bacteria to develop resistance to digestion by macrophages which is needed for human infection, thus enabling these pathogens to become potential human pathogens. The transfer of plasmids responsible for the coding of antibiotic resistance genes is mediated by protozoan species and may be relevant for public health (Brewer et al., 2011; Matsuo et al., 2010; Oguri et al., 2011; Schlimme et al., 1997).

The presence of *Tetrahymena thermophila* promoted the transfer of genes encoding for extended-spectrum β -lactamase (ESBLs) genotype CTX-M-27 between *E. coli* clinical strains (Oguri et al., 2011). The frequency of gene transfer between *E. coli* strains increased significantly (average 4.2×10^{-6}) in presence of *T. thermophila* compared with the frequency of gene transfer in absence of the ciliate (average frequency 1×10^{-10}).

In general, the intracellular stay and survival of foodborne pathogenic bacteria inside FLP is of major significance for **public health and food safety** (Gourabathini et al., 2008; Greub and Raoult, 2004; Thomas et al., 2010).

An enthusiastic heart finds opportunities everywhere

Paulo Coelho

Scientific aims

Scientific aims

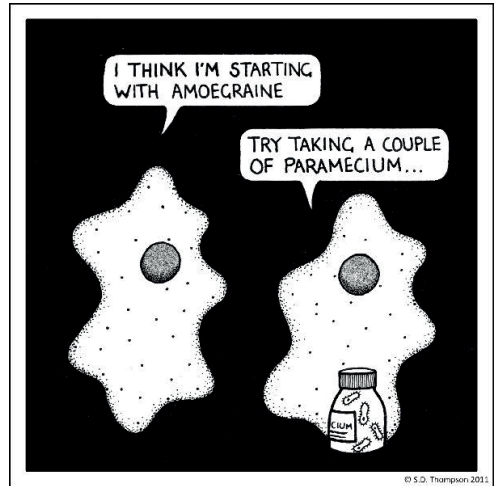
Interactions of free-living protozoa (FLP) have been implicated in the transmission and persistence of foodborne pathogenic bacteria. Some pathogenic bacteria have developed pre- and post-ingestional adaptations and are able to survive and grow inside FLP cells, enhancing their distribution to new habitats and hosts. Moreover, FLP can protect and shelter pathogenic bacteria against unfavourable physical and chemical conditions like desiccation, disinfectants and biocides. However, FLP are not routinely incorporated in microbiological surveys as they are generally considered to be harmless. As a result, data on the occurrence and diversity of FLP on and in food and in food-related environments is still scarce. Also information is completely lacking about the occurrence of FLP in livestock animals, which have been identified as main reservoirs for different foodborne pathogens. Moreover, validated protocols to recover FLP from the above matrices have not been developed.

This thesis forms part of a large research effort aimed at understanding the role of FLP in the persistence and transmission of pathogenic bacteria in food and food-related environments. It complements previous work carried out in the labs of the promotors which documented the presence and diversity of FLP in broiler houses, meat-cutting plants, domestic refrigerators and on vegetables (lettuce).

Since effective protocols are essential and required in the need for a profound insight in the 'FLP global picture', we first developed and evaluated protocols for recovering and quantifying FLP from the examined matrices. These protocols were then applied taking the **general aim to provide a detailed description of the diversity and occurrence of FLP in and on food products and in food-related environments**. More specifically, we examined dishcloths (chapter 1), vegetable sprouts (chapter 2) and animals (gastrointestinal tract and feces of pigs, chapter 3).

The **specific aims** of this research were:

- to assess the **occurrence, abundance and diversity of FLP** in combination with detection and enumeration of the most **common foodborne pathogens** and enumeration of the bacterial load on **dishcloths**. In addition, the potential impact of environmental factors on both FLP and bacterial presence and abundance were evaluated (chapter 1);
- to determine **FLP occurrence, abundance and diversity** and bacterial load on **vegetable sprouts**. Within-lot and between-lot variability were evaluated, and variation in protozoan community composition in relation to FLP numbers, species richness and environmental variables were analysed (chapter 2);
- to explore the **occurrence and diversity of FLP** in feces and throughout the **gastrointestinal tract of pigs** (chapter 3)



© S.O. Thompson 2011

I have no special talents I am only passionately curious
Albert Einstein

Chapter 1.

Co-occurrence of free-living protozoa and
foodborne pathogens on dishcloths:
implications for food safety

Adapted from: N. Chavatte, J. Baré, E. Lambrecht, I. Van Damme, M. Vaerewijck, K. Sabbe, K. Houf, 2014. Co-occurrence of free-living protozoa and foodborne pathogens on dishcloths: implications for food safety. *International Journal of Food Microbiology*. 191, 89-96.

Abstract

In the present study, the occurrence of free-living protozoa (FLP) and foodborne bacterial pathogens on dishcloths was investigated. Dishcloths form a potentially important source of cross-contamination with FLP and foodborne pathogens in food-related environments. First various protocols for recovering and quantifying FLP from dishcloths were assessed. The stomacher technique is recommended to recover flagellates and amoebae from dishcloths. Ciliates, however, were more efficiently recovered using centrifugation. For enumeration of free-living protozoa on dishcloths, the Most Probable Number method is a convenient method. Enrichment was used to assess FLP diversity on dishcloths (n=38). FLP were found on 89% of the examined dishcloths; 100% of these tested positive for amoebae, 71% for flagellates and 47% for ciliates. Diversity was dominated by amoebae: vahlkampfiids, vannellids, *Acanthamoeba* spp., *Hyperamoeba* sp. and *Vermamoeba vermiformis* were most common. The ciliate genus *Colpoda* was especially abundant on dishcloths while heterotrophic nanoflagellates mainly belonged to the genus *Bodo*, the glissomonads and cercozoans. The total number of FLP in used dishcloths ranged from 10 to 10^4 MPN/cm². Flagellates were the most abundant group, and ciliates the least abundant. Detergent use was identified as a prime determinant of FLP concentrations on used dishcloths. Bacterial load on dishcloths was high, with a mean total of aerobic bacteria of $7.47 \log_{10}$ cfu/cm². *Escherichia coli* was detected in 68% (26/38) of the used dishcloths, with concentrations up to $4 \log_{10}$ cfu/cm². Foodborne pathogens including *Staphylococcus aureus* (19/38), *Arcobacter butzleri* (5/38) and *Salmonella enterica* subsp. *enterica* ser. Halle (1/38) were also present. This study showed for the first time that FLP, including some opportunistic pathogens, are a common and diverse group on dishcloths. Moreover, important foodborne pathogens are also regularly recovered. This simultaneous occurrence makes dishcloths a potential risk factor for cross-contamination and a microbial niche for bacteria – FLP interactions.

Keywords

Free-living protozoa (FLP); foodborne pathogens; dishcloths; enumeration; diversity; Most Probable Number (MPN)

1 Introduction

Free-living protozoa (FLP) are unicellular heterotrophic eukaryotic organisms with a widespread distribution in aquatic (freshwater and marine) and terrestrial ecosystems (Hausmann et al., 2003). They are also present on food, like vegetables (Gourabathini et al., 2008; Vaerewijck et al., 2011), and in food-related environments, such as broiler houses (Baré et al., 2011, 2009; Snelling et al., 2005), meat-cutting plants (Vaerewijck et al., 2008), and domestic refrigerators (Vaerewijck et al., 2010).

Free-living protozoa are important predators of bacteria (Pernthaler, 2005; Sherr and Sherr, 2002). Some bacteria, however, are able to resist protozoan grazing, and can survive inside FLP cells. These include various foodborne pathogens such as *Campylobacter jejuni* (Axelsson-Olsson et al., 2005; Baré et al., 2010), *Escherichia coli* O157:H7 (Brown and Barker, 1999), *Listeria monocytogenes* (Zhou et al., 2007), *Salmonella* spp. (Gaze et al., 2003; Tezcanmerdol et al., 2004), *Staphylococcus aureus* (Huws et al., 2008), *Arcobacter butzleri* (Medina et al., 2014) and *Yersinia enterocolitica* (Lambrecht et al., 2013). However intraprotozoan survival and/or replication depends on various factors such as bacterial strain, environmental conditions, etc. (Schuppler, 2014; Vaerewijck et al., 2014). As a result, FLP can act as vectors, introducing pathogens into novel habitats, or as transmission routes towards hosts (Berk et al., 1998; Bouyer et al., 2007; Brandl et al., 2005; Matz and Kjelleberg, 2005; Snelling et al., 2008). In addition, they can also act as a protective niche or shelter for bacteria against harsh environmental conditions (Barker and Brown, 1994; King et al., 1988; Snelling et al., 2005), or even as training grounds and evolutionary cribs for foodborne pathogens (Molmeret et al., 2005), enhancing bacterial virulence and mediating bacterial gene transfer. This points towards a role of FLP in the epidemiology of foodborne pathogenic bacteria with significant implications for food safety and public health (Gourabathini et al., 2008; Greub and Raoult, 2004; Thomas et al., 2010; Vaerewijck et al., 2014).

Dishcloths are commonly used to clean surfaces, kitchen equipment and utensils, crockery and cutlery, etc., enhancing the potential for cross-contamination between food-related habitats (Kusumaningrum et al., 2003; Mattick et al., 2003). Foodborne bacteria are also commonly present in kitchens and households (Jackson et al., 2007; Macías-Rodríguez et al., 2013; Scott et al., 2008) and form an important source of foodborne illness (Luber, 2009; Newell et al., 2010). High bacterial concentrations (up to 5 log₁₀ cfu/ml) have been reported from dishcloths (Gorman et al., 2002). The moist conditions, presence of food residues, and storage at room temperature favours the survival and even growth of (pathogenic) bacteria, such as *Listeria* spp., *E. coli*, *Salmonella* spp., *S. aureus* and *C. jejuni* (Beumer et al., 1996; Gorman et al., 2002; Hilton and Austin, 2000; Josephson et al., 1997).

At present no information on the occurrence of FLP on dishcloths and the simultaneous occurrence of foodborne bacterial pathogens, is available. Furthermore, in contrast to bacteriological analysis, to date no standardized protocols for recovering and quantifying FLP from dishcloths are available.

The aims of the present study therefore were: (a) to develop and evaluate protocols for recovering and quantifying FLP from dishcloths; (b) to assess the occurrence, total number and diversity of FLP in used dishcloths; (c) to detect and enumerate bacteria in dishcloths, with special focus on most common foodborne pathogens; (d) to assess the simultaneous occurrence between foodborne pathogens and free-living protozoa on dishcloths; (e) to evaluate which factors have an impact on both FLP and bacterial presence and concentrations in used dishcloths.

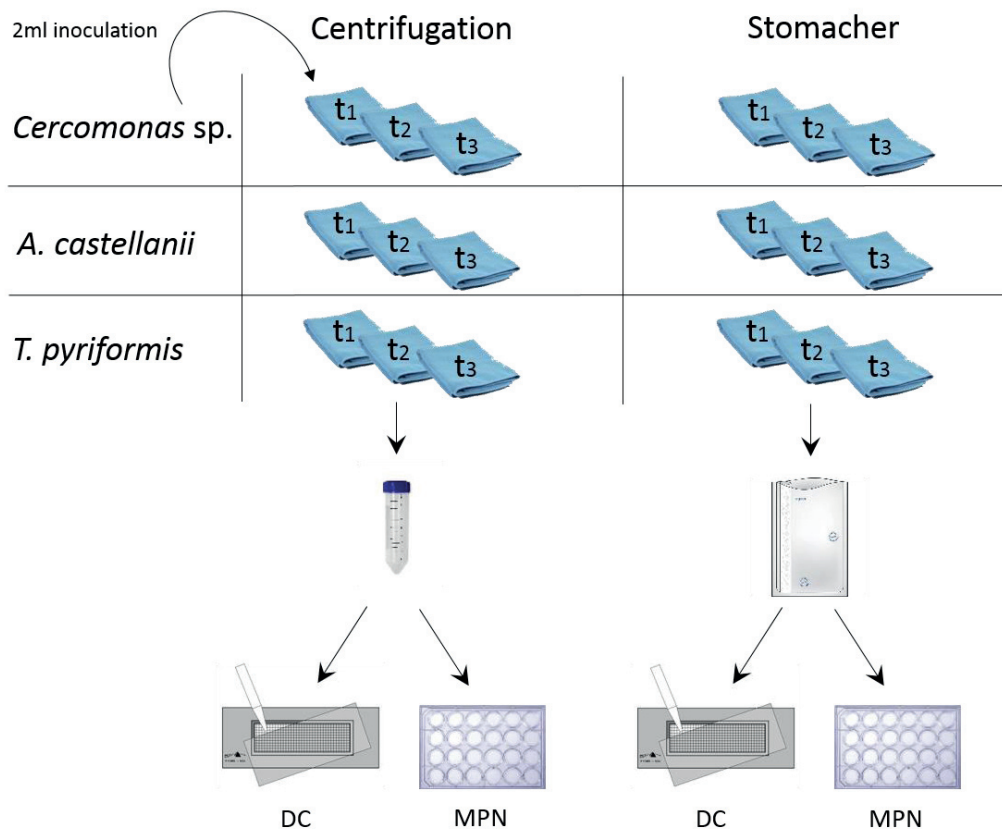


Figure 1. Overview of the experimental set-up for the evaluation of the recovery protocols. At experiment 1, for each recovery method (centrifugation, stomacher) and each model organism (*Cercomonas* sp., *A. castellanii*, *T. pyriformis*) 3 timepoints (t₁₋₃) were evaluated (n=18). For each timepoint enumeration was performed by direct counting (DC) and the Most Probable number (MPN) method, in triplicate and with the 3-tube test, respectively. At experiment 2, the same set-up was repeated for two timepoints (n=12).

2 Material and methods

2.1 Development and evaluation of two protocols for the recovery and quantification of FLP from dishcloths

In order to develop a protocol for recovering and quantifying FLP from dishcloths, spiking experiments with known concentrations of FLP were performed. Two recovery protocols were tested: (i) the centrifugation protocol which was optimized for the recovery of FLP and (ii) the stomacher protocol which is frequently used for bacteriological analysis (Lee, 2010; Sharma et al., 2009). For quantification of FLP from dishcloths, the Most Probable Number (MPN) method and a direct counting method were evaluated.

2.1.1 Cultivation of FLP

Three FLP species, representing the three main protozoan morphogroups, i.e. ciliates, flagellates and amoebae, were selected as model organisms. *Tetrahymena pyriformis* (ciliate, CCAP 1630/1W) and *Acanthamoeba castellanii* (amoeba, ATCC 30324) were cultivated axenically in 75 cm² tissue culture flasks (TPP AG, Trasadingen, Switzerland) in Proteose Peptone Yeast Extract Medium (PPY) (CCAP, Oban, UK, <http://www.ccap.ac.uk>) and Proteose peptone Yeast extract Glucose (PYG) (ATCC, <http://www.lgcstandards-atcc.org>), respectively. *Cercomonas* sp. (flagellate) was previously isolated from a meat-cutting plant (Vaerewijck et al., 2008) and cultivated non-axenically in 75 cm² tissue culture flasks in Page's Amoeba Saline (PAS, CCAP recipe), enriched with sterile, uncooked rice grains as a carbon source to stimulate bacterial growth (Patterson, 1998). *T. pyriformis* and *A. castellanii* were grown for 4 days at 25°C and *Cercomonas* sp. was grown for 5 days at 25°C. The protozoan cultures were centrifuged [*T. pyriformis* at 840 g for 10 min (Faulkner et al., 2008); *A. castellanii* and *Cercomonas* sp. at 540 g for 10 min (Vaerewijck et al., 2012)] and the supernatant was removed. The pellet was washed twice in PAS. The initial number of protozoan cells was determined using a Fuchs-Rosenthal counting chamber (Brand, Wertheim, Germany). For *A. castellanii*, enumeration and viability testing was assessed using the trypan blue exclusion assay (Gao et al., 1997). *T. pyriformis* and *Cercomonas* sp. were counted after fixation with 37% formaldehyde. The final number of organisms to be used in the spiking experiments was then adjusted to a final concentration of 1×10^6 cells/ml for *T. pyriformis* and *A. castellanii* and 1×10^4 cells/ml for *Cercomonas* sp.

2.1.2 Evaluation of the protocols

The protocols were evaluated by spiking known concentrations of each protozoan morphogroup onto sterile dishcloths (Fig. 1). By analogy with bacteriological dishcloth protocols (Koo et al., 2013; Lee, 2010), cotton dishcloths were cut into 6 cm x 6 cm segments (n=30), autoclaved at 110°C for 20 min and stored in sterile Petri dishes. Three ml of PAS was added to the sterile dishcloths, followed by spiking with 2 ml of the final concentration (see above) of the protozoan cultures. For the centrifugation method, the samples were transferred to a 50 ml test tube containing 20 ml PAS and centrifuged at 540 g for 5 min. After removal of the dishcloths, the remaining liquid was vortexed for 10 s and ten ml was used for enumeration of FLP (see below). For the stomacher method, samples were transferred to a stomacher bag, and homogenized for 2 min after addition of 20 ml PAS (Lee, 2010; Sharma et al., 2009).

The dishcloths were then carefully removed and the homogenate was vortexed for 10 s. Ten ml of the homogenate was used for enumeration. Free-living protozoa (*T. pyriformis*, *A. castellanii* and *Cercomonas* sp.) were enumerated in parallel by the Most Probable Number method (MPN; 3-tube test) (Blodgett, 2006; Ronn et al., 1995) and by a direct counting method. Both centrifuged and stomached suspensions were first vortexed to ensure homogeneity before further enumeration by MPN or direct counts.

For the MPN, suspensions were diluted in TSB/PAS (Tryptic Soy Broth diluted 1:1000 in PAS) to 10^{-5} for *T. pyriformis* and *A. castellanii* and to 10^{-4} for *Cercomonas* sp. and one ml was added in triplicate into 24 well microtiter plates (Ronn et al., 1995; Vaerewijck et al., 2011). Control wells were filled with one ml TSB/PAS only. The microtiter plates were incubated in the dark at $20 \pm 2^\circ\text{C}$. After one week incubation, the wells were examined microscopically for the presence of organisms (Ronn et al., 1995; Vaerewijck et al., 2010). The MPN was calculated using the US Food, Drug and Administration manual and tables (Blodgett, 2006), based on the following equation:

$$\sum_{j=1}^k \frac{g_j m_j}{1 - \exp(-\lambda m_j)} = \sum_{j=1}^k t_j m_j$$

Where $\exp(x)$ means e^x ,

λ is the concentration,

k denotes the number of dilutions,

g_j denotes the number of positive (or growth) tubes in the j th dilution,

m_j denotes the amount of the original sample put in each tube in the j th dilution,

t_j denotes the number of tubes in the j th dilution.

For direct counting, after fixation of the homogenate with 37% formaldehyde, one ml was transferred to a Sedgewick-Rafter counting chamber (Pyser-SGI Ltd., Kent, UK) and protozoan cells were counted using an Olympus CX41 microscope.

All experiments were performed in duplicate over time.

2.2 Occurrence, enumeration and diversity of FLP on used dishcloths

Based on results (see 3.1.) obtained from the spiking experiments, both recovery methods (centrifugation and stomacher) were applied to retrieve FLP from used dishcloths. For quantification of FLP from used dishcloths, only the MPN-method was applied. Direct counting was excluded as after fixation of the sample, it was not possible to differentiate all three protozoan groups (data not shown).

Used dishcloths (n=38) were collected from unrelated households (colleagues, staff, neighbours, friends, ...), stored in sterile bags and processed the same day. Each dishcloth was cut into 4 segments (approx. 15 cm x 15 cm). New, unused dishcloths (bought in sealed plastic bags) were included as controls.

One segment of the used and control dishcloths was processed with the centrifugation protocol in combination with the MPN method. The second segment, diagonally opposed of the first segment, was utilized for the stomacher protocol in combination with the MPN method. Both methodologies were applied as described above. After three to four days and after one week, the wells were examined for presence of FLP, which were then further identified (see below).

Parameter	Detection	Enumeration
Total aerobic bacteria		ISO4833 Decimal dilution series Pour plate 1 ml onto PCA Incubate at 30°C for 72h Colony count
<i>Listeria monocytogenes</i>	AFNOR AES-10/3-09/00 5 ml in 45 ml ½ Fraser broth Incubate at 30°C for 24h (±3h) Streak out onto ALOA Incubate at 37°C for 24h (±3h) Confirmation test (if necessary)	ISO 11290-2/A1 Decimal dilution series Pour plate 1 ml onto ALOA Incubate at 37°C for 24h (±3h) Colony count Confirmation test (if necessary)
<i>Staphylococcus aureus</i>	(Kim & Oh 2010): 5 ml in 45 ml TSB (+10% NaCl) Incubate at 37°C for 24h Streak out onto BPA Incubate at 37°C for 24h	ISO 6888-2 Decimal dilution series Pour plate 1 ml onto BPA Incubate at 37°C for 18-24h Colony count
<i>Salmonella</i> spp.	ISO 6579-FDAmd1 5 ml in 45 ml BPW Incubate at 37°C for 18h Pour 0.1 ml in 3 drops onto the MSRV plate Incubate at 41.5°C for 24h (±3h) Interpretation; if positive: Streak out onto XLD Incubate at 37°C for 24h (±3h) Confirmation test (if necessary)	(Nye 2002) Decimal dilution series Pour plate 1 ml onto XLD Incubate at 37°C for 16-24h Colony count
<i>Campylobacter</i> spp.	ISO 10272-1 5 ml in 45 ml Bolton broth Incubate micro-aerophilic at 41.5°C for 44h±4h Streak out onto mCCDA and CFA Incubate micro-aerophilic at 41.5°C for 44h±4h Confirmation tests (if necessary)	ISO 10272-2 Decimal dilution series Pour plate 1 ml onto mCCDA and CFA Incubate micro-aerophilic at 41.5°C for 48h Colony count Confirmation tests (if necessary)
<i>Escherichia coli</i>	AFNOR BRD-07/14 5 ml in 45 ml BPW Incubate at 37°C for 16-24h Streak out onto TBX Incubate at 44°C for 24h ±2h Confirmation tests (if necessary)	ISO 16649-2 Decimal dilution series Pour plate 1 ml onto TBX Incubate at 44°C for 21h (±3h) Colony count
<i>Yersinia enterocolitica</i>	ISO 10273 5 ml in 45 ml PSB broth Incubate at 25°C for 2 days 0.5ml PSB in 4.5ml 0.5% KOH Streak out onto CIN Incubate at 30°C for 24h(±2h) Confirmation tests (if necessary)	Decimal dilution series Pour plate 1 ml onto CIN Incubate at 30°C for 24h (±2h) Colony count
<i>Arcobacter</i> spp.	(Houf et al. 2001) 5 ml in 45 ml <i>Arcobacter</i> enrichment broth Incubate micro-aerobic at 28°C for 48h Streak out onto <i>Arcobacter</i> selective agar Incubate micro-aerobic at 28°C for 48h	(Houf et al. 2001) Pour plate 1 ml onto <i>Arcobacter</i> selective agar Incubate micro-aerobic at 28°C for 48h Colony count

Table 1: Overview of applied methods for bacteriological analyses (detection and enumeration) of dishcloths. PCA=plate count agar, ALOA=agar *Listeria* according to Ottoviani & Agosit, TSB=tryptic soy broth, BPA= Baird Parker agar, BPW=buffered peptone water, MSRV = modified semi-solid Rappaport-Vassiliadis agar, XLS=xylose lysine desoxycholate, mCCDA= modified charcoal-cefoperazone-deoxycholate agar, CFA= Campy food agar, TBX= tryptone bile X-glucuronide agar, PSB=peptone-sorbitol-bile, CIN=cefsulodin-irgasan-novobiocin.

A third dishcloth piece was applied for the morphological identification of FLP by an enrichment procedure. Therefore, the dishcloth pieces were transferred to a Petri dish (\varnothing 14 cm) containing 75 ml PAS. These Petri dishes were incubated in the dark at $20 \pm 2^\circ\text{C}$ for enrichment. Autoclaved dry rice grains were added to stimulate bacterial growth (Patterson, 1998). The enrichment cultures were examined after three to four days and after one week (see below).

The fourth segment of the used and control dishcloths was used for bacteriological analysis (see below). Further, a questionnaire was filled in to collect qualitative information on factors which may influence FLP numbers on the dishcloths: fabric type, number of days in use, usage for washing dishes or not, water and detergent usage and absence/presence of pets in the kitchen environment.

Free-living protozoa detected in the MPN (dishcloth segment one and two) or enrichment (segment three) cultures were identified on the basis of morphology and locomotion by light microscopy using standard taxonomic identification sources (Foissner and Berger, 1996; Jeuck and Arndt, 2013; Lee et al., 2005; Page, 1988; Patterson, 1998; Smirnov and Brown, 2004; Smirnov and Goodkov, 1999; Visvesvara and Schuster, 2008a, 2008b). Organisms were identified to the genus or species level where possible. All taxa were classified according to the recent eukaryote classification of Adl et al. (2012). Organisms that were not assignable to a known species or genus were assigned to a morphogroup (ciliate, flagellate or amoeba).

2.3 Bacteriological analysis of used dishcloths

The fourth segment of the used and control dishcloths was used for bacteriological analysis. Each dishcloth piece was analysed for the presence of the pathogenic bacteria: *Campylobacter* spp., *E. coli*, *L. monocytogenes*, *Salmonella* spp., *S. aureus* and *Y. enterocolitica* using normalized protocols (Table 1) and *Arcobacter* spp. based on Houf et al. (2001). In parallel, total aerobic bacteria (TAB) counts were performed. Each dishcloth piece was transferred to a stomacher bag and buffered peptone water was added to a final weight of 75 g to obtain a ratio of $1 \text{ ml} \sim 3 \text{ cm}^2$. The samples were homogenized for 2 min. Subsamples of the homogenate were used for detection and enumeration of (pathogenic) bacteria. For detection, 5 ml of the homogenate was enriched in 45 ml of a specific enrichment broth and streaked onto selective agar plates (LOD = 0.07 cfu/cm^2). For the enumeration, decimal dilution series were prepared and 1 ml of the homogenate was pour plated in selective agar plates (LOQ = 0.33 cfu/cm^2). More details are provided in Table 1. Suspected *Salmonella* colonies were biochemically (API test) and serologically identified, while suspected *Arcobacter* colonies were identified by multiplex PCR (Doudiah et al., 2010) and characterized by ERIC-PCR (Houf et al., 2002).

2.4 Data analysis

For quantitative analyses, all FLP and bacterial concentrations were expressed as MPN/cm² and cfu/cm², respectively. Bacterial concentrations were log₁₀ (x+1) transformed before further analyses. Data from samples below the limit of quantification (LOQ) were set to one-half of the enumeration threshold. Samples below the protozoan LOQ (0.075 MPN/cm² with the MPN methodology) were set to 0.04 MPN/cm², while samples below the bacterial LOQ (0.33 cfu/cm²) were set to 0.17 cfu/cm². For samples above the upper FLP LOQ (>6000 MPN/cm²), the highest MPN count (6000 MPN/cm²) was used. Enrichment and MPN cultures were considered FLP-positive if at least one viable protozoan was observed on at least one time point.

All statistical analyses were carried out using the statistical software package Stata/MP 12.1 (StataCorp, 2011).

The spiking experiments were performed in duplicate over time. Per condition, *i.e.* combination of recovery and quantification methods, three technical replicates were performed. To evaluate the effect of the recovery (centrifugation vs. stomacher) and quantification (MPN vs. direct counting) methodology for FLP from spiked dishcloths, negative binomial regression analyses were performed per morphogroup.

To evaluate the effect of dishcloth usage and kitchen practices on the total FLP concentration in the used dishcloths, negative binomial regressions were performed. To evaluate the effect of the total aerobic bacteria counts on the presence/absence of each FLP morphogroup in the used dishcloths, logistic regressions were executed.

In order to analyse patterns in FLP species composition (presence/absence of FLP species) and FLP numbers (log (x+1) transformed total numbers of ciliates, flagellates and amoebae) in relation to bacterial (TAB counts and presence/absence of pathogens) and environmental data (see 2.2.), multivariate (ordination) analyses were performed using the program CANOCO for Windows, version 4.5 (ter Braak and Smilauer, 1998). As a preliminary detrended correspondence analysis (DCA) suggested that the underlying response patterns in the FLP species composition data set were unimodal (length of gradient > 4, Jongman et al. (1995)), canonical correspondence analysis (CCA) was used to investigate the relationship between the FLP data and the bacteriological and environmental data. As for the FLP numbers data set the responses were linear (length of gradient < 2), redundancy analysis was used. Forward selection with Monte Carlo permutation testing was used to select the minimal set of bacteriological and environmental variables that contributed significantly and independently to explaining the variation in both FLP data sets (ter Braak and Smilauer, 1998).

Organism	Centrifugation		Stomacher	
	MPN	DC	MPN	DC
<i>Tetrahymena pyriformis</i>	4.72 x 10 ³ (8.59)	3.00 x 10 ³ (5.45)	9.17 x 10 ² (1.67)	5.42 x 10 ² (0.99)
<i>Acanthamoeba castellanii</i>	6.50 x 10 ¹ (0.12)	4.39 x 10 ² (0.8)	2.86 x 10 ² (0.52)	1.32 x 10 ³ (2.39)
<i>Cercomonas</i> sp.	4.73 x 10 ² (0.86)	4.03 x 10 ² (0.73)	1.16 x 10 ³ (2.1)*	4.97 x 10 ² (0.9)

Table 2. Mean (absolute) number of recovered protozoan organisms/cm² per recovery method (centrifugation, stomacher) and quantification method (mean probable number – MPN, direct counting - DC). Number of spiked organisms were 2 x 10⁶ cells (corresponding to an initial number of 5.5 x 10⁴ cells/cm²) for *T. pyriformis* and *A. castellanii*; and 2 x 10⁴ cells (corresponding to an initial number of 5.5 x 10² cells/cm²) for *Cercomonas* sp. Recovery efficiency between brackets (%).

3 Results

3.1 Development and evaluation of two protocols for the recovery and quantification of FLP from dishcloths

The three model organisms were recovered from dishcloths by two methods (centrifugation and stomacher) and quantified using two enumeration methods (MPN-method and direct counting). The initial numbers of organisms spiked per dishcloth segment were 2×10^6 cells for *T. pyriformis* and *A. castellanii* and 2×10^4 cells for *Cercomonas* sp. In general, both recovery methods showed a reduction in protozoan cells (Table 2). With exception of the number of recovered *Cercomonas* cells after applying the stomacher protocol in combination with the MPN method (*).

The recovery rate varied depending on the methods used and test organism. A statistically significant difference in total number of recovered organisms between both recovery methods was found for both *T. pyriformis* and *A. castellanii*. *T. pyriformis* was recovered in significantly higher numbers with the centrifugation method than with the stomacher protocol ($p < 0.001$), whereas for *A. castellanii*, significantly higher numbers were counted after usage of the stomacher method in comparison with the centrifugation protocol ($p < 0.001$). For the flagellate *Cercomonas* sp., no statistically significant differences between both recovery protocols were found.

No significant difference between the MPN method and direct counting (Sedgewick-Rafter) was observed for *T. pyriformis* ($p > 0.05$). Statistically higher numbers of *A. castellanii* were counted by direct counting in comparison to the MPN method ($p < 0.001$). The opposite was observed for *Cercomonas* sp. ($p < 0.05$).

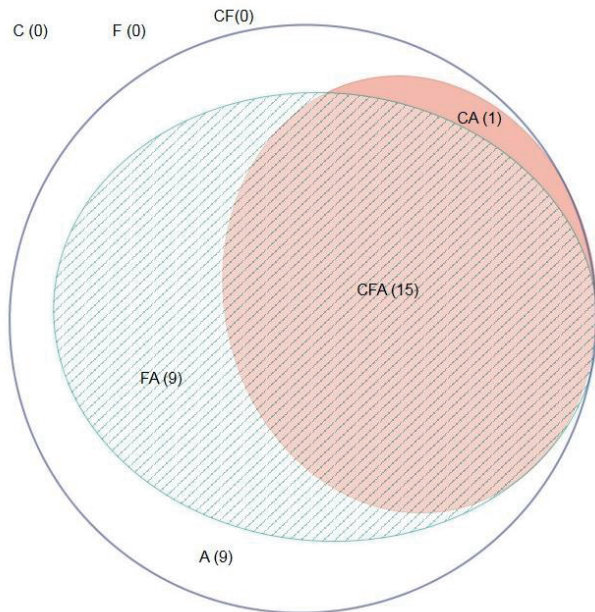


Figure 2. Distribution of the morphogroups in FLP-positive dishcloths, visualized by a proportional Venn diagram. C, F, and A are representing the presence of ciliates, flagellates and amoebae in dishcloths, respectively. CF, CA and FA are representing the presence of ciliates and flagellates, ciliates and amoebae and flagellates and amoebae in dishcloths, respectively. CFA represents the presence of all three morphogroups (ciliates, flagellates and amoebae) in dishcloths. The number between brackets represents the number of positive dishcloths for that specific morphogroup or morphogroup combination.

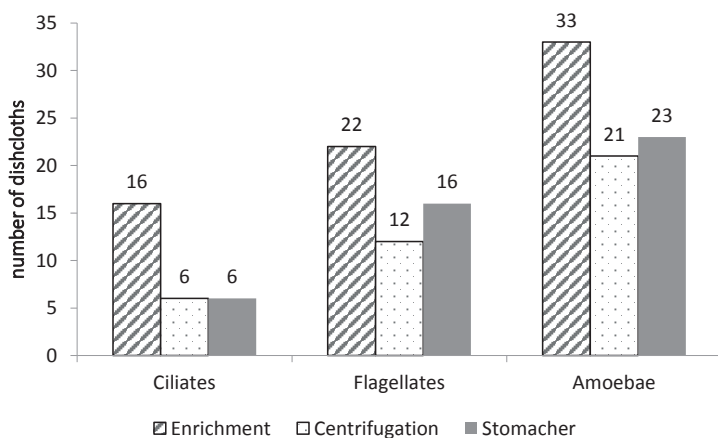


Figure 3. Number of FLP-positive dishcloths, per morphogroup and methodology.

3.2 Occurrence, enumeration and diversity of FLP on used dishcloths

A dishcloth was scored positive for the presence of FLP if ciliates, flagellates or amoebae were observed using either one of the recovery methods (centrifugation, stomacher) or the enrichment procedure. Free-living protozoa were present in 89% (34/38) of the used dishcloths, with 47% (16/34) of these testing positive for ciliates and 71% (24/34) for flagellates (Fig. 2). Amoebae were present in 100% (34/34) of the FLP positive dishcloths. No FLP were detected in the control dishcloths. Almost half (15/34) of the FLP-positive dishcloths contained all three morphogroups. No dishcloths contained only ciliates or flagellates, or the combination of these two groups without amoebae.

After one week, enrichment cultures, performed for FLP identification purposes, revealed more FLP positive dishcloths (33/34) compared to centrifugation (25/34) and stomacher (26/34) recovery methodologies. After enrichment, 16/16 dishcloths were positive for ciliates, while only 6/16 were positive using the centrifugation or stomacher protocols (Fig. 3). For flagellates, 22/24 dishcloths were positive after enrichment, while 12/24 and 16/24 dishcloths were positive using the centrifugation and stomacher method, respectively. The number of dishcloths positive for amoebae was 33/34 after enrichment, 21/34 after centrifugation, and 23/34 after using the stomacher protocol.

Numbers of FLP in used dishcloths were estimated using the MPN method. Total FLP numbers and numbers of ciliates, flagellates and amoebae in the used dishcloths were highly variable. Estimated total FLP numbers ranged from 0.11 to 8750 MPN/cm² and 0.11 to 6108 MPN/cm² for the centrifugation and stomacher protocols, respectively. In 26% (centrifugation) and 29% (stomacher) of the examined dishcloths the estimated total FLP numbers ranged from 10 to 10⁴ MPN/cm².

Flagellates were the most abundant group (Table 3) with mean numbers of 469 MPN/cm² (centrifugation) and 373 MPN/cm² (stomacher). Amoebae were recovered in higher numbers by the stomacher protocol compared to the centrifugation method (mean numbers 382 and 158 MPN/cm², respectively). The lowest numbers were recorded for ciliates, with 182 MPN/cm² for the centrifugation method and 5 MPN/cm² for the stomacher method.

In total 88 FLP (8 ciliates, 31 flagellates, and 49 amoebae) were identified to genus or species level. (Fig. 4 and Table S1). Centramoebida (e.g. *Acanthamoeba*) and Vannellida (e.g. *Vannella*) were frequently observed members of the Discosea. Together with *Hartmannella* and *Vermamoeba vermiformis* (Tubulinea), they were the most common Amoebozoa. Euglenozoa (e.g. *Bodo*) and Heterolobosea (e.g. vahlkampfiids) were representatives of the Discoba group. Ciliates all belonged to the Intramacronucleata (Alveolata), with *Colpoda* being the most often encountered genus. The flagellate groups glissomonads and cercomonads (Rhizaria) were general inhabitants of used dishcloths. *Hyperamoeba* sp. was present in 16 out of 34 amoebae-positive dishcloths. In addition to FLP, nematodes, rotifers, fungi, yeasts and molds were also detected in some dishcloths.

(n=34)	Minimum	Maximum	Mean	Standard Error
Centrifugation				
Ciliates	0,04	6000	182	176
Flagellates	0,04	6000	469	256
Amoebae	0,04	2750	158	89
Stomacher				
Ciliates	0,04	108	5	3
Flagellates	0,04	6000	373	206
Amoebae	0,04	6000	382	245

Table 3. Minimum, maximum, mean and standard error of the mean for each morphogroup and method; values are expressed as MPN/cm².

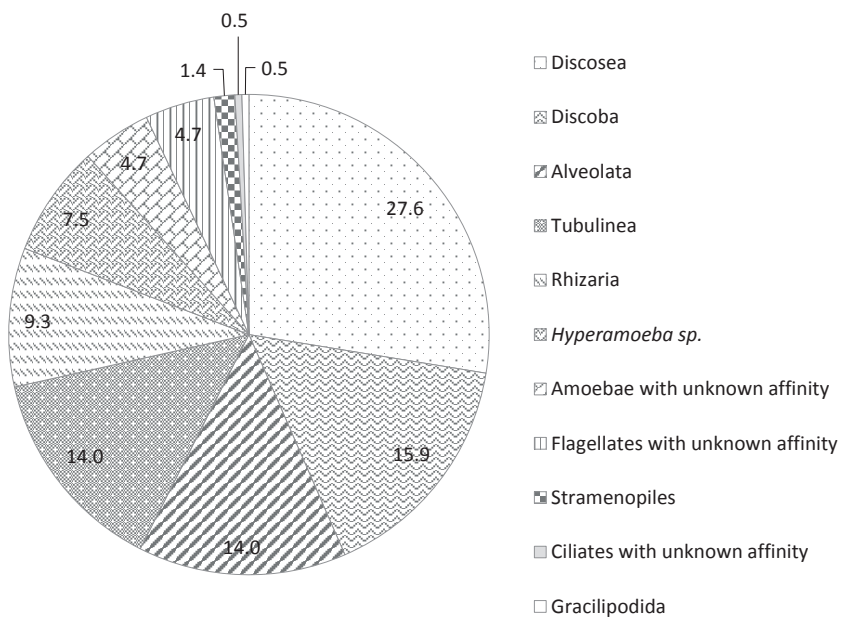


Figure 4. Relative proportion (%) of FLP taxonomic groups at first rank level, as described by Adl et al. (2012).

3.3 Bacteriological analysis of used dishcloths

All dishcloths were heavily contaminated with bacteria, with total aerobic bacteria (TAB) counts ranging from 4.36 to 8.93 \log_{10} cfu/cm², with a mean of $7.47 \pm 0.15 \log_{10}$ cfu/cm² (Fig. 5). *Escherichia coli* was found in 23 dishcloths, with concentrations ranging from 0.12 to 4.25 \log_{10} cfu/cm² (mean: $1.75 \pm 0.26 \log_{10}$ cfu/cm²).

Foodborne bacterial pathogens were detected in 22 dishcloths. From the four FLP-negative dishcloths, two also tested negative for foodborne pathogens. *Staphylococcus aureus* (n=19 positive dishcloths) were recovered in concentrations ranging from 0.12 to 3.48 \log_{10} cfu/cm² (mean: $0.93 \pm 0.24 \log_{10}$ cfu/cm²). *Salmonella* sp. (n=1) and *Arcobacter* sp. (n=5) were recovered from the collected dishcloths after enrichment. *Salmonella* colonies were biochemically and serologically confirmed as *Salmonella enterica* subsp. *enterica* ser. Halle. *Arcobacter* sp. was identified as *Arcobacter butzleri*. The five recovered *Arcobacter* isolates were all further characterized by ERIC-PCR as different strains. *Campylobacter* spp., *Listeria monocytogenes* and *Yersinia enterocolitica* were not detected in the examined dishcloths. No foodborne pathogens were present in the control dishcloths.

3.4 Evaluation of dishcloth usage and kitchen practices

All FLP-positive dishcloths harboured aerobic bacteria. With increasing numbers of aerobic bacteria, the possibility to find amoebae in the dishcloth increased significantly ($p = 0.023$), which was not the case for ciliates and flagellates. No significant differences in the total number of FLP were found between different fabric types, number of days in use, whether the dishcloths were also used to wash the dishes and whether there were pets in the kitchen environment allowed or not ($p > 0.05$). A negative relation was observed between detergent usage and the numbers of ciliates and amoebae ($p \leq 0.003$) and TAB counts on dishcloths ($p < 0.025$). No significant effect was found between detergent usage and the number of flagellates on dishcloths ($p > 0.05$).

