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

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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 cercomonads. The total number of FLP in used dishcloths ranged from 10 to 10^4 MPN/cm^2. 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/cm^2. *Escherichia coli* was detected in 68% (26/38) of the used dishcloths, with concentrations up to 4 $\log_{10}$ cfu/cm^2. 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.

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., 2009, 2011; 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 (Barker et al., 1999), *Listeria monocytogenes* (Zhou et al., 2007), *Salmonella* spp. (Gaze et al., 2003; Tezcan-Merdol 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 depend on various factors such as bacterial strain and environmental conditions (Schuppler, 2014; Vaerewijck et al., 2014). As a result, FLP can act as vectors, introducing pathogens into novel habitats, or as transmission routes toward 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 toward a role of FLP in the epidemiology of foodborne pathogenic bacteria with significant implications for food safety and public health

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(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; Macias-Rodriguez 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 $\log_{10}$ cfu/ml) have been reported from dishcloths (Gorman et al., 2002). The moist conditions, presence of food residues, and storage at room temperature favors 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.

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* (CCAP 1630/1W) and *Acanthamoeba castellanii* (ATCC 30324) were cultivated axenically in 75 cm^2 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. was previously isolated from a meat-cutting plant (Vaerewijck et al., 2008) and cultivated non-axenically in 75 cm^2 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. By analogy with bacteriological dishcloth protocols (Koo et al., 2013; Lee, 2010), cotton dishcloths were cut into 6 cm × 6 cm segments (n = 30), autoclaved at 110 °C for 20 min and stored in sterile Petri dishes. Three milliliters 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 10 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 milliliters 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; Rønn et al., 1995) and by a direct counting method. Both centrifuged and stomachered 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 1 ml was added in triplicate into 24 well microtiter plates (Rønn et al., 1995; Vaerewijck et al., 2011). Control wells were filled with 1 ml TSB/PAS only. The microtiter plates were incubated in the dark at 20 ± 2 °C. After one week of incubation, the wells were examined microscopically for the presence of organisms (Rønn 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:

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

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 jth dilution,
- m_j denotes the amount of the original sample put in each tube in the jth dilution,
- t_j denotes the number of tubes in the jth dilution.

For direct counting, after fixation of the homogenate with 37% formaldehyde, 1 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 in used dishcloths

Based on results (see Section 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