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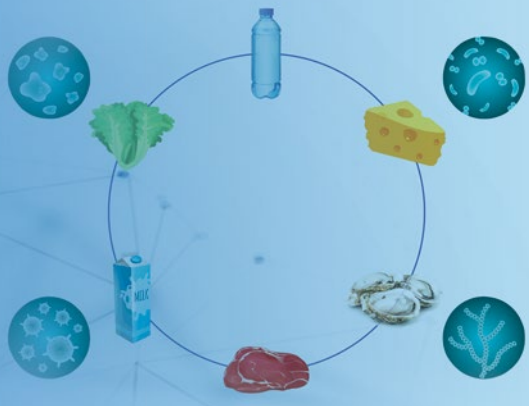

Marciane Magnani *Editor*

Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater

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Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater

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Dedication

I want to thank Prof. Anderson S. Sant'Ana for the invitation to edit this book and the authors who made it possible. I dedicate it to G. T. de Souza Pedrosa, L. I. G. de Oliveira, and W. K. A. da Costa.

Preface

Basic procedures for the enumeration of microorganisms have been used to check the microbiological quality of foods and beverages (so-called foods) and assess the impact of chemical and physical hurdles during their processing on the cultivable population of pathogenic and spoilage microorganisms. Besides, the enumeration of beneficial and technological microorganisms is the basis for establishing their feasibility for industrial application in foods. If the appropriate enumeration methodologies are used, classical approaches to risk assessment of bacteria, viruses, and protozoan in foods and beverages are possible. Most of the protocols for the enumeration of microorganisms in foods and water have been adapted from official methods. However, minor changes or technical errors may compromise their accuracy.

Furthermore, flow cytometry has emerged as an accurate alternative method to enumerate microorganisms in foods, but the detailed procedures are scarcely understood. This book gives a detailed and comprehensive description of methods and procedures used to detect and enumerate bacteria, yeast, viruses, and protozoan in distinct food matrices (fresh or processed) and freshwater to assess the quality of final products as well as to describe the behavior of microorganisms. The chapters present adapted procedures to specific food/beverage matrices, details of analytical techniques used to enumerate bacteria, mixed bacterial strains (naturally present or inoculated), yeasts, and protozoan. The use of surrogates as an alternative to enumerate the plate forming unit (PFU, supposed titer of the related virus) is also detailed. Specific procedures that allow the reduction of the working volume during analysis and the possible use of different culture media are emphasized. Each chapter introduces the topic and respective application, a list of materials and supplies, and a step-by-step description with tips to avoid mistakes that compromise the method's performance. At the end of the chapter, the key references for all information given will be listed to provide as efficiently as possible well-established protocols and procedures being used by laboratories in academia and industry. Overall, this book provides a basic understanding and enough guidance on the detection and enumeration of microorganisms in foods without disregarding the limitations or details in each specific procedure.

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Chapter 1

Survival of Pathogens on Surfaces and the Influence of Inoculating Matrix on Survival Capabilities

Matthew J. Igo and Donald W. Schaffner

Abstract

Survival of bacteria on biotic and abiotic surfaces is an important part in understanding food contamination. Temperature and relative humidity (RH) play important roles in influencing bacterial survival on surfaces. Surface type and inoculum diluent also appear to influence bacterial survival. This study examines how RH, temperature, and inoculum diluent affected the survival of *Enterobacter aerogenes* on stainless steel, polyvinyl chloride, and ceramic tile. While surface type had little effect on survival, temperature showed a clear effect. *E. aerogenes* survived better at 7 °C at 15% and 50% RH on all surfaces. Inoculum diluent composition influenced survival and allowed apparent growth under some high RH conditions. Understanding the impact that methods for the inoculation of surfaces have on bacterial survival will enable a better understanding of inconsistent research findings for the survival of bacteria on surfaces.

Key words Temperature, Relative humidity, Inoculum diluent, Surface type, *Enterobacter aerogenes*

1 Introduction

Cross-contamination of foods by pathogens present on contaminated surfaces can lead to foodborne illness, so understanding the survival of microorganisms on food contact surfaces is an important part of managing cross-contamination risk. The Centers for Disease Control and Prevention (CDC) estimates there are greater than nine million episodes of foodborne illness, including 56,000 hospitalizations, and 1300 deaths caused by known agents each year in the United States [1]. The CDC publishes reports that summarize data on surveillance for foodborne disease outbreaks in the United States. This report summarizes more than 30 contributing factors that may contribute to foodborne disease each year. Cross-contamination from surfaces (not including ill workers) is commonly a "top ten" contributing factor each year [2, 3]. The survival of pathogenic organisms on surfaces is an important driver of cross-contamination. Many pathogenic organisms including *Escherichia*

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coli and *Salmonella enterica* can survive for long periods of time on nonbiological surfaces [4–8]. Many factors influence the ability for these organisms to survive on surfaces, including temperature [4, 9–12], relative humidity (RH) [10, 13, 14], surface type [4, 9, 15], and microbial matrix [7, 16–18], while other factors can influence the transfer of and surviving bacteria from surfaces to food [16, 19].