The above results were confirmed by the direct ordination analyses (CCA and RDA respectively for FLP species presence/absence and morphogroup numbers) (data not shown). No significant relationships were found between variation in FLP community structure and the measured bacteriological and environmental factors. In contrast, variation in ciliate, flagellate and amoeba numbers were strongly negatively related to detergent use, while a weak but not significant ($p = 0.08$) positive relationship existed with the number of days a dishcloth had been in use.

An overview of the data gathered via the questionnaires is presented in Supplemental data - S2.

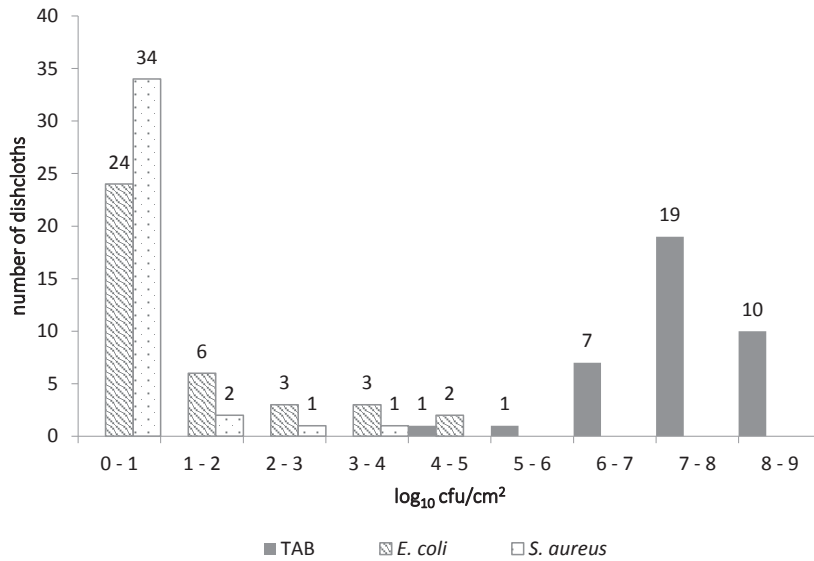


Figure 5. Bacterial load of dishcloths: total aerobic bacteria (TAB) and concentrations of *E. coli* and *S. aureus*. TAB: total aerobic bacteria (grey); *Escherichia coli* (hatched) and *Staphylococcus aureus* (dotted).

4 Discussion

The present study aimed to assess for the first time the occurrence, abundance and diversity of FLP in dishcloths in relation to bacteriological (TAB count, presence and abundance of bacterial foodborne pathogens) and environmental factors (e.g. number of days the dishcloths had been in use, use of detergent, etc.). In addition, protocols for recovering and quantifying FLP from dishcloths were developed and evaluated. This study showed that FLP, including some opportunistic pathogens, are a common and diverse microbial group on dishcloths. Important bacterial foodborne pathogens, such as *S. aureus*, *A. butzleri* and *Salmonella* were recovered from dishcloths.

Flagellates were recovered more efficiently than ciliates or amoebae. The lower recovery of amoebae may be ascribed to the high attachment capacity of amoebae. To obtain the total number of FLP (ciliates, flagellates and amoebae), the stomacher protocol in combination with the MPN method is recommended. The stomacher protocol is a broadly used and standardized method (Wu et al., 2003). However when focusing on ciliates in particular, centrifugation and stomacher protocols should be applied in parallel. For enumeration of free-living protozoa on dishcloths, the Most Probable Number method is a convenient method to estimate numbers of FLP as it is particularly useful for low concentrations of organisms (Blodgett, 2006).

Free-living protozoa form common and diverse communities on dishcloths (89% of the dishcloths FLP-positive). Amoebae were present in all FLP positive dishcloths, whereas ciliates and flagellates never occurred without amoebae. Flagellates were the most abundant group, which is in agreement with the studies of Vaerewijck et al. (2011) and Gourabathini et al. (2008) of FLP on food. The lowest numbers were counted for ciliates. Vaerewijck et al. (2011) also observed that ciliates were the least abundant group on butterhead lettuce.

The present study suggests that the use of detergent causes a significant reduction in numbers of ciliates, amoebae and TAB on dishcloths. Azizullah et al. (2011) tested the influence of detergent on motility, swimming velocity and cell shape of the freshwater flagellate *Euglena gracilis*. A strong impairment effect was seen immediately upon exposure to detergent, but with increasing exposure time this effect decreased, suggesting acclimatization to these stress conditions. Esteban and Tellez (1992) observed that ciliate numbers in wastewater treatment plants diminished with increasing detergent concentrations. However, further research is needed to assess the direct effect of detergent usage on the FLP and bacterial communities present in household dishcloths.

Most FLP species were detected after a few days of enrichment, suggesting they were present either in very low numbers of metabolically active cells or as resting stages (cysts) (Corliss, 2001). Free-living protozoa on dishcloths are frequently exposed to stress conditions such as desiccation, disinfection and cleaning treatments. Through cyst formation, various protozoan species can survive unfavourable conditions (Aksozek et al., 2002; Coulon et al., 2010; Dupuy et al., 2014; Sriram et al., 2008) which may also be the case on dishcloths.

The diversity of FLP in used dishcloths was the highest within the amoebae group, revealing the dominance of vahlkampfiids, vannellids, *Acanthamoeba* spp., *Hyperamoeba* sp. and *Vermamoeba vermiformis*.

Hyperamoeba sp. were present in almost half of the amoebae positive dishcloths. This species, being closely related to the myxogastric slime molds (Walker, 2003; Walochnik et al., 2004), has been isolated from human faeces, pond water and fecally contaminated soil (Karpov and Mylnikov, 1997; Zaman et al., 1999a). This amoeba has three life cycle stages: a flagellated stage, an amoeboid trophozoite and a cyst stage. Zaman et al. (1999) confirmed the presence of bacteria in the ectocyst of *Hyperamoeba* by using electron microscopy, which leads back to the possible role of FLP as vector and reservoir of foodborne bacteria. To date, *Hyperamoeba* sp. has never been observed in food-related environments.

In all ciliate positive dishcloths, *Colpoda* spp. (*Colpoda steinii* most common) were observed, making *Colpoda* a typical ciliate on dishcloths. *Colpoda* spp. are common and widely distributed terrestrial ciliates (Foissner and Berger, 1996) and are also able to form cysts to survive unfavourable conditions such as desiccation (Maeda et al., 2005). *Colpoda steinii* has also been recovered from spinach (Gourabathini et al., 2008).

Heterotrophic nanoflagellates were mainly *Bodo* spp., glissomonads and cercomonads. The genus *Bodo* is a member of the Kinetoplastida (Euglenozoa) and is found worldwide in freshwater and marine habitats.

Except for *Hyperamoeba* sp., the dominance of above mentioned protozoan species is somewhat similar to common taxa present in other food-related environments such as broiler houses (Baré et al., 2009), meat-cutting plants (Vaerewijck et al., 2008), and domestic refrigerators (Vaerewijck et al., 2010). These species are also detected on vegetables such as mushrooms (Napolitano, 1982), lettuce (Napolitano and Colletti-Eggolt, 1984; Vaerewijck et al., 2011), carrots (Sharma et al., 2004), radishes, tomatoes, cauliflowers and spinach (Rude et al., 1984).

The FLP diversity in used dishcloths is likely an underestimation of the true species richness, as cryptic species, *i.e.* species that are indistinguishable by morphology alone, were not detected based on this traditional approach (microscopy and culture) (Caron, 2009); and as ciliates and amoebae are often present as cysts (Corliss, 2001). Molecular based techniques like 18S rRNA gene sequencing may overcome this issue although molecular techniques have also their limitation (Forney et al., 2004; Nocker et al., 2007).

Moreover there is always a reduction in recovered cells, as shown in the spiking experiments. As a consequence the total number of FLP on used dishcloths is inevitably an underestimation of the real situation.

Detection and enumeration of bacterial load in dishcloths in combination with seven important foodborne pathogens has not been performed before. Published data, specifically about foodborne bacterial pathogens are also scarce, and mainly relate to spiking experiments (Diab-Elschahawi et al., 2010; Lee, 2010; Tian et al., 2012) and investigations into the occurrence of *Salmonella*, *Campylobacter*, *E. coli* and *S. aureus* in used dishcloths (Carrasco et al., 2008; Gorman et al., 2002; Hilton and Austin, 2000; Scott et al., 2008). Dishcloths examined in the present study were heavily contaminated. Similar results were also reported by Carrasco et al. (2008) who observed that used dishcloths carry the largest load of total coliforms and faecal coliforms compared to other kitchen surfaces.

In the present study, *E. coli* was frequently detected in used dishcloths, with concentrations up to 4.25 log₁₀ cfu/cm². *E. coli* is an indicator organism for hygiene and faecal contamination. Most *E. coli* are harmless and commensals of the intestinal tract, but some strains are pathogenic and causative agents of foodborne diseases. Washing hands, rinsing vegetables and fruits, and the fact that *E. coli* can survive desiccation for more than 24h (Bale et al., 1993; Mattick et al., 2003) can explain its frequent occurrence in kitchen environments and more specific in used dishcloths. Lee (2010) and Tian et al. (2012) showed that after inoculation of cultured cells on commercial available dishcloths *E. coli* grew very well at room temperature.

Besides direct pathogenic effects towards humans, FLP are also important in the ecology and epidemiology of foodborne bacterial pathogens, as described before. Interactions of *E. coli* with *Acanthamoeba* spp. and limax amoebae such as *Vermamoeba vermiformis* (previously named *Hartmannella vermiformis*) and *Vahlkampfia* (all present in the examined dishcloths) have been recorded (Alsam et al., 2005; Brown and Barker, 1999; Chekabab et al., 2012; Jung et al., 2007; Walochnik et al., 1998).

Staphylococcus aureus is a potential foodborne pathogen producing enterotoxins which can cause gastrointestinal problems after consumption of contaminated food. Milk and dairy products are important contamination sources (Balaban and Rasooly, 2000; Bianchi et al., 2014). In the present study, 50% (concentrations up to 10³ cfu/cm²) of the dishcloths harboured *S. aureus*, which is a higher prevalence than recorded by Hilton and Austin (2000) and (Scott et al., 2008). Survival and growth of *S. aureus* in *Acanthamoeba* spp. (Anacarso et al., 2012; Cardas et al., 2012; Huws et al., 2006) and *H. vermiformis* (Pickup et al., 2007), both organisms present on dishcloths, was observed.

Campylobacter spp. were not detected in dishcloths examined in the present study, which is in agreement with other studies (Gorman et al., 2002; Hilton and Austin, 2000).

Arcobacter is an emerging foodborne pathogen (Van den Abeele et al., 2014) which can cause human infections through the consumption of contaminated food such as poultry products, pork and beef (De Smet et al., 2012; Ho et al., 2006; Van Driessche and Houf, 2007). Apart from contaminated meat products, drinking water is also a potential contamination route (Jacob et al., 1993). The present study report for the first time *Arcobacter* presence in used dishcloths.

In the present study none of the dishcloths tested positive for pathogenic *L. monocytogenes*. This is in contrast with the study of Beumer et al. (1996), where *Listeria* was present in 37% of the dishcloths, of which 45% were *Listeria monocytogenes*.

One dishcloth tested positive for *Salmonella enterica* subsp. *enterica* ser. Halle. This particular serovar is associated with turtles. As indicated by obtained questionnaire data, the owner of the dishcloth kept turtles as pets.

Free-living protozoa can impact human health in a direct or indirect way. Some FLP organisms found in the present study, like *Acanthamoeba* spp., *Vahlkampfia* spp. and *Vermamoeba vermiformis*, are classified as opportunistic pathogenic amoebae, causing amoebic keratitis infections (Abedkhozasteh et al., 2013; Niyiyati et al., 2010). Further, FLP can indirectly cause infection pressure to human health by sheltering bacteria pathogenic to humans.

Free-living protozoa are often not incorporated in microbiological studies of food and food-related environments, as a result data of FLP in these environments are scarce. However, FLP are known vectors for (pathogenic) bacteria, and the present study elucidate the simultaneous presence of both FLP and bacteria in dishcloths. Further research on the impact of this finding on bacterial ecology and epidemiology is needed. The present study clearly demonstrates the reducing effect of detergent on both FLP and bacterial numbers, showing the importance of good hygiene measures in kitchen environments.

Acknowledgements

This work was supported by the Special Research Fund of Ghent University (BOF, Ghent, Belgium; grant 01J07111). Many thanks to M. Boonaert, S. Vangeenberghe and C. Van Lancker for the technical assistance. We thank all volunteers for providing their dishcloths and completing the questionnaire.

Supergroup	First rank	Second rank	Morphospecies/morphotype	
Amoebozoa	Tubulinea	Euamoebida	<i>Hartmannella cantabrigiensis</i> (= <i>Copromyxa cantabrigiensis</i> - Brown et al. 2011)	
			Hartmannellidae 1	
			Hartmannellidae 2	
		Leptomyxida		<i>Flabellula</i> sp. 1
				<i>Flabellula</i> sp. 2
				Flabellulidae 1
				Flabellulidae 2
				Gephyramoebidae 1
				Gephyramoebidae 2
				Gephyramoebidae 3
			Gephyramoebidae 4	
			Leptomyxidae	
			Arcellinida	<i>Vermamoeba vermiformis</i> (Page 1967)
	Discosea	Flabellinia		<i>Korotnevela</i> sp.
				<i>Korotnevela/Vexillifera</i> sp.
				<i>Vexillifera</i> sp.
				<i>Vannella platypodia</i> (~ <i>Ripella platypodia</i>)
				<i>Vannella</i> sp. 1
			<i>Vannella</i> sp. 2	
			<i>Vannella</i> sp. 3	
			Vannellidae	
		Longamoebia		Dermamoebidae 1
				Dermamoebidae 2
	<i>Mayorella</i> sp. 1			
	<i>Mayorella</i> sp. 2			
	<i>Acanthamoeba</i> sp. 1			
	<i>Acanthamoeba</i> sp. 2			
	<i>Acanthamoeba</i> sp. 3			
	<i>Acanthamoeba</i> sp. 4			
	<i>Acanthamoeba</i> sp. 5			
	Discosea 1			
	Discosea 2			
	Discosea 3			
	Discosea 4			
	Discosea 5			
	<i>Vexillifera/Acanthamoeba</i> sp.			
SAR	Gracilipodida		<i>Flamella</i> sp.	
	Stramenopiles	Chrysophyceae	<i>Monochrysis</i> sp.	
	Alveolata	Ciliophora		<i>Colpoda cucullus</i>
				<i>Colpoda inflata</i>
				<i>Colpoda</i> sp. 1
	Alveolata	Ciliophora		<i>Colpoda</i> sp. 2
				<i>Colpoda</i> sp. 3
				<i>Colpoda steinii</i>
			<i>Paracolpidium</i> sp.	
	Rhizaria	Cercozoa		(<i>Neo</i>) <i>Cercomonas</i> sp.
			Cercomonadidae	

Free-living protozoa on dishcloths

Supergroup	First rank	Second rank	Morphospecies/morphotype			
SAR	Rhizaria	Cercozoa	Cercomonadidae			
			<i>Paracercomonas/Metabolomonas/Cercomonas</i> sp.			
			Sandonidae 1			
			Sandonidae 2			
			Allapsidae			
			Neoheteromita 1			
			Neoheteromita 2			
			Neoheteromita 3			
			Sandona 1			
			Sandona 2			
			Sandona 3			
			Excavata	Discoba	Discicristata	<i>Bodo curvifilus/Bodo saltans</i>
						<i>Bodo designis</i>
						<i>Bodo globosus</i>
<i>Bodo saltans</i>						
<i>Bodo</i> sp.						
<i>Dimastigella trypaniformis</i>						
Euglenida sp.						
Euglenozoa						
<i>Neobodo saliens</i>						
Neobodonida sp.						
<i>Notosolenus/Petalomonas</i> sp.						
<i>Adelphamoebae/Naegleria</i> sp. 1						
<i>Adelphamoebae/Naegleria</i> sp. 2						
<i>Vahlkampfia</i> sp. 1						
<i>Vahlkampfia</i> sp. 2						
<i>Vahlkampfia</i> sp. 3						
Incertae sedis	Amoebae with unknown affinity		Amoeba A			
			Amoeba B			
			Amoeba C			
			Amoeba D			
			Amoeba E			
			Amoeba F			
			Amoeba G			
	<i>Hyperamoeba</i> sp.					
	Flagellates with unknown affinity		Flagellate A			
			Flagellate B			
			Flagellate C			
			Flagellate D			
			Flagellate E			
	Ciliates with unknown affinity			Ciliate A		

Table S1. Morphologically identified taxa, classified according to Adl et al. (2012).

Vragenlijst experiment 'micro-organismen in schotelvodden'

Naam:

Datum:

Beste,

Voor mijn doctoraatsonderzoek naar het voorkomen van micro-organismen in schotelvodden ben ik op zoek naar gebruikte schotelvodden (in dagelijkse context).

Zou ik dan ook mogen vragen om enkel een schotelvod die in de keuken (afwas, reinigen van keukenoppervlakken en -toestellen, ...) gebruikt werd, mee te geven.

Gelieve uw gebruikte schotelvod na het laatste gebruik uit te wringen zodanig dat deze niet meer overmatig nat is (niet lekkende vod) en te bewaren in het bijgevoegde plastic zakje (gesloten).

Hieronder volgen een aantal vragen omtrent het gebruik van de schotelvod. Gelieve deze vragen zo getrouw mogelijk in te vullen. Er is ook altijd mogelijkheid voorzien voor extra info te vermelden indien relevant. Deze informatie kan belangrijk zijn voor de interpretatie van de resultaten van het experiment.

1. Samenstelling schotelvod (meestal terug te vinden op het etiket en/of de verpakking):
 - 100% katoen (cotton)
 - microfiber (=polyester/polyamide)
 - cellulose/cotton
 - viscose
 - andere: _____

2. Datum 1^e gebruik schotelvod: _____

3. Datum (+ eventueel uur) laatste gebruik schotelvod: _____

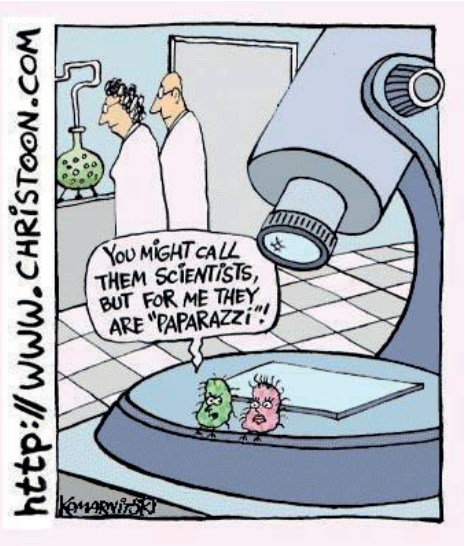
4. Hoeveel dagen gebruikt u een schotelvod gemiddeld: _____

5. Was uw schotelvod:
 - nieuw (rechtstreeks uit verpakking gehaald)
 - gewassen
 - andere: _____

6. Wat doet u met de schotelvod voordat u oppervlakken schoonmaakt:
 - nat maken met water
 - nat maken met water + detergent
 - detergent op aanbrengen
 - niets
 - andere: _____

7. Wat doet u met de schotelvod nadat u oppervlakken hebt schoongemaakt:
- uitspoelen met water
 - uitspoelen met water + detergent
 - niets
 - andere: _____
8. Indien u gebruik maakt van water, wat is de temperatuur van het water:
- koud water
 - lauw-warm water
 - heet water (onmogelijk met blote handen vast te nemen)
 - andere: _____
9. Indien u gebruik maakt van detergent, welke detergent (naam op verpakking) gebruikt u:
- _____
10. Wat doet u direct na gebruik van de schotelvod:
- volledig openplooiën en laten drogen
 - dichtplooiën/dubbelplooiën en laten drogen
 - in een 'bolletje' laten drogen
 - andere: _____
11. Waarvoor gebruikt u de schotelvod (meerdere antwoorden mogelijk):
- afwas
 - reinigen aanrecht/werkvlak
 - reinigen kookvuur
 - reinigen tafel
 - reinigen spoelbak/afdrupzone
 - reinigen keukentoestellen (bv. koffiezet, microgolfoven, oven, frigo)
 - andere: _____
12. Wat doet u met een vuile, gebruikte schotelvod (vod dat u niet meer wenst te gebruiken):
- wassen:
 - kookwas 95°C 60°C 40°C 30°C andere: _____
 - weggoeien
 - andere: _____
13. Heeft u een huisdier (dat ook in de keuken komt)?
- ja; specificeer: _____
 - nee

Supplemental data – S2. Overview of the data gathered via the questionnaires.



Logic will get you from A to B. Imagination will take you everywhere
Albert Einstein



Chapter 2.

Abundance, diversity and community
composition of free-living protozoa
on vegetable sprouts

Adapted from: N. Chavatte, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf, 2016. Abundance, diversity and community composition of free-living protozoa on vegetable sprouts. *Food Microbiology*. 55, 55-63.

Abstract

Interactions with free-living protozoa (FLP) have been implicated in the persistence of pathogenic bacteria on food products. In order to assess the potential involvement of FLP in this contamination, detailed knowledge on their occurrence, abundance and diversity on food products is required. In the present study, enrichment and cultivation methods were used to inventory and quantify FLP on eight types of commercial vegetable sprouts (alfalfa, beetroot, cress, green pea, leek, mung bean, red cabbage and rosabi). In parallel, total aerobic bacteria and *Escherichia coli* counts were performed. The vegetable sprouts harboured diverse communities of FLP, with *Tetrahymena* (ciliate), *Bodo saltans* and cercomonads (flagellates), and *Acanthamoeba* and *Vannella* (amoebae) as the dominant taxa. Protozoan community composition and abundance significantly differed between the sprout types. Beetroot harboured the most abundant and diverse FLP communities, with many unique species such as *Korotnevelia* sp., *Vannella* sp., *Chilodonella* sp., *Podophrya* sp. and *Sphaerophrya* sp. In contrast, mung bean sprouts were species-poor and had low FLP numbers. Sampling month and company had no significant influence, suggesting that seasonal and local factors are of minor importance. Likewise, no significant relationship between protozoan community composition and bacterial load was observed.

Keywords

Free-living protozoa; vegetable sprouts; diversity; protozoan community; microbiology

1 Introduction

In 2011, Europe witnessed one of the largest reported outbreaks of haemolytic uremic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC), also commonly referred to as verocytotoxin-producing *E. coli* (VTEC) and enterohaemorrhagic *E. coli* (EHEC) (Frank et al., 2011). In total, 4321 cases were reported, including 3469 EHEC and 852 HUS cases, with 50 deaths (Soon et al., 2013; Struelens et al., 2011). The source of contamination was initially thought to be cucumbers, tomatoes and leafy greens, resulting in the large-scale destruction of stocks of these vegetables. However, further investigations revealed a strong association between the consumption of unidentified vegetable sprouts and the outbreak (Buchholz et al., 2011). While the exact source of contamination of these vegetable sprouts is still an issue of debate, the outbreak resulted in increased awareness of vegetable sprouts as a potential source of foodborne illnesses. It has since been shown that sprout-associated outbreaks account for more illnesses and hospitalizations than other foods (Dechet et al., 2014).

Eliminating pathogens from vegetable sprout produce is problematic. The efficacy of various disinfection methods for eliminating pathogens from vegetable sprouts or their seeds is highly variable (Montville and Schaffner, 2004; Sikin et al., 2013). Even if a pathogen reduction with 2-5 log₁₀ cfu/g on the seeds can be achieved, the sprout production process itself stimulates microbial growth, and this will vary depending on seed or sprout type, pathogen identity, sample size and the materials and methods used. The effectiveness of sanitizing treatments is also hampered by the inability of antimicrobial compounds to reach viable pathogens inside seed and in particular inner sprout tissues due to the thick seed coat some seed types possess (Sikin et al., 2013). Seed coats are mechanically cracked to improve water uptake and thus more rapid germination of the seed during the sprouting process. These microscopic cracks allow bacteria to infiltrate; and some bacteria are able to escape from chemical treatments by entering a dormant stage (Holliday et al., 2001). The texture of the seed coat may also influence the amount of bacteria present on the seeds; e.g. wrinkled alfalfa seeds have more aerobic bacteria (which are more difficult to eliminate) than smooth seeds (Charkowski et al., 2001). Another cross-contamination source is the water used during the germination process. In addition, sprouting seeds release sugars and other organic molecules from the breakdown of endosperm into the irrigation water (Buchanan et al., 2000) which can also stimulate growth of bacteria.

Persistence of pathogenic bacteria in food related habitats has also been related to their association with free-living protozoa (FLP) (Brown and Barker, 1999). Free-living protozoa (heterotrophic microbial eukaryotes) are important bacterial consumers controlling bacterial biomass, and as such forming an important trophic link in aquatic and terrestrial food webs (Pernthaler, 2005; Sherr and Sherr, 2002). However, some bacteria developed pre- and post-ingestional adaptations and have become grazing resistant (Matz and Kjelleberg, 2005). As a result they are able to survive and grow inside FLP cells, potentially enhancing their transmission towards new habitats and hosts. Moreover, FLP (and their cysts) have been shown to protect and shelter pathogenic bacteria against harsh environmental conditions (Barker and Brown, 1994; King et al., 1988; Lambrecht et al., 2015b; Snelling et al., 2006b). FLP are common in natural and anthropogenic environments, including various food related environments, food products and drinking water (Vaerewijck et al., 2014).

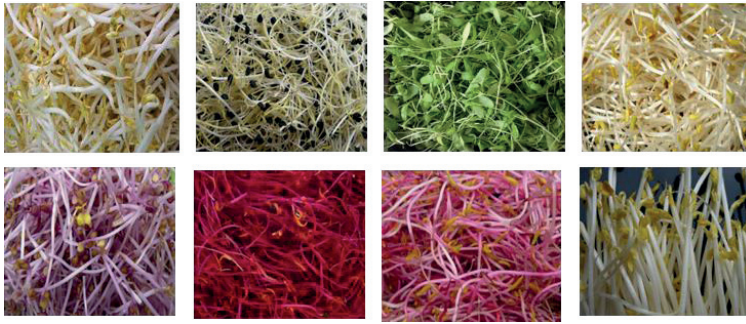


Figure 1. Pictures of the eight types of examined vegetable sprouts. First row, from left to right: mung bean, leek, cress and alfalfa sprouts. Second row: from left to right: red cabbage, beetroot, rosabi and green pea.

To date, FLP are not routinely incorporated in microbiological surveys as they are considered to be harmless. As a result, information on FLP on food is scarce and negligible. To our knowledge, no studies of the protozoan communities on vegetable sprouts have yet been performed. In the present study, we therefore (1) evaluated within-lot and between-lot variability in protozoan occurrence, abundance and diversity on eight types of vegetable sprouts; (2) determined the total FLP diversity on vegetable sprouts; (3) analysed variation in protozoan community composition in relation to FLP numbers, species richness and environmental variables; and (4) enumerated bacterial load on vegetable sprouts.

2 Material and methods

Eight types (Fig. 1) of vegetable sprouts: alfalfa (*Medicago sativa*), beetroot (*Beta vulgaris*), cress (*Lepidium sativum*), green pea (*Pisum sativum*), leek (*Allium ampeloprasum*), mung bean (*Vigna radiata*), red cabbage (*Brassica oleracea* convar. *capitata* var. *rubra*) and rosabi (*Beta vulgaris* conditiva); were purchased from six different retail stores or producers in Belgium between April and November 2014. In order to evaluate within-lot and between-lot variability in protozoan diversity and occurrence, 16 samples of a single lot ($n=16*8$ sprout types) and single samples of six different lots ($n=6*8$ sprout types), respectively, were examined for each of the eight sprout types. A single lot of vegetable sprouts germinated from one seed lot which is defined as a quantity of seeds originating from one field and harvested the same day. After purchase, all samples were stored at 2°C and processed within 24h and before end of shelf life.

2.1 Occurrence, abundance and diversity of FLP

For determining the occurrence and diversity of FLP on vegetable sprouts, two complementary methods were applied: (a) enrichment and (b) cultivation. (a) For the enrichment procedure, a stomacher protocol was first used for the recovery of FLP from the sprouts (Chavatte et al., 2014). Samples (10g) were transferred to a stomacher bag, and homogenized for 2 min after addition of Page's Amoeba Saline (PAS) to a final weight of 100g. Subsamples of the stomached suspension (homogenate) were used for enrichment (and also enumeration, see below). One millilitre of the homogenate was transferred to a 25 cm² tissue culture flask and PAS was added to a final volume of 15 ml. Sterile, uncooked rice grains were included as a carbon source to stimulate bacterial growth (Patterson, 1998). (b) For cultivation of FLP, one gram of each sprout type was directly transferred to a Petri dish containing 25 ml PAS. Culture flasks and Petri dishes were incubated in the dark at $20 \pm 2^\circ\text{C}$. The cultures were examined after three to four days and after one week for the presence of FLP. Repeated examinations are essential because of the sometimes rapid succession of FLP species in cultures. Free-living protozoa were identified on the basis of morphology and locomotion by inverted light microscopy (magnification x400) using standard taxonomic identification sources (Berger and Foissner, 2003; Jeuck and Arndt, 2013; Lee et al., 2005; Page, 1988; Patterson and Simpson, 1996; Smirnov and Brown, 2004; Smirnov and Goodkov, 1999). Organisms were identified to the genus or species level where possible. All taxa were classified according to the eukaryote classification of Adl et al. (2012). Organisms that were not assignable to a known species or genus were assigned to a morphogroup (ciliates, flagellates or amoebae) and for amoebae to a morphotype as specified by Smirnov and Goodkov (1999).

For enumeration of FLP the Most Probable Number (MPN) method (3-tube test) was applied. To ensure homogeneity, ten millilitres of the stomacher homogenate was vortexed for 10 s. Suspensions were serially diluted in TSB/PAS (Tryptic Soy Broth diluted 1:1000 in PAS) to 10^{-4} and one ml was added in triplicate into 24 well microtiter plates. Control wells were filled with one ml TSB/PAS only. The microtiter plates were incubated in the dark at $20 \pm 2^{\circ}\text{C}$. After three to four days and after one week incubation, the wells were examined microscopically for the presence of FLP (ciliates, flagellates and amoebae). The MPN was calculated using the US Food, Drug and Administration manual and tables (Blodgett, 2006).

2.2 Total aerobic bacteria and *Escherichia coli*

Ten millilitres of the homogenate was used for enumeration (direct plating) of total aerobic bacteria (TAB) and *E. coli*. The latter was used as an indicator organism for hygiene and fecal contamination. Serial dilutions were plated on Plate Count Agar (PCA, Bio-Rad, Hercules, California, USA) and Tryptone Bile X-glucuronide agar (TBX, Oxoid, Basingstoke, UK) for the enumeration of TAB and *E. coli*, respectively, using an Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain). Plates were incubated at 30°C for 48h (PCA) and at 44°C for 24h (TBX).

2.3 Data analysis

Results of the cultivation and enrichment methods were recorded as binary variables (presence/absence) for each of the morphogroups (ciliates, flagellates, amoebae). A sample was considered FLP positive if at least one of the morphogroups was observed using the enrichment and/or the cultivation method. Differences between methods were analysed using logistic regressions, including the sample as random effect. For all quantitative data analyses, FLP and bacterial concentrations were expressed as MPN/g and cfu/g, respectively. Protozoan data above the upper limit of quantification (LOQ) were set to the highest MPN count (110000 MPN/g). Bacterial concentrations were \log_{10} transformed and differences between sprout types were analysed using a linear regression analysis. Bonferroni corrections were applied to account for multiple testing. Statistical analyses were performed using the statistical software package Stata/MP 12.1 (StataCorp, 2011).

Sample	Ciliates			Flagellates			Amoebae		
	Number of samples positive after		Total positive*/total number of samples	Number of samples positive after		Total positive*/total number of samples	Number of samples positive after		Total positive*/total number of samples
	cultivation	enrichment		cultivation	enrichment		cultivation	enrichment	
Within-lot									
Alfalfa	0	0	0/16	16	16	16/16	14	13	16/16
Beetroot	16	15	16/16	16	16	16/16	15	15	16/16
Cress	9	2	11/16	16	14	16/16	16	16	16/16
Green pea	9	3	9/16	16	16	16/16	6	12	12/16
Leek	6	1	7/16	16	0	16/16	16	16	16/16
Mung bean	0	0	0/16	14	4	16/16	1	1	2/16
Red Cabbage	16	0	16/16	16	16	16/16	12	14	15/16
Rosabi	13	2	13/16	16	16	16/16	7	2	8/16
Overall positive/overall number of samples	69/128 (54%)	23/128 (18%)	72/128 (56%)	126/128 (98%)	98/128 (77%)	128/128 (100%)	87/128 (68%)	89/128 (70%)	101/128 (79%)
Between-lot									
Alfalfa	4	3	6/6	6	6	6/6	2	5	5/6
Beetroot	5	5	6/6	6	6	6/6	5	6	6/6
Cress	5	4	6/6	5	6	6/6	3	2	4/6
Green pea	5	5	5/6	6	6	6/6	2	5	5/6
Leek	6	1	6/6	6	5	6/6	5	5	6/6
Mung bean	0	1	1/6	1	6	6/6	0	4	4/6
Red Cabbage	3	0	3/6	6	6	6/6	5	6	6/6
Rosabi	5	3	5/6	6	6	6/6	2	2	3/6
Overall positive/overall number of samples	33/48 (69%)	22/48 (46%)	38/48 (79%)	42/48 (88%)	47/48 (98%)	48/48 (100%)	24/48 (50%)	35/48 (73%)	39/48 (81%)

Table 1. Occurrence of FLP on different sprout types (between- and within-lot), ordered per morphogroup and applied method (cultivation and enrichment). *Numbers presented as 'total positive' are cumulative numbers; a sample is scored positive if ciliates, flagellates or amoebae were observed using either the cultivation method or the enrichment method.

Multivariate ordination techniques were used to analyse variation in protozoan community composition (presence/absence) in relation to the diversity (species richness) and numbers (MPN) of the three protozoan morphogroups separately and all FLP together, TAB and *E. coli* counts, sprout type, company and sampling month. First, an in depth analysis of variability in composition between different sprout types was performed. To this end, per sprout type 16 samples of the same lot were examined for the presence and absence of protozoan taxa. These data were then compiled per sprout type, and analysed. All sprout lots except mung bean were obtained from the same company. A second, larger data set comprised 48 samples obtained from different sprout types from different lots obtained from different companies at different times of the year. To analyse and visualize the relationship between protozoan community composition, diversity and MPN in both data sets, Principal Components Analysis (PCA) with diversity and MPN parameters added as supplementary ('passive') variables were used. In the resulting PCA diagrams, only supplementary variables which were significantly ($p < 0,05$) correlated with the first or second PCA axis, were plotted. Only the 20 taxa which are best represented in the ordination space are shown. In addition, for the second data set, Redundancy Analysis (RDA)-based variation partitioning was used to determine to what degree TAB and *E. coli* counts, sprout type, company and sampling month were related to the variation in protozoan community composition. This approach allows evaluating to what degree each factor uniquely contributes in explaining the observed variation, how much they overlap, and whether their unique contributions are statistically significant (Monte Carlo Permutation Test – MCPT - with 4999 permutations). All analyses were implemented using Canoco 5 (Smilauer and Lepš, 2014).

3 Results

3.1 Occurrence and abundance of FLP

All examined vegetable sprouts harboured FLP, but differences in occurrence (Table 1) and abundance (Table 2) of the morphogroups were observed between the sprout types.

3.1.1 Within-lot variation

All within-lot samples ($n=8*16$) originated from the same producer (except for mung bean). Water, temperature and sprouting conditions were identical within each sprout type.

Overall, ciliates were present in 56% of the within-lot samples (72/128) (Table 1). All beetroot and red cabbage sprouts harboured ciliates, whereas alfalfa and mung bean samples scored negative for the presence of ciliates. Overall, significantly more within-lot samples were positive for ciliates using the cultivation method compared to the enrichment method (54% vs. 18%; $p < 0.001$). Using the cultivation method, ciliates were recovered in all (16/16) red cabbage samples, while no ciliates (0/16) were detected using the enrichment method.

In all within-lot samples (128/128) flagellates were present. Similar to the ciliates, significantly more within-lot samples were positive for flagellates using the cultivation method compared to the enrichment method (98% vs. 77%; $p < 0.001$). After enrichment, leek sprouts scored negative (0/16) for flagellates, whereas all samples were positive after applying the cultivation method (16/16).

	Ciliates		Flagellates		Amoebae		FLP	
	N	C	N	C	N	C	N	C
Within-lot								
Alfalfa	0	<3.0	16	1195 (230; 23980)	5	<3.0 (<3.0; 920)	16	1405 (230; 23980)
Beetroot	14	31 (<3.0; 230)	16	9330 (67; 46210)	16	67 (7.0; 4280)	16	9776 (717; 50720)
Cress	0	<3.0	16	230 (7.0; 2312)	16	4273 (2312; 14936)	16	4503 (2333; 15166)
Green pea	0	<3.0	16	919 (28; 4273)	10	16 (<3.0; 424)	16	930 (28; 4294)
Leek	0	<3.0	10	5.2 (<3.0; 2312)	16	>110000 (46208; >110000)	16	>110000 (46229; >110000)
Mung bean	0	<3.0	5	<3.0 (<3.0; 2110)	1	<3.0 (<3.0; 15)	5	<3.0 (<3.0; 2125)
Red Cabbage	0	<3.0	16	424 (21; 2312)	14	21 (<3.0; 424)	16	654 (35; 2736)
Rosabi	0	<3.0	16	22722 (2312; >110000)	2	<3.0 (<3.0; 15)	16	22724 (2312; >110000)
Between-lot								
Alfalfa	1	<3.0 (<3.0; 9.2)	6	16653 (35; >110000)	5	7.1 (<3.0; 9300)	6	16655 (38; >110000)
Beetroot	5	32 (<3.0; 919)	6	27664 (7668; 46208)	4	143 (<3.0; 2107)	6	28231 (7696; 48545)
Cress	3	44 (<3.0; 2312)	5	2706 (<3.0; 23978)	3	3.8 (<3.0; 4273)	5	4283 (<3.0; 24028)
Green pea	1	<3.0 (<3.0; 21)	3	460 (<3.0; >110000)	2	<3.0 (<3.0; 424)	3	672 (<3.0; >110000)
Leek	1	<3.0 (<3.0; 7.0)	3	10 (<3.0; 4300)	5	476 (<3.0; 2312)	6	1034 (21; 4300)
Mung bean	1	<3.0 (<3.0; 7.3)	3	4.4 (<3.0; 4300)	3	2.3 (<3.0; 30)	5	13 (<3.0; 4330)
Red Cabbage	0	<3.0	6	3292 (35; 14936)	5	19 (<3.0; 230)	6	3310 (42; 15166)
Rosabi	1	<3.0 (<3.0; 7.3)	6	35093 (14936; >110000)	4	5.2 (<3.0; 21)	6	35107 (14936; >110000)

Table 2. Estimated numbers (MPN/g) of ciliates, flagellates, amoebae and FLP per sprout type (within- and between-lot); N: number of samples above the limit of quantification; C: median (minimum; maximum) MPN/g based on all samples of each sprout type (n=16 for within-lot samples and n=6 for between-lot samples); Lower limit of quantification: <3.0 MPN/g; upper limit of quantification: >110000 MPN/g.

In total, 79% (101/128) of the within-lot samples were positive for amoebae. Amoebae were less common on mung bean (12.5%) and rosabi sprouts (50%). More green pea samples were positive for amoebae after using the enrichment method (12/16) compared to the cultivation method (6/16).

All sprout samples (except mung bean) had FLP numbers above the lower LOQ, with median numbers ranging between 654 MPN/g (red cabbage) and >110000 MPN/g (leek) (Table 2). Lowest FLP numbers were observed for mung bean, for which 81% (13/16) of the samples had FLP numbers equal or below 3.0 MPN/g.

3.1.2 Between-lot variation

Flagellates were present in 100% of the samples (48/48), amoebae in 81% of the samples (39/48) and ciliates in 79% of the examined samples (38/48) (Table 1). Significantly more samples were positive for ciliates using the cultivation method (69%) compared to the enrichment method (46%) ($p=0.023$). Contrarily, for flagellates and amoebae, more samples were positive after enrichment than after cultivation, with 98% vs. 88% ($p=0.084$) for flagellates and 73% vs. 50% for amoebae ($p=0.019$). Ciliates were less common in mung bean and red cabbage with only 17% (1/6) and 50% (3/6) positive samples, respectively.

Median FLP numbers per sprout type ranged from 13 MPN/g (mung bean) to 35107 MPN/g (rosabi) (Table 2). Flagellates were the most abundant morphogroup with median concentrations above 10000 MPN/g for alfalfa, beetroot and rosabi. Flagellates were less abundant in leek and mung bean, with concentrations below 4300 MPN/g. Ciliates were the least abundant morphogroup, with median concentrations below 3.0 MPN/g for all sprout types.

3.2 Diversity of free-living protozoa

In total 72 FLP (30 ciliates, 20 flagellates and 22 amoebae) were identified to species, genus or morphotype level (Table S1). All identified ciliates were classified as Intramacronucleata (Alveolata) with *Tetrahymena* sp. as a common representative for most sprout types (50% positive within-lot samples; 31% positive between-lot samples). *Bodo saltans*, belonging to the Euglenozoa (Discoba), was a frequently observed flagellate (75% positive within-lot samples; 54% positive between-lot samples). Cercomonads (Rhizaria, Cercozoa) were common on several sprout types. Amoebal diversity was dominated by *Acanthamoeba* sp. (Centramoebida) and *Vannella* sp. (Vannellida), both members of Discosea (75% positive within-lot samples; 35% positive between-lot samples for both species). The distribution of the morphogroups per sprout type for within-lot and between-lot samples are presented in Fig. 2.

Species diversity was highest in beetroot samples, with 28 and 41 morphospecies for within-lot and between-lot samples, respectively. Cress sprouts (between-lot samples) were also species-rich, with 35 morphospecies in total, of which 17 were ciliate species. The lowest diversity was observed for mung bean, both for within-lot (one flagellate species) and between-lot samples (five morphospecies). No ciliates were detected in the within-lot samples of alfalfa and mung bean sprouts. In contrast, ciliate richness (13 morphospecies) was high for beetroot sprouts of within-lot samples.

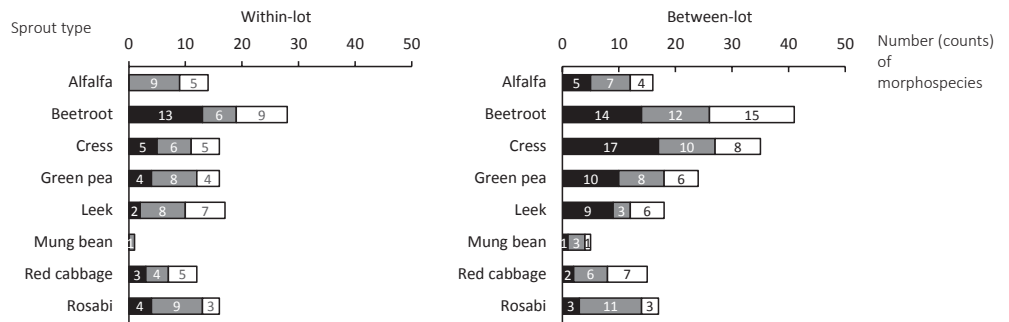


Figure 2. Number of morphospecies per sprout type (between- and within-lot; total diversity on all samples per sprout type) and morphogroup: ciliates (black); flagellates (grey) and amoebae (white).

PCA of the first data set [note that for the FLP data, all samples per lot and per sprout type were compiled, so this analysis contains 8 samples (one per sprout type) and 52 taxa in total, Fig. 3] revealed a clear distinction between the beetroot samples and the other sprout types, with the former harbouring a significantly more diverse ciliate and amoeba community, and significantly higher ciliate numbers (CIL_MPN). Characteristic taxa on beetroot are amoebae morphospecies *Korotnevela* sp., *Vannella* sp., *Hartmannella* sp.; and ciliate species *Paracolpidium* sp., *Chilodonella* sp., *Podophrya* sp., *Sphaerophrya* sp. and *Hypotrichia* sp., most of which were only found in association with this sprout type. Along the second PCA axis, cress and leek sprouts are separated from the other sprout types, with typical morphotypes and -species such as the acanthopodial and branched morphotypes of amoebae, the ciliate *Colpoda cucculus* and significantly higher amoeba numbers (AM_MPN). Alfalfa, rosabi, green pea and red cabbage sprouts on the other hand are characterized by generally lower diversity and MPN, and only a few typical species such as *Bodo elegans* (flagellate) and *Tetrahymena* sp. (ciliate). Mung bean, which was the only lot originating from another company, only held one species, a flagellate with unknown affinity.

PCA of the second, more extensive data set (for this data set, there were samples from 6 lots per sprout type, yielding a total of 48 samples and 63 taxa, Fig. 4) generally confirmed the results of the first analysis. Beetroot sprouts are generally found on the right side of the diagram, and are characterized by significantly higher richness of the three protozoan morphogroups. All other sprout types are mainly negatively characterized by lower richness (as confirmed by the arrows of the 20 best represented taxa which all point to the right). Most sprout types, except green pea and alfalfa, cluster more or less together, suggesting that they are rather similar in species composition. Along the second axis, red cabbage and leek are separated from cress and rosabi, with mung bean, green pea and alfalfa taking a more or less intermediate position. Flagellate and FLP numbers tend to be highest in beetroot en rosabi sprouts. Characteristic taxa on beetroot are the amoeba morphospecies *Vannella* sp., and ciliate species *Cyclidium* sp. and *Chilodonella* sp.

Variation partitioning revealed that there is no significant relationship between protozoan community composition and TAB and *E. coli* counts. When partitioning the variation over sprout type, sampling month and company, it appeared that sprout type uniquely (i.e. after partialling out the effects of sampling month and company) and significantly (MCPT, $p < 0,001$) explained 27,3 % of the variation in protozoan community composition. Neither sampling month nor company had a significant unique contribution, underscoring the importance of sprout type, irrespective of the company or lot it was derived from.

Figure 3. Principal Components Analysis (PCA) of the first data set (8 samples, 52 FLP taxa) showing the variability in protozoan community composition on the different vegetable sprout types. Protozoan abundance and diversity measures which are significantly ($p < 0.05$) correlated with PCA axis 1 or 2 are also shown as supplementary variables. Only the 20 species best represented by the first two axes are shown. AM_MPN and CIL_MPN represent amoeba and ciliate numbers (Most Probable Number, MPN), respectively; am_rich, cil_rich and FLP_rich represent amoeba, ciliate and total FLP richness, respectively.

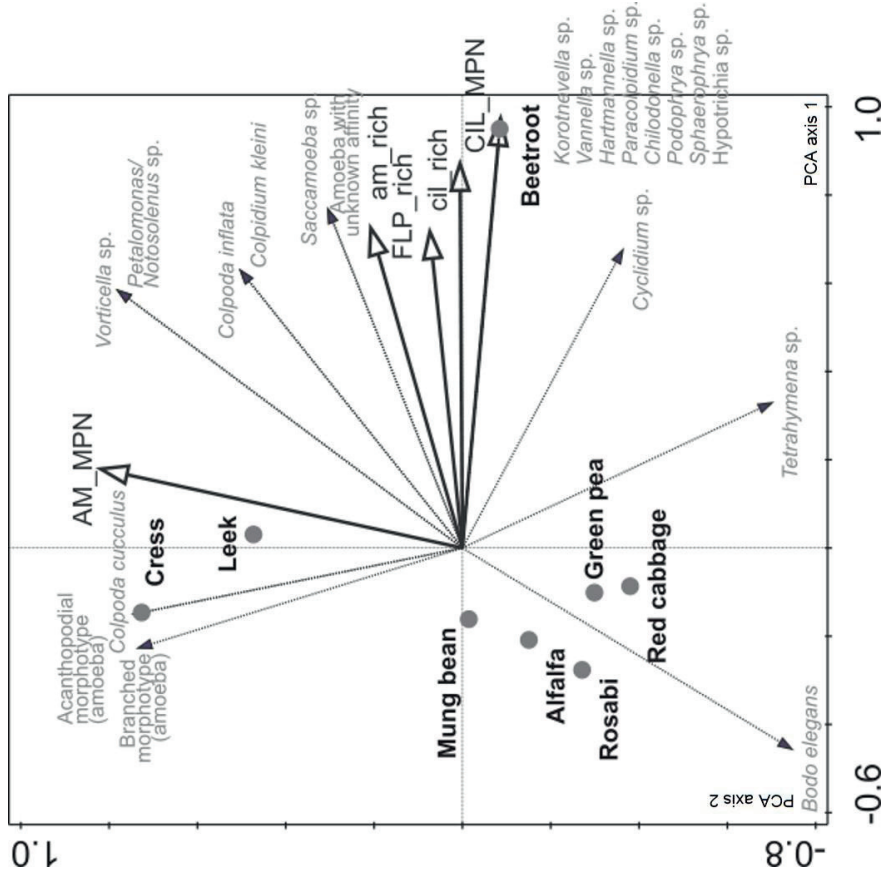
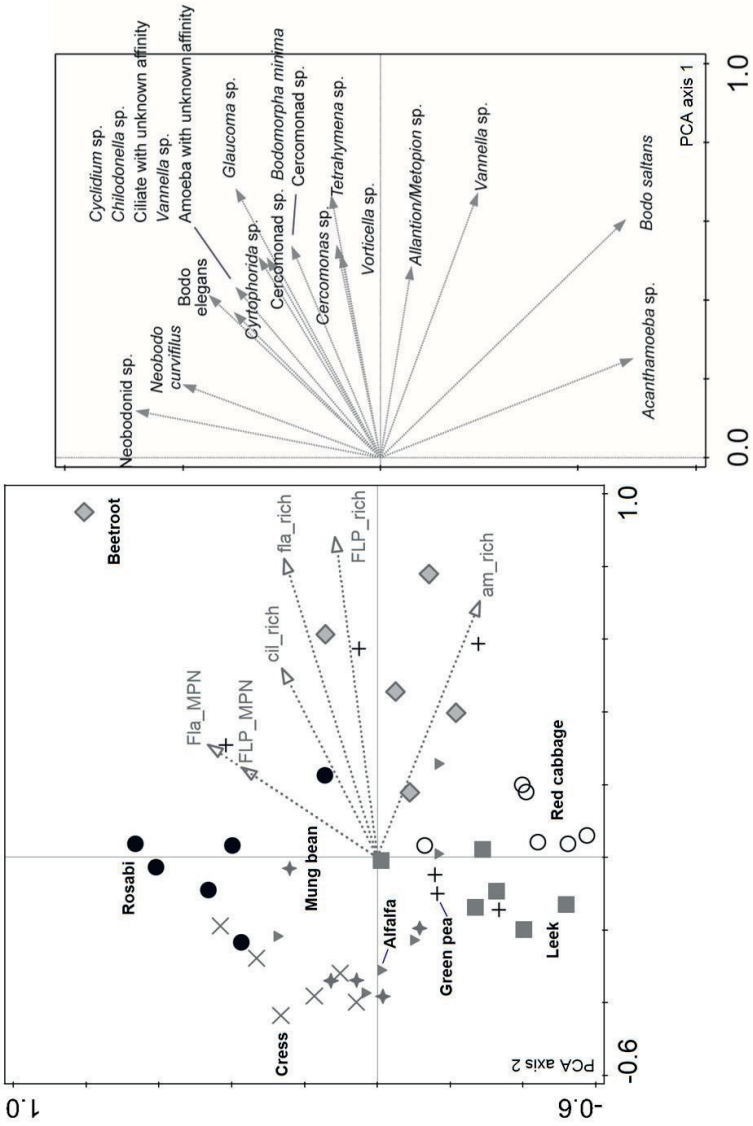


Figure 4. Principal Components Analysis (PCA) of the second data set (48 samples, 62 FLP taxa) showing the variability in protozoan community composition on the different vegetable sprout types. Protozoan abundance and diversity measures which are significantly ($p < 0.05$) correlated with PCA axis 1 or 2 are also shown as supplementary variables. Only the 20 species best represented by the first two axes are shown. Left: sample plot; right: species and supplementary variable plot. Fla_MPN and FLP_MPN represent flagellate and total FLP numbers (Most Probable Number, MPN), respectively; am_rich, cil_rich, fla_rich and FLP_rich represent amoeba, ciliate, flagellate and total FLP richness, respectively. Alfalfa samples are visualized by ▼, beetroot samples by ◆, cress samples by ×, green pea samples by +, leek samples by ■, mung bean samples by ▲, red cabbage samples by ○ and rosabi samples by ●



3.3 Bacterial load on vegetable sprouts

Mean total aerobic bacteria (TAB) counts on vegetable sprouts ranged from 4.68 log₁₀ cfu/g (red cabbage) to 9.26 log₁₀ cfu/g (leek) for within-lot samples; and from 5.85 log₁₀ cfu/g (green pea) to 9.33 log₁₀ cfu/g (leek) for between-lot samples. For green pea TAB counts (mean 6.84 log₁₀ cfu/g) of between-lot samples were significantly lower ($p < 0.001$) compared to all other sprout types.

For *Escherichia coli*, counts were below the limit of detection (<10 cfu/g) in 100% of the within-lot samples and in 98% of the between-lot samples.

4 Discussion

The present study shows that (FLP) form diverse communities on vegetable sprouts, comprising a variety of ciliated, flagellated and amoeboid morphotypes. Likewise, bacterial numbers are high, demonstrating that despite disinfection measures (see below), FLP and bacteria persist in commercial vegetable sprouts. Total aerobic bacteria counts ranged between 10⁶ and 10⁹ cfu/g which is comparable with the study of Waje et al. (2009) where concentrations of total aerobic bacteria and coliforms on alfalfa, mung bean and red cabbage sprouts were between 10⁴ and 10⁸ cfu/g. Interestingly, the numbers of environmental FLP (up to 10⁵ MPN/g) and total aerobic bacteria counts (between 10⁶-10⁹ cfu/g) approximate MOI (multiplicity of infection) of 1:100 used to study bacteria-FLP interactions in *in vitro* cultures (Lambrecht et al., 2015a; Olofsson et al., 2013). Most sprout-associated outbreaks are caused by *Salmonella* or *Escherichia coli* (Dechet et al., 2014). In the present study, counts of *E. coli* were mostly below the limit of detection. Although initial contamination levels can be low (0.1 log₁₀ cfu/g), numbers of bacteria can reach 10⁸ cfu/g during the germination process (Landry et al., 2014) increasing the health risks.

The most dominant FLP on vegetable sprouts were *Tetrahymena* sp., *Bodo saltans*, cercomonads, *Acanthamoeba* sp. and *Vannella* spp. All are ubiquitous in aquatic and terrestrial habitats but also in anthropogenic environments. *Acanthamoeba* and *Tetrahymena* are commonly used as model organisms in FLP-bacteria interaction experiments, and are known vectors for pathogenic bacteria such as *Campylobacter jejuni* and *Salmonella enterica* (Anacarso et al., 2012; Brandl et al., 2005; Vaerewijck et al., 2014). *Bodo saltans* is a common kinetoplastid in aquatic environments (Arndt et al., 2003; Patterson and Simpson, 1996) but also in refrigerators (Vaerewijck et al., 2010), on butterhead lettuce (Gourabathini et al., 2008; Vaerewijck et al., 2011), in poultry houses (Baré et al., 2009) and in meat-cutting plants (Vaerewijck et al., 2008). *Vannella* spp. are fan-shaped gymnamoebae (Smirnov et al., 2011, 2002) which typically occur in aquatic habitats including drinking water systems (Delafont et al., 2013; Thomas and Ashbolt, 2011) and wastewater (Ramirez et al., 2014). Cercomonads are gliding biflagellated organisms that are abundant and diverse in freshwater and soil (Bass et al., 2009).

Data on FLP on food is limited to a few recent studies by Gourabathini et al. (2008) and Vaerewijck et al. (2011) who investigated the occurrence of FLP on lettuce, spinach and butterhead lettuce, respectively. Napolitano (1982), Napolitano and Colletti-Eggolt (1984) and Rude et al. (1984) also recovered amoebae from vegetables and mushrooms. In the present study flagellates were the most abundant morphogroup on vegetable sprouts, which corroborates the findings of Gourabathini et al. (2008) and Vaerewijck et al. (2011). Ciliates were recovered more frequently with the cultivation method, while amoebae were more abundant after enrichment of the stomachered homogenate. This may be due to the fact that amoebae have a higher attachment capacity and may therefore be better recovered after stomaching. Chavatte et al. (2014) also reported differences in the recovery of ciliates and amoebae from dishcloths depending on the method used. It is hypothesized that stomaching can rupture the cell body of ciliates. Besides, protozoan abundance is inversely correlated with their cell size (Fenchel and Finlay, 2004); since ciliates are relatively large protozoan species, this might be a possible explanation for their lower abundances. The culturing conditions as well can be selective for certain FLP species as some species may not grow in the selected enrichment medium (Fenchel et al., 1997; Smirnov, 2003). Despite the fact that the MPN method is a commonly used enumeration method (Ronn et al., 1995; Vaerewijck et al., 2011), in combination with the traditional light microscopy approach it will tend to underestimate FLP numbers (Caron, 2009; Chavatte et al., 2014). Unfortunately, to date no gold standard for protist species detection and enumeration exists (Boenigk et al., 2012). The use of complementary methods (stomaching vs. direct incubation, cultivation vs. enrichment) is thus recommended in order to obtain a more complete FLP inventory.

Interestingly, protozoan community composition appears to be strongly determined by sprout type, as evidenced by clustering per sprout type in the PCA diagram of the second, more extensive data set (Fig. 4), and the results of the variation partitioning. Sampling month and company however had no significant effect, suggesting that in the present study seasonal and local (company-specific) factors are of minor importance. Beetroot harboured the most diverse and abundant FLP communities, especially ciliates and amoebae, with many unique species such as *Korotnevelia* sp., *Vannella* sp., *Chilodonella* sp., *Podophrya* sp. and *Sphaerophrya* sp. In contrast, mung bean sprouts were species poor and had low FLP numbers. The most likely explanation for the observed discrepancies lies in different microniche requirements of the FLP species. Differences in the composition of the resident bacteria on the sprouts can select for different FLP through selective grazing or through the production of specific bacterial metabolites. *Acanthamoeba* has been shown to selectively feed on Gram-negative bacteria such as *Escherichia coli* and *Enterobacter aerogenes* (Khan et al., 2014). The presence of certain pheromones/terpenes produced by bacteria has been hypothesized to attract or repel specific FLP (Cane and Ikeda, 2012; Kleerebezem et al., 1997). However, while it is known that the dominant bacteria on vegetable sprouts are enterobacteria, pseudomonads and lactic acid bacteria (Robertson et al., 2002; Weiss et al., 2007), no data exist on possible differences in bacterial community composition between sprout species. As in the present study as well no data were obtained on bacterial community composition on the sprout species, it is as yet not possible to evaluate its impact on FLP community composition. It is unlikely that the observed differences in FLP composition between the sprout species are sampling artefacts, as all sprout species were sampled using the same methods (cf. above). However, it cannot be ruled out that differences in vegetable sprouts characteristics like plant surface texture, chemical composition and exudates may influence in a direct or indirect way (e.g. by stimulating growth of specific bacteria), the recovery of specific groups of FLP.

Possible sources of contamination of FLP and bacteria on vegetable sprouts are manifold. In addition to insufficiently disinfected seeds (Beuchat and Scouten, 2002) (see also introduction), other contamination pathways can occur between seed production and sprout consumption. The sprouting process is water based (hydroponic culture) which provides ideal conditions for bacterial multiplication (Taormina et al., 1999), especially when ground water is used, which has a higher bacterial load than treated (chlorinated) tap water. Temperature as well is an important variable during the production process. While in most production areas temperature is kept constant between 7-17°C, production area temperatures between 20 and 40°C may occur (Weiss et al., 2007), which greatly enhance protozoan and bacterial growth. Internalization of pathogens in vegetable sprouts and the development of biofilms on sprouts, even after treatment, have been observed (Fett, 2000; Fransisca et al., 2011), despite the short shelf life span (3-10 days at refrigerator temperatures) of sprouts. Biofilms constitute ideal habitats for FLP (Brown and Barker, 1999; Dopheide et al., 2008; Matz and Kjelleberg, 2005).

The production process takes two (e.g. alfalfa) to six (e.g. leek) weeks, depending on the sprout type (personal communication provided by a vegetable sprouts producer). The first step is minimizing microbial growth by rinsing and soaking the seeds in water with antimicrobial agents such as free chlorine or hydrogen peroxide (concentrations ranging between 20 – 200 ppm). This soaking step is followed by a stage during which seeds germinate in dry conditions. In the last step seeds grow to maturity in tanks or rotary drums. The choice for tanks or rotary drums is also sprout dependent. In tanks (leek and green pea) the vegetable sprouts are irrigated regularly for cooling as a reaction to the heat released from the sprouting seedbeds and to prevent desiccation. Rotary drums combine a spindle movement, a stationary moment and the addition of water and antimicrobial agents. Rotary drums have the advantage that hard seed coats (beetroot) are removed and entanglement of roots (alfalfa, mung bean, red cabbage, and rosabi) is prevented due to the spindle movement. An exception to the above process is cress, which is grown to maturity in open ground greenhouses. Finally the vegetable sprouts are washed in water with antimicrobial agents and dry centrifuged before packaging.

The data obtained in the present study contribute to the growing knowledge on the occurrence and diversity of FLP on fresh food products. It is shown that vegetable sprouts harbour abundant and diverse FLP communities, which through their interactions with bacteria, including pathogenic ones, will impact the diversity and dynamics of the sprout-associated microbiota, and as such constitute potentially important key players in the survival and transmission of pathogenic bacteria on vegetable sprouts or on fresh produce in general.

Acknowledgements

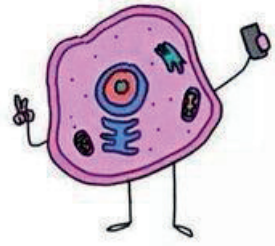
This work was supported by the Special Research Fund of Ghent University (BOF, Ghent, Belgium; grant 01J07111). Special thanks to Nancy Tibergyn and Henk Goesaert for their valuable cooperation in this study. Many thanks to Martine Boonaert, Sandra Vangeenberghe and Carine Van Lancker for the technical support and assistance.

Free-living protozoa on vegetable sprouts

Supergroup	First rank	Second rank	Morphospecies/ morphotype	Sprout type										
				Alfalfa	Beetroot	Cress	Green pea	Leek	Mung bean	Red cabbage	Rosabi			
Amoebozo a	Discosea	Flabellinia	Dactylopodial morphotype					+						
			Fan-shaped morphotype 1	+	+									
			Fan-shaped morphotype 2		+	+	+				+			
			<i>Koratnevela</i> sp.		+									
			<i>Ripella</i> sp.		+	+					+			
			<i>Vannella</i> sp. 1	+	+	+	+	+		+	+			
			<i>Vannella</i> sp. 2		+	+								
			<i>Vannella</i> sp. 3		+									
			<i>Vannella</i> sp. 4		+									
			<i>Vexillifera</i> sp.				+							
			Himatismenida		<i>Cochliopodium</i> sp.		+	+	+	+		+		
			Longamoebia		<i>Acanthamoeba</i> sp.	+	+	+	+	+		+		
					Acanthapodial morphotype			+						
				Tubulinea	Euamoebida	<i>Hartmannella</i> sp.		+					+	+
			<i>Saccamoeba</i> sp.				+			+				+
					Leptomyxida	Branched morphotype	+		+		+			
						<i>Paraflabellula</i> sp.		+	+	+				
Excavata	Discoba	Discicristata	<i>Bodo elegans</i>	+	+	+	+				+	+		
			<i>Bodo saltans</i>	+	+	+	+	+	+		+			
			<i>Dimastigella</i> sp.								+			
			<i>Eubodonida</i> sp.	+										
			<i>Euglenida</i> sp.			+	+					+	+	
			<i>Neobodo curvifilus</i>	+	+	+	+	+			+	+		
			<i>Neobodonida</i> sp.	+	+					+		+		
			<i>Parabodonida</i> sp.	+	+									
			<i>Peranema</i> sp.					+						
			<i>Petalomonas/ Notosolenus</i> sp.				+	+				+		
			<i>Vahlkampfia</i> sp.	+	+					+		+		
			Sar	Alveolata	Ciliophora	<i>Aspidisca</i> sp.			+					
<i>Chiladonella</i> sp. 1		+				+	+	+			+			
<i>Chiladonella</i> sp. 2		+												
<i>Cinetochilum</i> sp.						+								
<i>Colpidium kleini</i>		+				+								
<i>Colpoda cucculus</i>						+								
<i>Colpoda inflata</i>		+				+								
<i>Colpoda</i> sp.	+					+	+	+	+					
<i>Colpoda steinii</i>	+					+			+			+		
<i>Cyclidium</i> sp.		+				+					+			
<i>Cyrtophoria</i> sp.		+												
<i>Epistylis</i> sp.									+					
<i>Glaucoma</i> sp.		+				+	+					+		
<i>Heliophrya</i> sp.		+												
Oligohymenophorea sp. 1	+	+				+	+	+				+		
Oligohymenophorea sp. 2							+							
Oligohymenophorea sp. 3						+	+							

Supergroup	First rank	Second rank	Morphospecies/ morphotype	Sprout type								
				Alfalfa	Beetroot	Cress	Green pea	Leek	Mung bean	Red cabbage	Rosabi	
			<i>Hypotrachia</i> sp. 1		+	+	+					
			<i>Hypotrachia</i> sp. 2			+		+				
			<i>Paracolpidium</i> sp.		+	+	+					
			<i>Peritrichia</i> sp.	+	+							
			<i>Podophrya</i> sp.		+							
			<i>Sphaerophrya</i> sp.		+	+	+					
			<i>Tetrahymena</i> sp.		+	+	+	+			+	+
			<i>Uronema</i> sp.		+	+	+	+				+
			<i>Urotricha</i> sp.		+							
			<i>Vorticella</i> sp. 1	+	+	+		+				
			<i>Vorticella</i> sp. 2		+			+				
	Rhizaria	Cercozoa	<i>Allantion/Metopion</i> sp.	+	+	+	+	+			+	+
			<i>Bodomorpha minima</i>	+	+							+
			<i>Cercomonas</i> sp. 1		+	+	+	+				+
			<i>Cercomonas</i> sp. 2		+	+	+	+				+
			<i>Cercomonadida</i> sp.1	+	+	+	+	+			+	+
			<i>Cercomonadida</i> sp.2		+	+	+	+				+
			<i>Glissomonadida</i> sp.	+		+	+		+			+
			<i>Paracercomonas</i> sp.									+
	Amoebae with unknown affinity		Amoeba A		+							
			Amoeba B		+			+	+	+		
			Amoeba C			+	+				+	+
	Ciliates with unknown affinity		Ciliate A		+							
			Ciliate B					+				
	Flagellates with unknown affinity		Flagellate A						+			
			Flagellate B								+	
			<i>Hyperamoeba</i> sp.		+			+				

Table S1. Morphologically identified taxa, classified according to Adl et al. (2012).

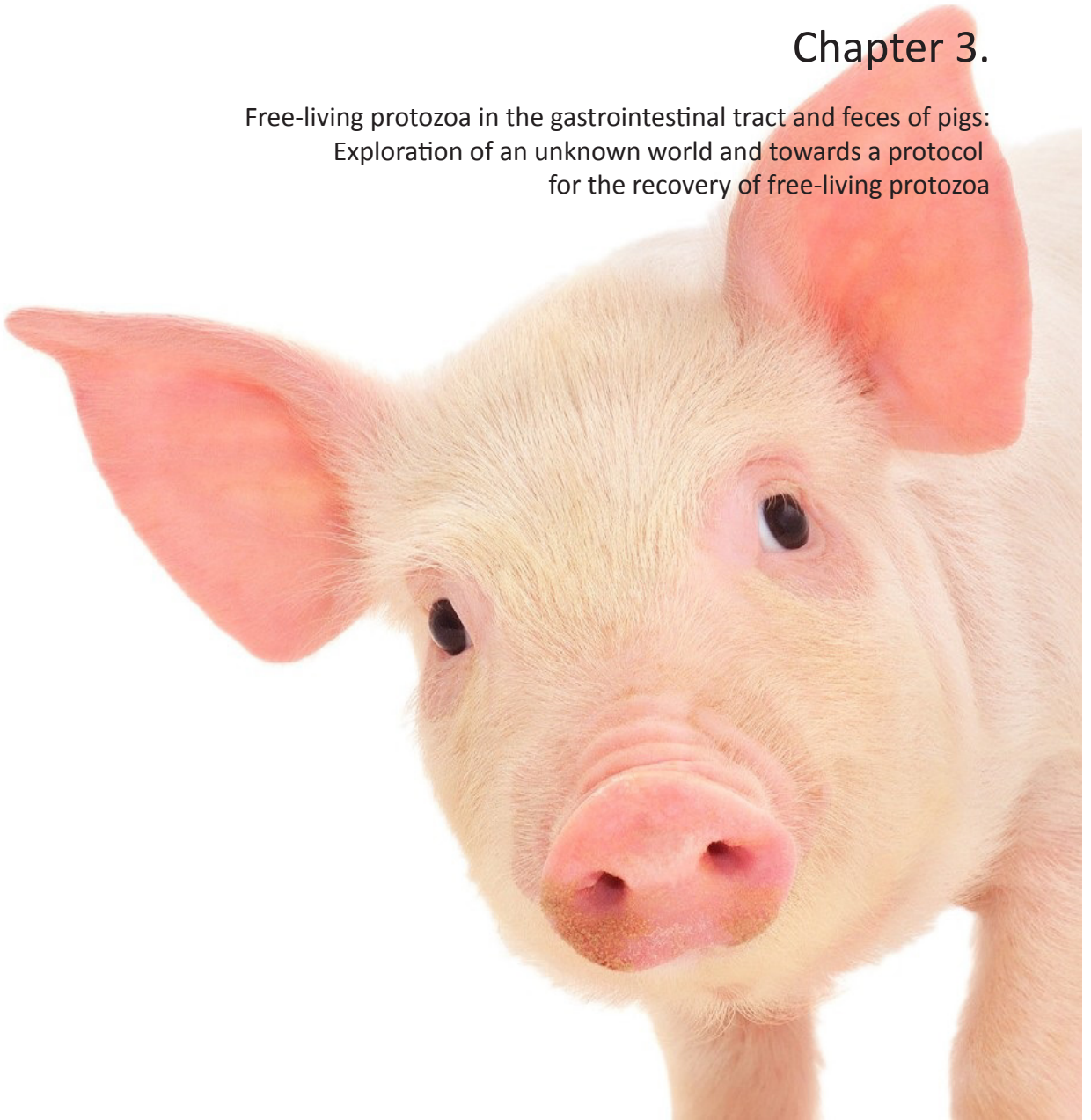


Cell-fie

You never fail until you stop trying
Albert Einstein

Chapter 3.

Free-living protozoa in the gastrointestinal tract and feces of pigs:
Exploration of an unknown world and towards a protocol
for the recovery of free-living protozoa



N. Chavatte, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf. Free-living protozoa in the gastrointestinal tract and feces of pigs: Exploration of an unknown world and towards a protocol for the recovery of free-living protozoa.

Submitted to *Veterinary Parasitology*

Abstract

Associations with free-living protozoa (FLP) have been implicated in the persistence of foodborne pathogenic bacteria in food-related environments. To date however no information is available on the presence of FLP in the gastrointestinal tract (GIT) of pigs, which represents an important reservoir for zoonotic foodborne bacteria and hence a potential location for associations with FLP. This is at least partly due to the lack of adequate protocols to recover FLP from intestinal content and feces. In the present study different protocols to recover FLP from the porcine GIT and feces were tested. The most effective protocols were then applied to explore the presence of live FLP in the pig GIT and feces. A filtration based protocol was identified as the most suitable method to recover viable FLP from the porcine GIT and feces. Cultivable FLP were recovered from different parts of the GIT, suggesting at least a transient presence of FLP in this habitat. Free-living amoebae species (*Acanthamoeba* spp., *Hyperamoeba* sp., *Vannella* sp., *Vermamoeba vermiformis*, hartmannellids and vahlkampfiids) but also ciliates (*Colpoda* sp. and *Tetrahymena/Glaucoma* lookalike) and flagellates (cercomonads, bodonids and glissomonads) were recovered and cultured from pig intestinal content. *Acanthamoeba hatchetti* and *Filamoeba sinensis* were isolated for the first time from pig intestinal content. Despite high gastric acidity, non-cyst forming amoeba species were also detected which suggests survival of their trophozoites in the animal GIT.

Keywords

Free-living protozoa; free-living amoebae; gastrointestinal tract; feces; pigs

1 Introduction

Studies dealing with intestinal protozoa of mammals are mainly focused on parasitic protozoa like *Giardia*, *Cryptosporidium*, *Entamoeba* and *Toxoplasma* (Geurden et al., 2008; Hammes et al., 2007; Robertson et al., 2010; Solaymani-Mohammadi and Petri, 2006). Notwithstanding their importance as causative agents of disease in both animals and humans, virtually nothing is known about the presence of free-living protozoa (FLP) in the gastrointestinal tract and feces of mammals.

Free-living protozoa, *i.e.* unicellular heterotrophic eukaryotes which do not have an obligate parasitic life cycle, are widespread in aquatic and soil habitats. Recent research has revealed that they can play an important role in the transmission and persistence of foodborne pathogenic bacteria in different environments (Vaerewijck et al., 2014). While FLP and bacteria typically show a predator-prey interaction, some bacteria resist protozoan uptake and digestion and are able to survive and even grow inside FLP (Matz and Kjelleberg, 2005). Free-living protozoa can thus act as a shelter and protect intracellular bacteria against harsh environmental conditions (King et al., 1988; Lambrecht et al., 2015a). Moreover, FLP are so called Trojan horses, enabling internalized bacteria to pass the first line mammal immune system (Barker and Brown, 1994; Greub and Raoult, 2004).

Though most FLP are harmless for mammals, some taxa are opportunistic pathogens, such as the well-studied free-living amoeba (FLA) genera *Acanthamoeba*, *Naegleria* and *Balamuthia*. Information about the presence of FLP in mammals is limited to a few studies and reports on FLA recovered from stool and feces (see below). More than 80% of healthy human individuals have been tested seropositive for antibodies against *Acanthamoeba polyphaga* (Chappell et al., 2001), indicating that FLA are more common than suspected. *Acanthamoeba* spp. and other emerging opportunistic pathogenic FLA species like *Hartmannella* sp., *Hyperamoeba* sp. and *Vahlkampfia* sp. have been recovered from human stool of both healthy and infected individuals (Bradbury and Forbes, 2013; de Moura et al., 1985; Mergeryan, 1991; Zaman et al., 1999a, 1999b). *Acanthamoeba* strains belonging to the most pathogenic genotype T4, have also been isolated from cattle and squirrel feces (Lorenzo-Morales et al., 2007b; Niyati et al., 2009).

Pigs have a similar microbiome as humans and are susceptible to the same (though not all) enteric pathogens (Zhang et al., 2013). The gastrointestinal tract of pigs is also an important reservoir for zoonotic foodborne bacteria like *Salmonella enterica* Typhimurium (Mikkelsen et al., 2004), *Yersinia enterocolitica* (Van Damme et al., 2015), *Campylobacter* spp. (Aguilar et al., 2014; Madden et al., 2007), verotoxigenic *Escherichia coli* (VTEC) (Dixit et al., 2004; Mainil and Daube, 2005), and *Arcobacter* spp. (Van Driessche et al., 2004). With pork being one of the most frequently consumed kinds of meat (Devine, 2003), control of foodborne pathogens in the pig reservoir is important for public health. As pigs are omnivorous and likely to be exposed to environmental FLP, the gastrointestinal tract creates an ideal niche and meeting place for foodborne pathogens and FLP, favouring the close association between both microbial groups.

To date, virtually nothing is known about the presence of viable FLP in the porcine gastrointestinal tract and their excretion in the feces. Partly due to lack of protocols to recover FLP from intestinal content, thorough research of this microbial group in this habitat is impeded. The hypothesis of the present study is that besides the ubiquitous dispersal of FLP in the environment, FLP are also present and passing through the gastrointestinal tract of pigs and consequently, due to their co-occurrence with foodborne pathogens in this environment, are potentially important for the persistence and transmission of pathogenic bacteria in this habitat.

The aim of the present study is to explore the presence of FLP in the pig gastrointestinal tract and feces. However, as standardized protocols implemented in parasitic research, are not yet applied to recover FLP from intestinal content, a suitable protocol to recover FLP from the porcine gastrointestinal tract and feces is developed and tested.

2 Material and methods

2.1 Development and testing of protocols for the recovery of FLP from the porcine gastrointestinal tract and feces

A comprehensive review of literature was conducted in search of existing protocols. Methods commonly applied in parasitic research for the examination of stool and feces samples and methods implemented for the recovery of FLA from water and soil samples were selected as a starting point for the development of a suitable recovery protocol for FLP from pig intestinal content. Four methodologies, newly developed or adapted and optimized where necessary, were tested on artificially inoculated fecal samples: (1) a filtration based design; (2) a sedimentation based design; (3) Ludox and magnesium sulphate density gradient centrifugation (based on Heip et al. (1985) and Dryden et al. (2005)); and (4) formalin-ethyl acetate sedimentation concentration (modified from a protocol by Garcia (2007) for *ova* and parasite evaluation in stool specimens).

Three FLP organisms, representing the three main morphogroups, were selected for the artificial inoculation: *Tetrahymena pyriformis* (ciliate, CCAP 1630/1W), *Acanthamoeba castellanii* (amoeba, ATCC 30324) and *Cercomonas* sp. (flagellate, environmental isolate from a meat-cutting plant, 2008). Cultivation of FLP was performed as previously described by Chavatte et al. (2014).

Three fecal samples (originating from different pigs) were aseptically collected during defecation, individually packed in plastic containers, transported to the lab and processed the same day. A minimum of 10 g of feces was put in a sterile Petri dish followed by artificial inoculation with 2 ml of either a single species or a mixed species cell suspension (*T. pyriformis*, *A. castellanii* and *Cercomonas* sp.). The final inoculation concentration of the model organisms was 10^5 cells/ml for *T. pyriformis* and *A. castellanii* and 10^4 cells/ml for *Cercomonas* sp. for the single species suspension, and 10^5 cells/ml (1:1:1) for the mixed species cell suspension.

Inoculated samples were stored for 1 h at room temperature. Samples were then transferred to a stomacher bag (with filter), Page's Amoeba Saline (PAS, CCAP recipe) was added to a final weight of 100 g and samples were homogenized for 2 min. Subsamples of the homogenate were immediately used for the filtration and sedimentation protocols listed below. Non-inoculated samples were included as controls.

2.1.1 Filtration

Filtration of samples over small pore size filters (1.5 μm) is a commonly used technique to recover FLA from water and soil samples (Pernin et al., 1998; Rahdar et al., 2012). The filtration based adapted protocol included three filtration steps. The first two exclude fecal debris, the final intercepts FLP cells, allowing bacterial cells to pass through.

Fifty ml of the homogenate was filtered consecutively over a 0.5 mm woven wire mesh filter (VWR, Belgium), a 200 μm polyethyleneterephthalat cell strainer (PluriSelect, HISS Diagnostics, Germany) and a 1.6 μm glass microfiber filter (VWR, Belgium). The 1.6 μm filter was then put in a 50 ml tube filled with 20 ml PAS and placed on a lab shaker for 5 min at 350 rpm. The suspension and filter were transferred to a Petri dish and autoclaved dry rice grains were added as a nutrient source. Sealed Petri dishes were incubated at 25°C and 7°C. Samples were examined daily (inverted microscopy, magnification x400) for the presence of FLP. The samples were washed on a regular basis and sub cultured (1:10) with PAS.

2.1.2 Sedimentation

The sedimentation protocol is based on the observation that amoeba sink to the bottom of a conical tube, while motile ciliates and flagellates remain in the supernatant. Further cultivation is done by means of inoculation on Non-Nutrient Agar (NNA) plates for amoebae and in liquid culture for ciliates and flagellates. Inoculation on NNA plates seeded with heat-killed *Escherichia coli* is a culturing method to recover FLA from water and soil, proposed by Page (1988).

Fifty ml of the homogenate was collected in a 50 ml polypropylene conical tube (Novolab, Belgium) and incubated for 2 h at room temperature. Twenty ml of the supernatant was removed and transferred to a Petri dish with addition of sterile dry rice grains. Sealed Petri dishes were incubated at 25°C and 7°C. In addition, 30 μl of the sediment was streaked onto NNA plates seeded with *E. coli* and incubated at 25°C. Samples were examined daily (inverted microscopy, magnification x400) for the presence of FLP. The samples were washed on a regular basis and sub cultured (1:10) with PAS.

2.1.3 Ludox and magnesium sulphate density gradient centrifugation

Density gradient centrifugation or flotation techniques are commonly used to recover parasite eggs and oocysts from stool or feces (Dryden et al., 2005). Furthermore, it is applied to extract meiofauna from marine sediments (Heip et al., 1985) and ciliates from coastal soils (Zhao et al., 2012). Based on the protocols described by Heip et al. (1985) and Dryden et al. (2005), 10 g of artificially inoculated feces was used to test a density gradient centrifugation protocol with Ludox or magnesium sulphate (MgSO_4) as flotation medium. Ludox (a colloidal silica polymer, specific gravity 1.18 g/cm^3) or MgSO_4 (specific gravity 1.28 g/cm^3) was added (solution) to the fecal samples (final volume 250 ml) and gently mixed prior to centrifugation for 12 min at 2072g. The floating phase was separated and Ludox or MgSO_4 was added to the sediment phase. This procedure was repeated two times. Both phases were examined for the presence of FLP.

2.1.4 Formalin-ethyl acetate sedimentation concentration

The protocol described by Garcia (2007) to recover *ova* and parasites from stool specimens was adapted and applied to 10 g of artificially inoculated feces. In a first step, the artificially inoculated feces was added to a round bottom tube and mixed thoroughly with formalin. After 1 h of fixation, the feces-formalin mixture was filtered through a wet gauze. After addition of 0.85% NaCl the samples were centrifuged for 10 min at 540g. The supernatant was removed and the precipitate was washed with 0.85% NaCl and re-suspended in formalin. Afterwards, ethyl acetate was added, tubes were shaken vigorously and the samples were centrifuged for 10 min at 540g. Ethyl acetate is used as an extractor of debris and fat from the feces and should leave the protozoan cells at the bottom of the suspension. This resulted in four layers: a small layer of precipitate at the bottom, a layer of formalin, a plug of fecal debris on top of the formalin layer and a layer of ethyl acetate at the top. A small amount of the bottom layer was put on a slide and examined for the presence of FLP using microscopy (magnification x400).

2.2 Exploration of FLP in the porcine gastrointestinal tract and feces

During the development of the protocols, microscopic observations indicated that inoculation of samples with *Tetrahymena pyriformis* resulted in samples with lower bacterial load, most probably due to the high grazing rate of *Tetrahymena*. As a result, it was much easier to distinguish and identify other protozoan species present in the initial fecal samples. For our protozoan analyses, we therefore also decided, in addition to the 4 recovery protocols outlined above, to also inoculate part of the original samples with a cell suspension of *T. pyriformis* (see section 2.1.) and use these samples for protozoan detection and identification.