Stainless steel, ceramic tile, and polyvinyl chloride (PVC) are surfaces commonly found in homes, restaurants, and food processing facilities. Surface free energy, hydrophobicity, and porosity have all been shown to effect bacteria attachment and biofilm formation, which can be important factors in bacterial survival on surfaces [20–27]. Stainless steel has been shown to promote biofilm formation and allow microorganisms to survive longer vs. other metals, which can in turn promote cross-contamination in food processing facilities [8, 28, 29]. The survival of pathogenic organisms on ceramic tile has been shown to be a potential cause of foodborne disease outbreaks [30]. PVC is a thermoplastic that is widely used in a variety of ways in food facilities and produce packinghouses, including as a food contact surface [31]. Understanding survival of bacteria on these surfaces can help to create a better understanding of when cross-contamination can occur to help manage the risk foodborne disease [32].

The suspending diluent (commonly a buffer) used to inoculate a microbial suspension onto a food or surface in a laboratory experiment is generally assumed to have a minimal effect on the experimental results. Typical suspending matrices (e.g., dilute peptone or phosphate-buffered saline) reduce osmotic stress to the suspended cells. The concentration of peptone in a buffer can affect microbial survival on surfaces [16, 33]. Studies have evaluated the ability of different peptone buffers to recover and subsequently culture bacteria like *E. coli* and *S. enterica* from foods [33–35]. *S. enterica* survival on surfaces has been shown to be greater when suspended in Tryptic Soy Broth (TSB) rather than when suspended in phosphate buffered saline (PBS), likely due to TSB's nutrient content [15].

2 Materials

2.1 Preparation of Surfaces

Stainless steel (0.018″ thickness, 16 gauge), polyvinyl chloride (1/8″ thickness), and ceramic tile purchased online or locally and were cut to 5 cm × 5 cm tiles for use in this study. Many other surfaces such as wood, cardboard, and rubber have also been used in other survival studies, and preparation of these surfaces are typically similar [9, 36, 37]. Reused tiles may need to be wiped with a clean paper towel to remove any visible dust or dirt. Tiles were then wrapped in aluminum foil to keep the surfaces separated

and prevent possible contamination if the surfaces needed to be moved or handled. Foil can be cut into squares about twice the size of the surfaces in order to wrap the foil completely around the tiles. Tiles wrapped in foil were placed into autoclavable containers and then autoclaved for 15 min at 250 °C. Tiles were removed from the autoclave and allowed to cool. The foil should prevent most moisture that condenses on the surface from the autoclave, but some additional drying may be necessary before inoculation. Tiles were sprayed with 70% ethanol and allowed to dry to minimizes any cross-contamination after sterilization. The tiles can be either left in the sterilized tin foil or put into an open sterile petri dish, so that they could be flipped over, and both sides could be sprayed with ethanol. A nitrile glove or sterile tongs should be used when handling surfaces to prevent contamination from bacteria on the hands.

2.2 Preparation of Culture Media

Bacteria culture and recovery media may be prepared several days in advance and stored at refrigerated temperatures. TSB is a general growth media for mesophilic bacteria, it can be made by combining 25 g of media per 1 L of distilled water. Media should then be heated on a hot plate until boiling and then allowed to boil for 10 min and autoclaved for 15 min at 250 °C. Once removed and sealed, it can be stored at room temperature for up to 1 month and potentially longer at refrigeration temperature. In this study, the microorganism used for testing was resistant to nalidixic acid, which was added in the correct concentration to all media prior to use. The addition of antimicrobials can allow for the increase of shelf life of the media, prevent contamination of the culture media, and prevent recovery of any accidental surface contaminants.