2.2.1 Recovery of FLP from pig feces

Based on results of the artificially inoculated samples (see below, section 3.1.), the filtration and sedimentation protocols were selected for analyses of FLP in fecal samples. Fecal samples originating from six different pigs (including three samples also used in the development and testing of the protocols) were collected and processed as described in section 2.1. Subsamples of this homogenate were used for either the filtration or sedimentation method.

2.2.2 Recovery of FLP from the pig gastrointestinal tract

Based on the results of section 3.2.1., the filtration method was selected to recover FLP from the content of the gastrointestinal tract of pigs. Gastrointestinal tracts of seven pigs, originating from three different pig herds collected at three different time points, were collected *post mortem* from the slaughterhouse and processed the same day. In the laboratory, a maximum of 10 g of content of each stomach, ileum, caecum, colon and rectum was aseptically collected, transferred to a stomacher bag, and further processed and incubated as described in section 2.1.1. for the fecal samples. For each homogenate pH values were measured. To mimic the gastrointestinal tract environment of pigs, samples were incubated at 37°C micro-aerobically (6% CO₂, 6% H₂, 4% O₂, 84% N₂) and at 25°C. Samples were examined daily (inverted microscopy, magnification x400) for the presence of FLP and washed and sub cultured (1:10) with PAS in order to isolate FLP species at a further stage.

When bacterial load was too high, a gentamycin (20 µg/ml PAS, 24h, 25°C) treatment was applied to suppress bacterial growth (Anacarso et al., 2012). Preliminary tests (data not shown) indicated that PYG/PAS medium (proteose peptone yeast extract glucose diluted in 1:1000 PAS) ensured the balance between stimulating protozoan growth and suppressing bacterial growth. Finally, FLP cells were transferred to 25 cm² tissue culture flasks (TPP AG, Trasadingen, Switzerland) in PYG/PAS (1:1000) medium for further cultivation, followed by identification.

2.2.3 Morphological identification of FLP

Free-living protozoa detected in the purified cultures were first identified on the basis of morphology and locomotion by inverted microscopy (magnification x400) using standard taxonomic identification sources (Berger and Foissner, 2003; Jeuck and Arndt, 2013; Lee et al., 2005; Page, 1988; Patterson and Simpson, 1996; Smirnov and Brown, 2004; Smirnov and Goodkov, 1999). Organisms that could not be assigned to a known species or genus were assigned to a morphogroup (ciliates, flagellates or amoebae) and where possible further analysed by molecular based methods.

2.2.4 Molecular biological identification of FLP

Extraction, amplification and sequencing of 18S rDNA

The FLP species recovered from the GIT were isolated for further molecular based identification. Amoebal cells were hand-picked and added to 6-well plates (TPP AG, Trasadingen, Switzerland). The clonal isolates were then further grown in PYG/PAS medium (1:1000), and purified by washing (PAS) and serial dilutions (0.5:1). Cells were removed from the bottom of the 6-well plates by cell scraping (Cell Scraper, TPP), transferred to 25 cm² tissue culture flasks and further cultivated in PYG/PAS medium (1:1000). Cultures were further purified by adding 30 µl of the samples to NNA plates seeded with heat-killed *E. coli*. FLA detected on the plates were cut out of the agar and sub cultured onto new NNA plates seeded with heat-killed *E. coli*. Afterwards, a small piece of agar containing FLA was removed and transferred to 25 cm² tissue culture flasks in PYG/PAS medium (1:1000) for cultivation. Finally, trophozoites of amoebae were harvested by centrifugation (300g, 5 min). A collection of the isolated trophozoites and cysts (if applicable) was stored at -20°C. DNA extraction was performed using the procedure as described by Muyzer et al. (1993) and extracted DNA is stored at -20°C.

18S rDNA was amplified either by a single PCR with the following universal eukaryotic primers (Schroeder et al., 2001): SSU1_F (5'-CTG GTT GAT TCT GCC AGT-3') and SSU2_R (5'-TGA TCC TTC YGC AGG TTC AC-3') or by two separate PCR reactions with the following primer sets (as such the complete 18S region should be covered): (i) SSU1_F (5'-CTG GTT GAT TCT GCC AGT-3') and SSU1_R (5'-CGG CCA TGCACC ACC-3'); and (ii) SSU2_F (5'-CGG TAA TTC CAG CTC C-3') and SSU2_R (5'-TGA TCC TTC YGC AGG TTC AC-3'). The PCR amplification consisted of initialization at 94°C for 3 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 90 s, followed by the final elongation for 10 min at 72°C. As applied in FLA studies of Nassonova et al. (2010) and Kudryavtsev (2014), a fragment of the mitochondrial gene, cytochrome c oxidase (*cox1*) was also amplified using COX1_F (5'-CAA CCA YAA AGA TAT WGG TAC-3') and COX1_R (5'-AAA CTT CWG GRT GAC CAA AAA-3'). Cycles of PCR were as following: initialization at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min and elongation at 72°C for 90 s, followed by the final elongation for 5 min at 72°C. For both PCR amplifications, the PCR mixture contained 2.5 U AmpliTaq DNA polymerase, 1X PCR buffer I (Life Technologies, N8080152), 0.5 µM primers, 10 ng template DNA, 200 µM (each) dNTP's (Life Technologies), 8 nM Bovine Serum Albumin (BSA, Sigma) and Milli-Q water. All PCR products were purified with a PCR purification kit (Qiagen) and quality controlled for the length of amplification on a 2% agarose gel (Life Technologies).

Purified PCR amplicons were ligated in a pGEM-T Easy Vector and transformed into JM109 competent cells according to the manufacturer's recommendations (pGEM-T Easy Vector System II kit, Promega, Cat. No. A3610). White colonies (i.e. successful transformants) were selected and the cloned fragments were amplified as recommended.

PCR products (5 µl) were sent for sequencing (Macrogen Europe, Amsterdam, The Netherlands). The obtained sequences were edited and aligned using BioNumerics 7.1. The length of the amplified sequences ranged from 680 bp (*cox1*) up to 2000 bp (SSU). SSU rDNA and *cox1* sequences were compared with available DNA sequences in the NCBI GenBank using BLASTn searches.

3 Results

3.1 Development and testing of protocols for the recovery of FLP from pig feces

The artificially inoculated FLP species were only recovered and detected with the filtration or sedimentation methods. *Tetrahymena pyriformis* and *A. castellanii* were recovered from all artificially inoculated samples incubated at 25°C, but not from those incubated at 7°C. *Cercomonas* sp. was not recovered at all, irrespective of temperature or method, suggesting that this organism may not survive inoculation in the fecal samples or may not be detected.

The samples obtained after streaking fecal precipitate onto NNA plates seeded with heat-killed *E. coli* and incubated at 25°C did not result in the recovery of any FLP species. Molds covered the plates after a few days of incubation. The samples obtained after applying the Ludox and magnesium sulphate density gradient centrifugation as well as the samples obtained after applying the formalin-ethyl acetate sedimentation concentration did not reveal any protozoan species.

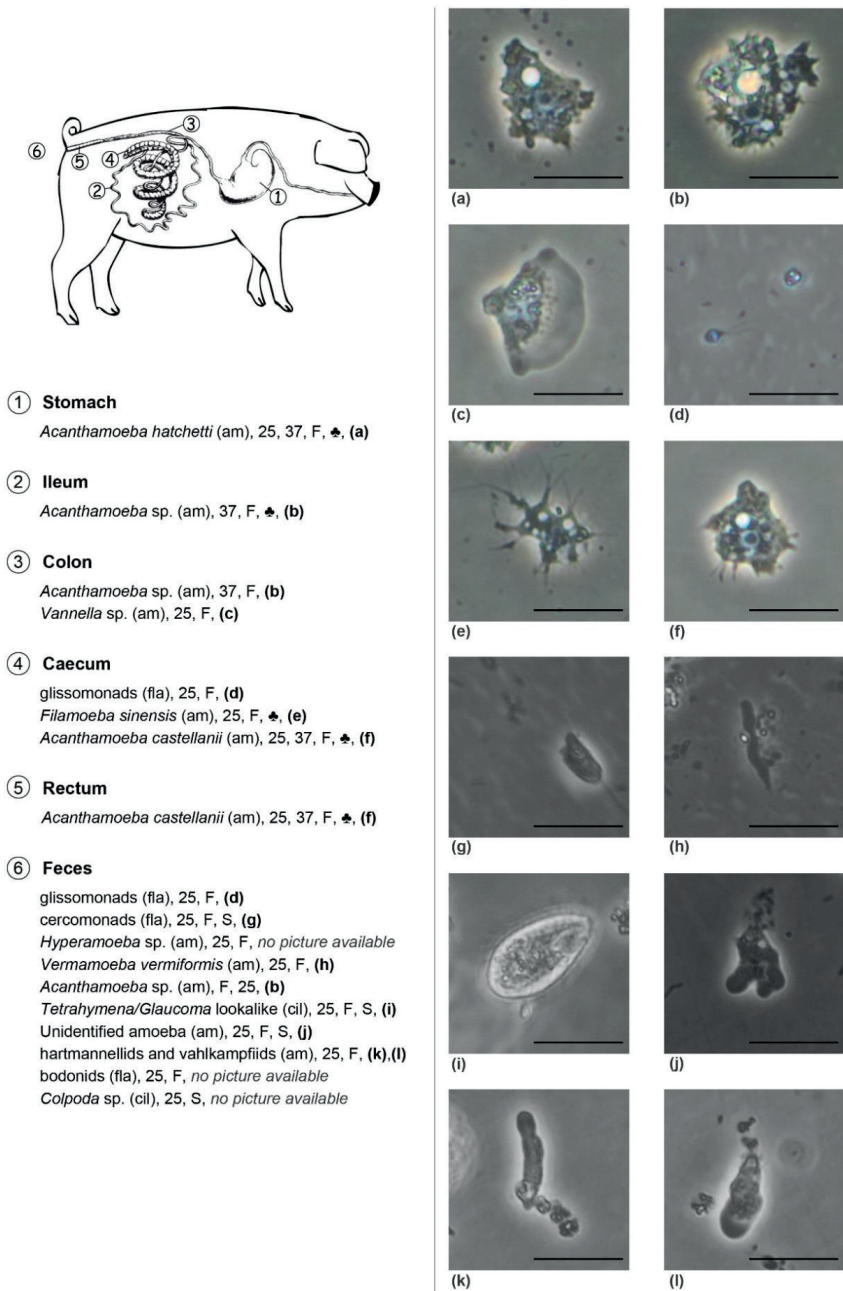


Figure 1. Overview of FLP species isolated from the porcine gastrointestinal tract and feces. Recovered FLP species are summarized per sampling point, i.e. (1) stomach, (2) ileum, (3) colon, (4) caecum, (5) rectum and (6) feces. Morphogroup, incubation temperature and applied recovery method specified: am: amoeba; cil: ciliate; fla: flagellate; 25: incubation at 25°C; 37: incubation at 37°C; F: recovered through filtration; S: recovered through sedimentation. Species identified based on 18S rDNA sequencing are marked with '♣'; letter in bold refers to the corresponding picture of the species (shown at the right); scale bar = 20 µm; inverted microscopy magnification x400

3.2 Exploration of FLP in the porcine gastrointestinal tract and feces

3.2.1 FLP in pig feces

An overview of the isolated FLP species is provided in Figure 1 and Table S1. All examined samples were FLP positive. The artificially inoculated samples incubated at 25°C and used during the development and testing of the protocols (section 2.1.), also harboured other FLP species. Amoebae with unknown affinity (trophozoites and cysts) with an eruptive movement were recovered from the fecal samples when the filtration method was applied. After using the sedimentation method, *Vermamoeba vermiformis*, *Hyperamoeba* sp. and amoebae with unknown affinity, both trophozoites and cysts, were recovered.

Regarding the other pig feces samples, after filtration and three days of incubation at 25°C, amoebal cysts and flagellates were commonly observed. At least two flagellate morphospecies (flagellate 1 and flagellate 2) were present. On the basis of the morphological characteristics, (see Table S1) these flagellates were identified as glissomonads. Other observed flagellates were *Cercomonas* sp. and bodonids. *Tetrahymena/Glaucoma* lookalike ciliate species were frequently observed. Amoebae species like vahlkampfiids, hartmannellids, *Hyperamoeba* sp., *Vermamoeba vermiformis* and *Acanthamoeba* spp. were detected. In all samples, an unidentified cyst-forming amoeba species was detected.

Fewer protozoan species were recovered after applying the sedimentation method compared to the filtration method. After three days of incubation at 25°C, *Tetrahymena/Glaucoma* lookalike and *Colpoda* sp. ciliate species, the flagellated *Cercomonas* sp., *Hyperamoeba* sp., *Acanthamoeba* spp. and the unidentified cyst-forming amoeba species were recovered. The ciliate *Colpoda* sp. was detected after sedimentation but not after filtration.

3.2.2 FLP in the pig gastrointestinal tract

Protozoan species were detected in five of the seven sampled gastrointestinal tracts. Free-living amoebae were the most common, and recovered from stomach, ileum, caecum, colon and rectum and at both incubation temperatures, 25°C and 37°C (Fig. 1). Mean pH values ranged from 4.6 ±0.79 (stomach) to 7.0 ±0.00 (rectum). Free-living protozoa were identified by means of morphology (inverted microscopy) and/or 18S rDNA sequences. FLP identification was done on FLP species isolated from the purified FLP cultures. A detailed overview of the recovered species with corresponding phenotypic features is provided in Table S1.

Amoebae species were detected in the stomach samples of one pig, both incubated at 25°C and 37°C. Trophozoites had a typical *Acanthamoeba* morphology and cysts had multiple endocyst arms. The nucleotide comparison revealed 99% identity with the SSU rDNA sequences of *Acanthamoeba hatchetti*. In the ileum samples of another pig, incubated at 37°C, flattened trophozoites with numerous pseudopodia and double layered cysts of an amoeba were observed. The SSU rDNA sequences revealed a closest match (95% similarity) with the sequence of *Acanthamoeba* sp. In the colon of one pig, FLP were recovered at both incubation temperatures. *Vannella* spp. were identified morphologically and trophozoites of *Acanthamoeba* sp. were observed, which encysted after two weeks of incubation.

Flagellates were observed in the caecum of one pig, at 25°C incubation. Cells were <10 µm and microscopic identification led to a strong association with the group of glissomonads. In the caecal content of another pig (incubation 25°C), a flattened, expanded, slender branched amoeba was observed. The cysts of this species were single walled and smooth. The SSU rDNA sequences matched (99% similarity) with the sequence of *Filamoeba sinensis*. Caecal content of another pig (incubation temperatures 25°C and 37°C) harboured amoebae with irregularly outlined trophozoites and double layered cysts. Cox1 sequences matched (88% similarity) with the sequence of *Acanthamoeba castellanii*. In the rectum samples of one pig, incubated at 25°C and 37°C, trophozoites with a typical *Acanthamoeba* shape and cysts with multiple endocyst arms were observed. Both Cox1 (97% similarity) and SSU rDNA (99% similarity) sequences matched with the sequence of *Acanthamoeba castellanii*.

4 Discussion

The present study demonstrates for the first time the presence of free-living protozoa in the porcine gastrointestinal tract (GIT) and feces. Free-living protozoa were recovered from different parts of the GIT, covering the whole length of the tract from stomach till rectum. No clear relationship can be demonstrated between the presence of a specific FLP species and the location in the GIT or feces. At least a transient passage of FLP through the porcine GIT is proposed; the potential of FLP to attach to and colonize the gastrointestinal tract remains uncertain. In *Acanthamoeba* several adhesins, like mannose-binding proteins, mediating the adhesion of *Acanthamoeba* cells to corneal epithelial cells, have been identified (Garate et al., 2004). Whether these adhesins can also be involved in the attachment of *Acanthamoeba* to intestinal epithelial cells is unknown.

In the present study, different methods were developed and tested for their ability to recover FLP from pig feces. Based on the results, filtration of the intestinal content is identified as the most suitable protocol to recover FLP from the porcine GIT and feces. The advantage of using a filtration method is that viable and cultivable FLP species can be recovered, which greatly enhances morphology-based identification. Sedimentation was less suitable due to the presence of impurities and fecal debris in the samples and a high fungal and bacterial load, which hampered detection and isolation of FLP cells. The remaining high fungal and bacterial load in the samples after applying the sedimentation protocol also resulted in the ineffectiveness of plating the sediment on heat-killed *E. coli* coated NNA plates. Density gradient centrifugation, a flotation technique, was not successful in the recovery of FLP from pig feces. The chance of success of flotation techniques rely on the specific gravity (SG) of the solutions which must be greater than that of the target cells (Dryden et al., 2005). Data about the SG of FLP trophozoites and cysts was not found in literature, but the SG of parasitic protozoan species like *Giardia*, *Cryptosporidium* and *Toxoplasma* are between 1.00 g/cm³ and 1.12 g/cm³ (Dumètre et al., 2012). Despite the assumption that the selected flotation solutions Ludox and magnesium sulphate had an appropriate SG for FLP (1.18 g/cm³ and 1.28 g/cm³ respectively), density gradient centrifugation was ineffective for the recovery of FLP trophozoites and cysts. The sedimentation concentration technique, a routine procedure used for the examination of oocysts and ova of parasitic protozoa in stool and feces (Garcia, 2007), was also unsuccessful for the recovery of FLP from pig feces. Staining and fixation techniques, as applied in the latter protocols, also kill the FLP and usually cause loss of cell shape and structure which renders identification problematic.

The present study suggested that artificial inoculation of the samples with *Tetrahymena* species had a positive effect on the differentiation and recognition of FLP species. One hypothesis might be that *Tetrahymena* spp., which are bacterial consumers, can reduce bacterial growth in the incubated samples. By grazing on bacteria and organic matter present in the gastrointestinal tract and feces, the likelihood to detect FLP is increased and microscopic visualization enhanced. On the contrary, *Tetrahymena* may compete or prey upon other FLP species, which may result in a decreased number of observed FLP species.

Free-living amoebae species like *Hyperamoeba* sp., *Vannella* sp., *Vermamoeba vermiformis* and vahlkampfiids were isolated from the pig fecal samples. *Hyperamoeba* sp., closely related to myxogastric slime molds (Walker, 2003; Walochnik et al., 2004) was previously recovered from dishcloths, human feces and fecally contaminated soils (Chavatte et al., 2014; Karpov and Mylnikov, 1997; Zaman et al., 1999a). *Vannella* spp. are fan-shaped gymnamoeba which occur in anthropogenic and aquatic environments like wastewater (Ramirez et al., 2014), healthcare facilities (Cateau et al., 2014); but also food and food-related environments like vegetable sprouts (Chavatte et al., 2016), dishcloths (Chavatte et al., 2014) and poultry houses (Baré et al., 2009). *Vermamoeba vermiformis* and *Vahlkampfia* sp. are recognized as emerging pathogenic amoebae which can cause amoebic keratitis (Abedkhozasteh et al., 2013; Niyiyati et al., 2010). Flagellates were also recovered from the fecal samples. These were identified as gliding biflagellated protozoa (Rhizaria, Cercozoa) which typically occur in freshwater and soil; and included cercomonads and glissomonads, the latter is an order within the family of Heteromitidae, described by Howe et al. (2009).

Acanthamoeba was the most encountered taxon isolated from the porcine gastrointestinal tract. *Acanthamoeba hatchetti*, isolated from a pig stomach, is a species which was first isolated from brackish and ocean sediments (Sawyer et al., 1977). These amoebae are pathogenic to mice after intranasal inoculation and have been isolated from a horse with severe amoebic placentitis (Begg et al., 2014). Infections in pigs have not yet been reported. *Filamoeba sinensis* was recovered from a pig caecum. The genus *Filamoeba* was first described by Page (1967) as a freshwater amoeba with filose, sometimes branching pseudopods. *Filamoeba* sp. were previously isolated from hot-water piping systems (Dykova et al., 2009). The species *Filamoeba sinensis* was first isolated from the gills of the Prussian Carp (*Carassius gibelio*). To our knowledge this is the first report of *F. sinensis*, ten years after the initial description of this species by Dykova et al. (2005). *Acanthamoeba* and *Filamoeba* species were the most abundant FLA species in soil under desert shrubs (Rodriguez-Zaragoza et al., 2005). These species belong to the group of omnivore amoebae, feeding on bacteria, fungi, algae and yeasts and are adapted to survive extreme environmental conditions like long periods of desiccation.

Free-living amoebae have been reported from water and soil habitats covering a wide range of temperature, salinity and pH conditions (Schuster and Visvesvara, 2004a). According to Khan (2006), *Acanthamoeba* spp. survive and grow at pH range 4 to 12. However, Schuster and Visvesvara (2004c) state that trophozoites are not able to survive passage through the stomach, and that due to the high gastric acidity an intestinal infection with FLA only occur via cysts. In the present study both trophozoites and cysts of amoebae were recovered from the stomach (pH 4.6 ± 0.79) of one pig. Trophozoites of *Vannella* sp. were isolated from the colon of another pig, but no cysts could be detected. The most common freshwater species *Vannella simplex* does not form cysts (Smirnov et al., 2002). This strongly points towards the survival of FLA trophozoites in the animal gastrointestinal tract, or at least in the porcine GIT, supporting the hypothesis of Khan (2006). Survival of FLA trophozoites is also supported by the study of Ramirez et al. (2014), who recovered non-cyst forming FLA like *Vannella*, *Korotnevella*, *Mayorella*, *Thecamoeba* and *Vexillifera* from textile wastewater systems. The trophozoites of these FLA species were able to survive this harsh environment with chemical residues like fabric dyes and fixers. In a study of Thamprasert et al. (1993), *Acanthamoeba* sp. was detected in the ulcerated stomach wall of a woman, who died as a result of a perforated ulcer. They believe that amoebae invaded the stomach wall secondarily, due to the reduction of gastric acidity.

In the present study, species recovery of the gastrointestinal tract and feces samples was highly variable and some samples were FLP negative. Sampling and culturing conditions can be selective for certain FLP species and some species may not grow or multiply in the selected enrichment medium (Fenchel et al., 1997; Smirnov, 2003). As only ten g of intestinal content was examined, this may play a role in the lower FLP species diversity of the porcine GIT and feces. Moreover, the developed and tested methods provided qualitative data. Consequently, the recovered FLP species only illustrate the minimum species richness in pig GIT and feces.

Free-living protozoa are a potential pathway of infection for foodborne pathogens and FLA may play a role in the introduction of pathogenic bacteria in the gastrointestinal tract of animals and humans, as suggested by Bradbury and Forbes (2013). Due to associations with FLP, foodborne pathogens are able to tolerate low pH and able to survive the passage through the human and animal gastrointestinal tract. At lower pH values, survival of *Campylobacter jejuni* was higher when the bacteria were co-incubated with *Acanthamoeba* spp. Moderate acidic conditions (pH 4-5) might trigger bacterial motility, and adhesion and internalization into amoebae (Axelsson-Olsson et al., 2010).

In conclusion, this research demonstrated that FLP are common in 'FLP unknown habitats' like the gastrointestinal tract of pigs. This calls attention to the potential role of FLP in the persistence and transmission of pathogenic bacteria. Internalized bacteria can be released in the environment through induction of host cell lysis or through egestion of food vacuoles (vesicles) or its content (pellets) (Gourabathini et al., 2008; Greub and Raoult, 2004; Raghu Nadhanan and Thomas, 2014). Bacteria inside these vesicles or pellets appear to be even more resistant to external stress factors (Denoncourt et al., 2014). However, our understanding of the ecological and epidemiological role of free-living protozoa in food, food related-, or animal environments is still limited. Whether there is a true colonization or transient passage of free-living protozoa in the gastrointestinal tract must be elucidated. Another pressing research question is whether opportunistic pathogenic FLP like *Acanthamoeba* spp. contribute to a higher prevalence of porcine gastrointestinal pathologies. Later on, the potential role of pigs in the transmission of opportunistic pathogenic FLP and their associated intracellular pathogenic bacteria towards humans and animals might be assessed.

Acknowledgements

This work was supported by the Special Research Fund of Ghent University (BOF, Ghent, Belgium; grant 01J07111). Special thanks to Sofie D'hondt for the molecular analyses and her valuable cooperation in this study. Many thanks to J. Vandenneuvel, C. Van Lancker and other colleagues for collecting the gastrointestinal tracts, and for the technical support and assistance. Thanks to S. De Wannemacker for the help with the illustration.

Origin	mean pH ± SD	Recovery method	Incubation conditions	Identification based on	Morphogroup	Identification	Phenotypic features	Cysts		GenBank accession no.	Percentage similarity, E-value
								Trophozoites	Cysts		
Stomach	4.6 ± 0.79	filtration	25°C & 37°C*	microscopy and 18S rDNA sequencing	amoeba	<i>Acanthamoeba hatchetti</i> (Longamoebia, Discosea)	cell size ±25 µm; amoeba with typical <i>Acanthamoeba</i> features, triangular shape	diameter ±16-18 µm; multiple endocyst arms, ectocyst triangular or quadrangular shape	AF251937	Ident 99%, E-value 0.0	
Ileum	6.2 ± 0.76	filtration	37°C*	microscopy and 18S rDNA sequencing	amoeba	<i>Acanthamoeba</i> sp. (Longamoebia, Discosea)	cell size up to 25 µm; flattened amoeba with numerous subpseudopodia	diameter ±11 µm; reticulate shape, double layered	AF352388	Ident 95%, E-value 0.0	
Colon	6.3 ± 0.49	filtration	25°C	microscopy	amoeba	<i>Vampella</i> sp. (Fiabellinia, Discosea)	cell size B ± 21-28 µm; flabellate shape, anterior broad smooth hyaline margin; floating form with multiple pointed pseudopodia	nd			
		filtration	37°C*	microscopy	amoeba	<i>Acanthamoeba</i> sp. (Longamoebia, Discosea)	cell size ± 27-30 µm; typical <i>Acanthamoeba</i> shape, obvious nucleus and contractile vacuole	nd			
Caecum	5.9 ± 0.38	filtration	25°C	microscopy	flagellate	glissomonads (Cercosoa, Rhizaria)	cell size 2-5 µm; small gliding cells, oval shape, posterior end rounded, jiggly movement	nd			
		filtration	25°C	microscopy and 18S rDNA sequencing	amoeba	<i>Filamoeba sinensis</i> (Graecilipodida, Amoebozoa)	cell size ±15-20 µm, extended form up to 45 µm long; irregular shape, flattened, expanded, slender branched cells; pseudopodia long and fine, ending in filamentous subpseudopodia (up to 18 µm long)	diameter ± 10 µm; spherical, single walled, smooth, no pores	A1714369	Ident 99%, E-value 0.0	

Origin	mean pH ± SD	Recovery method	Incubation conditions	Identification based on	Morphogroup	Identification	Phenotypic features	Cysts		GenBank accession no.	Percentage similarity, E-value
								Trophozoites	Cysts		
Caecum	5.9 ± 0.38	filtration	25°C & 37°C*	microscopy and 18S rDNA sequencing	amoeba	<i>Acanthamoeba castellanii</i> (Longamoebia, Discosoa)	cell size ±27 µm, irregular outline, slender acanthopodia	diameter ± 17µm; double layered	U12386 (Cox1)	ident 88%, E-value 0.0	
Rectum	7.0 ± 0.00	filtration	25°C & 37°C*	microscopy and 18S rDNA sequencing	amoeba	<i>Acanthamoeba castellanii</i> (Longamoebia, Discosoa)	cell size 27-30 µm; tapering, slender subpseudopodia	diameter 10-15 µm, reticulate shape, multiple endocyst arms	U12389 (Cox1); GU001160 (SSU)	ident 97%, E-value 0.0 (Cox1); ident 99%, E-value 0.0 (SSU)	
Feces	/	filtration	25°C	microscopy	flagellate	glissomonads (Cercosoa, Rhizaria)	flagellate 1: cell size ±10 µm; 1 anterior flagellum projecting forwards, agitated movement, changeable cell shape	nd			
						glissomonads (Cercosoa, Rhizaria)	flagellate 2: cell size <10 µm; constant cell shape, oval cell body, posterior protoplasmic tail	nd			
		filtration	25°C	microscopy	amoeba	hartmannellids (Euamoebida, Tubulinea)	cell size L ±22 µm, B ±4 µm; limax amoeba, during locomotion hyaline cap present	nd			
		filtration	25°C	microscopy	amoeba	vahlkampffids (Discristata, Discoba)	cell size L ±19 µm, B ±8 µm; eruptive, rapid movement, limax amoeba, only amoeboid cells observed	nd			
		filtration	25°C	microscopy	flagellate	bodonids (Discristata, Discoba)	cell size <10 µm; ovoid, kidney shaped cells, anterior flagellum, attached to the substrate, jerky movement	nd			
		filtration & sedimentation	25°C	microscopy	flagellate	cercomonads (Cercosoa, Rhizaria)	cell size 12-15 µm, pseudopodia present, anterior flagellum waving	nd			

Origin	mean pH \pm SD	Recovery method	Incubation conditions	Identification based on	Morphogroup	Identification	Phenotypic features	GenBank accession no.	Percentage similarity, E-value						
Feces	/	filtration & sedimentation	25°C	microscopy	amoeba	<i>Hyperamoeba</i> sp. (polyphyletic, taxonomic position unclear)	Trophozoites flagellated form up to 20 μ m long, elongate shape, anterior flagellum, anterior end pointed, uroid present	nd	diameter 8-12 μ m; spherical with irregular outline						
							Cysts			nd	nd				
												microscopy	amoeba	<i>Vermamoeba vermiformis</i> (<i>Euamoebida</i> , <i>Tubulinea</i>)	cell size L \pm 28 μ m, B \pm 4 μ m; elongate, limax amoeba, dark grey coloured
													amoeba	<i>Acanthamoeba</i> sp. (Longamoebia, Discosea)	cell size \pm 27-30 μ m, obvious acanthopodia and contractile vacuole, multiple food vacuoles
												microscopy	ciliate	<i>Tetrahymena/Glaucocystis</i> lookalike (Ciliophora, Alveolata)	cell size \pm 30 μ m, B \pm 15 μ m, pyriform, ovoid cell shape, oral apparatus subapical, longitudinal rows of cilia
												microscopy	amoeba	Unidentified amoeba	cell size L \pm 18-22 μ m, B \pm 7-9 μ m; monopodial, limax amoeba, dark grey coloured; no clear distinction between hyaloplasm and cytoplasm, eruptive movement
												microscopy	ciliate	<i>Colpoda</i> sp. (Ciliophora, Alveolata)	cell size L \pm 30 μ m, reniform cell shape, convex and concave side, oral cilia grouped, contractile vacuole

Table S1: Overview of FLP species isolated from the porcine gastrointestinal tract and feces. Recovered FLP species are listed per sampling point. Detailed descriptions provided: mean pH \pm SD (n=7); applied recovery method; incubation temperature, * micro-aerobic; identification based on microscopy and 18S rDNA sequencing; morphogroup; identification; phenotypic features of trophozoites and cysts; GenBank accession no; percentage similarity and E-value. nd = not detected

*Die Welt des Kleinen auch
Ist wunderbar und groß,
Und aus dem Kleinen
Bauen sich die Welten*

*The world of tiny creatures
Is wondrous to behold,
Yet from such small beginnings
Our living world evolved.*

Christian Gottfried Ehrenberg

General discussion

General discussion

The aim of this thesis is to describe the **occurrence and diversity of FLP** in a food-related environment (dishcloths, chapter 1), on fresh produce (vegetable sprouts, chapter 2), and in animals (gastrointestinal tract and feces of pigs, chapter 3), and where possible interpret the observed patterns in relation to selected environmental conditions. To this end, **protocols for recovering and quantifying FLP** from the specified matrices were developed, tested and evaluated.

1 Methodological remarks

Protocols to recover FLP from the above-mentioned matrices were either newly **developed or adapted from existing protocols**. A comparative overview of these protocols is provided in Figure 1.

Based on artificially inoculated samples, different methods were evaluated for their ability to **recover and quantify** FLP from the examined matrices. Detection of **viable FLP** is crucial in the complete process of recovering and identifying protozoan species in environmental samples, as it enables differentiation of cell shape and cell structures, enhancing microscopic identification.

Recovery

In chapter 1, optimized centrifugation and stomacher protocols to recover FLP from dishcloths were evaluated. Despite the fact that both recovery protocols had low recovery efficiencies (see Table 1 below), they both were suitable (i.e. all three morphogroups were retrieved viable and cultivable) to recover FLP from dishcloths.

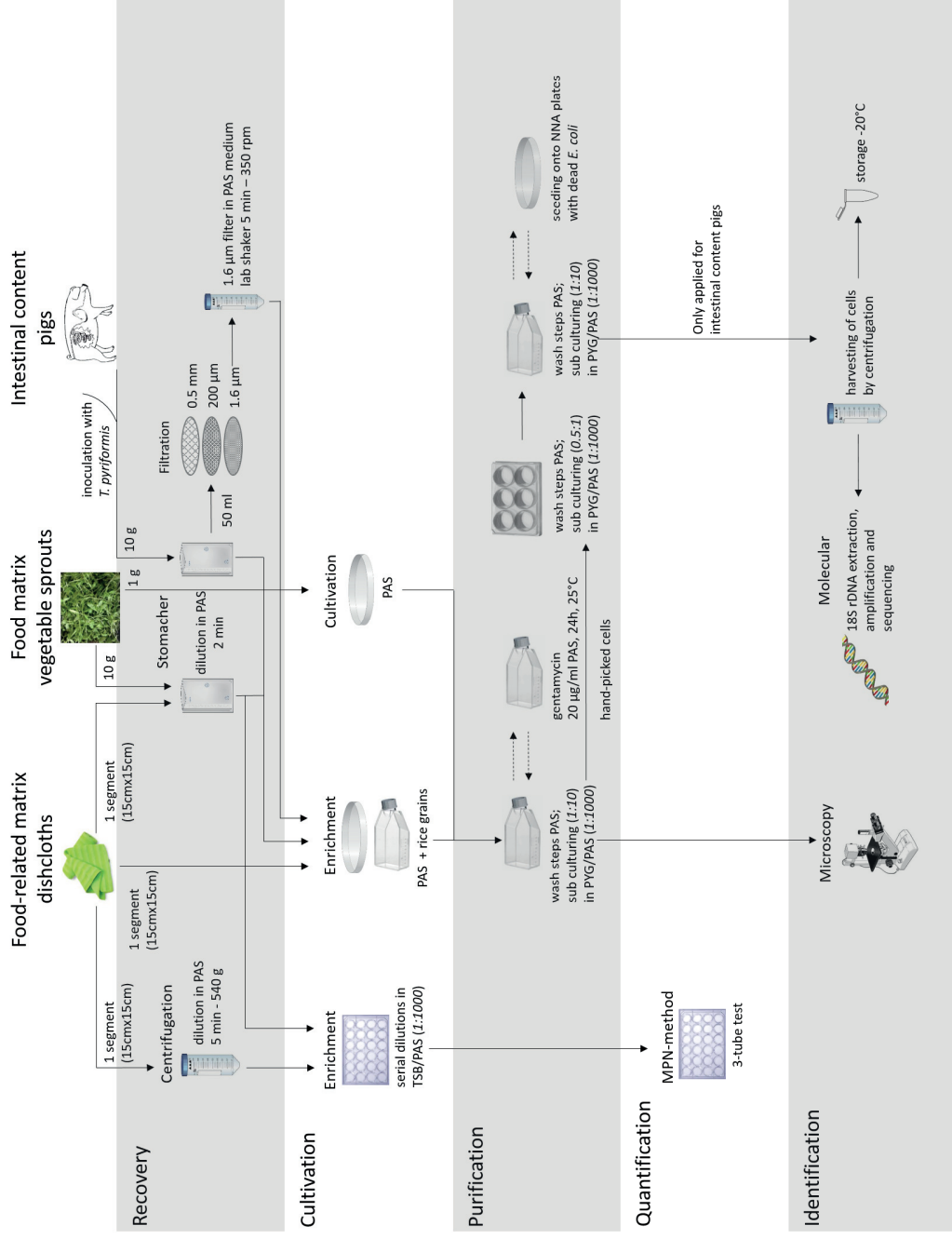
Based on the results of chapter 1 and the fact that the **stomacher** protocol is also a broadly used and standardized protocol in bacteriological analyses of food matrices, it was decided to apply this protocol for the recovery of FLP from vegetable sprouts (chapter 2) and as a starting point to recover FLP from pig intestinal content (chapter 3).

In chapter 3, four methodologies: (1) a filtration based design, (2) a sedimentation based design; (3) density gradient centrifugation and (4) sedimentation concentration, were adapted where necessary and evaluated for the recovery of FLP from pig intestinal content.

Live FLP were only recovered and detected with the filtration and sedimentation methods. However, sedimentation was less suitable due to the presence of impurities and fecal debris in the samples and a high fungal and bacterial load (see chapter 3). **Filtration** of the samples is a solution for the latter problem and was thus identified as the most suitable protocol to recover FLP from the porcine gastrointestinal tract and feces.

Due to the use of fixatives which kill FLP and as such cause loss of cell shape and structure, density gradient centrifugation and sedimentation concentration were not successful for the recovery of FLP from pig feces.

Figure 1. Schematic overview of the developed and applied protocols for the recovery of FLP from dishcloths (a food-related matrix), vegetable sprouts (a food matrix) and pig intestinal content.



In general, recovery efficiencies of FLP from the above mentioned matrices ranged between 0.12% to 8.59% (Table 1) as shown in our experiments with the artificially inoculated samples (chapter 1). Depending on the applied method, differences in the recovery efficiency of specific morphogroups were observed. Ciliates were recovered in higher numbers from dishcloths with the centrifugation protocol than with the stomacher protocol, whereas for amoebae significantly higher numbers were counted with the stomacher method. For flagellates, no statistically significant differences between both recovery methods were found. The lower recovery of amoebae may be due to the high attachment capacity of amoebae which may therefore be better recovered after stomaching. However, the paddle action of the stomaching method might rupture the cell body of ciliates.

Vaerewijck et al. (2011, 2010) evaluated different sampling strategies to recover FLP from lettuce leaves and domestic refrigerators. Swabbing of the surface with cotton wools was suitable to recover FLP from plastic surfaces but showed that amoebae were less effectively recovered compared to flagellates and ciliates.

Evaluation of the recovery efficiency of *Acanthamoeba polyphaga* (amoeba), *Chilomonas paramecium* (flagellate) and *T. pyriformis* (ciliate) from cotton wools after swabbing inoculated (inoculation levels 1×10^5 , 1×10^4 and 1×10^3 cells/100 cm²) plastic coupons, showed a 2 log reduction in protozoan cells for an inoculation level of 1×10^5 cells. Protozoa were rarely recovered at inoculation levels ≤ 100 cells/100 cm² (Vaerewijck et al. 2010).

Besides swabbing, washing, scraping, excision and homogenization were also evaluated and applied to recover FLP from lettuce leaves. The highest number of taxa was obtained after washing, with a recovery efficiency of 10%. Though, differences in the FLP diversity retrieved with the different methods were not statistically significant.

Recovery efficiencies are highly variable and differ depending on the applied protocols, examined volumes, target organisms and applied enumeration method. The same observations were reported for the recovery of viruses and parasitic protozoa from different food matrices.

For the detection of foodborne viruses such as Norovirus (NoV) and hepatitis A virus (HAV) in many food products, different extraction protocols exist (Stals et al., 2012). The elution-concentration protocol is broadly used for extraction of foodborne viruses from carbohydrate- and water-based foods like fruits and vegetables, fat- and protein-based fruits (ready-to-eat products) and shellfish.

Virus recovery efficiencies ranged between 5% and 90%, depending on the food matrix, the type of elution (alkaline or neutral) and concentration (e.g. PEG precipitation). For raspberries and forest fruit mix, the recovery efficiency of NoV genogroup I (GI) ranged between 7.4% and 61.1%, when recovering 10^4 RNA copies per 10 g (Stals et al., 2011). In comparison, concentration of NoV and HAV particles eluted from raspberries and soft fruits by means of ultracentrifugation, resulted in recovery efficiencies of 0.1% (NoV) and 2.5% (HAV) (Rzezutka et al., 2006, 2005).

Highly variable recovery efficiencies were also observed when recovering *Cryptosporidium* oocysts and *Giardia* cysts from seeded shellfish by means of immunomagnetic separation (IMS) (Schets et al., 2013). For oysters, median recoveries were 4.0% and 5.3% for *Giardia* and *Cryptosporidium* (oo)cysts, respectively. Median recoveries of 45% (*Cryptosporidium* oocysts) and 82% (*Giardia* cysts) were observed in mussels.

Experimentally contaminated (mean dose of 408 (oo)cysts/g) basil and raspberries were analyzed for the presence of *Toxoplasma gondii*, *C. parvum* and *G. intestinalis* (oo)cysts. IMS coupled to detection by real-time PCR resulted in variable performances (Hohweyer et al., 2016). For basil, mean recovery efficiencies ranged between 2% and 35% (LOD < 3 oocysts/g for *Cryptosporidium* and *Giardia*, and LOD < 1 oocyst/g for *Toxoplasma*). On raspberries, mean recovery rates were between 2.5% and 21% (LOD < 1 (oo)cyst/g).

Cultivation

Cultivation of FLP (i.e. enrichment or cultivation s.s.) was needed in order to quantify (enumeration) and/or identify (occurrence and diversity) FLP from the examined matrices and therefore applied in all studies.

Cultivation by means of direct incubation (without homogenization) of parts of the matrix (dishcloths and vegetable sprouts), resulted in more FLP positive samples (see chapters 1 and 2). This may be explained by the fact that organisms are not subjected to any mechanical handling. In addition, more volume of sample was examined by direct incubation (1 g) compared to enrichment (0.1 g). The disadvantage of direct incubation is that quantification of the observed FLP is not possible and only FLP diversity of the cultures can be examined.

Most FLP species were only detected after a few days of enrichment, suggesting they were present either in very low numbers or as encysted forms and/or stimulation of growth and excystment by the enrichment medium was necessary. Frequent exposure to chemical and physical stresses (changes in temperature, oxygen and pH, desiccation, disinfection and cleaning treatments), which is typical for the examined matrices, can trigger encystment of many FLP species (Corliss, 2001; Khan, 2006; Lloyd, 2014).

Nonetheless, culturing methods have disadvantages. The chosen medium or culturing conditions are not optimal for all organisms and can be selective for certain FLP species, and consequently only a limited number of species are able to grow (Fenchel et al., 1997; Smirnov, 2003). Organisms may be killed during the preparation of the samples (e.g. stomacher, vortex) or organisms may outcompete each other and prey upon each other.

Organism	Centrifugation		Stomacher	
	MPN	DC	MPN	DC
<i>Tetrahymena pyriformis</i>	4.72 x 10 ³ (8.59)	3.00 x 10 ³ (5.45)	9.17 x 10 ² (1.67)	5.42 x 10 ² (0.99)
<i>Acanthamoeba castellanii</i>	6.50 x 10 ¹ (0.12)	4.39 x 10 ² (0.8)	2.86 x 10 ² (0.52)	1.32 x 10 ³ (2.39)
<i>Cercomonas</i> sp.	4.73 x 10 ² (0.86)	4.03 x 10 ² (0.73)	1.16 x 10 ³ (2.1)	4.97 x 10 ² (0.9)

Table 1. Mean (absolute) number of recovered protozoan organisms/cm² per recovery method (centrifugation, stomacher) and quantification method (mean probable number – MPN, direct counting - DC). Number of spiked organisms were 2 x 10⁶ cells (corresponding to an initial number of 5.5 x 10⁴ cells/cm²) for *T. pyriformis* and *A. castellanii*; and 2 x 10⁴ cells (corresponding to an initial number of 5.5 x 10² cells/cm²) for *Cercomonas* sp. Recovery efficiency between brackets (%).

Purification

Food and food-related matrices are rich in organic material. In order to differentiate and identify FLP organisms, further purification of the samples by regular washing and subculturing with PAS, was necessary. When bacterial load was too high, a gentamycin treatment was applied, though this treatment will also indirectly influence FLP as most environmental FLP species are difficult to render axenic, suggesting they require the presence of other organisms to survive. With respect to the molecular identification of FLP, extra steps (i.e. washing and subculturing of hand-picked cells + seeding onto NNA plates, see Fig. 1) were performed to purify the samples. Samples with a high amount of organic debris and/or high fungal and bacterial load, are sources of contamination and will hamper molecular based identification of FLP species (Thomas et al., 2015).

Quantification

In chapter 1, the Most Probable Number (MPN) method and a direct counting method were evaluated for their ability to quantify FLP from dishcloths.

According to Foissner (1999b) and Berthold & Palzenberger (1995) **direct counting** methods (on living cells) are reliable for enumeration of (active) soil protozoa. However, they also stated that direct counts are inappropriate for naked amoebae and flagellates because many of them are very small and adhere strongly to the (soil) particles. In the present study, due to the large amount of samples direct counting after fixation was required. Our observations revealed that after fixation of the homogenate it was not possible to differentiate the three morphogroups. Fixation causes loss of cell shape and important cell structures like cilia and flagella which renders differentiation problematic. Consequently, direct counting was excluded as a quantification method in further experiments.

Subsamples of the homogenate (after centrifugation or stomacher) were used to evaluate enumeration of FLP by the **MPN method**. After incubation, all three morphogroups were observed in the wells and thus the most probable number of FLP on dishcloths could be estimated (see chapter 1). Consequently the MPN method was also applied to enumerate FLP numbers of vegetable sprouts (chapter 2).

The MPN method is a dilution culture technique and particularly useful when dealing with low numbers of microbial organisms (Blodgett, 2006). Despite the fact that the MPN method is a commonly used enumeration method (Ronn et al., 1995; Vaerewijck et al., 2011), it only provides an estimation of the number of active and encysted protozoa. As stated above, in the present study there was always a reduction in the recovered cells (a reduction of 1 log up to 3 log). Besides, systematic errors (dilution errors, observation errors) may occur and the MPN method has a limited precision (Foissner, 1999b). Consequently, in combination with traditional light microscopy, it will tend to underestimate FLP numbers in samples compared to environmental FLP numbers (Caron, 2009; Chavatte et al., 2014). Today no suitable alternative exists and despite the disadvantages, the MPN method is the most convenient method to enumerate FLP from food or food-related environments.

Identification

After cultivation and purification, the FLP were identified on the basis of phenotypic criteria (mainly morphology and locomotion) by inverted light microscopy. Only in chapter 3, DNA-based identification of some FLP species, recovered from the gastrointestinal tract of pigs, was performed to compare with the light microscopy identification. The choice for a largely morphology-oriented approach was mainly based on lab expertise and the fact that 'omics' approaches for eukaryotic micro-organisms were still not well developed for routine application at the start of this PhD research. Cultivation-independent and cultivation-dependent molecular microbial screening techniques previously applied in the lab (DGGE, T-RFLP) also have their shortcomings (Foster et al., 2012). For example, Baré et al. (2011, 2009) and Vaerewijck et al. (2011, 2010, 2008) revealed that more taxa were observed by microscopy compared to DGGE. Amoebae were mostly underestimated in molecular data (probably due to the use of general eukaryotic primers) but frequently observed in enrichment cultures. On the other hand, some small flagellate groups (e.g. *Spumella*) are hard to identify microscopically but were revealed via DGGE analysis. According to Vaerewijck et al. (2011) these discrepancies occur due to sampling errors, primer bias or PCR artefacts. Moreover, as only prominent bands on DGGE gels are excised and sequenced, which may result in underrepresentation of species. Underestimation of organisms may be also due to the small amount (e.g. subsamples of 2 ml) of the cultures used for DNA extraction and thus species low in number may be missed. The latter can be solved by using the complete sample (concentration of the sample by means of centrifugation) as applied in the study of Baré et al. (2009).

The level of knowledge about global FLP diversity is limited, which restricts the thorough identification of FLP species. This is also due to the limited number of standard identification keys. Identification is based on microscopic observations (morphology and locomotion). Therefore distinct morphological features (cell shape, presence of flagella, number of cilia, ...) must be recognized by the taxonomist or biologist which is mostly time-consuming and difficult due to the small size of protozoan cells. Moreover, identification based on morphology alone, does not detect cryptic species.

Molecular-based methods may present a solution, but have drawbacks. In amplicon analysis, there is the possibility to have bias introduced by primers and/or amplification and persistence of free DNA can hamper the interpretation of community responses to environmental changes (Urich et al., 2008). Taxon-specific protocols and primers must be designed to get the most sequences within that taxon and to exclude other eukaryotic sequences such as fungi (Lara and Acosta-Mercado, 2012). But since FLP are a paraphyletic group, this makes it difficult to develop primers which cover all taxa within amoebae, ciliates and flagellates.

Until today, knowledge of the protist genotypic diversity is still limited but growing. High-throughput methods, such as next generation sequencing (NGS) technologies are promising procedures to reduce this gap in genetic FLP diversity.

In this thesis, NGS have not been applied. Only very recently environmental studies are implementing NGS for estimating protist diversity (Foster et al., 2012; Geisen et al., 2015; Santoferrara et al., 2014; Stoeck et al., 2014). 454 pyrosequencing and Illumina sequencing technologies, with the v4 and v9 variable regions of the SSU rRNA gene as sequencing targets, are more and more applied in environmental studies to estimate microbial eukaryotic diversity (Dunthorn et al., 2012). **Metagenomics**, i.e. environmental and community genomics, is the sequence-based analysis of the collective genome of microorganisms collected in environmental samples (Solieri et al., 2013). **Metatranscriptomics** (transcription of genes in environmental samples) is based on using retrotranscribed environmental RNA instead of amplified DNA. Only preferential gene sequences from active organisms, which contain more RNA, will be revealed (Urich et al., 2008).

The application of NGS in the area of food microbiology is starting to become established, with recently the exploration of the sensitivity of a metagenomic shotgun sequencing method for the detection of STEC on fresh bagged spinach (Leonard et al., 2015). For sure, metagenomic sequencing will open up potentials in the near future (Solieri et al., 2013).

Molecular environmental diversity screening methods have their own technical and conceptual **limitations**, like representative genomic DNA, suitable primers, meaningful taxonomic units of species. Regarding free-living protozoan data, the construction of 18S rDNA libraries are seriously limited since the so-called 'universal' primers are not always universal (Forney et al., 2004). Molecular techniques (based on SSU rRNA genes) should be used, bearing in mind, the biology and ecology of the organisms. Most environmental studies will reveal new species and this requires an ecological and morphological background. Sequence relatedness does not necessarily mean biological or ecological similarity (Lara and Acosta-Mercado, 2012). Other promising 'genes' are nuclear Internal Transcribed Spacer (ITS) and the **mitochondrial markers COI** (cytochrome oxidase, *cox 1* and cytochrome *b*, *cyt b*) (Barth et al., 2006; Chantangsi et al., 2007; Diggles and Adlard, 1997). The COI genes are present in all mitochondriated eukaryotes, have a homologous function, are readily amplified and show a high level of variation, making them useful to detect species divergence (Lara and Acosta-Mercado, 2012).

In summary, molecular biology and traditional morphology and ecology should be combined and basic work on traditional microbial ecology, aiming to isolate and cultivate species, should continue in the hope to link genomic data and morphology to ecosystem functions (Goñi et al. (2014). Consequently, in order to make an inventory of environmental FLP communities a substantial effort in classical protistology, *i.e.* species culturing and description, must be carried out. Parallel use of complementary methods is recommended in order to obtain a more complete FLP inventory.

To conclude, to investigate the occurrence and abundance of FLP communities on food or food-related environments, homogenization (stomacher) in combination with the MPN method is recommended. When only FLP diversity of a sample is targeted, direct cultivation of the samples is more successful. However, when striving for a more complete description of the occurrence, abundance and diversity of FLP, the use of both complementary methods is advised. Additionally, matrices with a high amount of organic debris may require an additional filtration step.

2 Diversity, occurrence and abundance of free-living protozoa on dishcloths, vegetable sprouts and in the porcine gastrointestinal tract and feces

This PhD work presents the first evidence that FLP, including some (opportunistic) pathogens, are a **common and diverse microbial group** in various food-related habitats, viz. dishcloths, vegetable sprouts and in the porcine gastrointestinal tract and feces, comprising a variety of amoeboid, ciliated and flagellated morphotypes.

Diversity

The morphogroup of the **ciliates** (Alveolata, Ciliophora) were mainly Hymenostomatia (*Tetrahymena*, *Glaucoma*, *Colpidium*), Peritrichia (*Epistylis*, *Vorticella*), Colpodida (*Colpoda*), Cyrtophoria (*Chilodonella*), Oligohymenophorea (*Cyclidium*) and Hypotrichia.

Flagellates included mainly Rhizaria, represented by Cercozoa (cercomonads and glissomonads) and Excavata, including Euglenozoa (*Bodo*, *Petalomonas*, *Notosolenus*, eubodonids, neobodonids, euglenids).

Amoebae were represented by the super-group Amoebozoa, including Tubulinea (*Hartmannella*, *Saccamoeba*, *Vermamoeba vermiformis*), Flabellinia (*Vannella*, *Ripella*, *Korotnevela*, *Vexillifera*) and Centramoebida (*Acanthamoeba*), but also included Heterolobosea (*Vahlkampfia*) belonging to the Excavata. *Incertae sedis*, *Hyperamoeba* was observed in all three matrices.

An overview of recovered FLP species from dishcloths, pig intestinal content and other food-related environments like broiler houses, meat-cutting plants and domestic refrigerators is reported in Table 1.

Protozoan diversity recovered from vegetable sprouts and other vegetables is shown in Table 2.

Although dishcloths, vegetable sprouts and the porcine gastrointestinal tract and feces are remarkably different habitats, the overall genus composition was very similar. These habitats are characterized by a **core community**, composed of *Acanthamoeba* spp., vannellids, hartmannellids, vahlkampfiids, *Vermamoeba vermiformis*, and *Hyperamoeba* sp. for amoebae; *Colpoda* spp. and *Tetrahymena* spp. for ciliates; and the most encountered flagellates were *Bodo* spp., glissomonads and cercomonads.

Interestingly, the representatives of the core community of dishcloths, vegetable sprouts and the porcine gastrointestinal tract and feces were also observed in other food and food-related environments (Baré et al., 2010, 2009; Vaerewijck et al., 2011, 2010, 2008).

Broiler houses, meat-cutting plants, domestic refrigerators, dishcloths and pig intestinal content have following taxa in common: Euamoebida (*Hartmannella/Saccamoeba*), Vannellida (*Vannella/Ripella*), *Vahlkampfia* and Euglenozoa (e.g. *Bodo*).

Supergroup	First rank	Second rank	Third rank	Identified taxa	Food-related environment					
					Broiler houses	Meat-cutting plants	Domestic refrigerators	Dishcloths	Pig intestinal content	
Amoebozoa	Tubulinea	Euamoebida		<i>Hartmannella, Saccamoeba</i> spp.	+	+	+	+	+	
				<i>Leptomyxa</i> spp.	+	+		+		
				<i>Flabellula</i> spp.	+			+		
				gephyramoebids				+		
				<i>Cryptodiffugia</i> sp.	+	+				
	Arcellinida			<i>Echinamoeba exundans</i>						
				<i>Vermamoeba vermiformis</i>				+		
	Discosa	Flabellinia		<i>Vannella, Ripella</i> spp.	+	+	+	+	+	
				<i>Platyamoeba placida</i>		+		+		
				<i>Koratnevela, Vexillifera</i> spp.		+		+		
<i>Cochilopodium</i> sp.				+	+		+			
<i>Mayorella</i> spp.				+			+			
Himatismeniida	Longamoebia		dermamoebids.				+			
			<i>Thecamoeba</i> spp.	+	+		+			
			<i>Acanthamoeba</i> spp.	+	+	+	+	+		
			discosae spp.				+			
			<i>Mastigamoeba/Mastigella</i> sp.	+		+				
Opisthokonta	Archamoebae	Mastigamoebaeae	<i>Protostelium mycophaga</i>			+				
			<i>Flamella</i> sp.			+				
	Gracillipodiida			<i>Filamoeba sinensis</i>				+		
				craspedids	+					
	Holozoa	Choanomomada		<i>Diaphanoeca grandis</i>		+				
				<i>Stephanoeca alpicostata</i>		+				
	Nucleomycea			<i>Nuclearia</i> sp.			+			
				bicosoecids						
	SAR	Stramenopiles	Bicosoecida		<i>Saprolegnia parasitica</i>					
					<i>Spumella</i> spp.	+		+		
Alveolata		Protalveolata	Ciliophora	<i>Monochrysis</i> sp.				+		
				<i>Colpodella</i> spp.				+		
				<i>Cyclidium, Tetrahymena, Glaucoma, Vorticella</i> spp.*	+	+	+	+	+	
				<i>Colpoda, Paracolpidium</i> spp. dinozoan sp.				+		
Rhizaria		Cercozoa		<i>Cercomonas, Heteromita</i> spp.	+	+	+	+	+	
				cercomonads				+		

Supergroup	First rank	Second rank	Third rank	Identified taxa	Food-related environment					
					Broiler houses	Meat-cutting plants	Domestic refrigerators	Dishcloths	Pig intestinal content	
SAR	Rhizaria	Cercozoa	Glissomonadida	<i>Sandona, Neoheteromita, Alliapsa</i> spp.*					+	
				glissomonads imbricateans					+	
				<i>Spongamonas minima</i> thecophiloseans	+		+			
				<i>Chloromonas</i> sp., <i>Polytama uvella</i>						
Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae	<i>Vahlkampfia</i> spp.	+				+	
				<i>Adelephamoeba/Naegleria</i> sp.					+	
				<i>Petalomonas, Peranema, Natosolenus, Bodo</i> spp.*	+		+		+	
				<i>Dimastigella trypaniformis</i> <i>Neobodo</i> spp. euglenids					+	
Excavata	Discoba	Discicristata	Heterolobosea	<i>Oxnerella</i> sp.			+			
Incertae sedis Eukaryotes	Centrohelida	Heterophryidae								
				Ciliates with unknown affinity					+	
				Flagellates with unknown affinity					+	
				Amoebae with unknown affinity					+	
				<i>Hyperamoeba</i> sp.						+

Table 1. Overview of protozoan taxa detected in five food-related environments. Compilation of identifications obtained by microscopy and sequencing of 18S rRNA gene fragments. Data obtained from the dishcloths (Chavatte et al., 2014) and pig intestinal content, in combination with Baré et al. (2010, 2009), Snelling et al. (2006a, 2005) and Vaerewijck et al. (2011, 2010, 2008). Classification according to Adl et al. (2012). *some taxa are clustered as represented in the corresponding study. The use of nameless ranked systematics was proposed by Adl et al. (2005); the highest ranks or supergroups of eukaryotes may represent the basis groupings similar to traditional kingdoms.

Supergroup	First rank	Second rank	Third rank	Identified taxa	Vegetables				
					Vegetable sprouts	Lettuce	Spinach	Mushrooms	Carrots, radishes, tomatoes, cauliflowers
Amoebozoa	Tubulinea	Euamoebida		<i>Hartmannella</i> spp.	+	+			+
				<i>Saccamoeba</i> spp.	+	+			
				<i>Paraffabellula</i> sp.	+				
				Branched morphotype	+				
				<i>Vermamoeba vermiformis</i>	+				+
				<i>Ripella</i> spp.	+			+	
				<i>Vannella</i> spp.	+			+	+
				Fan-shaped morphotype	+				
				<i>Koratrevella</i> sp.	+				
				<i>Vexillifera</i> sp.	+				
				Dactylopodial morphotype	+				
				<i>Cochliopodium</i> sp.	+			+	
				<i>Mayorella</i> sp.	+			+	
				<i>Acanthamoeba</i> spp.	+			+	+
				Acanthopodial morphotype	+				
Chromalveolata SAR	Cryptophyceae Stramenopiles Alveolata	Goniomonadales Chrysophyceae Ciliophora	Intramacronucleata	<i>Gonimonas truncata</i>	+	+			
				<i>Spumella</i> spp.	+				
				<i>Aspidisca</i> sp.	+	+			
				<i>Chilodonella</i> spp.	+	+			
				<i>Cinetochilum</i> sp.	+	+			
				<i>Colpidium kleini</i>	+				
				<i>Colpoda</i> spp.	+			+	
				<i>Cyclidium</i> sp.	+			+	
				cyrtophorids	+				
				<i>Epistylis</i> sp.	+				
				<i>Glaucoma</i> sp.	+			+	
				<i>Heliophrya</i> sp.	+				
				hypotrichs	+				+
				oligohymenophorids	+				
				<i>Paracolpidium</i> sp.	+			+	
<i>Paramecium</i> sp.	+								
peritrichs	+								

Protozoan diversity on vegetable sprouts, in total 72 FLP (30 ciliates, 20 flagellates, 22 amoebae), was noticeably higher when compared to the FLP diversity of other vegetables, e.g. on lettuce leaves (Vaerewijck et al., 2011) 28 taxa were identified. Species diversity (only amoebae) of mushrooms and other vegetables were also remarkably lower (5-2). The most likely explanation is that the sampling effort (examined number of samples) of the present study (n=176 vegetable sprout samples vs 64 lettuce leaf samples) was much higher, which resulted in a higher species diversity. Foissner (2008) emphasized that undersampling is the main cause of underestimating protist diversity, e.g. 70% of the actually present species will be missed.

The most common taxa of above mentioned food and food-related habitats were also commonly found in natural soil and aquatic (benthic) environments. Striking similarities were found with the active protist communities in soils (Domonell et al., 2013; Geisen et al., 2015; Geisen et al., 2014). It must be emphasized that, in above mentioned studies, the comparison is based both on the cultivation-dependent approach (*i.e.* identification based on morphology alone) and with a metatranscriptomic approach. Keeping the morphospecies and cryptic species concept in mind, this will lead to different observations and thus identifications.

For amoebae, the super-group Amoebozoa, including Tubulinea (Euamoebida e.g. hartmannellids, Leptomyxida, Arcellinida) and the Discosea (vannellids and Centramoebida, *Acanthamoeba*) and Excavata (Heterolobosea, e.g. vahlkampfiids), were the major groups. For flagellates, Rhizaria (Cercozoa, mainly cercozoans and glissomonads), Straminopiles (Chrysophyceae and Bicosoecida) and Excavata (Bodonida and Euglenida). Ciliates were dominated by Ciliophora (Colpodea, Spirotrichea and Oligohymenophorea).

Occurrence

Besides biotic factors, which are usually difficult to measure and quantify, the occurrence of FLP is primarily influenced by abiotic factors. The examined matrices can generally be characterized as habitats that are prone to variable stresses. The dishcloths are subjected to long periods of desiccation and addition of chemical factors like detergent. FLP on vegetable sprouts are highly influenced by temperature and must manage refrigerator temperatures (7°C or even lower at the producer site), while the gastrointestinal tract of pigs is especially characterized by low to moderate acidity (mean pH of the stomach was 4.6).

In spite of these stress factors, highly diverse protozoan communities were detected in the examined matrices. The core community highlighted genera classified as generalist and euryoecious, *i.e.* taxa that are found in a wide variety of ecological niches and can tolerate a broad range of abiotic conditions (pH, salinity, temperature, humidity).

Free-living amoebae (FLA) of the core community, *Acanthamoeba* spp., vannellids, hartmannellids and vahlkampfiids are able to survive and grow in a variety of habitats (Rodríguez-Zaragoza, 1994), from highly acidic lakes (Amaral-Zettler, 2012), waste water treatment systems (Ramirez et al., 2014), thermal saline baths (Zbikowska et al., 2013) to drinking water (Delafont et al., 2013).

Flagellates belonging to the cercomonads are typical inhabitants of soil and freshwater, marine and brackish habitats (Brabender et al., 2012; Howe et al., 2009) but were also found in high mountain lakes (Triadó-Margarit and Casamayor, 2012). *Bodo* spp. (Jeuck and Arndt, 2013; Lee et al., 2005) are typical freshwater euglenids but were also recovered from Antarctic melt water (Dietrich and Arndt, 2004).

The genus *Colpoda* are dominant soil ciliates (Foissner et al., 2011) but were recently found in epiphytic bromeliads (Duran-Ramirez et al., 2015).

The robustness of some FLP species was also demonstrated by *in vitro* experiments. The work of Vaerewijck et al. (2012) revealed that the effect of benzalkonium chloride, an active ingredient of disinfectants, was highly dependent of exposure time and concentration. Cells exposed to 50 mg/l for 5 min under clean conditions did not have a significant effect on trophozoites of *Acanthamoeba polyphaga*. However, the treatment caused a slight decrease in cell number to about 70% of the original population size. However, less than 10% amoebal cells remained viable after exposure to 100 mg/l for 5 min of this quaternary ammonium salt.

Our own research (data not shown) gave a first indication that induced stress factors like acidification (pH 3 for 1h), osmotic stress (0.1M NaCl for 2h) and oxidative stress (0.01M H₂O₂ for 15 min) did not have a detectable effect on trophozoite morphology, cell integrity and viability of *A. castellanii*. On the other hand, heating of trophozoites at 55°C for 5 min did cause distortion and detachment of *A. castellanii* trophozoites. Our findings correspond with the study of Chang et al. (2013), who demonstrated that superheating of trophozoites (75°C) and cysts (95°C) of *A. castellanii* resulted in a significant decrease in amoebal cells. Stress essays performed in our lab (data not shown), indicated that treatment of cysts with sodium dodecyl sulfate, a detergent, resulted in loosening of the (normally) clustered cysts. Treatment of cysts with biocidal, a disinfectant, resulted in a wrinkled, dehydrated/desintegrated look of the cysts. However, none of these stress treatments resulted in cell death.

Biocides like chlorhexidine (CHA) and polyhexamethylene biguanide (PHMB), both have trophocidal and cysticidal effects (Aksozek et al., 2002; Turner et al., 2000)(Aksozek et al., 2002; Turner et al., 2000). Oxidizing agents like free and combined chlorine and hydrogen peroxide had contrasting efficacies against trophozoites and cysts (Siddiqui et al., 2008; Storey et al., 2004). Other potential trophocidals and cysticidals are ozone and peracetic acid (PAA). Though, concentrations and contact time are crucial factors in the efficacy of these treatments (Hughes and Kilvington, 2001). Besides chemical treatments, heat, UV radiation and other physical treatments offer perspectives as being cysticidal and/or trophocidal (Thomas et al., 2010).

Abundance

All examined matrices harboured FLP but differences in occurrence and abundance of the morphogroups were observed (see chapters 1 and 2). Total numbers of FLP in the examined matrices were highly variable and estimated FLP numbers were up to 10^4 MPN/cm² (dishcloths) and 10^5 MPN/g (vegetable sprouts).

Overall, flagellates were the most abundant morphogroup and the lowest numbers were counted for ciliates.

As stated earlier, these total FLP numbers are an underestimation of the FLP numbers of environmental samples, partly due to the applied enumeration method but also due to the fact that the examined samples were diluted.

Species abundance is inversely correlated with body volume; e.g. 1 ml of fresh or marine water contains 10^6 bacteria, 10^3 protists and 10 zooplankton individuals (Fenchel and Finlay, 2004).

Most studies of protists in natural environments are mainly focusing on species diversity, while only a limited number of studies also quantify protists. In comparison with another food environment, the total number of FLP on lettuce leaves was up to 10^5 MPN/g with also flagellates as the most abundant and ciliates being less abundant (Vaerewijck et al., 2011). These numbers are also within the range (10^4 individuals.g⁻¹ soil) reported for protists in natural soils (Domonell et al., 2013; Finlay et al., 2000; Geisen et al., 2014).

3 Direct or indirect impact on food safety and public health

Free-living protozoa can influence human health in a **direct or indirect way**. In this thesis, (opportunistic) pathogens like *Acanthamoeba* spp. and other emerging pathogenic amoebae *Hartmannella* spp., *Vermamoeba vermiformis*, *Vahlkampfia* spp. and *Hyperamoeba* sp. were recovered from the examined matrices. Although the exact proportion of pathogenic vs. non-pathogenic free-living amoebae of these environments is unknown, the above mentioned amoebae are main representatives of the core community defined in this thesis and these FLA were also observed in other food and food-related environments. Consequently, their ecological role is potentially high, but due to the limited available information on infectious dose and occurrence of infections, the direct implications for human health remains uncertain.

The **most encountered taxon** throughout this dissertation was *Acanthamoeba*. This is probably one of the best studied FLP genera and identifications based on sequencing 18S rRNA genes distinguished 20 genotypes to date (Corsaro et al., 2015). Pathogenicity of specific strains is confirmed and human disease is mostly associated with genotype T4, which is the most common strain isolated from the environment (Maciver et al., 2013). *Acanthamoeba* species are causative agents of *Acanthamoeba* keratitis (AK), a sight-threatening infection of the cornea. Although AK is mainly diagnosed in contact lens wearers, *Acanthamoeba* spp. can also cause infection in non-contact lens wearers. AK is considered a rare disease with an estimated prevalence of 1-9/100000 (Lorenzo-Morales et al., 2015). The infectious dose for *Acanthamoeba* keratitis in mice are 100-1000 trophozoites. *Acanthamoeba* is a dominant gymnamoeba species in aquatic and soil habitats and has the ability to survive diverse environments (Khan, 2006; Tanveer et al., 2013). Moreover, *Acanthamoeba* spp. are known vectors for pathogenic bacteria (Anacarso et al., 2012; Salah et al., 2009; Yousuf et al., 2013), viruses (Lorenzo-Morales et al., 2007a; Marciano-Cabral and Cabral, 2003; Scheid and Schwarzenberger, 2012; Suzan-Monti et al., 2007) and cryptosporidia (Scheid and Schwarzenberger, 2011).

Detection and enumeration of bacterial load in dishcloths in combination with seven important foodborne pathogens has been performed for the first time (chapter 1). Bacterial load on dishcloths was high, with concentrations up to 10^9 cfu/cm². Potential bacterial **foodborne pathogens**, such as *Escherichia coli*, *Staphylococcus aureus*, *Arcobacter butzleri* and *Salmonella* were recovered from dishcloths. Concentrations of these pathogens were up to 10^4 cfu/cm². None of the dishcloths tested positive for *Campylobacter* or *Listeria monocytogenes*.

Vegetable sprouts (Dechet et al., 2014) and the gastrointestinal tract of pigs are also important reservoirs for foodborne bacteria (Aguilar et al., 2014; Botteldoorn et al., 2003; Mikkelsen et al., 2004; Van Damme et al., 2015; Van Driessche et al., 2004).

The **simultaneous occurrence** of FLP and foodborne pathogens on food and in food-related environments creates a microbial niche for bacteria-FLP interactions, is a potential risk factor for cross-contamination and consequently may have implications for food safety and public health.

FLP are potentially important key players in the **survival and transmission** of pathogenic bacteria on food and in food-related environments.

Despite cleaning and disinfection measures, FLP and bacterial communities **persist** in these environments. Baré et al. (2011) showed that protozoan communities persisted across consecutive rearing cycles in broiler houses, despite stringent biosecurity measures. Contamination of these broiler houses with *Campylobacter jejuni* is a huge problem and a major source of human campylobacteriosis. The exact epidemiology is as yet unknown, but the results obtained in the research of Baré et al. (2010) suggested a potential role of FLP as environmental hosts and transmission route for *C. jejuni* in broiler houses. Besides associations with *Campylobacter*, species-specific **interactions** (Cirillo et al., 1997) of other foodborne pathogens such as *Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica* and *Staphylococcus aureus* with *Acanthamoeba* spp., *Vermamoeba vermiformis* and *Vahlkampfia* spp. have been confirmed (Anacarso et al., 2012; Brandl et al., 2005; Gourabathini et al., 2008; Khan and Siddiqui, 2014; Lambrecht et al., 2013; Vaerewijck et al., 2014; Vieira et al., 2016; Wildschutte and Lawrence, 2007).

Many protozoan species are able to form **cysts**, which can also be beneficial for **intracellular pathogenic bacteria**. In the co-cultivation study of Lambrecht et al. (2015a), *in vitro* experiments and transmission electron microscopy confirmed that important foodborne pathogens like *Salmonella enterica* and *Listeria monocytogenes* survived inside cysts (cytosol) of *A. castellanii* for up to 21 days and 14 days respectively, even after exposure of the cysts to antibiotics treatment (100 µg/ml gentamicin) and high acidic conditions (pH 0.2). Moreover, these foodborne pathogenic bacteria grew successfully in media, after induction of excystment of the protozoan host and once the conditions were optimally again.

Free-living amoebae and **waterborne pathogens** share the same ecological niche as demonstrated by the isolation of *Acanthamoeba*, *Vermamoeba*, *Vahlkampfia*, *Naegleria*, *Echinamoeba*, *Protacanthamoeba*, and mycobacteria and *Legionella* from drinking water systems, which can also present a health risk (Delafont et al., 2014, 2013; Taylor et al., 2009; Thomas and Ashbolt, 2011). Environmental water samples harboured 69% cultivable FLA, of which 88% harboured nontuberculous mycobacteria, indicating the role of FLA in the persistence of mycobacteria in water systems (Delafont et al., 2014). In industrial waters, *Legionella* spp., the causative agent of Legionnaires' disease, always co-occurred with *Acanthamoeba*, which is a suitable host and shelter for the pathogenic bacteria (Scheikl et al., 2014). Survival and/or replication of mycobacteria (Adékambi et al., 2006; Delafont et al., 2014; Wheat et al., 2014) and *Vibrio cholerae* (Henst et al., 2015; Shanan et al., 2011) in trophozoites and cysts of *Acanthamoeba* have been reported. Free-living amoeba may increase the virulence of *Legionella* spp. and *Mycobacterium* spp. and other amoeba resisting microorganisms, often responsible for lung infections and acute respiratory illness (Craun et al., 2010; Thomas and Ashbolt, 2011).

Recent studies have been focusing on the potential interactions of **FLA and viruses** (norovirus, pandoravirus, mimivirus, coxsackievirus, adenovirus, etc.) and the fact that these amoebae act as **vehicles and reservoirs** for human viruses (Antwerpen et al., 2015; Hsueh and Gibson, 2015; Mattana et al., 2006; Scheid and Schwarzenberger, 2012; Sharma et al., 2015). Viruses bound to the cell surface can internalize and survive inside the amoebal cytosol and inside the cysts (Scheid and Schwarzenberger, 2012). Noroviruses are cause of foodborne outbreaks and commonly associated with ready to eat food or fresh produce (Callejón et al., 2015). Co-occurrence of FLP, noroviruses and foodborne pathogens on fresh produce (Chavatte et al., 2016; Dechet et al., 2014; Gourabathini et al., 2008; Vaerewijck et al., 2011), and the fact that these foods are consumed raw, increase the health risks.

Data gathered in chapter 3, demonstrated the presence of FLP in the **gastrointestinal tract of pigs** which may have an indirect impact on food safety. Bradbury and Forbes (2013) suggested that FLA may play a role in the introduction of pathogenic bacteria in the gastrointestinal tract of mammals. Moreover, growth and survival of *H. pylori* was favoured in the presence of *Acanthamoeba castellanii* (Winięcka-Krusnell et al., 2002). Associations of FLP with animal and human pathogens like *Helicobacter suis*, cause of gastric ulcers, might be important, but are yet unknown.

4 Future perspectives

The knowledge gathered during this PhD resulted in improved FLP sampling protocols and provides new insights into the diversity and occurrence of protozoan communities on food and in food-related environments. These examined matrices are representative for a wide range of food and food-related matrices. Moreover, the developed sampling protocols can be used as a starting point for further experiments.

In contrast to ample bacterial diversity studies characterising environmental bacterial communities, the description of protist diversity across different environments is lagging behind. Moreover, there is an urgent need for describing FLP communities from food and food-related environments. This increased knowledge will contribute to unravel their ecological significance and function in these environments and in the persistence of (foodborne) pathogenic bacteria. The core community (composed of *Acanthamoeba* spp., vannelids, hartmannellids, vahlkampfiids, *Vermamoeba vermiformis*, and *Hyperamoeba* sp. *Colpoda* spp. and *Tetrahymena* spp., and *Bodo* spp., glissomonads and cercozoans), in the present study observed on dishcloths, vegetable sprouts, and in the gastrointestinal tract of pigs, but also detected in broiler houses, meat-cutting plants, domestic refrigerators and on lettuce, to my opinion, must fulfill an ecological role in these microbiomes which needs to be elucidated. Lukeš et al. (2015) and Parfrey et al. (2014) emphasized that eukaryotes play important, but largely unrecognized roles in multiple ecosystems, e.g. the human intestinal gut ecosystem. The eukaryome can have strong effects on the composition and dynamics of the microbiome, with potential consequences for human health. This eukaryome vs. microbiome idea might be ported to FLP vs. pathogenic bacteria relationships and interactions in food and food-related environments. It is worth testing which effect removal and addition of environmentally isolated FLP communities have on the 'in-house' microbiome, e.g. from food processing areas.

A frequently asked challenge is to provide data on the co-occurrence of FLP and foodborne pathogens detected *in situ* on food or in food-related environments. Until today, Gourabathini et al. (2008) exclusively provided evidence that protozoa (*Tetrahymena* spp. and *Glaucoma* sp.) isolated from fresh produce (spinach and lettuce) were able to sequester internalized *Salmonella enterica* and *E. coli* O157:H7 in vesicles. Moreover, these human pathogens were able to multiply in, and exit from these protozoan vesicles. They provided evidence that vesicle formation can occur directly on leaves of wet produce. Although the conditions of an experimental set-up and *in vitro* protozoan cultures are far from those that occur in natural samples due to the complex influence of biotic and abiotic factors, this remains an initial and essential step in scientific research.

Another challenge is to optimize and validate the protocols developed during this dissertation. How can the recovery efficiency of the protocols be increased, ensuring that they are specific and sensitive enough to recover viable and cultivable FLP, including all three morphogroups, from environmental, food, or food-related environments? Can the recovery efficiency be improved anyway or are the results obtained during this dissertation satisfactory? I believe in continuous improvement.

As stated above, a potential pathway is metagenomic sequencing, that is a powerful tool to explore microbial communities by means of their whole genomes (Foster et al., 2012). For example next generation sequencing of amplicon libraries can detect OTUs that were either dominant or rare in different samples and may reveal an unknown component of diversity (Santoferrara et al., 2014).

In combination with morphological observations, sequences technology, as applied in NGS, enable high-throughput sampling and will increase the knowledge of the global FLP diversity (Santoferrara et al., 2014).

Keeping in mind that reference databases for rDNA sequences still need to expand, as there is a very limited number of sequenced FLP relative to what exists in nature (Foster et al., 2012). Many organisms in the databases are still unclassified and exist as OTUs only (Santoferrara et al., 2014). Therefore, further morphological research is still needed. Moreover according to Foissner (2008) more than 50% of protist morphological diversity is still undescribed.

Regarding methods for quantifying FLP, real-time quantitative PCR (qPCR) and fluorescence-activated cell sorting (FACS), are potential alternative procedures to enumerate protozoan cells in environmental samples. FACS was evaluated in the review of Dumétre and Dardé (2003) as a promising method to detect the parasitic protozoan *Toxoplasma gondii* oocysts in environmental samples.

To conclude, free-living protozoa contribute significantly to the biota and function of food and food-related environments and are of ecological importance. Moreover, the ecological and epidemiological role of free-living protozoa in food, food-related environments, or in animals is largely overlooked, still in its infancy and far from completely unravelled. Information about these microorganisms is elusive and in order to reduce the knowledge gap on this underestimated group the need for further research is underscored.

This PhD research, contributed to a better understanding of the ecological role of FLP in food and food-related environments, allowing scientists to appreciate the diversity of FLP and be more aware that **free-living protozoa do count**.

References

References

- Abedkhojasteh, H., Niyiyati, M., Rahimi, F., Hei-Dari, M., Farnia, S., Rezaeian, M., 2013. First report of *Hartmannella* keratitis in a cosmetic soft contact lens wearer in Iran. *Iran. J. Parasitol.* 8, 481–485.
- Adékambi, T., Salah, S. Ben, Khelif, M., Raoult, D., Drancourt, M., 2006. Survival of environmental mycobacteria in *Acanthamoeba polyphaga*. *Appl. Environ. Microbiol.* 72, 5974–5981.
- Adl, S.M., Simpson, A.G.B., Farmer, M. a., Andersen, R. a., Anderson, O.R., Barta, J.R., Bowser, S.S., Brugerolle, G., Fensome, R. a., Fredericq, S., James, T.Y., Karpov, S., Kugrens, P., Krug, J., Lane, C.E., Lewis, L. a., Lodge, J., Lynn, D.H., Mann, D.G., Mccourt, R.M., Mendoza, L., Moestrup, Ø., Mozley-Standridge, S.E., Nerad, T. a., Shearer, C. a., Smirnov, A. V., Spiegel, F.W., Taylor, M.F.J.R., 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.* 52, 399–451.
- Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., Bowser, S.S., Brown, M.W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., Gall, L. Le, Lynn, D.H., McManus, H., Mitchell, E. a D., Mozley-Stanridge, S.E., Parfrey, L.W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C.L., Smirnov, A., Spiegel, F.W., 2012. The revised classification of eukaryotes. *J. Eukaryot. Microbiol.* 59, 429–493.
- Aguilar, C., Jiménez-Marín, Á., Martins, R.P., Garrido, J.J., 2014. Interaction between *Campylobacter* and intestinal epithelial cells leads to a different proinflammatory response in human and porcine host. *Vet. Immunol. Immunopathol.* 162, 14–23.
- Aksozek, A., McClellan, K., Howard, K., Niederkorn, J.Y., Alizadeh, H., 2002. Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. *J. Parasitol.* 88, 621–623.
- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D., 1994. *Molecular biology of the cell*, Third edit. ed. Garland Publishing - Taylor and Francis Group.
- Alsam, S., Sissons, J., Dudley, R., Khan, N.A., 2005. Mechanisms associated with *Acanthamoeba castellanii* (T4) phagocytosis. *Parasitol. Res.* 96, 402–409.
- Amaral-Zettler, L. a., 2012. Eukaryotic diversity at pH extremes. *Front. Microbiol.* 3, 1–17.
- Anacarso, I., de Niederhäusern, S., Messi, P., Guerrieri, E., Iseppi, R., Sabia, C., Bondi, M., 2012. *Acanthamoeba polyphaga*, a potential environmental vector for the transmission of food-borne and opportunistic pathogens. *J. Basic Microbiol.* 52, 261–268.
- Antwerpen, M., Georgi, E., Zoeller, L., Woelfel, R., Stoecker, K., Scheid, P., 2015. Whole-genome sequencing of a Pandoravirus isolated from keratitis-inducing *Acanthamoeba*. *Genome Announc* 3, 2–3.
- Arndt, H., Hausmann, K., Wolf, M., 2003. Deep-sea heterotrophic nanoflagellates of the Eastern Mediterranean Sea: Qualitative and quantitative aspects of their pelagic and benthic occurrence. *Mar. Ecol. Prog. Ser.* 256, 45–56.
- Axelsson-Olsson, D., Svensson, L., Olofsson, J., Salomon, P., Waldenström, J., Ellström, P., Olsen, B., 2010. Increase in acid tolerance of *Campylobacter jejuni* through coinubation with amoebae. *Appl. Environ. Microbiol.* 76, 4194–4200.