2.3 Preparation of Bacterial Strains

A high cell concentration of overnight broth culture was prepared to use for inoculation of the surfaces. Our lab has previously used *E. aerogenes* strain B199A, a nonpathogenic microorganism [38] that has shown attachment characteristics similar to *S. enterica* on chicken skin [39], which was used for all experiments (Vivolac Cultures, Indianapolis, Ind). This strain is resistant to nalidixic acid and control experiments showed that no nalidixic acid-resistant *E. aerogenes* were found on any surfaces after disinfection. While this study used an organism that was resistant to an antimicrobial, microorganisms that are not resistant to antimicrobials may also be used in surface survival experiments as sterilization of the surfaces should prevent any background microbiota from contaminating the results. Strain selection appears to be important for duration of survival in microorganisms, including on surfaces, and there should be some care in researching the appropriate strains to use before experimentation.

Cultures for our experiments were prepared in a similar manner to that described previously [38, 39]. A frozen stock of *E. aerogenes* in 80% glycerol solution was streaked onto Tryptic Soy Agar (Difco, BD, Sparks, MD) containing 50 μg/mL of nalidixic acid (Sigma Chemical Co., St. Louis, Mo.), referred to as TSA-na in order to select for a single isolated colony. These plates can be wrapped in parafilm and stored in a refrigerator for use up to 2 weeks. One colony was grown *overnight* in 10 mL TSB (Difco, BD, Sparks, MD), containing 50 μg/mL of nalidixic acid and incubated at 37 °C for 24 h, consistent with methods used previously for this organism [19, 40]. Inoculum matrices were of the three different types described below. Cells were harvested from the *overnight* culture in TSA-na by centrifuging at 5000 × *g* for 10 min and washed twice in either 0.1% peptone water (Difco, BD), 1% phosphate buffered saline solution (Difco, BD), or sterile distilled water. Typically, a final concentration of 10^8 CFU/mL will be achieved on a nonselective media, such as TSA.

2.4 Preparation of Controlled Environment

Saturated salt solutions can be kept in the bottom of glass desiccators to control for environmental RH with the inoculated surfaces stored above the solutions. A small amount of petroleum jelly can be applied around the lid to ensure tight seal on the glass desiccators. In our study, lithium chloride or potassium carbonate (each 230 g) was slowly mixed into 100 mL of heated water to create saturated salt solutions at 15% and 50% RH, respectively. Potassium sulfate salt (250 g) was mixed into 100 mL of water to create a 100% RH environment. Salt solutions were placed in the bottom of glass desiccators (Thermo Fisher Scientific, Waltham, MA) and given 24 h for the RH to stabilize. A list of additional salts that can be used to achieve a variety of relative humidities was published by Greenspan in 1976 [41]. Data loggers purchased from LASCAR Electronics (Erie, PA) for RH and temperature were used to monitor the environment. Loggers were sensitive to 0.5(±1) °C and 1 (±2)% RH. Desiccators were stored on the lab bench to represent room temperature (21 °C), and desiccators were also stored in a walk-in refrigeration unit to achieve a cool (7 °C) storage temperature. Desiccators that were held at room temperature in the lab were found to have very consistent storage temperatures, within ±1 °C, and storage temperature inside of the walk-in refrigerator showed even less variability. RH inside of the desiccators was also fairly consistent, ±3%. There were changes in the RH when the chambers were opened, but the humidity stabilized within 1 h. Incubators that control for both temperature and RH can achieve more precise control; however, these are much more expensive than salt and glass desiccators.

3 Methods

3.1 Survival Based on Surface and Temperatures

In our study, three surface types (stainless steel, PVC, and ceramic tile) were inoculated with 100 μL containing ~10^8 CFU/mL in 0.1% peptone of *overnight* culture after centrifuging and washing. The coupons allowed to dry for approximately 2 h at room temperature and ambient RH for an initial concentration of ~10^7 CFU per coupon. Coupons can also be placed in a biosafety cabinet which will dry the surfaces quicker because of the air flow. Dried coupons were then placed in previously equilibrated desiccators containing saturated salt solutions at 15, 50, or 100% RH. Desiccators were placed either on the bench top (21 °C) or in a walk-in cooler (7 °C). Tiles were removed from the desiccators at ten time points (from 0 to 21 days). Cell recovery time points may need to be adjusted based on the storage conditions of the coupons, where slower cell decline should have time points taken further out, and conditions that show a more rapid cell decline should take time points in a shorter period of time in order to adequately capture the cell decline. Tiles were removed by using a gloved hand in order to prevent any potential contamination of the surfaces. Some care should be taken to not touch the site of inoculation, in order to prevent premature removal of cells. Each coupon was placed in a sterile 207-mL Whirl-Pak sampling bag (Nasco, Fort Atkinson, WI) and filled with 10 mL of 0.1% peptone water. The rub-shake method was used for 1 min to detach the microorganisms from the surfaces [4]. A clear site of inoculation can typically be seen on the coupons, and it is important to rub well on this spot to properly detach all cells from the surface. Dilutions were plated on TSA-na plates and incubated at 37 °C for 24 h and colonies were counted. Populations were expressed in log CFU per surface.