- Axelsson-Olsson, D., Waldenstrom, J., Broman, T., Olsen, B., Holmberg, M., 2005. Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 71, 987–992.
- Azizullah, A., Richter, P., Häder, D.P., 2011. Toxicity assessment of a common laundry detergent using the freshwater flagellate *Euglena gracilis*. *Chemosphere* 84, 1392–1400.
- Balaban, N., Rasooly, A., 2000. Staphylococcal enterotoxins. *Int. J. Food Microbiol.* 61, 1–10.
- Bale, M.J., Bennett, P.M., Beringer, J.E., Hinton, M., 1993. The survival of bacteria exposed to desiccation on surfaces associated with farm-buildings. *J. Appl. Bacteriol.* 75, 519–528.
- Barbeau, J., Buhler, T., 2001. Biofilms augment the number of free-living amoebae in dental unit waterlines. *Res. Microbiol.* 152, 753–760.
- Baré, J., Houf, K., Verstraete, T., Vaerewijck, M., Sabbe, K., 2011. Persistence of free-living protozoan communities across rearing cycles in commercial poultry houses. *Appl. Environ. Microbiol.* 77, 1763–1769.
- Baré, J., Sabbe, K., Huws, S., Vercauteren, D., Braeckmans, K., van Gremberghe, I., Favoreel, H., Houf, K., 2010. Influence of temperature, oxygen and bacterial strain identity on the association of *Campylobacter jejuni* with *Acanthamoeba castellanii*. *FEMS Microbiol. Ecol.* 74, 371–381.
- Baré, J., Sabbe, K., Van Wichelen, J., Van Gremberghe, I., D'hondt, S., Houf, K., 2009. Diversity and habitat specificity of free-living protozoa in commercial poultry houses. *Appl. Environ. Microbiol.* 75, 1417–1426.
- Barker, J., Brown, M.R.W., 1994. Trojan Horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiology* 140, 1253–1259.
- Barth, D., Krenek, S., Fokin, S.I., Berendonk, T.U., 2006. Intraspecific genetic variation in *Paramecium* revealed by mitochondrial cytochrome c oxidase I sequences. *J. Eukaryot. Microbiol.* 53, 20–25.
- Bass, D., Howe, A.T., Mylnikov, A.P., Vickerman, K., Chao, E.E., Edwards Smallbone, J., Snell, J., Cabral, C., Cavalier-Smith, T., 2009. Phylogeny and Classification of Cercomonadida (Protozoa, Cercozoa): *Cercomonas*, *Eocercomonas*, *Paracercomonas*, and *Cavernomonas* gen. nov. *Protist* 160, 483–521.
- Begg, A.P., Todhunter, K., Donahoe, S.L., Krockenberger, M., Šlapeta, J., 2014. Severe amoebic placentitis in a horse caused by an *Acanthamoeba hatchetti* isolate identified using next-generation sequencing. *J. Clin. Microbiol.* 52, 3101–3104.
- Begon, M., Townsend, C., Harper, J., 2006. *Ecology - From individuals to ecosystems*, fourth edit. ed. Blackwell Publishing.
- Beijerinck, M., 1913. De infusies en de ontdekking der bakteriën., in: *Jaarboek van de Koninklijke Aakademie Voor Wetenschappen*. Müller, Amsterdam, pp. 1–28.
- Belanche, A., de la Fuente, G., Newbold, C.J., 2014. Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiol. Ecol.* 90, 663–677.
- Berger, H., Foissner, W., 2003. Illustrated guide and ecological notes to ciliate indicator species (*Protozoa, Ciliophora*) in running waters, lakes, and sewage plants., in: *Handbuch Angewandte Limnologie*. p. 160.

- Berk, S.G., Ting, R.S., Turner, G.W., Ashburn, R.J., 1998. Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. Appl. Environ. Microbiol. 64, 279–286.
- Bertelli, C., Greub, G., 2012. Lateral gene exchanges shape the genomes of amoeba-resisting microorganisms. Front. Cell. Infect. Microbiol. 2, 1–15.
- Berthold, A., Palzenberger, M., 1995. Comparison between direct counts of active soil ciliates (Protozoa) and most probable number estimates obtained by Singh's dilution culture method. Biol. Fertil. Soils 19, 348–356.
- Beuchat, L.R., Scouten, a. J., 2002. Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Appl. Microbiol. 92, 382–395.
- Beumer, R.R., te Giffel, M.C., Spoorenberg, E., Rombouts, F.M., 1996. *Listeria* species in domestic environments. Epidemiol. Infect. 117, 437–442.
- Bianchi, D.M., Gallina, S., Bellio, A., Chiesa, F., Civera, T., Decastelli, L., 2014. Enterotoxin gene profiles of *Staphylococcus aureus* isolated from milk and dairy products in Italy. Lett. Appl. Microbiol. 58, 190–196.
- Blaschek, H.P., Wang, H.H., Agle, M.E., 2007. Biofilms in the food environment, First edit. ed. Blackwell Publishing.
- Blodgett, R., 2006. US Food and Drug Administration online bacteriological analytical manual appendix 2: Most Probable Number from serial dilutions.
- Boenigk, J., Arndt, H., 2002. Bacterivory by heterotrophic flagellates: Community structure and feeding strategies. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 81, 465–480.
- Boenigk, J., Ereshefsky, M., Hoef-Emden, K., Mallet, J., Bass, D., 2012. Concepts in protistology: Species definitions and boundaries. Eur. J. Protistol. 48, 96–102.
- Boenigk, J., Stadler, P., Wiedroither, A., Hahn, M.W., 2004. Strain-specific differences in the grazing sensitivities of closely related ultramicrobacteria affiliated with the *Polynucleobacter* cluster. Appl. Environ. Microbiol. 70, 5787–5793.
- Botteldoorn, N., Heyndrickx, M., Rijpens, N., Herman, L., 2003. Detection and characterization of verotoxigenic *Escherichia coli* by a VTEC/EHEC multiplex PCR in porcine faeces and pig carcass swabs. Res. Microbiol. 154, 97–104.
- Bouyer, S., Imbert, C., Rodier, M.H., Héchard, Y., 2007. Long-term survival of *Legionella pneumophila* associated with *Acanthamoeba castellanii* vesicles: Brief report. Environ. Microbiol. 9, 1341–1344.
- Brabender, M., Kiss, Á.K., Domonell, A., Nitsche, F., Arndt, H., 2012. Phylogenetic and morphological diversity of novel soil cercoconad species with a description of two new genera (*Nucleocercomonas* and *Metabolomonas*). Protist 163, 495–528.
- Bradbury, R.S., Forbes, B. a., 2013. Free-Living amoebae recovered from human stool samples in strongyloides agar culture. J. Clin. Microbiol. 52, 699–700.
- Brandl, M.T., Rosenthal, B.M., Haxo, a. F., Berk, S.G., 2005. Enhanced survival of *Salmonella enterica* in vesicles released by a soilborne *Tetrahymena* species. Appl. Environ. Microbiol. 71, 1562–1569.

- Brewer, M.T., Xiong, N., Dier, J.D., Anderson, K.L., Rasmussen, M. a., Franklin, S.K., Carlson, S. a., 2011. Comparisons of *Salmonella* conjugation and virulence gene hyperexpression mediated by rumen protozoa from domestic and exotic ruminants. *Vet. Microbiol.* 151, 301–306.
- Bridier, a., Briandet, R., Thomas, V., Dubois-Brissonnet, F., 2011. Resistance of bacterial biofilms to disinfectants: a review. *Biofouling* 27, 1017–1032.
- Bridier, A., Sanchez-Vizueté, P., Guilbaud, M., Piard, J.-C., Naïtali, M., Briandet, R., 2015. Biofilm-associated persistence of food-borne pathogens. *Food Microbiol.* 45, 167–178.
- Brindley, N., Matin, A., Khan, N.A., 2009. *Acanthamoeba castellanii*: High antibody prevalence in racially and ethnically diverse populations. *Exp. Parasitol.* 121, 254–256.
- Brown, M.R.W., Barker, J., 1999. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends Microbiol.* 7, 46–50.
- Buchanan, B.B., Gruissem, W., Jones, R.L., 2000. *Biochemistry & molecular biology of plants*. American Society of Plant Physiologists.
- Buchholz, U., Bernard, H., Werber, D., Bohmer, M., Remschmidt, C., Wilking, H., Deleré, Y., an der Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., Faber, M., Frank, C., Fricke, G., Greiner, M., Höhle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Lubber, P., Rosner, B., Stark, K., Kühne, M., 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *N. Engl. J. Med.* 365, 1763–1770.
- Burki, F., 2014. The eukaryotic tree of life from a global phylogenomic perspective. *Cold Spring Harb. Perspect. Biol.* 6, a016147–a016147.
- Callejón, R.M., Rodríguez-Naranjo, M.I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M.C., Troncoso, A.M., 2015. Reported foodborne outbreaks due to fresh produce in the United States and European union: trends and causes. *Foodborne Pathog. Dis.* 12, 32–8.
- Cane, D.E., Ikeda, H., 2012. Exploration and mining of the bacterial terpenome. *Acc. Chem. Res.* 45, 463–472.
- Cardas, M., Khan, N.A., Alsam, S., 2012. *Staphylococcus aureus* exhibit similarities in their interactions with *Acanthamoeba* and ThP1 macrophage-like cells. *Exp. Parasitol.* 132, 513–8.
- Carlson, S. a., Sharma, V.K., McCuddin, Z.P., Rasmussen, M. a., Franklin, S.K., 2007. Involvement of a *Salmonella* genomic island 1 gene in the rumen protozoan-mediated enhancement of invasion for multiple-antibiotic-resistant *Salmonella enterica* serovar typhimurium. *Infect. Immun.* 75, 792–800.
- Caron, D. a., 2009. Past presidents address: Protistan biogeography: Why all the fuss? *J. Eukaryot. Microbiol.* 56, 105–112.
- Carrasco, L., Mena, K.D., Mota, L.C., Ortiz, M., Behravesh, C.B., Gibbs, S.G., Bristol, J.R., Mayberry, L., Cardenas, V.M., 2008. Occurrence of faecal contamination in households along the US-Mexico border. *Lett. Appl. Microbiol.* 46, 682–687.

- Catarino, L.M., Serrano, M.G., Cavazzana, M., Almeida, M.L., Kaneshina, E.K., Campaner, M., Jankevicius, J. V., Teixeira, M.M.G., Itow-Jankevicius, S., 2001. Classification of trypanosomatids from fruits and seeds using morphological, biochemical and molecular markers revealed several genera among fruit isolates. *FEMS Microbiol. Lett.* 201, 65–72.
- Cateau, E., Delafont, V., Hechard, Y., Rodier, M.H., 2014. Free-living amoebae: What part do they play in healthcare-associated infections? *J. Hosp. Infect.* 87, 131–140.
- Chang, C.W., Lu, L.W., Kuo, C.L., Hung, N.T., 2013. Density of environmental *Acanthamoeba* and their responses to superheating disinfection. *Parasitol. Res.* 112, 3687–3696.
- Chantangsi, C., Lynn, D.H., Brandl, M.T., Cole, J.C., Hetrick, N., Ikononi, P., 2007. Barcoding ciliates: A comprehensive study of 75 isolates of the genus *Tetrahymena*. *Int. J. Syst. Evol. Microbiol.* 57, 2412–2425.
- Chappell, C.L., Wright, J. a, Coletta, M., Newsome, A.L., 2001. Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects 8, 724–730.
- Charkowski, A., Sarreal, C., Mandrell, R., 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. *J. Food Prot.* 64, 1292–1298.
- Chavatte, N., Baré, J., Lambrecht, E., Van Damme, I., Vaerewijck, M., Sabbe, K., Houf, K., 2014. Co-occurrence of free-living protozoa and foodborne pathogens on dishcloths: implications for food safety. *Int. J. Food Microbiol.* 191, 89–96.
- Chavatte, N., Lambrecht, E., Van Damme, I., Sabbe, K., Houf, K., 2016. Abundance, diversity and community composition of free-living protozoa on vegetable sprouts. *Food Microbiol.* 55, 55–63.
- Chekabab, S.M., Daigle, F., Charette, S.J., Dozois, C.M., Harel, J., 2012. Survival of enterohemorrhagic *Escherichia coli* in the presence of *Acanthamoeba castellanii* and its dependence on Pho regulon. *Microbiologyopen* 1, 427–37.
- Cirillo, J.D., Falkow, S., Tompkins, L.S., Bermudez, L.E., 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infect. Immun.* 65, 3759–3767.
- Clarke, M., Lohan, A.J., Liu, B., Lagkouvardos, I., Roy, S., Zafar, N., Bertelli, C., Schilde, C., Kianianmomeni, A., Bürglin, T.R., Frech, C., Turcotte, B., Kopec, K.O., Synnott, J.M., Choo, C., Paponov, I., Finkler, A., Heng Tan, C.S., Hutchins, A.P., Weinmeier, T., Rattei, T., Chu, J.S.C., Gimenez, G., Irimia, M., Rigden, D.J., Fitzpatrick, D. a, Lorenzo-Morales, J., Bateman, A., Chiu, C.-H., Tang, P., Hegemann, P., Fromm, H., Raoult, D., Greub, G., Miranda-Saavedra, D., Chen, N., Nash, P., Ginger, M.L., Horn, M., Schaap, P., Caler, L., Loftus, B.J., 2013. Genome of *Acanthamoeba castellanii* highlights extensive lateral gene transfer and early evolution of tyrosine kinase signaling. *Genome Biol.* 14, R11.
- Clement, B.G., Kehl, L.E., DeBord, K.L., Kitts, C.L., 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J. Microbiol. Methods* 31, 135–142.
- Corliss, J.O., 2001. Protozoan cysts and spores, in: *Encyclopedia of Life Sciences*. pp. 1–8.
- Corsaro, D., Walochnik, J., Köhler, M., Rott, M.B., 2015. *Acanthamoeba* misidentification and multiple labels: redefining genotypes T16, T19, and T20 and proposal for *Acanthamoeba micheli* sp. nov. (genotype T19). *Parasitol. Res.*

- Coulon, C., Collignon, A., McDonnell, G., Thomas, V., 2010. Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. *J. Clin. Microbiol.* 48, 2689–2697.
- Craun, G.F., Brunkard, J.M., Yoder, J.S., Roberts, V. a., Carpenter, J., Wade, T., Calderon, R.L., Roberts, J.M., Beach, M.J., Roy, S.L., 2010. Causes of outbreaks associated with drinking water in the United States from 1971 to 2006. *Clin. Microbiol. Rev.* 23, 507–528.
- Crosbie, P.B.B., Munday, B.L., 1999. Environmental factors and chemical agents affecting the growth of the pathogenic marine ciliate *Uronema nigricans*. *Dis. Aquat. Organ.* 36, 213–219.
- Culbertson, C., Smith, J., Minner, J., 1958. *Acanthamoeba*: observations on animal pathogenicity. *Science* (80-). 127, 1506.
- Dawson, D., 2005. Foodborne protozoan parasites. *Int. J. Food Microbiol.* 103, 207–227.
- de Man, J.C., 1975. The probability of most probable numbers. *Eur. J. Appl. Microbiol.* 1, 67–78.
- de Moura, H., Salazar, H.C., Fernandes, O., Lisboa, D.C., de Carvalho, F.G., 1985. Amebas de vida livre no intestino humano evidencias de parasitismo. *Rev. Inst. Med. Trop. Sao Paulo* 27, 150–156.
- De Smet, S., De Zutter, L., Houf, K., 2012. Spatial distribution of the emerging foodborne pathogen *Arcobacter* in the gastrointestinal tract of pigs. *Foodborne Pathog. Dis.* 9, 1097–103.
- Dechet, A.M., Herman, K.M., Chen Parker, C., Taormina, P., Johanson, J., Tauxe, R. V, Mahon, B.E., 2014. Outbreaks caused by sprouts, United States, 1998-2010: lessons learned and solutions needed. *Foodborne Pathog. Dis.* 11, 635–44.
- Delafont, V., Brouke, A., Bouchon, D., Moulin, L., Héchard, Y., 2013. Microbiome of free-living amoebae isolated from drinking water. *Water Res.* 47, 6958–6965.
- Delafont, V., Cambau, E., Joyeux, M., Bouchon, D., Moulin, L., 2014. First Evidence of Amoebae – Mycobacteria Association in Drinking Water Network.
- Denoncourt, A.M., Paquet, V.E., Charette, S.J., 2014. Potential role of bacteria packaging by protozoa in the persistence and transmission of pathogenic bacteria. *Front. Microbiol.* 5, 1–11.
- Devine, R., 2003. Meat consumption trends in the world and the European union. *INRA Prod. Anim.* 16, 325–327.
- Diab-Elschahawi, M., Assadian, O., Blacky, A., Stadler, M., Pernicka, E., Berger, J., Resch, H., Koller, W., 2010. Evaluation of the decontamination efficacy of new and reprocessed microfiber cleaning cloth compared with other commonly used cleaning cloths in the hospital. *Am. J. Infect. Control* 38, 289–292.
- Dietrich, D., Arndt, H., 2004. Benthic heterotrophic flagellates in an Antarctic melt water stream. *Hydrobiologia* 529, 59–70.
- Diggles, B., Adlard, R., 1997. Intraspecific variation in *Cryptocaryon irritans*. *J. Eukaryot. Microbiol.* 44, 25–32.
- Dijk, R., van den Berg, D., Beumer, R.R., de Boer, E., Dijkstra, A.F., Kalkman, P., Stegeman, H., Uyttendaele, M., Veenendaal, H.R., 2012. *Microbiologie van voedingsmiddelen - Methoden, principes en criteria*, Vierde druk. ed. Drs. S Wanders, Rotterdam.

- Dixit, S.M., Gordon, D.M., Wu, X.Y., Chapman, T., Kailasapathy, K., Chin, J.J.C., 2004. Diversity analysis of commensal porcine *Escherichia coli* - Associations between genotypes and habitat in the porcine gastrointestinal tract. *Microbiology* 150, 1735–1740.
- Domonell, A., Brabender, M., Nitsche, F., Bonkowski, M., Arndt, H., 2013. Community structure of cultivable protists in different grassland and forest soils of Thuringia. *Pedobiologia (Jena)*. 56, 1–7.
- Dopheide, A., Lear, G., Stott, R., Lewis, G., 2008. Molecular characterization of ciliate diversity in stream biofilms. *Appl. Environ. Microbiol.* 74, 1740–1747.
- Dorny, P., Praet, N., Deckers, N., Gabriel, S., 2009. Emerging food-borne parasites. *Vet. Parasitol.* 163, 196–206.
- Doudah, L., De Zutter, L., Vandamme, P., Houf, K., 2010. Identification of five human and mammal associated *Arcobacter* species by a novel multiplex-PCR assay. *J. Microbiol. Methods* 80, 281–286.
- Dragesco, A., Dragesco, J., Coste, F., Gasc, C., Romestand, B., Raymond, J.C., Bouxi, G., 1995. *Philasterides dicentrarchi* n. sp., (Ciliophora, Scuticociliatida), a histophagous opportunistic parasite of *Dicentrarchus labrax* (Linnaeus, 1758), a reared marine fish. *Eur. J. Protistol.* 31, 327–340.
- Dryden, M.W., Payne, P. a, Ridley, R., Smith, V., 2005. Comparison of common fecal flotation techniques for the recovery of parasite eggs and oocysts. *Vet. Ther.* 6, 15–28.
- Dumètre, A., Aubert, D., Puech, P.-H., Hohweyer, J., Azas, N., Villena, I., 2012. Interaction forces drive the environmental transmission of pathogenic protozoa. *Appl. Environ. Microbiol.* 78, 905–12.
- Dumètre, A., Dardé, M.-L., 2003. How to detect *Toxoplasma gondii* oocysts in environmental samples? *FEMS Microbiol. Rev.* 27, 651–661.
- Dunthorn, M., Klier, J., Bunge, J., Stoeck, T., 2012. Comparing the hyper-variable V4 and V9 regions of the Small Subunit rDNA for assessment of ciliate environmental diversity. *J. Eukaryot. Microbiol.* 59, 185–187.
- Dupuy, M., Berne, F., Herbelin, P., Binet, M., Berthelot, N., Rodier, M.H., Soreau, S., Héchar, Y., 2014. Sensitivity of free-living amoeba trophozoites and cysts to water disinfectants. *Int. J. Hyg. Environ. Health* 217, 335–339.
- Duran-Ramirez, C.A., Garcia-Franco, J.G., Foissner, W., Mayen-Estrada, R., 2015. Free-living ciliates from epiphytic tank bromeliads in Mexico. *Eur. J. Protistol.* 51, 15–33.
- Dyall, S.D., Brown, M.T., Johnson, P.J., 2004. Ancient invasions: from endosymbionts to organelles. *Science* 304, 253–257.
- Dykova, I., Figueras, A., 1994. Histopathological changes in turbot *Scophthalmus maximus* due to a histophagous ciliate. *Dis. Aquat. Organ.* 18, 5–9.
- Dykova, I., Hrubá, L., Kostka, M., Peckova, H., 2009. *Filamoeba* sp. isolated from hot-water piping system, a host of *Legionella* closely related to *Legionella micdadei*. *Acta Protozool.* 48, 319–325.
- Dyková, I., Lom, J., 2004. Advances in the knowledge of amphizoic amoebae infecting fish. *Folia Parasitol. (Praha)*. 51, 81–97.

- Dyková, I., Pecková, H., Fiala, I., Dvořáková, H., 2005. *Filamoeba sinensis* sp. n., a second species of the genus *Filamoeba* Page, 1967, isolated from gills of *Carassius gibelio* (Bloch, 1782). *Acta Protozool.* 44, 75–80.
- Elsner, J.J., Marzolf, E.R., Goldman, C.R., 1990. Phosphorus and nitrogen limitation of phytoplankton growth in the freshwaters of North-America: a review and critique of experimental enrichments. *Can. J. Fish. Aquat. Sci.* 47, 1468–1477.
- Esteban, G., Tellez, C., 1992. The influence of detergents on the development of ciliate communities in activated sludge. *Water, Air Soil Pollut.* 61, 185–190.
- Esteban, G.F., Clarke, K.J., Olmo, J.L., Finlay, B.J., 2006. Soil protozoa—An intensive study of population dynamics and community structure in an upland grassland. *Appl. Soil Ecol.* 33, 137–151.
- Faulkner, G., Berk, S.G., Garduno, E., Ortiz-Jimenez, M.A., Garduno, R.A., 2008. Passage through *Tetrahymena tropicalis* triggers a rapid morphological differentiation in *Legionella pneumophila*. *J. Bacteriol.* 190, 7728–7738.
- Fenchel, T., 2014. Protozoa and oxygen. *Acta Protozool.* 53, 3–12.
- Fenchel, T., Esteban, G., Finlay, B., 1997. Local versus global diversity of microorganisms: Cryptic diversity of ciliated protozoa. *Oikos* 80, 220–225.
- Fenchel, T., Finlay, B., 2008. Oxygen and the spatial structure of microbial communities. *Biol. Rev.* 83, 553–569.
- Fenchel, T., Finlay, B.J., 2006. The diversity of microbes: resurgence of the phenotype. *Philos. Trans. R. Soc. B Biol. Sci.* 361, 1965–1973.
- Fenchel, T., Finlay, B.J., 2004. The Ubiquity of Small Species: Patterns of Local and Global Diversity. *Bioscience* 54, 777.
- Fernandez-Leborans, G., 2010. Epibiosis in Crustacea: an overview. *Crustaceana* 83, 549–640.
- Fett, W.F., 2000. Naturally occurring biofilms on alfalfa and other types of sprouts. *J. Food Prot.* 63, 625–632.
- Fett, W.F., Cooke, P.H., 2005. A survey of native microbial aggregates on alfalfa, clover and mung bean sprout cotyledons for thickness as determined by confocal scanning laser microscopy. *Food Microbiol.* 22, 253–259.
- Finlay, B.J., 2002. Global dispersal of free-living microbial eukaryote species. *Science* 296, 1061–1063.
- Finlay, B.J., 1990. Physiological ecology of free-living protozoa., in: Marshall, K. (Ed.), *Advances in Microbial Ecology*. Plenum Publishing Corporation, New York, pp. 1–35.
- Finlay, B.J., Black, H.I., Brown, S., Clarke, K.J., Esteban, G.F., Hindle, R.M., Olmo, J.L., Rollett, a, Vickerman, K., 2000. Estimating the growth potential of the soil protozoan community. *Protist* 151, 69–80.
- Finlay, B.J., Corliss, J.O., Esteban, G., Fenchel, T., 1996. Biodiversity at the Microbial Level: The Number of Free-Living Ciliates in the Biosphere. *Q. Rev. Biol.* 71, 221.
- Finlay, B.J., Esteban, G., 1998. Freshwater protozoa: biodiversity and ecological function. *Biodivers. Conserv.* 7, 1163–1196.
- Finlay, B.J., Esteban, G.F., Fenchel, T., 2004. Protist diversity is different? *Protist* 155, 15–22.

- Foissner, W., 2008. Protist diversity and distribution: Some basic considerations. *Biodivers. Conserv.* 17, 235–242.
- Foissner, W., 2006. Biogeography and dispersal of micro-organisms: A review emphasizing protists. *Acta Protozool.* 45, 111–136.
- Foissner, W., 1999a. Protist diversity: estimates of the near-imponderable. *Protist* 150, 363–368.
- Foissner, W., 1999b. Soil protozoa as bioindicators: Pros and cons, methods, diversity, representative examples. *Agric. Ecosyst. Environ.* 74, 95–112.
- Foissner, W., Berger, H., 1996. A user-friendly guide to the ciliates (Protozoa, Ciliophora) commonly used by hydrobiologists as bioindicators in rivers, lakes, and waste waters, with notes on their ecology. *Freshw. Biol.* 35, 375–482.
- Foissner, W., Stoeck, T., Agatha, S., Dunthorn, M., 2011. Intra-class evolution and classification of the Colpodea (Ciliophora). *J. Eukaryot. Microbiol.* 58, 397–415.
- Forney, L.J., Zhou, X., Brown, C.J., 2004. Molecular microbial ecology: Land of the one-eyed king. *Curr. Opin. Microbiol.* 7, 210–220.
- Foster, J. a., Bunge, J., Gilbert, J. a., Moore, J.H., 2012. Measuring the microbiome: Perspectives on advances in DNA-based techniques for exploring microbial life. *Brief. Bioinform.* 13, 420–429.
- Fouque, E., Trouilhé, M.C., Thomas, V., Hartemann, P., Rodier, M.H., Hécharde, Y., 2012. Cellular, biochemical, and molecular changes during encystment of free-living amoebae. *Eukaryot. Cell* 11, 382–387.
- Fowler, M., Carter, R., 1965. Acute pyogenic meningitis probably due to *Acanthamoeba* sp: a preliminary report. *Br. Med. J.* 2, 740–743.
- Frank, C., Werber, D., Cramer, J.P., Askar, M., Faber, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M.J., Follin, P., King, L. a, Rosner, B., Buchholz, U., Stark, K., 2011. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *N. Engl. J. Med.* 1771–80.
- Fransisca, L., Zhou, B., Park, H., Feng, H., 2011. The effect of calcinated calcium and chlorine treatments on *Escherichia coli* O157:H7 87-23 population reduction in radish sprouts. *J. Food Sci.* 76, 404–412.
- Gao, L.Y., Harb, O.S., Kwaik, Y.A., 1997. Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infect. Immun.* 65, 4738–4746.
- Garate, M., Cao, Z., Bateman, E., Panjwani, N., 2004. Cloning and characterization of a novel mannose-binding protein of *Acanthamoeba*. *J. Biol. Chem.* 279, 29849–29856.
- Garcia, L.S., 2007. Macroscopic and microscopic examination of fecal specimens, in: *Diagnostic Medical Parasitology*. ASM Press, Washington, DC, pp. 782–930.
- García, M.T., Jones, S., Pelaz, C., Millar, R.D., Abu Kwaik, Y., 2007. *Acanthamoeba polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after disinfection. *Environ. Microbiol.* 9, 1267–1277.
- Gast, R.J., Sanders, R.W., Caron, D. a., 2009. Ecological strategies of protists and their symbiotic relationships with prokaryotic microbes. *Trends Microbiol.* 17, 563–569.

- Gaze, W.H., Burroughs, N., Gallagher, M.P., Wellington, E.M.H., 2003. Interactions between *Salmonella typhimurium* and *Acanthamoeba polyphaga*, and Observation of a New Mode of Intracellular Growth within Contractile Vacuoles. *Microb. Ecol.* 46, 358–369.
- Geisen, S., Bandow, C., Römbke, J., Bonkowski, M., 2014. Soil water availability strongly alters the community composition of soil protists. *Pedobiologia (Jena)*. 57, 205–213.
- Geisen, S., Tveit, A.T., Clark, I.M., Richter, A., Svenning, M.M., Bonkowski, M., Urich, T., 2015. Metatranscriptomic census of active protists in soils. *ISME J.* 9, 2178–2190.
- Gelman, B.B., Popov, V., Chaljub, G., Nader, R., Rauf, S.J., Nauta, H.W., Visvesvara, G.S., 2003. Neuropathological and ultrastructural features of amebic encephalitis caused by *Sappinia diploidea*. *J. Neuropathol. Exp. Neurol.* 62, 990–998.
- Geurden, T., Thomas, P., Casaert, S., Vercruyse, J., Claerebout, E., 2008. Prevalence and molecular characterisation of *Cryptosporidium* and *Giardia* in lambs and goat kids in Belgium. *Vet. Parasitol.* 155, 142–145.
- Goñi, P., Fernández, M.T., Rubio, E., 2014. Identifying endosymbiont bacteria associated with free-living amoebae. *Environ. Microbiol.* 16, 339–349.
- Gorman, R., Bloomfield, S., Adley, C.C., 2002. A study of cross-contamination of food-borne pathogens in the domestic kitchen in the Republic of Ireland. *Int. J. Food Microbiol.* 76, 143–150.
- Gourabathini, P., Brandl, M.T., Redding, K.S., Gunderson, J.H., Berk, S.G., 2008. Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Appl. Environ. Microbiol.* 74, 2518–2525.
- Greub, G., Raoult, D., 2004. Microorganisms resistant to free-living amoebae. *Clin. Microbiol. Rev.* 17, 413–433.
- Hadas, E., Derda, M., Winięcka-Krusnell, J., Sulek-Piatkowska, A., 2004. *Acanthamoeba* spp. as vehicles of pathogenic bacteria. *Acta Parasitol.* 49, 276–280.
- Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108.
- Hamnes, I.S., Gjerde, B.K., Forberg, T., Robertson, L.J., 2007. Occurrence of *Cryptosporidium* and *Giardia* in suckling piglets in Norway. *Vet. Parasitol.* 144, 222–233.
- Hamp, V., Hug, L., Leigh, J.W., Dacks, J.B., Lang, B.F., Simpson, A.G.B., Roger, A.J., 2009. Phylogenomic analyses support the monophyly of Excavata and resolve relationships among eukaryotic “supergroups”. *Proc. Natl. Acad. Sci. U. S. A.* 106, 3859–64.
- Hausmann, K., Hulsmann, N., Radek, R., 2003. *Protistology*, 3rd comple. ed. E. Schweizerbart’sche Verlagsbuchhandlung, Stuttgart, Germany.
- Heip, C., Vincx, M., Vranken, G., 1985. The ecology of marine nematodes. *Oceanography and marine biology - An annual review* 23.
- Henst, C. Van Der, Scignari, T., Maclachlan, C., Blokesch, M., 2015. An intracellular replication niche for *Vibrio cholerae* in the amoeba *Acanthamoeba castellanii* 1–14.

- Hilton, a. C., Austin, E., 2000. The kitchen dishcloth as a source of and vehicle for foodborne pathogens in a domestic setting. *Int. J. Environ. Health Res.* 10, 257–261.
- Ho, H.T.K., Lipman, L.J.A., Gastra, W., 2006. *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! *Vet. Microbiol.* 115, 1–13.
- Hohweyer, J., Cazeaux, C., Travaillé, E., Languet, E., Dumètre, A., Aubert, D., Terryn, C., Dubey, J.P., Azas, N., Houssin, M., Loïc, F., Villena, I., La Carbona, S., 2016. Simultaneous detection of the protozoan parasites *Toxoplasma*, *Cryptosporidium* and *Giardia* in food matrices and their persistence on basil leaves. *Food Microbiol.* 57, 36–44.
- Holliday, S.L., Scouten, A.J., Beuchat, L.R., 2001. Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *J. Food Prot.* 64, 1489–1495.
- Hoof, K., Devriese, L.A., De Zutter, L., Van Hoof, J., Vandamme, P., 2001. Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. *Int. J. Food Microbiol.* 71, 189–196.
- Hoof, K., Zutter, L. De, Hoof, J. Van, Vandamme, P., 2002. Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. *Appl. Environ. Microbiol.* 68, 2172–2178.
- Howe, A.T., Bass, D., Vickerman, K., Chao, E.E., Cavalier-Smith, T., 2009. Phylogeny, taxonomy, and astounding genetic diversity of Glissomonadida ord. nov., the dominant gliding zooflagellates in soil (Protozoa: Cercozoa). *Protist* 160, 159–189.
- Hsueh, T.Y., Gibson, K.E., 2015. Interactions between Human Norovirus Surrogates and *Acanthamoeba* spp. *Appl. Environ. Microbiol.* 81, 4005–4013.
- Hughes, R., Kilvington, S., 2001. Comparison of hydrogen peroxide contact lens disinfection systems and solutions against *Acanthamoeba polyphaga*. *Antimicrob. Agents Chemother.* 45, 2038–2043.
- Huws, S. a., McBain, a. J., Gilbert, P., 2005. Protozoan grazing and its impact upon population dynamics in biofilm communities. *J. Appl. Microbiol.* 98, 238–244.
- Huws, S. a., Morley, R.J., Jones, M. V., Brown, M.R.W., Smith, A.W., 2008. Interactions of some common pathogenic bacteria with *Acanthamoeba polyphaga*. *FEMS Microbiol. Lett.* 282, 258–265.
- Huws, S.A., Smith, A.W., Enright, M.C., Wood, P.J., Brown, M.R.W., 2006. Amoebae promote persistence of epidemic strains of MRSA. *Environ. Microbiol.* 8, 1130–1133.
- Jackson, V., Blair, I.S., McDowell, D. a., Kennedy, J., Bolton, D.J., 2007. The incidence of significant foodborne pathogens in domestic refrigerators. *Food Control* 18, 346–351.
- Jacob, J., Lior, H., Feuerpfeil, I., 1993. Isolation of *Arcobacter butzleri* from a drinking water reservoir in Eastern Germany. *Int. J. Hyg. Environ. Med.* 193, 557–562.
- Jeuck, A., Arndt, H., 2013. A short guide to common heterotrophic flagellates of freshwater habitats based on the morphology of living organisms. *Protist* 164, 842–860.
- Jongman, R.H.G., ter Braak, C.J.F., Van Tongeren, O.F.R., 1995. Data analysis in community and landscape ecology. Cambridge University Press, Cambridge, United Kingdom.

- Josephson, K.L., Rubino, J.R., Pepper, I.L., 1997. Characterization and quantification of bacterial pathogens and indicator organisms in household kitchens with and without the use of a disinfectant cleaner. *J. Appl. Microbiol.* 83, 737–750.
- Jung, S.Y., Matin, A., Kim, K.S., Khan, N.A., 2007. The capsule plays an important role in *Escherichia coli* K1 interactions with *Acanthamoeba*. *Int. J. Parasitol.* 37, 417–423.
- Jürgens, K., Wickham, S. a., Rothhaupt, K.O., Santer, B., 1996. Feeding rates of macro- and microzooplankton on heterotrophic nanoflagellates. *Limnol. Oceanogr.* 41, 1833–1839.
- Kalina, L. V, Page, F.C., 1992. Culture and preservation of naked amoebae. *Acta Protozool.* 31, 115–126.
- Karpov, S. a., Mylnikov, A.P., 1997. Ultrastructure of the colourless flagellate *Hyperamoeba flagellata* with special reference to the flagellar apparatus. *Eur. J. Protistol.* 33, 349–355.
- Katz, L.A., 2012. Origin and Diversification of Eukaryotes. *Annu. Rev. Microbiol.* 66, 411–427.
- Khan, N., 2009. *Acanthamoeba* - Biology and pathogenesis. Caister Academic Press, Norfolk.
- Khan, N., Iqbal, J., Siddiqui, R., 2014. Taste and smell in disease. *Acta Protozool.* 53, 139–144.
- Khan, N.A., 2006. *Acanthamoeba*: Biology and increasing importance in human health. *FEMS Microbiol. Rev.* 30, 564–595.
- Khan, N.A., Siddiqui, R., 2014. Predator vs aliens: bacteria interactions with *Acanthamoeba*. *Parasitology* 141, 869–74.
- Kilvington, S., Price, J., 1990. Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *J. Appl. Bacteriol.* 68, 519–525.
- King, C.H., Shotts, E.B., Wooley, R.E., Porter, K.G., 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Appl. Environ. Microbiol.* 54, 3023–3033.
- Kleerebezem, M., Quadri, L.E., Kuipers, O.P., de Vos, W.M., 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol.* 24, 895–904.
- Koo, O.K., Martin, E.M., Story, R., Lindsay, D., Ricke, S.C., Crandall, P.G., 2013. Comparison of cleaning fabrics for bacterial removal from food-contact surfaces. *Food Control* 30, 292–297.
- Kudryavtsev, A., 2014. *Paravannella minima* n. g. n. sp. (Discosea, Vannellidae) and distinction of the genera in the vannellid amoebae. *Eur. J. Protistol.* 50, 258–269.
- Kusumaningrum, H.D., Riboldi, G., Hazeleger, W.C., Beumer, R.R., 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *Int. J. Food Microbiol.* 85, 227–236.
- Lambrecht, E., Baré, J., Chavatte, N., Bert, W., Sabbe, K., Houf, K., 2015a. Protozoan cysts act as survival niche and protective shelter for foodborne pathogenic bacteria. *Appl. Environ. Microbiol.* AEM.01031–15.
- Lambrecht, E., Baré, J., Claeys, M., Chavatte, N., Bert, W., Sabbe, K., Houf, K., 2015b. Transmission electron microscopy sample preparation protocols for the ultrastructural study of cysts of free-living protozoa. *Biotechniques* 58, 181–188.

- Lambrecht, E., Baré, J., Van Damme, I., Bert, W., Sabbe, K., Houf, K., 2013. Behavior of *Yersinia enterocolitica* in the presence of the bacterivorous *Acanthamoeba castellanii*. *Appl. Environ. Microbiol.* 79, 6407–6413.
- Landry, K.S., Chang, Y., McClements, D.J., McLandsborough, L., 2014. Effectiveness of a novel spontaneous carvacrol nanoemulsion against *Salmonella enterica* Enteritidis and *Escherichia coli* O157: H7 on contaminated mung bean and alfalfa seeds. *Int. J. Food Microbiol.* 187, 15–21.
- Lara, E., Acosta-Mercado, D., 2012. A molecular perspective on ciliates as soil bioindicators. *Eur. J. Soil Biol.* 49, 107–111.
- Lee, S.Y., 2010. Survival and growth of *Escherichia coli* on various commercial dish sponge/dishcloths and inhibitory effect of UV sterilization with or without moderate heat. *J. Food Saf.* 30, 721–731.
- Lee, W.J., Simpson, A.G.B., Patterson, D.J., 2005. Free-living heterotrophic flagellates from freshwater sites in Tasmania (Australia), a field survey. *Acta Protozool.* 44, 321–350.
- Leonard, S.R., Mammel, M.K., Lacher, D.W., Elkins, C.A., 2015. Application of metagenomic sequencing to food safety: Detection of shiga toxin-producing *Escherichia coli* on fresh bagged spinach. *Appl. Environ. Microbiol.* 81, 8183–8191.
- Leslie, M., 2015. Deep-ocean microbe is closest living relative of complex cells. *Science.* 348, 615–616.
- Lloyd, D., 2014. Encystment in *Acanthamoeba castellanii*: A review. *Exp. Parasitol.* 145, S20–S27.
- Lorenzo-Morales, J., Coronado-Álvarez, N., Martínez-Carretero, E., Maciver, S.K., Valladares, B., 2007a. Detection of four adenovirus serotypes within water-isolated strains of *Acanthamoeba* in the Canary Islands, Spain. *Am. J. Trop. Med. Hyg.* 77, 753–756.
- Lorenzo-Morales, J., Khan, N.A., Walochnik, J., 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite* 22, 10.
- Lorenzo-Morales, J., López-Darías, M., Martínez-Carretero, E., Valladares, B., 2007b. Isolation of potentially pathogenic strains of *Acanthamoeba* in wild squirrels from the Canary Islands and Morocco. *Exp. Parasitol.* 117, 74–79.
- Loret, J.F., Greub, G., 2010. Free-living amoebae: Biological by-passes in water treatment. *Int. J. Hyg. Environ. Health* 213, 167–175.
- Luber, P., 2009. Cross-contamination versus undercooking of poultry meat or eggs - which risks need to be managed first? *Int. J. Food Microbiol.* 134, 21–28.
- Lukeš, J., Stensvold, C.R., Jirků-Pomajbíková, K., Wegener Parfrey, L., 2015. Are Human Intestinal Eukaryotes Beneficial or Commensals? *PLoS Pathog.* 11, 7–12.
- Macías-Rodríguez, M.E., Navarro-Hidalgo, V., Linares-Morales, J.R., Olea-Rodríguez, M. a., Villarruel-López, A., Castro-Rosas, J., Gómez-Aldapa, C. a., Torres-Vitela, M.R., 2013. Microbiological safety of domestic refrigerators and the dishcloths used to clean them in Guadalajara, Jalisco, Mexico. *J. Food Prot.* 76, 984–990.
- Maciver, S.K., Asif, M., Simmen, M.W., Lorenzo-Morales, J., 2013. A systematic analysis of *Acanthamoeba* genotype frequency correlated with source and pathogenicity: T4 is confirmed as a pathogen-rich genotype. *Eur. J. Protistol.* 49, 217–221.