3.2 Survival Based on Diluent Type

Survival of *E. aerogenes* in different diluent types was evaluated on only stainless steel. Cultures were washed with either 0.1% peptone, 1% PBS, or sterile distilled water and inoculated onto stainless steel surfaces and placed in a desiccator containing saturated salt solutions at either 15, 50, 100% RH as described above. Different inoculating media and food slurries have also been used to inoculate surfaces in order to test bacteria survival. Slurries of food products have also been used to mimic a potential real-world scenario of contamination of a surface from a food product [18, 42]. Typically, inoculating media that have greater nutrient concentration will allow for greater survival of bacteria [16, 18]. Desiccators were placed on the lab benchtop (21 °C), and tiles were sampled at ten time points (from 0 to 21 days) for peptone and PBS samples and over 10 more frequent time points (from 0 to 168 h) for sterile distilled water samples. Surfaces were placed in sterile Whirl-Pak bags containing 10 mL of the same

diluent that was used for inoculation, and the rub-shake method as previously described was used to detach microorganisms from surface then diluted and plated on the TSA-na plates. Colonies were counted and expressed as log CFU per surface.

3.3 Survival at Different Starting Concentrations at a High Humidity

E. aerogenes was inoculated onto stainless steel coupons at starting concentrations of ~2, 4, and 6 log CFU/surface with 0.1% peptone and 1% PBS and ~3, 4, and 5 log CFU/surface using distilled water and placed in desiccators containing saturated potassium sulfate salt solutions to ensure 100% RH. It is important to remember to dilute the initial inoculum in the same matrix that the cells were washed in to prevent possible inconsistencies of results. It is important to note that the concentration put onto the surface will be greater that the concentration recovered after the initial drying time, and the reduction may be >90% under some conditions. We would recommend testing the initial concentration after drying if a more dilute inoculum culture is being used to determine the initial cell concentration on the surfaces. Coupons were removed from the desiccators at ten time points (from 0 to 21 days) for 0.1% peptone and 1% PBS or 10 more frequent time points (from 0 to 7 days). Each coupon was placed in a sterile Whirl-Pak bag with 10 mL of 0.1% peptone water or PBS. The rub-shake method as previously described was again applied for 1 min to detach the bacteria from the surfaces as described above. Dilutions were plated on TSA-na plates and incubated at 37 °C for 24 h and colonies were counted. Populations were expressed in log CFU per surface.

4 Conclusions

Bacteria survival on abiotic surfaces is important in understanding how food can become contaminated due to cross contamination. Cross contamination of foods can potentially lead to illness after consumption of the food. Survival of bacteria can be greatly influenced based on the methodology used to inoculate the surfaces. Important variable includes how the bacteria are grown and the matrix that suspends the inoculated organism. The storage conditions of the surfaces including temperature and RH can also influence survival. These factors should be considered before beginning any experimentation. The methods listed above can help create consistent research practices in order to obtain the best results.

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Chapter 2

Enumeration of Viable Cells of Bacteria in Food and Water with Flow Cytometry

Jossana Pereira de Sousa Guedes and Evandro Leite de Souza

Abstract

Flow cytometry (FC) can be used to enumerate viable bacteria in food, beverage, and water samples. The combination of the fluorescent dyes thiazole orange (TO) and propidium iodide (PI) makes it possible to identify cells with membrane damage, investigate cell viability, identify live, dead, and injured cell subpopulations, and enumerate these cells. FC requires short analysis time, has high reliability and ease of use, in addition to provide results comparable with the classical plate count method.

Key words Flow cytometry, Cell count, Viability, Membrane damage, Bacteria

1 Introduction

Viability of an individual microorganism can be considered as a spectrum of physiological states. In some of these physiological states, the cells should become not capable of division and producing visible colonies on solid media or detectable turbidity in classical growth-based viability assays. Therefore, considering that some cells should exist in viable but not cultivable (VBNC) state, the method to enumerate bacterial cells should enable the quantification of this cell population and demonstrate that they are viable [1, 2]. VBNC are injured cells but still metabolically active and capable of growing when the stress conditions are removed [3].