- Madden, R.H., Moran, L., Scates, P., 2007. Diversity of *Campylobacter coli* genotypes in the lower porcine gastrointestinal tract at time of slaughter. *Lett. Appl. Microbiol.* 45, 575–580.
- Maeda, H., Akematsu, T., Fukui, R., Matsuoka, T., 2005. Studies on the resting cyst of ciliated protozoan *Colpoda cucculus*: resistance to temperature and additional inducing factors for en- or excystment. *J. Protozool. Res.* 15, 7–13.
- Mainil, J.G., Daube, G., 2005. Verotoxigenic *Escherichia coli* from animals, humans and foods: Who's who? *J. Appl. Microbiol.* 98, 1332–1344.
- Marciano-Cabral, F., Cabral, G., 2003. *Acanthamoeba* spp. as agents of disease in humans. *Clin. Microbiol. Rev.* 16, 273–307.
- Margulis, L., Schwarz, K., 1988. *Five Kingdoms: an illustrated guide to the phyla of life on earth.*, 2nd editio. ed. Freeman, New York.
- Marie, D., Shi, X.L., Rigaut-Jalabert, F., Vaulot, D., 2010. Use of flow cytometric sorting to better assess the diversity of small photosynthetic eukaryotes in the English Channel. *FEMS Microbiol. Ecol.* 72, 165–178.
- Marie, D., Zhu, F., Balagué, V., Ras, J., Vaulot, D., 2006. Eukaryotic picoplankton communities of the Mediterranean Sea in summer assessed by molecular approaches (DGGE, TTGE, QPCR). *FEMS Microbiol. Ecol.* 55, 403–415.
- Martínez, a. J., Visvesvara, G.S., 2001. *Balamuthia mandrillaris* infection. *J. Med. Microbiol.* 50, 205–207.
- Martinez, A., 1980. Is *Acanthamoeba* encephalitis an opportunistic infection? *Neurology* 30, 567–574.
- Maschio, V.J., Chies, F., Carlesso, A.M., Carvalho, A., Rosa, S.P., Van Der Sand, S.T., Rott, M.B., 2015. *Acanthamoeba* T4, T5 and T11 isolated from mineral water bottles in Southern Brazil. *Curr. Microbiol.* 70, 6–9.
- Matsuo, J., Oguri, S., Nakamura, S., Hanawa, T., Fukumoto, T., Hayashi, Y., Kawaguchi, K., Mizutani, Y., Yao, T., Akizawa, K., Suzuki, H., Simizu, C., Matsuno, K., Kamiya, S., Yamaguchi, H., 2010. Ciliates rapidly enhance the frequency of conjugation between *Escherichia coli* strains through bacterial accumulation in vesicles. *Res. Microbiol.* 161, 711–719.
- Mattana, A., Serra, C., Mariotti, E., Delogu, G., Fiori, P.L., Cappuccinelli, P., 2006. *Acanthamoeba castellanii* promotion of in vitro survival and transmission of coxsackie b3 viruses. *Eukaryot. Cell* 5, 665–71.
- Mattick, K., Durham, K., Domingue, G., Jørgensen, F., Sen, M., Schaffner, D.W., Humphrey, T., 2003. The survival of foodborne pathogens during domestic washing-up and subsequent transfer onto washing-up sponges, kitchen surfaces and food. *Int. J. Food Microbiol.* 85, 213–226.
- Matz, C., Bergfeld, T., Rice, S.A., Kjelleberg, S., 2004. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. *Environ. Microbiol.* 6, 218–226.
- Matz, C., Jürgens, K., 2005. High motility reduces grazing mortality of planktonic bacteria. *Appl. Environ. Microbiol.* 71, 921–929.
- Matz, C., Kjelleberg, S., 2005. Off the hook - how bacteria survive protozoan grazing. *Trends Microbiol.* 13, 302–307.

- Medina, G., Flores-Martin, S., Fonseca, B., Otth, C., Fernandez, H., 2014. Mechanisms associated with phagocytosis of *Arcobacter butzleri* by *Acanthamoeba castellanii*. *Parasitol. Res.* 113, 1933–1942.
- Medlin, L.K., Metfies, K., Mehl, H., Wiltshire, K., Valentin, K., 2006. Picoeukaryotic plankton diversity at the Helgoland time series site as assessed by three molecular methods. *Microb. Ecol.* 52, 53–71.
- Mergeryan, H., 1991. The prevalence of *Acanthamoeba* in the human environment. *Rev. Infect. Dis.* 13, 390–391.
- Merhej, V., Royer-Carenzi, M., Pontarotti, P., Raoult, D., 2009. Massive comparative genomic analysis reveals convergent evolution of specialized bacteria. *Biol. Direct* 4, 13.
- Mikaelyan, A., Strassert, J.F.H., Tokuda, G., Brune, A., 2014. The fibre-associated cellulolytic bacterial community in the hindgut of wood-feeding higher termites (*Nasutitermes* spp.). *Environ. Microbiol.* 16, 2711–2722.
- Mikkelsen, L.L., Naughton, P.J., Mette, S., Jensen, B.B., Hedemann, M.S., 2004. Effects of physical properties of feed on microbial ecology and survival of *Salmonella enterica* serovar Typhimurium in the pig gastrointestinal tract. *Appl. Environ. Microbiol.* 70, 3485–3492.
- Moliner, C., Fournier, P.E., Raoult, D., 2010. Genome analysis of microorganisms living in amoebae reveals a melting pot of evolution. *FEMS Microbiol. Rev.* 34, 281–294.
- Molmeret, M., Horn, M., Wagner, M., Santic, M., Abu Kwaik, Y., 2005. Amoebae as training grounds for intracellular bacterial pathogens. *Appl. environmental Microbiol.* 71, 20–28.
- Montville, R., Schaffner, D.W., 2004. Analysis of published sprout seed sanitization studies shows treatments are highly variable. *J. Food Prot.* 67, 758–765.
- Mulisch, M., 2003a. Morphogenesis and reproduction, in: *Protistology*. pp. 256–271.
- Mulisch, M., 2003b. Nuclei and sexual reproduction, in: *Protistology*. pp. 241–255.
- Munday, B.L., O'Donoghue, P.J., Watts, M., Rough, K., Hawkesford, K., 1997. Fatal encephalitis due to the scuticociliate *Uronema nigricans* in sea-caged, southern bluefin tuna *Thunnus maccoyii*. *Dis. Aquat. Organ.* 30.
- Muyzer, G., De Waal, E.C., Uitterlinden, a. G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- Nagata, T., Kirchman, D.L., 1991. Release of dissolved free and combined amino acids by bacterivorous marine flagellates. *Limnol. Oceanogr.* 36, 433–443.
- Napolitano, J.J., 1982. Isolation of amoebae from edible mushrooms. *Appl. Environ. Microbiol.* 44, 255–257.
- Napolitano, J.J., Colletti-Eggolt, C., 1984. Occurrence of amoebae on oak leaf lettuce (*Lactuca sativa* var. *crispa*) and Boston lettuce (*L. sativa* var. *capitata*). *J. Protozool.* 31, 454–455.
- Nassonova, E., Smirnov, A., Fahrni, J., Pawlowski, J., 2010. Barcoding amoebae: comparison of SSU, IS and COI genes as tools for molecular identification of naked lobose amoebae. *Protist* 161, 102–115.

- Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threlfall, J., Scheutz, F., der Giessen, J. Van, Kruse, H., 2010. Food-borne diseases - The challenges of 20 years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* 139, S3–S15.
- Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Martín-Navarro, C.M., Mohebbali, M., Maghsood, A.H., Farnia, S., Valladares, B., Rezaeian, M., 2010. First report of a mixed infection due to *Acanthamoeba* genotype T3 and *Vahlkampfia* in a cosmetic soft contact lens wearer in Iran. *Exp. Parasitol.* 126, 89–90.
- Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Mohebbali, M., Maghsood, A.H., Motevalli-Haghi, A., Martín-Navarro, C.M., Farnia, S., Valladares, B., Rezaeian, M., 2009. Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. *Exp. Parasitol.* 121, 242–245.
- Nocker, A., Burr, M., Camper, A.K., 2007. Genotypic microbial community profiling: A critical technical review. *Microb. Ecol.* 54, 276–289.
- Nolte, V., Pandey, R.V., Jost, S., Medinger, R., Ottenwälder, B., Boenigk, J., Schlötterer, C., 2010. Contrasting seasonal niche separation between rare and abundant taxa conceals the extent of protist diversity. *Mol. Ecol.* 19, 2908–2915.
- Oguri, S., Matsuo, J., Hayashi, Y., Nakamura, S., Hanawa, T., Fukumoto, T., Mizutani, Y., Yao, T., Akizawa, K., Suzuki, H., Shimizu, C., Matsuno, K., Kamiya, S., Yamaguchi, H., 2011. Ciliates promote the transfer of the gene encoding the extended-spectrum β -lactamase CTX-M-27 between *Escherichia coli* strains. *J. Antimicrob. Chemother.* 66, 527–530.
- Olofsson, J., Axelsson-Olsson, D., Brudin, L., Olsen, B., Ellström, P., 2013. *Campylobacter jejuni* actively invades the amoeba *Acanthamoeba polyphaga* and survives within non digestive vacuoles. *PLoS One* 8.
- Olofsson, J., Griekspoor Berglund, P., Olsen, B., Ellstrom, P., Axelsson-Olsson, D., 2015. The abundant free-living amoeba, *Acanthamoeba polyphaga*, increases the survival of *Campylobacter jejuni* in milk and orange juice. *Infect. Ecol. Epidemiol.* 5, 1–7.
- Otterholt, E., Charnock, C., 2011. Identification and phylogeny of the small eukaryote population of raw and drinking waters. *Water Res.* 45, 2527–2538.
- Page, F.C., 1988. A new key to freshwater and soil gymnamoebae. Freshwater Biological Association, Ambleside, United Kingdom.
- Page, F.C., 1967. *Filamoeba nolandii* n. g., n. sp., a filose amoeba. *Trans. Am. Microsc. Soc.* 86, 405–411.
- Parfrey, L.W., Walters, W.A., Lauber, C.L., Clemente, J.C., Berg-Lyons, D., Teiling, C., Kodira, C., Mohiuddin, M., Brunelle, J., Driscoll, M., Fierer, N., Gilbert, J.A., Knight, R., 2014. Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. *Front. Microbiol.* 5, 1–13.
- Patterson, D.J., 1998. Free-living freshwater protozoa: a colour guide. Manson Publishing Ltd, London.
- Patterson, D.J., Simpson, a. G.B., 1996. Heterotrophic flagellates from coastal marine and hypersaline sediments in Western Australia. *Eur. J. Protistol.* 32, 423–448.
- Pedrós-Alió, C., 2006. Marine microbial diversity: can it be determined? *Trends Microbiol.* 14, 257–263.

- Pernin, P., Pélandakis, M., Rouby, Y., Faure, A., Siclet, F., 1998. Comparative recoveries of *Naegleria fowleri* amoebae from seeded river water by filtration and centrifugation. *Appl. Environ. Microbiol.* 64, 955–959.
- Pernthaler, J., 2005. Predation on prokaryotes in the water column and its ecological implications. *Nat. Rev. Microbiol.* 3, 537–546.
- Pfister, G., Auer, B., Arndt, H., 2002. Pelagic ciliates (Protozoa, Ciliophora) of different brackish and freshwater lakes — a community analysis at the species level. *Limnol. - Ecol. Manag. Inl. Waters* 32, 147–168.
- Pickup, Z.L., Pickup, R., Parry, J.D., 2007. Growth of *Acanthamoeba castellanii* and *Hartmannella vermiformis* on live, heat-killed and DTAF-stained bacterial prey. *FEMS Microbiol. Ecol.* 61, 264–272.
- Poitelon, J.B., Joyeux, M., Welté, B., Duguet, J.P., Peplies, J., Dubow, M.S., 2009. Identification and phylogeny of eukaryotic 18S rDNA phylotypes detected in chlorinated finished drinking water samples from three Parisian surface water treatment plants. *Lett. Appl. Microbiol.* 49, 589–595.
- Raghu Nadhanan, R., Thomas, C.J., 2014. *Colpoda* secrete viable *Listeria monocytogenes* within faecal pellets. *Environ. Microbiol.* 16, 396–404.
- Rahaman Mizan, M.F., Jahid, I.K., Ha, S.-D., 2015. Microbial biofilms in seafood: a food-hygiene challenge. *Food Microbiol.* 49, 41–55.
- Rahdar, M., Niyiyati, M., Salehi, M., Fegghi, M., Makvandi, M., Pourmehdi, M., Farnia, S., 2012. Isolation and genotyping of *Acanthamoeba* strains from environmental sources in Ahvaz city, Khuzestan province, Southern Iran. *J. Parasitol.* 7, 22–26.
- Ramirez, E., Robles, E., Martinez, B., Ayala, R., Sainz, G., Martinez, M.E., Gonzalez, M.E., 2014. Distribution of free-living amoebae in a treatment system of textile industrial wastewater. *Exp. Parasitol.* 145, S34–S38.
- Rivera, F., Ramírez, E., Bonilla, P., Calderón, a, Gallegos, E., Rodríguez, S., Ortiz, R., Zaldivar, B., Ramírez, P., Durán, a, 1993. Pathogenic and free-living amoebae isolated from swimming pools and physiotherapy tubs in Mexico. *Environ. Res.* 62, 43–52.
- Robertson, L.J., Gjerde, B.K., Furusetth Hansen, E., 2010. The zoonotic potential of *Giardia* and *Cryptosporidium* in Norwegian sheep: A longitudinal investigation of 6 flocks of lambs. *Vet. Parasitol.* 171, 140–145.
- Robertson, L.J., Johannessen, G.S., Gjerde, B.K., Loncarevic, S., 2002. Microbiological analysis of seed sprouts in Norway. *Int. J. Food Microbiol.* 75, 119–126.
- Rodríguez-Zaragoza, S., 1994. Ecology of free-living amoebae. *Crit. Rev. Microbiol.* 20, 225–241.
- Rodríguez-Zaragoza, S., Mayzlish, E., Steinberger, Y., 2005. Vertical distribution of the free-living amoeba population in soil under desert shrubs in the Negev Desert, Israel. *Appl. Environ. Microbiol.* 71, 2053–2060.
- Ronn, R., Ekelund, F., Christensen, S., 1995. Optimizing soil extract and broth media for MPN-enumeration of naked amoebae and heterotrophic flagellates in soil. *Pedobiologia (Jena).* 39, 10–19.
- Rose, J.M., Caron, D. a., Sieracki, M.E., Poulton, N., 2004. Counting heterotrophic nanoplanktonic protists in cultures and aquatic communities by flow cytometry. *Aquat. Microb. Ecol.* 34, 263–277.

- Rowbotham, T.J., 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* 33, 1179–1183.
- Rude, R., Jackson, G., Bier, J., Sawyer, T., Risty, N., 1984. Survey of fresh vegetables for nematodes, amoebae, and *Salmonella*. *J. Assoc. Off. Anal. Chem.* 67, 613–615.
- Rzezutka, A., Alotaibi, M., D'Agostino, M., Cook, N., 2005. A centrifugation-based method for extraction of Norovirus from raspberries. *J. Food Prot.* 68, 1923–1925.
- Rzezutka, A., D'Agostino, M., Cook, N., 2006. An ultracentrifugation-based approach to the detection of hepatitis A virus in soft fruits. *Int. J. Food Microbiol.* 108, 315–320.
- Salah, I.B., Ghigo, E., Drancourt, M., 2009. Free-living amoebae, a training field for macrophage resistance of mycobacteria. *Clin. Microbiol. Infect.* 15, 894–905.
- Santoferrara, L.F., Grattepanche, J.D., Katz, L. a., Mcmanus, G.B., 2014. Pyrosequencing for assessing diversity of eukaryotic microbes: Analysis of data on marine planktonic ciliates and comparison with traditional methods. *Environ. Microbiol.* 16, 2752–2763.
- Sapp, J., 2009. *The new foundations of evolution: On the tree of life*. Oxford University Press, New York.
- Sawyer, T.K., Visvesvara, G.S., Harke, B. a, 1977. Pathogenic amoebas from brackish and ocean sediments, with a description of *Acanthamoeba hatchetti*, n. sp. *Science* 196, 1324–1325.
- Scheid, P., Schwarzenberger, R., 2012. *Acanthamoeba* spp. as vehicle and reservoir of adenoviruses. *Parasitol. Res.* 111, 479–85.
- Scheid, P., Zöller, L., Pressmar, S., Richard, G., Michel, R., 2008. An extraordinary endocytobiont in *Acanthamoeba* sp. isolated from a patient with keratitis. *Parasitol. Res.* 102, 945–950.
- Scheid, P.L., Schwarzenberger, R., 2011. Free-living amoebae as vectors of cryptosporidia. *Parasitol. Res.* 109, 499–504.
- Scheikl, U., Sommer, R., Kirschner, A., Rameder, A., Schrammel, B., Zweimüller, I., Wesner, W., Hinker, M., Walochnik, J., 2014. Free-living amoebae (FLA) co-occurring with legionellae in industrial waters. *Eur. J. Protistol.* 50, 422–429.
- Schets, F.M., van den Berg, H.H.J.L., de Roda Husman, A.M., 2013. Determination of the recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts from seeded bivalve mollusks. *J. Food Prot.* 76, 93–8.
- Schlimme, W., Marchiani, M., Hanselmann, K., Jenni, B., 1997. Gene transfer between bacteria within digestive vacuoles of protozoa. *FEMS Microbiol. Ecol.*
- Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I. a., Seal, D. V., Markus, M.B., Fuerst, P. a., Byers, T.J., 2001. Use of subgenomic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* spp. from humans with keratitis and from sewage sludge. *J. Clin. Microbiol.* 39, 1903–1911.
- Schuppler, M., 2014. How the interaction of *Listeria monocytogenes* and *Acanthamoeba* spp. affects growth and distribution of the food borne pathogen. *Appl. Microbiol. Biotechnol.* 1–10.

- Schuster, F.L., Visvesvara, G.S., 2004a. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int. J. Parasitol.* 34, 1001–1027.
- Schuster, F.L., Visvesvara, G.S., 2004b. Opportunistic amoebae: Challenges in prophylaxis and treatment. *Drug Resist. Updat.* 7, 41–51.
- Schuster, F.L., Visvesvara, G.S., 2004c. Amebae and ciliated protozoa as causal agents of waterborne zoonotic disease. *Vet. Parasitol.* 126, 91–120.
- Scott, E., Duty, S., Callahan, M., 2008. A pilot study to isolate *Staphylococcus aureus* and methicillin-resistant *S aureus* from environmental surfaces in the home. *Am. J. Infect. Control* 36, 458–460.
- Shanan, S., Abd, H., Hedenström, I., Saeed, A., Sandström, G., 2011. Detection of *Vibrio cholerae* and *Acanthamoeba* species from same natural water samples collected from different cholera endemic areas in Sudan. *BMC Res. Notes* 4, 109.
- Sharma, A., Pandey, R., Pandey, K., 2004. A report on the occurrence of amphizoic amoebae from carrot. *Flora Fauna* 10, 141–143.
- Sharma, M., Eastridge, J., Mudd, C., 2009. Effective household disinfection methods of kitchen sponges. *Food Control* 20, 310–313.
- Sharma, V., Colson, P., Chabrol, O., Scheid, P., Pontarotti, P., Raoult, D., 2015. Welcome to pandoraviruses at the “Fourth TRUC” club. *Front. Microbiol.* 6, 1–11.
- Sherr, E.B., Sherr, B.F., 2002. Significance of predation by protists in aquatic microbial food webs. *Antonie Van Leeuwenhoek* 81, 293–308.
- Sherr, E.B., Sherr, B.F., 1994. Bacterivory and herbivory: key roles of phagotrophic protists in pelagic food webs. *Microb. Ecol.* 28, 223–235.
- Siddiqui, R., Ortega-Rivas, A., Khan, N.A., 2008. *Balamuthia mandrillaris* resistance to hostile conditions. *J. Med. Microbiol.* 57, 428–31.
- Sigee, D.C., 2005. *Freshwater Microbiology*.
- Sikin, A.M., Zoellner, C., Rizvi, S.S.H., 2013. Current intervention strategies for the microbial safety of sprouts. *J. Food Prot.* 76, 2099–123.
- Singh, T., Coogan, M.M., 2005. Isolation of pathogenic *Legionella* species and legionella-laden amoebae in dental unit waterlines. *J. Hosp. Infect.* 61, 257–262.
- Smilauer, P., Lepš, J., 2014. *Multivariate Analysis of Ecological Data using CANOCO 5*. Cambridge University Press, Cambridge.
- Smirnov, A. V., Chao, E., Nasonova, E.S., Cavalier-Smith, T., 2011. A revised classification of naked lobose amoebae (Amoebozoa: Lobosa). *Protist* 162, 545–570.
- Smirnov, A. V., 2003. Optimizing methods of the recovery of gymnamoebae from environmental samples: a test of ten popular enrichment media , with some observations on the development of cultures. *Protistology* 3, 45–57.

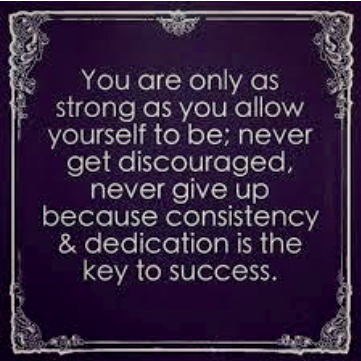
- Smirnov, A. V, Brown, S., 2004. Guide to the methods of study and identification of soil gymnamoebae. *Protistology* 3, 148–190.
- Smirnov, A. V, Goodkov, A. V, 1999. An illustrated list of basic morphotypes of Gymnamoebia (Rhizopoda, Lobosea). *Protistology* 1, 20–29.
- Smirnov, A., Nassonova, E., Holzmann, M., Pawlowski, J., 2002. Morphological, ecological and molecular studies of *Vannella simplex* Wohlfarth-Bottermann 1960 (Lobosea, Gymnamoebia), with a new diagnosis of this species. *Protist* 153, 367–377.
- Snelling, W.J., McKenna, J.P., Hack, C.J., Moore, J.E., Dooley, J.S.G., 2006a. An examination of the diversity of a novel *Campylobacter* reservoir. *Arch. Microbiol.* 186, 31–40.
- Snelling, W.J., McKenna, J.P., Lecky, D.M., Dooley, J.S.G., 2005. Survival of *Campylobacter jejuni* in waterborne protozoa. *Appl. Environ. Microbiol.* 71, 5560–71.
- Snelling, W.J., Moore, J.E., McKenna, J.P., Lecky, D.M., Dooley, J.S.G., 2006b. Bacterial-protozoa interactions; an update on the role these phenomena play towards human illness. *Microbes Infect.* 8, 578–587.
- Snelling, W.J., Stern, N.J., Lowery, C.J., Moore, J.E., Gibbons, E., Baker, C., Dooley, J.S.G., 2008. Colonization of broilers by *Campylobacter jejuni* internalized within *Acanthamoeba castellanii*. *Arch. Microbiol.* 189, 175–179.
- Sogin, M.L., 1991. Early evolution and the origin of eukaryotes. *Curr. Opin. Genet. Dev.* 1, 457–463.
- Sogin, M.L., Elwood, H.J., Gunderson, J.H., 1986. Evolutionary diversity of eukaryotic small-subunit rRNA genes. *Proc. Natl. Acad. Sci. U. S. A.* 83, 1383–1387.
- Sogin, M.L., Gunderson, J.H., Elwood, H.J., Alonso, R. a, Peattie, D. a, 1989. Phylogenetic meaning of the kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*. *Science* 243, 75–77.
- Solaymani-Mohammadi, S., Petri, W. a., 2006. Zoonotic implications of the swine-transmitted protozoal infections. *Vet. Parasitol.* 140, 189–203.
- Solieri, L., Dakal, T.C., Giudici, P., 2013. Next-generation sequencing and its potential impact on food microbial genomics. *Ann. Microbiol.* 63, 21–37.
- Soon, J.M., Seaman, P., Baines, R.N., 2013. *Escherichia coli* O104: H4 outbreak from sprouted seeds. *Int. J. Hyg. Environ. Health* 216, 346–354.
- Sriram, R., Shoff, M., Booton, G., Fuerst, P., Visvesvara, G.S., 2008. Survival of *Acanthamoeba* cysts after desiccation for more than 20 years. *J. Clin. Microbiol.* 46, 4045–4048.
- Stals, A., Baert, L., De Keuckelaere, A., Van Coillie, E., Uyttendaele, M., 2011. Evaluation of a norovirus detection methodology for ready-to-eat foods. *Int. J. Food Microbiol.* 145, 420–425.
- Stals, A., Baert, L., Van Coillie, E., Uyttendaele, M., 2012. Extraction of food-borne viruses from food samples: A review. *Int. J. Food Microbiol.* 153, 1–9.
- StataCorp, 2011. Stata: Release 12, Statistical Software. StataCorp LP, College Station, Texas.

- Stoeck, T., Breiner, H.W., Filker, S., Ostermaier, V., Kammerlander, B., Sonntag, B., 2014. A morphogenetic survey on ciliate plankton from a mountain lake pinpoints the necessity of lineage-specific barcode markers in microbial ecology. *Environ. Microbiol.* 16, 430–444.
- Storey, M. V., Winięcka-Krusnell, J., Ashbolt, N.J., Stenstrom, T.-A., 2004. The efficacy of heat and chlorine treatment against thermotolerant *Acanthamoebae* and *Legionellae*. *Scand. J. Infect. Dis.* 36, 656–662.
- Struelens, M.J., Palm, D., Takkinen, J., 2011. Enteroaggregative, Shiga toxin-producing *Escherichia coli* O104:H4 outbreak: new microbiological findings boost coordinated investigations by European public health laboratories. *Eurosurveillance* 16, 1–3.
- Suzan-Monti, M., La Scola, B., Barrassi, L., Espinosa, L., Raoult, D., 2007. Ultrastructural characterization of the giant volcano-like virus factory of *Acanthamoeba polyphaga* Mimivirus. *PLoS One* 2, e328.
- Tanveer, T., Hameed, A., Muazzam, A.G., Jung, S.-Y., Gul, A., Matin, A., 2013. Isolation and molecular characterization of potentially pathogenic *Acanthamoeba* genotypes from diverse water resources including household drinking water from Khyber Pakhtunkhwa, Pakistan. *Parasitol. Res.* 112, 2925–2932.
- Taormina, P.J., Beuchat, L.R., Slutsker, L., 1999. Infections associated with eating seed sprouts: An international concern. *Emerg. Infect. Dis.* 5, 626–634.
- Taylor, M., Ross, K., Bentham, R., 2009. *Legionella*, protozoa, and biofilms: interactions within complex microbial systems.
- ter Braak, C.J.F., Smilauer, P., 1998. Canoco reference manual and user's guide to Canoco for Windows: software for canonical community ordination (version 4). Microcomputer Power, Ithaca, New York.
- Tezcan-merdol, D., Ljungstro, M., Winięcka-krusnell, J., Linder, E., Engstrand, L., Rhen, M., 2004. Uptake and replication of *Salmonella enterica* in *Acanthamoeba rhyodes*. *Appl. Environ. Microbiol.* 70, 3706–3714.
- Thamprasert, K., Khunamornpong, S., Morakote, N., 1993. *Acanthamoeba* infection of peptic ulcer. *Ann. Trop. Med. Parasitol.* 87, 403–405.
- Thomas, J.M., Ashbolt, N.J., 2011. Do free-living amoebae in treated drinking water systems present an emerging health risk? *Environ. Sci. Technol.* 45, 860–869.
- Thomas, V., Clark, J., Doré, J., 2015. Fecal microbiota analysis: an overview of sample collection methods and sequencing strategies. *Future Microbiol.* 10, 1485–1504.
- Thomas, V., Herrera-Rimann, K., Blanc, D.S., Greub, G., 2006. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl. Environ. Microbiol.* 72, 2428–2438.
- Thomas, V., McDonnell, G., Denyer, S.P., Maillard, J.Y., 2010. Free-living amoebae and their intracellular pathogenic microorganisms: Risks for water quality. *FEMS Microbiol. Rev.* 34, 231–259.
- Tian, J.Q., Bae, Y.M., Choi, N.Y., Kang, D.H., Heu, S., Lee, S.Y., 2012. Survival and growth of foodborne pathogens in minimally processed vegetables at 4 and 15 °C. *J. Food Sci.* 77, 2011–2013.
- Triadó-Margarit, X., Casamayor, E.O., 2012. Genetic diversity of planktonic eukaryotes in high mountain lakes (Central Pyrenees, Spain). *Environ. Microbiol.* 14, 2445–2456.

- Turner, N.A., Russell, A.D., Furr, J.R., Lloyd, D., 2000. Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *J. Antimicrob. Chemother.* 46, 27–34.
- Urich, T., Lanzén, A., Qi, J., Huson, D.H., Schleper, C., Schuster, S.C., 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS One* 3.
- Vaerewijck, M.J.M., Baré, J., Lambrecht, E., Sabbe, K., Houf, K., 2014. Interactions of foodborne pathogens with free-living protozoa: potential consequences for food safety. *Compr. Rev. Food Sci. Food Saf.* 13, 924–944.
- Vaerewijck, M.J.M., Sabbe, K., Baré, J., Houf, K., 2011. Occurrence and diversity of free-living protozoa on butterhead lettuce. *Int. J. Food Microbiol.* 147, 105–111.
- Vaerewijck, M.J.M., Sabbe, K., Baré, J., Houf, K., 2008. Microscopic and molecular studies of the diversity of free-living protozoa in meat-cutting plants. *Appl. Environ. Microbiol.* 74, 5741–5749.
- Vaerewijck, M.J.M., Sabbe, K., Baré, J., Spengler, H.-P., Favoreel, H.W., Houf, K., 2012. Assessment of the efficacy of benzalkonium chloride and sodium hypochlorite against *Acanthamoeba polyphaga* and *Tetrahymena* spp. *J. Food Prot.* 75, 541–546.
- Vaerewijck, M.J.M., Sabbe, K., Van Hende, J., Baré, J., Houf, K., 2010. Sampling strategy, occurrence and diversity of free-living protozoa in domestic refrigerators. *J. Appl. Microbiol.* 109, 1566–1578.
- Valster, R.M., Wullings, B. a., Bakker, G., Smidt, H., Van Der Kooij, D., 2009. Free-living protozoa in two unchlorinated drinking water supplies, identified by phylogenetic analysis of 18S rRNA gene sequences. *Appl. Environ. Microbiol.* 75, 4736–4746.
- Valster, R.M., Wullings, B. a., Van Der Kooij, D., 2010. Detection of protozoan hosts for *Legionella pneumophila* in engineered water systems by using a biofilm batch test. *Appl. Environ. Microbiol.* 76, 7144–7153.
- Van Damme, I., Berkvens, D., Vanantwerpen, G., Baré, J., Houf, K., Wauters, G., De Zutter, L., 2015. Contamination of freshly slaughtered pig carcasses with enteropathogenic *Yersinia* spp.: Distribution, quantification and identification of risk factors. *Int. J. Food Microbiol.* 204, 33–40.
- Van den Abeele, A.-M., Vogelaers, D., Van Hende, J., Houf, K., 2014. Prevalence of *Arcobacter* species among Humans, Belgium, 2008–2013. *Emerg. Infect. Dis.* 20, 1746–1749.
- Van Driessche, E., Houf, K., 2007. Discrepancy between the occurrence of *Arcobacter* in chickens and broiler carcass contamination. *Poult. Sci.* 86, 744–751.
- Van Driessche, E., Houf, K., Vangroenweghe, F., Nollet, N., De Zutter, L., Vandamme, P., Van Hoof, J., 2004. Occurrence and strain diversity of *Arcobacter* species isolated from healthy Belgian pigs. *Res. Microbiol.* 155, 662–666.
- Vanormelingen, P., Verleyen, E., Vyverman, W., 2008. The diversity and distribution of diatoms: From cosmopolitanism to narrow endemism. *Biodivers. Conserv.* 17, 393–405.
- Vieira, A., Seddon, A.M., Karlyshev, A. V., 2016. *Campylobacter* – *Acanthamoeba* interactions 933–947.
- Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol. Med. Microbiol.* 50, 1–26.

- Visvesvara, G.S., Schuster, F.L., 2008a. Opportunistic free-living amebae, Part II. Clin. Microbiol. Newsl. 30, 159–166.
- Visvesvara, G.S., Schuster, F.L., 2008b. Opportunistic free-living amoebae, part I. Clin. Microbiol. Newsl. 30, 151–158.
- Visvesvara, G.S., Schuster, F.L., Martinez, A.J., 1993. *Balamuthia mandrillaris*, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. J. Eukaryot. Microbiol. 40, 504–514.
- Vossbrinck, C.R., Maddox, J. V, Friedman, S., Debrunner-Vossbrinck, B.A., Woese, C.R., 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature 326, 411–414.
- Waje, C.K., Jun, S.Y., Lee, Y.K., Kim, B.N., Han, D.H., Jo, C., Kwon, J.H., 2009. Microbial quality assessment and pathogen inactivation by electron beam and gamma irradiation of commercial seed sprouts. Food Control 20, 200–204.
- Walker, G., 2003. An ultrastructural and molecular study of *Hyperamoeba dachnaya*, n. sp., and its relationship to the mycetozoon slime moulds. Eur. J. Protistol. 39, 319–336.
- Walochnik, J., Michel, R., Aspöck, H., 2004. A molecular biological approach to the phylogenetic position of the genus *Hyperamoeba*. J. Eukaryot. Microbiol. 51, 433–440.
- Walochnik, J., Picher, O., Aspöck, C., Ullmann, M., Sommer, R., Aspöck, H., 1998. Interactions of “Limax amoebae” and gram-negative bacteria: experimental studies and review of current problems. Tokai J. Exp. Clin. Med. 23, 273–278.
- Weiss, A., Hertel, C., Grothe, S., Ha, D., Hammes, W.P., 2007. Characterization of the cultivable microbiota of sprouts and their potential for application as protective cultures. Syst. Appl. Microbiol. 30, 483–493.
- Wheat, W.H., Casali, A.L., Thomas, V., Spencer, J.S., Lahiri, R., Williams, D.L., McDonnell, G.E., Gonzalez-Juarrero, M., Brennan, P.J., Jackson, M., 2014. Long-term survival and virulence of *Mycobacterium leprae* in amoebal cysts. PLoS Negl. Trop. Dis. 8, e3405.
- Whittaker, R., 1969. New concepts of kingdoms of organisms. Evolutionary relations are better represented by new classifications than by the traditional two kingdoms. Science (80-). 163, 150–160.
- Whittaker, R.H., Margulis, L., 1978. Protist classification and the kingdoms of organisms. Biosystems. 10, 3–18.
- Wildschutte, H., Lawrence, J.G., 2007. Differential *Salmonella* survival against communities of intestinal amoebae. Microbiology 153, 1781–1789.
- Wildschutte, H., Wolfe, D.M., Tamewitz, A., Lawrence, J.G., 2004. Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella. Proc. Natl. Acad. Sci. U. S. A. 101, 10644–10649.
- Winiacka-Krusnell, J., Wreiber, K., von Euler, A., Engstrand, L., Linder, E., 2002. Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scand. J. Infect. Dis. 34, 253–256.
- Woese, C.R., Kandler, O., Wheelis, M.L., 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. U. S. A. 87, 4576–4579.
- Wu, V.C.H., Jitareerat, P., Fung, D.Y.C., 2003. Comparison of the pulsifier and the stomacher for recovering microorganisms in vegetables 11, 145–152.

- Xiong, N., Brewer, M.T., Day, T. a., Kimber, M.J., Barnhill, A.E., Carlson, S. a., 2010. Evaluation of the pathogenicity and virulence of three strains of *Salmonella* organisms in calves and pigs. *Am. J. Vet. Res.* 71, 1170–1177.
- Yousuf, F., Siddiqui, R., Khan, N., 2013. *Acanthamoeba castellanii* of the T4 genotype is a potential environmental host for *Enterobacter aerogenes* and *Aeromonas hydrophila*. *Parasit. Vectors* 6, 169.
- Zaman, V., Zaki, M., Howe, J., Ng, M., Leipe, D.D., Sogin, M.L., Silberman, J.D., 1999a. *Hyperamoeba* isolated from human feces: description and phylogenetic affinity. *Eur. J. Protistol.* 35, 197–207.
- Zaman, V., Zaki, M., Manzoor, M., 1999b. *Acanthamoeba* in human faeces from Karachi. *Ann. Trop. Med. Parasitol.* 93, 189–191.
- Zbikowska, E., Walczak, M., Krawiec, A., 2013. Distribution of *Legionella pneumophila* bacteria and *Naegleria* and *Hartmannella* amoebae in thermal saline baths used in balneotherapy. *Parasitol. Res.* 112, 77–83.
- Zhang, Q., Widmer, G., Tzipori, S., 2013. A pig model of the human gastrointestinal tract. *Gut Microbes* 4, 193–200.
- Zhao, F., Xu, K., He, Y., 2012. Application of the Ludox-QPS method for estimating ciliate diversity in soil and comparison with direct count and DNA fingerprinting. *Eur. J. Soil Biol.* 49, 112–118.
- Zhou, X., Elmore, J., Call, D.R., 2007. Interactions between the environmental pathogen *Listeria monocytogenes* and a free-living protozoan (*Acanthamoeba castellanii*). *Environ. Microbiol.* 9, 913–922.



You are only as strong as you allow yourself to be; never get discouraged, never give up because consistency & dedication is the key to success.

Summary

Summary

Free-living protozoa (FLP) are unicellular heterotrophic eukaryotes, comprising ciliated, flagellated and amoebal life forms. They are important bacterial consumers controlling bacterial biomass and forming an important trophic link in aquatic and terrestrial food webs. FLP are common in both natural and anthropogenic environments.

Interactions of FLP have been implicated in the transmission and persistence of foodborne pathogenic bacteria. Some pathogenic bacteria have developed pre- and post-ingestional adaptations and are able to survive and grow inside FLP cells, enhancing their distribution to new habitats and hosts. Moreover, FLP can protect and shelter pathogenic bacteria against unfavourable physical and chemical conditions like desiccation, disinfectants and biocides.

Information about the **occurrence and diversity of FLP** on and in food and in food-related environments is **scarce**. Information about the occurrence of FLP in livestock animals, which have been identified as main reservoirs for different foodborne pathogens is completely lacking. This is due to the fact that FLP are not routinely incorporated in microbiological surveys as they are generally considered to be harmless. In addition, validated protocols to recover and quantify FLP have not yet been developed for most food (-related) matrices.

The **general aim** of this doctoral thesis is to **provide a detailed description of the diversity and occurrence of FLP in and on food products and in food-related environments**. More specifically, we examined dishcloths, vegetable sprouts and the gastrointestinal tract and feces of pigs. Since effective protocols are essential and required in this whole process, we first developed and evaluated **protocols for recovering and quantifying FLP** from the examined matrices.

In the **first chapter** special attention was paid to the **co-occurrence** of **FLP** and important **foodborne pathogens** on **dishcloths**. Dishcloths are commonly used to clean kitchen surfaces, equipment and utensils, and form a potentially important source of cross-contamination with FLP and foodborne pathogens in food-related environments like domestic kitchens.

The occurrence, abundance and diversity of FLP in combination with detection and enumeration of the most common foodborne pathogens and enumeration of the bacterial load on used dishcloths was performed. **Homogenisation** (stomacher) in combination with the **Most Probably Number (MPN) method** is recommended to recover and enumerate FLP from dishcloths.

FLP were present on **89% of the examined dishcloths**, 100% of these tested positive for amoebae, 71% for flagellates and 47% for ciliates. **Estimated FLP numbers** in used dishcloths ranged from **10 to 10⁴ MPN/cm²**. Enrichment was used to assess **FLP diversity** revealing that amoebae like vahlkampfiids, vannellids, *Acanthamoeba* spp., *Hyperamoeba* sp. and *Vermamoeba vermiformis* were dominant on dishcloths. Heterotrophic flagellates were mainly glissomonads, cercomonads and the genus *Bodo*, while *Colpoda* was a common ciliate taxon on dishcloths.

Bacterial load on dishcloths was high, with a **mean total aerobic bacteria of 7.47 log₁₀ cfu/cm²**. *Escherichia coli* was detected in 68% of the used dishcloths, with concentrations up to 4 log₁₀ cfu/cm². Moreover, important **foodborne pathogens** such as *Staphylococcus aureus*, *Arcobacter butzleri* and *Salmonella enterica* subsp. *enterica* ser. Halle were recovered. Qualitative and quantitative data analysis of factors which have an impact on both FLP and foodborne pathogen occurrence and numbers revealed that **detergent use** caused a significant reduction in numbers of ciliates, amoebae and total aerobic bacteria and as such was a prime determinant of FLP and total aerobic bacteria concentrations on dishcloths.

There is increasing awareness of **vegetable sprouts** as a potential source of **foodborne illnesses** and that sprout-associated outbreaks account for more illnesses and hospitalizations than other foods. Therefore, the objective of the **second chapter** was to evaluate, by means of **enrichment and cultivation** methods, within-lot and between-lot **variability of protozoan species composition** and enumeration of **bacterial load** on eight types of vegetable sprouts (alfalfa, beetroot, cress, green pea, leek, mung bean, red cabbage and rosabi). Variation in protozoan community composition in relation to FLP numbers, species richness and environmental variables was analysed.

Vegetable sprouts harboured **diverse communities of FLP**, with *Tetrahymena* (ciliate), *Bodo saltans* and cercomonads (flagellates), and *Acanthamoeba* and *Vannella* (amoebae) as the dominant taxa. **Protozoan community composition** and abundance **significantly differed** between the **sprout types**. Beetroot harboured the most abundant and diverse FLP communities, with many unique species such as *Korotnevela* sp., *Vannella* spp., *Chilodonella* sp., *Podophrya* sp. and *Sphaerophrya* sp. In contrast, mung bean sprouts were species-poor and had low FLP numbers.

Sampling month and company had **no significant influence** on protozoan community composition, suggesting **that seasonal and local factors** are of minor importance. Likewise, no significant relationship between community composition and bacterial load was observed.

On vegetable sprouts total **FLP numbers** were up to **10⁵ MPN/g** and bacterial load was high (**total aerobic bacteria** concentrations up to **10⁹ cfu/g**), while counts of *E. coli* were mostly below the limit of detection. Depending on the **recovery method** used, **differences** in the recovery of **specific morphogroups** was observed. Ciliates were recovered more frequently with the cultivation method, while amoebae were more abundant after enrichment of the stomached homogenate. Consequently, the use of **complementary methods** is recommended in order to obtain a more complete FLP inventory.

The **gastrointestinal tract (GIT) of pigs** is an important **reservoir for zoonotic foodborne bacteria** and creates an ideal niche and meeting place for foodborne pathogens and FLP. Information about the presence of viable FLP in the porcine GIT and their excretion in the feces is inexistent. This is partly due to lack of protocols to recover FLP from intestinal content.

Chapter three focused on the development of a **recovery protocol** to explore the presence and diversity of FLP in feces and throughout the GIT of pigs. Four methodologies, newly developed or adapted and optimized where necessary, were tested on artificially inoculated fecal samples.

Filtration of the intestinal content was identified as the most suitable protocol to recover FLP from the GIT and feces. Free-living protozoa were recovered from different parts of the GIT which suggests at least a **transient passage of FLP** through the **porcine GIT**.

Free-living amoebae species like *Hyperamoeba* sp., *Vannella* sp., *Vermamoeba vermiformis*, hartmannellids and vahlkampfiids, but also ciliates likes *Colpoda* sp. and a *Tetrahymena/Glaucoma* lookalike, and flagellates like cercoconads, bodonids and glissomonads, were recovered and cultured from pig intestinal content. *Acanthamoeba* was the most often encountered taxon isolated from the porcine GIT. *Acanthamoeba hatchetti* and *Filamoeba sinensis* were isolated for the first time from pig intestinal content. Despite high gastric acidity, **non-cyst forming amoeba species** were also detected which suggests survival of their trophozoites in the animal GIT.

The data obtained during this PhD research provide **new insights** about the **occurrence and diversity of FLP in and on food, in food-related environments and in animals**. The developed **protocols** can form the basis for the development of protocols for further studies of free-living protozoa in similar environments.

Free-living protozoa, including some opportunistic pathogens, form **common and diverse communities** on dishcloths, vegetable sprouts and in the GIT of pigs. This occurrence creates a microbial niche for (pathogenic) bacteria-FLP interactions, and as such is a potential risk factor for cross-contamination with potential implications for food safety and public health.

Samenvatting

Samenvatting

Vrijlevende protozoa zijn ééncellige, heterotrofe eukaryoten en omvatten een verscheidenheid aan **ciliaten**, **flagellaten** en **amoeben**. Vrijlevende protozoa zijn alom vertegenwoordigd in natuurlijke en antropogene omgevingen en worden als belangrijke bacteriële predatoren beschouwd. Hierdoor vormen ze een essentiële schakel in zowel het aquatisch als het terrestrisch voedselweb.

Recent onderzoek heeft aangetoond, dat sommige **voedselpathogenen** in **associatie** met vrijlevende protozoa in diverse ongunstige milieus kunnen overleven. Sommige bacteriën ontwikkelden extracellulaire overlevingsmechanismen en kunnen hierdoor de opname door vrijlevende protozoa verhinderen. Andere ziekteverwekkende bacteriën ontwikkelden intracellulaire mechanismen en kunnen overleven en zich zelfs vermenigvuldigen binnenin de protozoaire cel. Vrijlevende protozoa beschermen geïnternaliseerde bacteriën tegen ongunstige fysische en chemische omstandigheden zoals uitdroging, ontsmettingsmiddelen en biociden. Daarenboven kunnen ze fungeren als vector en verspreidingsroute voor bepaalde soorten bacteriën wat de verspreiding naar andere gastheren en habitats bewerkstelligt. Al deze interacties kunnen zorgen voor een verhoogde overleving en verspreiding van bacteriële (voedsel)pathogenen.

Informatie over de aanwezigheid van **vrijlevende protozoa** in voedsel en voedselgerelateerde omgevingen is **bepikt**. De kennis van het voorkomen van vrijlevende protozoa in landbouwdieren, die een belangrijk reservoir voor voedselpathogenen vormen, is nihil. Dit is te wijten aan het feit dat vrijlevende protozoa over het algemeen onschadelijk zijn én aan het gebrek aan gestandaardiseerde staalnamemethoden om ze uit deze matrices te halen en te kwantificeren.

Het **hoofddoel** van deze thesis was een beter inzicht te krijgen in **het voorkomen en de diversiteit van vrijlevende protozoa** in een **voedselgerelateerde omgeving** (schotelvodden), in **voedsel** (kiemscheuten) en in **dieren** (maag-darmkanaal en mest van varkens).

Geschikte **staalnamemethoden** om vrijlevende protozoa uit deze omgevingen te halen, zijn een essentiële vereiste in dit hele proces. Tijdens dit doctoraatsonderzoek werden verschillende staalnamemethoden om vrijlevende protozoa uit bovenstaande matrices te halen en te kwantificeren, ontwikkeld en geëvalueerd.

In het **eerste hoofdstuk** werd de focus gelegd op het samen voorkomen van vrijlevende protozoa én voedselpathogenen in **schotelvodden**. Schotelvodden worden frequent gebruikt voor het reinigen van keukenoppervlakken en keukengerei en kunnen zodoende een belangrijke bron van kruisbesmetting met vrijlevende protozoa en voedselpathogenen in huishoudens vormen. In dit onderzoek werden de **aanwezigheid**, de **diversiteit** en de **aantallen vrijlevende protozoa** op schotelvodden bepaald. Daarnaast werden de aan- of afwezigheid en de aantallen van belangrijke **voedselpathogenen** en het totaal kiemgetal van schotelvodden bepaald. **Homogenisatie** (stomacher) in combinatie met de **'Most Probable Number' (MPN) telmethode** is aanbevolen om vrijlevende protozoa uit schotelvodden te halen en te kwantificeren.

Vrijlevende protozoa werden terug gevonden in **89% van de onderzochte schotelvodden**. Van deze positieve schotelvodden waren allen positief voor amoeben, 71% bevatte flagellaten en 47% was positief voor ciliaten. Het geschatte **aantal vrijlevende protozoa** in schotelvodden varieerde tussen 10 en **10⁴ MPN/cm²**.

Door middel van een aanrijkmethode werd de **diversiteit** aan vrijlevende protozoa in schotelvodden onderzocht. Amoeben zoals vahlkampfiiden, vannelliden, *Acanthamoeba* spp., *Hyperamoeba* sp. en *Vermamoeba vermiformis* waren de meest dominante soorten. Heterotrofe flagellaten waren voornamelijk glissomonads, cercomonads en *Bodo*. De groep van ciliaten werd gedomineerd door *Colpoda*.

Het **totaal kiemgetal** van schotelvodden was met een gemiddelde waarde van **7.47 log₁₀ kolonievormende eenheden (kve)/cm²** zeer hoog. In 68% van de schotelvodden werd *Escherichia coli* gedetecteerd, met concentraties tot 4 log₁₀ kve/cm². **Belangrijke voedselpathogenen** zoals *Staphylococcus aureus*, *Arcobacter butzleri* en *Salmonella enterica* subsp. *enterica* ser. Halle werden eveneens in schotelvodden teruggevonden. Kwalitatieve en kwantitatieve gegevens over factoren die mogelijk een rol spelen in de aanwezigheid van vrijlevende protozoa en voedselpathogenen op schotelvodden toonden aan, dat het **gebruik van detergent** een significant reducerend effect had op de aantallen van ciliaten, amoeben en op het totaal kiemgetal.

De laatste decennia is het duidelijk geworden dat **kiemscheuten** een rol kunnen spelen in **voedselinfecties**. Scheutgerelateerde voedselinfecties zorgen voor een hoger aantal zieken en hospitalisaties. Om deze reden werd in **hoofdstuk 2** door middel van **aanrijkmethode- en cultivatiemethoden** de variatie in de **soortensamenstelling** van **vrijlevende protozoa** binnen een lot en tussen verschillende loten kiemscheuten (luzerne, rode biet, tuinkers, erwtasperge, prei, soja, rode kool en rosabi) nagegaan. Eveneens werd het **totaal kiemgetal** van deze kiemscheuten bepaald. De variatie in de soortensamenstelling van vrijlevende protozoa in relatie tot aantallen vrijlevende protozoa, soortenrijkdom en omgevingsvariabelen werd onderzocht.

Dit onderzoek toonde aan, dat kiemscheuten een enorme **diversiteit** aan vrijlevende protozoa bezitten, waarbij de taxa *Tetrahymena* (ciliaat), *Bodo* en cercomonads (flagellaten), en *Acanthamoeba* en *Vannella* (amoeben) vaak voorkwamen. Op gebied van de soortensamenstelling en aantallen van vrijlevende protozoa werden opmerkelijke **verschillen** tussen de **soorten kiemscheuten** vastgesteld. Op rodebietenscheuten werden de hoogste diversiteit en aantallen vrijlevende protozoa teruggevonden. Waaronder enkele unieke soorten zoals *Korotnevela* sp., *Vannella* spp., *Chilodonella* sp., *Podophrya* sp. en *Sphaerophrya* sp. Sojascheuten daarentegen waren soortenarm en bevatten lage aantallen vrijlevende protozoa.

De maand en het bedrijf waarin de stalen genomen werden, hadden geen significant effect op de soortenrijkdom en aantallen, waardoor men kan concluderen dat **seizoenale en lokale factoren** van **ondergeschikt belang** waren aan de samenstelling van vrijlevende protozoogemeenschappen van de onderzochte kiemscheuten. Er kon geen relatie aangetoond worden tussen de aanwezigheid van bepaalde protozoogemeenschappen en het totaal kiemgetal van kiemscheuten.

De onderzochte kiemscheuten bereikten **concentraties** van **vrijlevende protozoa** tot **10⁵ MPN/g**. Het **totaal kiemgetal** was hoog met concentraties tot **10⁹ kve/g**, in tegenstelling tot concentraties van *Escherichia coli* die in de meeste gevallen onder de telbare detectielimiet lagen.

Afhankelijk van de **gebruikte methode** werden bepaalde morfogroepen meer of minder teruggevonden. Ciliaten werden vaker teruggevonden wanneer de cultivatiemethode toegepast werd, terwijl amoeben na aanrijking van het homogenaat meer werden teruggevonden. Om een vollediger beeld van de protozoagemeenschappen op kiemscheuten te verkrijgen is het aan te raden om verschillende **complementaire methoden** toe te passen.

Het **maag-darmkanaal van varkens** vormt een belangrijk reservoir voor voedselpathogenen en kan als dusdanig een ideale ontmoetingsplaats zijn voor pathogene bacteriën en vrijlevende protozoa. Kennis over de aanwezigheid van vrijlevende protozoa in het maag-darmkanaal van varkens is onbestaande. Dit is deels te wijten aan het gebrek aan gestandaardiseerde staalnamemethoden om vrijlevende protozoa uit deze omgeving te halen.

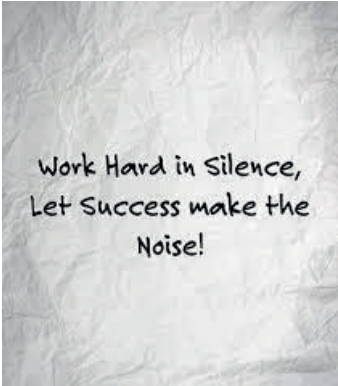
Om de aanwezigheid en de diversiteit van vrijlevende protozoa in het maag-darmkanaal van varkens aan te tonen, werd in **hoofdstuk 3** de focus gelegd op de **ontwikkeling van een protocol** om vrijlevende protozoa uit varkensmest (en bijgevolg ook het maag-darmkanaal) te halen. Vertrekkende van bestaande methoden, werden 4 methoden aangepast en uitgetest op artificieel geïnoculeerde varkensmest.

Filtratie van de darminhoud was de meest effectieve methode om vrijlevende protozoa uit het **maag-darmkanaal** en mest van **varkens** te halen. In verschillende delen van het maag-darmkanaal werden vrijlevende protozoa teruggevonden, wat ten minste een doorstroming van vrijlevende protozoa doorheen het maag-darmkanaal van varkens suggereert.

Vrijlevende amoeben zoals *Hyperamoeba* sp., *Vannella* sp., *Vermamoeba vermiformis*, hartmannelliden en vahlkampfiiden; maar ook ciliaten zoals *Colpoda* sp. en een *Tetrahymena/Glaucoma* lookalike; en flagellaten zoals cercomonads, bodoniden en glissomonads werden teruggevonden én succesvol gecultiveerd. *Acanthamoeba* was het vaakst voorkomend taxon en desondanks de hoge zuurtegraad van het maag-darmkanaal werden ook niet-cystevormende amoeben teruggevonden. Dit laatste kan wijzen op de overleving van de vegetatieve celvormen van vrijlevende protozoa in deze ongunstige omgeving.

Dit doctoraatsonderzoek draagt bij tot een **verhoogde kennis** van het voorkomen van vrijlevende protozoa in voedsel, voedselgerelateerde omgevingen en in dieren. De ontwikkelde **methoden** kunnen fungeren als **model** voor verder onderzoek naar protozoa in soortgelijke matrices.

Vrijlevende protozoa, waaronder sommige opportunistische pathogenen, vormen **diverse en soortenrijke gemeenschappen** op schotelvodden, kiemscheuten en in het maag-darmkanaal van varkens. Daarnaast werden **effectieve methoden** voor het terugvinden van vrijlevende protozoa uit bovenstaande matrices **ontwikkeld en geëvalueerd**. De aanwezigheid van vrijlevende protozoa in voedsel, voedselgerelateerde en dierlijke omgevingen zou een niche kunnen creëren voor mogelijke associaties tussen pathogene bacteriën en vrijlevende protozoa, zorgt bijgevolg voor een verhoogd risico van kruisbesmetting en kan belangrijke gevolgen hebben voor de voedselveiligheid en de volksgezondheid.



Work Hard in Silence,
Let Success make the
Noise!

Curriculum vitae

Curriculum vitae

Natascha Chavatte werd geboren op 25 augustus 1978 te Geraardsbergen. Na het beëindigen van haar secundaire studies Techniek-Wetenschappen aan het Maria-Jozef Instituut te Geraardsbergen, startte ze in 1996 met de studies Biologie aan de Universiteit Gent. Zij behaalde in 2001 het diploma van Licentiaat in de Biologie (optie Dierkunde) met onderscheiding. Na een aantal jaren gewerkt te hebben als clinical data manager binnen de farmaceutische industrie maakte zij in februari 2013 een carrière-switch en startte als bursaal van het Bijzonder Onderzoeksfonds van de UGent haar doctoraatsonderzoek bij de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid van de faculteit Diergeneeskunde. Dit onderzoek handelde over het voorkomen en de diversiteit van vrijlevende protozoa in voedsel en voedselgerelateerde omgevingen. De doctoraatsthesis vormt een bijdrage aan het ecologische luik betreffende het belang van vrijlevende protozoa en hun interacties met voedselpathogenen in bovengenoemde omgevingen. In 2015 won zij de Best Poster Presentation Award op het FLAM congres en de Dafra Pharma Award for Best Presentation op de BSPP annual meeting. Tevens vervulde zij in 2015 het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de UGent. Natascha Chavatte is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij nam actief deel aan meerdere nationale en internationale congressen.

Scientific publications

N. Chavatte, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf. Free-living protozoa in the gastrointestinal tract and feces of pigs: Exploration of an unknown world and towards a protocol for the recovery of free-living protozoa. *Submitted to Veterinary Parasitology*.

N. Chavatte, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf, 2016. Abundance, diversity and community composition of free-living protozoa on vegetable sprouts. *Food Microbiology*. 55, 55-63.

N. Chavatte, J. Baré, E. Lambrecht, I. Van Damme, M. Vaerewijck, K. Sabbe, K. Houf, 2014. Co-occurrence of free-living protozoa and foodborne pathogens on dishcloths: implications for food safety. *International Journal of Food Microbiology*. 191, 89-96.

E. Lambrecht, J. Baré, **N. Chavatte**, W. Bert, K. Sabbe, K. Houf, 2015. Protozoan cysts act as survival niche and protective shelter for foodborne pathogenic bacteria, *Applied and Environmental Microbiology*, 81 (16), 5604-5612.

E. Lambrecht, J. Baré, M. Claeys, **N. Chavatte**, W. Bert, K. Sabbe, K. Houf, 2015. Transmission electron microscopy sample preparation protocols for the ultrastructural study of cysts of free-living protozoa. *BioTechniques*. 58, 181-188.

Oral presentations

XVI International Meeting on the Biology and Pathogenicity of Free-Living Amoebae, 18-22 May 2015, Alghero, Sardinia, Italy. **N. Chavatte**, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf. Occurrence and diversity of free-living protozoa on sprouts.

Annual Scientific Meeting of the Belgian Society for Parasitology and Protistology, 13 November 2014, Antwerp, Belgium. **Chavatte N.**, Baré J., Lambrecht E., Van Damme I., Vaerewijck M., Sabbe K., Houf K. Free-living protozoa on dishcloths: occurrence, diversity and possible implications for food safety.

33rd Annual Meeting of the German Society for Protozoology, 12-15 February 2014, Essen, Germany. **N. Chavatte**, J. Baré, E. Lambrecht, K. Sabbe, K. Houf. Occurrence of free-living protozoa and foodborne pathogens in dishcloths.

8th Benelux Congress of Zoology, 23-24 November 2001, Nijmegen, The Netherlands. **N. Chavatte**, K. Hostens. Structural patterns and seasonality of the hyperbenthos of the Oosterschelde and the Westerschelde (Southern Bight of the North Sea).

Poster presentations

19th Conference on Food Microbiology, 18 & 19 September 2014, Brussels, Belgium. **N. Chavatte**, J. Baré, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf. Free-living protozoa on dish cloths: occurrence, diversity and possible implications for food safety.

19th Conference on Food Microbiology, 18 & 19 September 2014, Brussels, Belgium. E. Lambrecht, J. Baré, **N. Chavatte**, K. Sabbe, K. Houf. Protozoan cysts: a potential vector and shelter for foodborne pathogens.

34th Annual Meeting of the German Society for Protozoology, 3-6 March 2015, Magdeburg, Germany. E. Lambrecht, J. Baré, **N. Chavatte**, K. Sabbe, K. Houf. Protozoan cysts: a potential vector and shelter for foodborne pathogens.

34th Annual Meeting of the German Society for Protozoology, 3-6 March 2015, Magdeburg, Germany. Lambrecht E., Baré J., **Chavatte N.**, Sabbe K., Houf K. Transmission electron microscopy sample preparation protocols for the ultrastructural study of cysts of free-living protozoa.

XVI International Meeting on the Biology and Pathogenicity of Free-Living Amoebae, 18-22 May 2015 Alghero, Sardinia, Italy. **N. Chavatte**, J. Baré, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf. Free-living protozoa on dishcloths: occurrence, diversity and possible implications for food safety.

XVI International Meeting on the Biology and Pathogenicity of Free-Living Amoebae, 18-22 May 2015 Alghero, Sardinia, Italy. Lambrecht E., Baré J., **Chavatte N.**, Sabbe K., Houf K. Transmission electron microscopy sample preparation protocols for the ultrastructural study of cysts of free-living protozoa.

Scientific awards

Dafra Pharma Award for Best Presentation

Annual Scientific Meeting of the Belgian Society for Parasitology and Protistology, 13 November 2014, Antwerp, Belgium. Chavatte N., Baré J., Lambrecht E., Van Damme I., Vaerewijck M., Sabbe K., Houf K. Free-living protozoa on dishcloths: occurrence, diversity and possible implications for food safety.

Best poster presentation award

XVI International Meeting on the Biology and Pathogenicity of Free-Living Amoebae, 18-22 May 2015 Alghero, Sardinia, Italy. N. Chavatte, J. Baré, E. Lambrecht, I. Van Damme, K. Sabbe, K. Houf. Free-living protozoa on dishcloths: occurrence, diversity and possible implications for food safety.

“i fall. i rise. i make mistakes. i live.
i learn. i've been hurt but i'm alive.
i'm human. i'm not perfect but
i'm thankful.”

DON'T EVER LET
ANYONE DULL
YOUR
sparkle

MARKUP.COM

Dankjewel

Dankjewel

Ik was (en ben eigenlijk nog altijd) een 'masker van het zusterhuis'...
Daar begon het allemaal...

Nooit gedacht... dat ik na mijn secundair Techniek-Wetenschappen ging slagen als 'biologe' aan den unief. Wat ben ik blij dat ik toen die stap heb durven zetten.

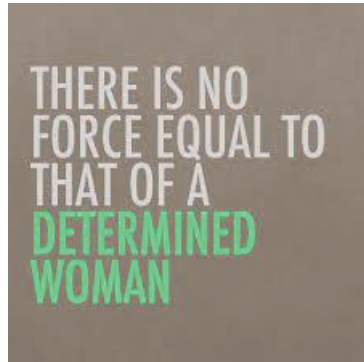
Nooit gedacht... dat ik mijn vorig leven als clinical data manager aan de kant zou schuiven en een totale switch zou maken voor dit avontuur. Nog vaak denk ik terug aan de leute die ik had met de collega's van DICE/Veeda. Elsie en Kelly, jullie hebben voor altijd een speciaal plaatsje in mijn hart.
<3

Nooit gedacht... dat ik tijdens mijn kennismakingsgesprek met Kurt, Koen, Julie en Ellen, hen kon overtuigen van mijn motivatie en gedrevenheid. Ik zag mezelf als iemand (een 'oudere') die maar weinig kans maakte, maar ik had niets te verliezen, dus ging ik er gewoon voor. Als ze konden kiezen tussen een oudere of een jongere pas afgestudeerde wetenschapper, gingen ze wel gaan voor de laatste, dacht ik. Niets was minder waar... Daarom **Kurt en Koen**, bedankt voor het vertrouwen en het geloven in mij, voor mij was dit echt een 'once in a lifetime chance and experience'. Uit de grond van mijn hart, bedankt!!!

Julie, als mijn begeleidster heb jij me met hart en ziel geïntroduceerd in de boeiende wereld van de vrijlevende protozoa. Jouw grondige kennis vond ik bewonderenswaardig. Jouw deur stond altijd open voor mij, je was er altijd om goeie raad en tips te geven, mét de glimlach. Super dikke merci! Jammer dat je 'jouw poulain' niet tot op het einde hebt kunnen volgen...

Ellen, wij waren, euh zijn voor altijd, de 'protozoa girls'. Wat ben ik blij dat jij in mijn team, of beter 'ons team' zat. Je hebt me samen met Julie ingewijd in de wondere protozoa wereld, van jou heb ik de labo-kneepjes geleerd; complementair op alle vlakken verliep onze samenwerking vlekkeloos en waren we een geoliede machine. Zonder jou was de harmonie weg in onze bureau, zonder jou geen koekjes of knabbels in onze bureau, zonder jou was het iets hectischer om de studenten te begeleiden (studenten begeleiden is jouw tweede natuur). Jouw kritische blik, waardevolle opmerkingen en suggesties, tilden mijn papers en doctoraat een trapje hoger... zonder jou was mijn boekje nooit wat het nu is geworden... dank je voor alles! Nu is het wachten op jouw doctoraat, you go girl! Protozoa girls rule!

Francesca, grazie mille per la tua spontaneità! I love your italian way of being yourself. Thank you for the nice (too short) time in the office, but also thank you for our 'escapes' during lunch break. Whether it was Close-Up or Colruyt ;-), you are my 'shopping' partner in crime; but above all: la tua amicizia è un regalo molto speciale. Per sempre nel mio cuore.



Ook mijn andere LHT collega's wil ik bedanken voor de tijd samen aan de vakgroep. **Inge, Glynnis, Bavo, Tomasz, Wauter en Gerty**; als doctoraatsstudenten (excuseer, ondertussen zijn de helft al post-docs) vormden wij een klein maar fijn groepje waar Bavo en Tomasz zorgden voor wat mannelijk testosteron, gelukkig maar 😊. Een speciaal dankjewel voor Inge die me met veel raad en daad heeft bijgestaan, vooral dan het laatste jaar. Dankjewel Inge!

Wat ik ook nooit zal vergeten zijn de gezellige momenten tijdens de koffiepauze waar er vaak over koetjes en kalfjes werd gepraat maar ook soms serieuze gesprekken gevoerd werden (met een Delacre koekje in de hand). Martine, Carine, Sandra en Soetkin bedankt om me direct op te nemen in de groep van dames.

Martine, Sandra en Jeroen, jullie wil ik ook expliciet bedanken voor alle praktische hulp en Jeroen eveneens voor zijn bijdrage in testosteron. Martine, jij hebt me ingewijd in de wereld van de voedselpathogenen, thanks! **Carine**, ook jij bedankt! Ik zie jou als de lieve 'mama' voor de studenten, voor jou is er geen werk teveel; jij staat er elke dag met de glimlach.

Het begon dus allemaal in **Gent**... En als ik aan Gent denk, denk ik aan een fantastische studententijd waar **Katrien** en **Sofie** een belangrijk aandeel in hadden. Man, man, man, we hebben er echt wel van geprofiteerd, van het zorgeloze studentenleven. De anekdotes en memorabele momenten zijn eindeloos. **Klaartje**, jij was ook vaak van de partij, jouw droge gevoel voor humor werkt aanstekelijk. Ondertussen mogen we al spreken van Katrien & Joris en Simon en van Klaartje & Diederik en Paulien. Jaja, we breiden uit, hopelijk mogen we nog veel fijne momenten samen beleven!

Sofietje & Bart, Gus en Luk. Jullie zijn een straffe madam en ne straffe vent. Sofie, jij was mijn partner in crime in Gent (ik ga niet in detail treden), maar o wat hebben we veel leuke gekend! En nu met de kindjes erbij wordt het nog meer een gezellige en soms drukke bedoening.

Als ik aan Gent denk, moet ik zeker ook een dankjewel zeggen voor **Kris & Els, Sam en Lars**. Jullie huisje in de Herfststraat zal ik nooit vergeten, de babysitavonden, de gezellige aperitiefjes... De laatste jaren zien we mekaar minder vaak, maar als we een date hebben, dan zitten we direct weer op hetzelfde spoor, mo how seg 😊 Ook een expliciet dankjewel voor **Kris**, mijn thesisbegeleider, die bij mij de passie voor onderzoek aangewakkerd heeft en het is met de jaren alleen maar erger geworden; ook dankjewel om toentertijd in mij te geloven en mij het 'zetje' te geven om te 'doctoreren'.

'Wacko' vriendjes: Lieven & Marieke, Joke & Wim, Bart & Griet... En uiteraard ook de kindjes: Gus & Renée, Fran & Luz, Lars & Stan. Alle gekheid op een stokje! Daarmee is al veel gezegd:-)

De avonden en weekendjes in jullie compagnie is altijd 'totally chill' met hier en daar een vleugje droge humor, muziek en vaak de gekste ideeën eerst. Ik denk dan spontaan aan de Bazaar weekends, je kon het zo gek niet bedenken of er bestond een workshop van. Ik hou van jullie creativiteit en 'gekheid', dat werkt inspirerend én ontspannend... Bedankt!

Van studeren in Gent gaan we naar de studententijd in Leuven. Koens' 'buddy' was (en blijft voor altijd) 'de Pieter', eigenlijk een maat die Koen al kende van in't Karmelieten, maar die ik pas leren kennen heb in Leuven. Pieter is Pieter, op zijn manier zijn gewone zelve en vooral 'no-nonsense'. En dat kan ik best wel pruimen.

Pieter & Svenja, Tim en Arno, jullie zijn onze 'verste' (lees: Humbeek ligt juste niet tegen Windeke) vrienden, maar de avondjes (én weekends) met jullie zijn (meestal) rustig, maar aangenaam! We komen graag naar Humbeek, al is het maar om te smullen van jullie pasta en om de paarden te bewonderen (zeker ons Oona), maar bovenal omdat we graag een babbelke doen met jullie, een kaartje leggen en ons op ons gemakken voelen (de schoenen gaan steevast uit).

Van Humbeek gaan we terug naar home sweet home, Scheldewindeke. Hettingen, daar is waar onze 'cava' staat. Hettingen is ook **Hettingen Feest! Comité: Ann & Johan, Els & Rony, Kris & Véro, Petra & Kris**. Jullie/wij zijn een fantastisch team dat dit jaar voor het zesde jaar op rij ons straatfeest gaan organiseren en dat is elk jaar een succes! Dat is vooral te wijten aan het feit dat jullie harde werkers zijn, het hart op de juiste plaats hebben, dat we complementair zijn, maar vooral dat we er nog altijd zelf heel veel plezier aan beleven! Bedankt voor de leuke voorbereidingsavonden, 'Hettingen feesten', ... Dat we nog vele jaren 'mogen mogen'... Ook dankjewel voor de (grote) kinderen: Jens & Brecht, Kaat & Kim, Luca & Tomma, Rania & Rens! Opvolging is verzekerd!

'Music is life' is een slogan op mijn lijf geschreven. **Muziek** is voor mij belangrijk, geeft me heel vaak een energie boost en dreef me letterlijk vooruit tijdens het schrijven van mijn papers en dit 'boekje'. Op het labo werkte ik soms met oortjes in. Misschien wat asociaal, alhoewel, we 'tetterden' nog genoeg, eh Ellen ;-). Daarom was tijdens het schrijven van deze thesis thuis werk zo zalig, als ik even nood had aan een pauze, zette ik mijn muziek vollen bak en brulde ik mee, of liet ik me even gaan in een wilde zotte dans; zalig! even alle remmen los was dat...

Didas, Martha, Elikunda, Oforo and Herieli, thanks for the hospitality and including us as a member of your family. Asante sana!

En dan komen we dichterbij waar mijn hartje ligt...

Isabelle & Ives, Griet & Benoit, Mie & Wim, Tine; mijn chiro-vriendjes die me zo nauw aan het hart liggen....

Wat hebben we samen al fijne tijden beleefd en nu nog! We zijn een beetje zoals rode wijn, het wordt 'a gelijk' nog beter met de jaren. Ons chiro-verleden heeft ons gemaakt tot wie we nu zijn... Een leutige bende! Wat wij al meegemaakt hebben, daar kunnen we (komedie)boeken over schrijven. Ons weekendjes met de kindjes Wieter & Norah, Floris, Nell & Klaasje, Marieke & Lowie, dat voor mij pure ontspanning! Alhoewel er recentelijk ook een andere wind aan het waaien is, zijnde mannen-weekends... De vrouwen komen ook nog zelle... Enfin, mercikiesj veur de liefde en de vriendskap, en aske wiltj ei...

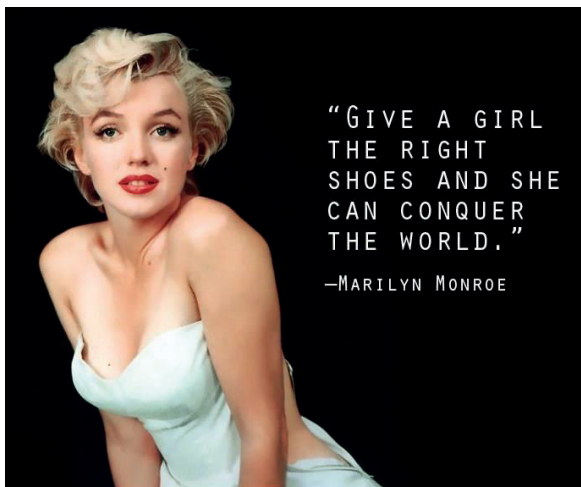
Een speciaal dankjewel voor Isabelle, meter van ons Asa. Samen hebben we al een lange weg afgelegd: gaande van chiro-oudersfeesten (in bloemekeskleedjes), romantische 'meebleit' films in de cinema, tot leren dansen in de kelder bij jou thuis... waar is den tijd... En dan heb ik het niet over onze fantastische en onvergetelijke kampen samen. Je bent een prachtige madam! Bedankt voor de vriendschap en om meter te zijn van ons Asa!

Intermezzo - Antwoord van een vriendin op de vraag wat ik gedaan heb voor mijn doctoraat...

... je hebt van die vrijlevende beesten onderzocht op vodden, scheuten en in de kak en den buik van de veirkisj... Ze zaten overal. Vooral en zeker op de schotelvodden. Detergent is een doder en zorgt voor meer properiteit. Bieten zijn vuil maar sojascheuten zijn te vreten en de biestjes in de darmen kunnen zelfs door dat kanaal ed en kunnen ook zo makkelijk in het eten terechtkomen. Gij hebt geprobeerd om op de beste manieren de beestjes te vangen. Voor de varkens werd dit nog maar weinig gedaan omdat er weinig goeie of gebruikte methoden zijn. Met uw onderzoek heb je willen aantonen dat die beesten er zijn en dat het wel degelijk nodig is dat er nog verder onderzoek naar gebeurt want dat het wel "gevaarlijk" kan zijn voor de gezondheid van de mens. Maar er moet wel nog verder onderzoek gedaan worden ook naar de beste manier om ze te vangen...

Jürgen & Annelies, Kris & Véro, jullie zijn pas later op ons levenspad komen wandelen; maar ik zou me een leven zonder jullie al niet meer kunnen voorstellen.

Kris en Véro, Luca en Tomma, het begon allemaal met de uitnodiging om te klinken op het feit dat jullie huis met de grond was gelijk gemaakt. Dat was best een gezellige avond en toen is ook het idee voor Hettingen Feest! ontstaan. Een aantal jaren later hebben we er al skivakanties, weekendjes maar vooral heel veel ontspannende momenten opzitten. Ik geniet van de aperitiefjes met jullie, de vrijdagavonden in t Neerhof, het altijd welkom zijn... Jullie zijn niet meer 'gewoon' burens, maar goeie vrienden. Thanks voor de vele leuke!



Jürgen en Annelies, ik vergeet nooit onze eerste ontmoeting bij ons thuis. Jullie waren Koen 's collega's en het klikte heel goed met hem dus wou Koen jullie eens uitnodigen bij ons thuis zodat ook ik jullie kon leren kennen. Na die avond had ik het gevoel dat we mekaar kenden van een vorig leven, wij zaten meteen op dezelfde golflechte... en dit gevoel is met de jaren alleen maar versterkt... Annelies, jij en ik delen dezelfde liefde en passie voor dansen, lekker gek doen, kleren en schoenen... of hebben we dit laatste aan Jürgen te danken? Ja... Jürgen, maatje, jij bent me der eentje... jij hebt me echt wel geïntroduceerd in de 'high heels world', het pokeren, de champagne... maar vooral de 'geniet van het moment' levenswijsheid (of hoe kan ik dit verwoorden?), het moet niet altijd even serieus zijn. Ondertussen hebben we er al een aantal fantastische en zeer ontspannende reizen opzitten, veel avondjes uit, veel lekker eten en drinken, veel serieuze gesprekken maar ook en vooral heel veel plezier. Ondertussen is Koen ook peter van Lotte (bij deze ook een dikke zoen voor Lukas en Lotte)... Ondertussen zijn jullie mijn beste maatjes...Allright!

Mie & Wim, Marieke en mijn **petekindje Lowie!** With all my heart...

En nu ga ik eerst beginnen met de kleinsten van den hoop: Lowie.

Lowie... jij bent een super lieve schat, stoer maar met een o zo klein hartje! Meter Tasch is trots op de flinke 'man' die jij nu (al) bent! Ik kijk al uit naar en ben benieuwd naar welke grote en lieve vent jij gaat worden! Ik hoop dat we samen nog vele leuke momenten mogen beleven! Marieke, lieve en mooie meid, je bent een super grote zus!

Mieke en Wim... hoe dichter bij jouw hartje, hoe moeilijker het wordt om uit te drukken hoeveel mensen voor je betekenen, maar jullie weten zelf wel wat we aan mekaar hebben. Op jullie kan heel ons gezinnetje rekenen. Zonder Mieke en Wim mankeert er een puzzelstukje uit ons levensplaatje. Jullie zijn niet voor niets meter Mieke en peter Wim! Oona en Asa zijn gek op jullie! Bedankt voor de leuke reizen samen, de weekendjes, de ontspannende momenten, het zonder gène kunnen zagen en klagen tegen mekaar, kortom, het mogen delen van leuke en minder leuke momenten...

Mijn schoonouders, **Jos & Georges** wil ik vooral bedanken omdat hun deur vanaf dag één altijd voor mij openstond, het was en is nog steeds geen enkel probleem om een stoel (of vier) bij te schuiven en bordjes bij te zetten. Karibu! Oprecht bedankt ook voor al hetgeen jullie doen voor onze kindjes, jullie steun en hulp is onvoorwaardelijk. Oona en Asa hebben geluk met zo'n lieve oma en opa! Asante sana!

Jan & Tanja, Stien & Cas

Jan en Tanja... ook nu moet ik ver terug gaan in de tijd....Wat dan spontaan in mij opkomt is de knuffel die ik van jou kreeg Jan tijdens de meisjesbezoekdag van het kamp in 1999, het jaar dat Koen voor zijn thesis in Tanzania verbleef. Ik had het moeilijk toen en gedurende die paar seconden/minuten was jij er voor mij en dat deed deugd... Vanaf toen wist ik, ik heb een schoonbroer die er staat voor mij/ons als het erop aan komt... Bedankt Jan, om de 'groten' te zijn voor onze 'kleinen' en de zotte peter Jan te zijn van ons Oona. Tanja, ook al zaten we al jaren samen in de chiro, jou heb ik pas echt leren kennen toen we samen in de leiding stonden. Je bent een toffe, creatieve en ook wel een zotte (voor Oona en Asa, tante) Tanja. Merci voor al de mode-, huis-, tuin- en keukentips die ik van jou krijg. Merci ook voor de ongedwongen babbels over de dagelijkse dingen.

Stien en Cas, jullie wil ik bedanken om zo'n fantastisch nichtje en neefje te zijn van Oona en Asa!

Ah ja, en ook bedankt voor de fijne skivakantie dit jaar! Jullie waren geweldig!

Uit het diepste van mijn hart wil ik mijn '**Oete**' bedanken.... Eigenlijk bestaan er geen woorden om te zeggen wat jij betekent voor mij... Jij was er toen ik geboren was, jij was er toen mijn marraine gestorven was, jij was er toen mijn ouders gescheiden zijn. Jouw huis werd mijn warme thuis... voor altijd...

Jij bent er nog altijd voor mij en ik hoop nog vele jaren... Ik wil hiervoor expliciet een dikke merci zeggen aan **Jo en Carine**. Op gebied van gezondheid waren de laatste jaren voor nonkel heel zwaar, zeker na zijn hartoperatie en vooral nu nog, heeft hij immens veel steun en hulp van jullie. Ergens brengt dat ook rust in mijn hoofd en hart, wetende dat nonkel in goede handen is. Oprecht bedankt!

En dan ... **Koen, Oona en Asa**... oh boy, ik heb het aan mijne rekker, nu krijg ik het lastig...of ligt dit aan het late uur?... of door het glas rode wijn?

Oona en Asa, mijn meisjes, mijn prinsessen, mijn twee lieve schatten van dochters! Jullie zijn echt super meiden, mama is zo trots op jullie! Oona, jouw enthousiasme, behulpzaamheid en gevoel voor rechtvaardigheid zijn onbeschrijfelijk. Asa, jouw aanhoudend optimisme en jouw opgewekt doorheen de dag fladderen, werkt aanstekelijk. Jullie toveren vaak een glimlach op mijn gezicht. Bedankt om 'gewoon' Oona en Asa te zijn!

Ik verval in clichés, maar de laatste weken was ik waardeloos als moeder én als vrouw... als een echte stress kip liep ik rond, I know, en dat allemaal om van dit 'boekje' iets moois te maken. Mama heeft zich vaak schuldig gevoeld wanneer ze nog maar eens naar haar bureau trok ipv samen met jullie leuke dingen te doen... Om nog maar te zwijgen wat Koen heeft moeten aanhoren en doorstaan... Koeni... wat zou ik doen zonder jou? Jij bent mijn steun, mijn troost, mijn lach, mijn traan, maar bovenal de papa van mijn kinderen én mijn man... Op jou kan ik altijd rekenen, jouw hulp in het huishouden, jouw papa zijn voor Oona en Asa, jouw 'Koen' zijn, brengt harmonie in ons gezin. Koeni... wat zou ik doen zonder jou? Dit doctoraat zou er in elk geval niet gekomen zijn... Toen ik drie jaar geleden met dit 'zotte' idee (doorgestuurd door jou by the way) afkwam, om te starten aan een doctoraat bij de diergeneeskunde, was jij de eerste om mij aan te moedigen. Jij wist dat dit mijn 'droom' was en dat ik er dus moest voor gaan. Zot he, en hier zijn we dan! Bedankt om het te verduren, zonder klagen of zagen: het kunnen aanhoren van mijn constant streven naar verbetering, van mijn opnieuw checken van..., van mijn volgens mij kwam jij vaak zotter dan ikzelf, van al de eisen die ik mezelf oplegde... Zeker tijdens die 'laatste loodjes' was ik echt 'mezelf' niet meer, maar nu... het doctoraat ligt er, de nieuwe uitdaging is een feit (lees: de nieuwe job bij Primoris is een schot in de roos), de lente is in het land, het leven lacht ons toe, kortom vanaf nu is het (weer) GENIETEN!!! Carpe diem! *Let's dance, put on your red shoes and dance the blues...*

Nooit gedacht... dat ik mij zo trots kon voelen.

Wat ooit een verre droom leek, is nu realiteit geworden... de max!

Ik draag dit doctoraat op aan 'mijnen Oete' ...

Being happy doesn't mean that everything is perfect. It means that you've decided to look beyond the imperfections - Gerard Way