The use of flow cytometry (FC) makes it possible to evaluate physiological functions in bacterial cells [4], in addition to enumerate viable bacteria in food, beverage, and water samples [5–7]. FC detects individual particles, based on the combination of light signals scattered emitted by each cell and can be used to obtain the number of live and dead cells [8]. It has been already reported the possibility of enumerating viable probiotic cells on total cell population, with a good correlation between plate count method and FC. The enumeration of viable probiotic cells is important

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because their metabolic activity brings benefits to the host [7]. FC is also accepted as a tool for water quality assessment through enumeration of waterborne microorganisms [8, 9]. However, the relationship between the heterotrophic plate count and FC in water is still not clear [10].

The advantages of FC for enumeration of viable cells of bacteria include the reduction of analysis time, high reliability, ease of use, and versatility, besides to provide comparable results with the classical plate count method [11, 12]. Information on viability state of bacteria using fluorescent dyes can be drawn based on various cell characteristics, such as membrane potential and permeability, and metabolic and respiratory activity [2, 13]. Regardless of the relationship between plate count method and FC, it is necessary to use non-growth-dependent methods, such as FC, to quantify both viable and VBNC cell populations [3, 14].

2 Materials

1. Brain heart infusion agar (BHIA).
2. Phosphate-buffered saline (PBS; 50 mM K_2HPO_4/KH_2PO_4; pH 7.4).
3. Fluorochromes: Thiazole orange (TO), and propidium iodide (PI).
4. Solvents: Dimethyl sulfoxide (DMSO), and absolute ethyl alcohol 99.5% (v/v).

2.1 Inoculum Preparation

1. Centrifuge (4500 × *g*, 15 min, 4 °C) a 3-mL aliquot from a food or water sample (*see* **Note 1**).
2. Wash twice and resuspend the sample into a tube with the same volume (3 mL) of PBS (pH 7.4) to obtain a concentrated cell suspension.
3. Adjust the optical density (OD) of the concentrated cell suspension in a spectrophotometer to provide a viable count of approximately 10^8 colony-forming units per milliliter (CFU/mL) when pour-plated on agar.
4. Use a sufficient aliquot of bacterial inoculum to desired food or water sample volume for a final viable count between 10^5 and 10^6 CFU/mL.

2.2 Dye Concentrated Solutions

1. Prepare dye solutions at room temperature and protected from light (*see* **Note 2**).
2. TO concentrated solution: prepare a 10 mg/mL solution in DMSO (*see* **Note 2**).
3. Use PI ready-made water solution with 1 mg/mL.

3 Methods

3.1 Staining Procedure

1. Centrifuge each sample (4500 × *g*, 15 min, 4 °C), wash twice, resuspend in PBS (pH 7.4), and label with the different fluorochromes.
2. Use unstained bacterial cell sample in PBS (pH 7.4) as negative control for TO and PI staining.
3. Use ethanol-fixed cells as a positive control for PI staining. For a 5-mL aliquot, add 500 μL of bacteria cell suspension and 1 mL of icy saline into 3.5 mL of icy ethanol, and incubate for 30 min at 4 °C.
4. Add 1 μL of TO concentrated solution (10 mg/mL in DMSO) into a microcentrifuge tube with 1 mL of the cell suspension in PBS (10 μg/mL; pH 7.4), vortex gently to mix and incubate for at least 5 and 15 min for Gram-positive and Gram-negative bacteria, respectively, in the dark at 37 °C. Add 10 μL of PI concentrated solution (1 mg/mL in water) into a microcentrifuge tube with 1 mL of the Gram-positive cell suspension in PBS (10 μg/mL; pH 7.4), vortex gently to mix and incubate for at least 5 min in the dark at 30 °C. Add 1 μL of PI concentrated solution (1 mg/mL in water) into a microcentrifuge tube with 1 mL of the Gram-negative cell suspension in PBS (1 μg/mL; pH 7.4), vortex gently to mix, and incubate for at least 15 min in the dark at 37 °C. For double staining, add 1 μL of each TO and PI concentrated solutions in the same microcentrifuge tube with 1 mL of the cell suspension in PBS (pH 7.4), vortex gently to mix and incubate for at least 5 and 15 min for Gram-positive and Gram-negative bacteria, respectively, in the dark at 30 or 37 °C (*see* **Notes 3** and **4**).
5. Centrifuge cell suspension from each sample (4500 × *g*, 15 min, 4 °C) after incubation period with the fluorochromes, wash twice, and resuspend in PBS (pH 7.4).

3.2 Acquisition Settings and Data Collection

1. Select a 96-well plate and create a template. Name the plate and the samples according to the well location.
2. Define acquisition settings (fluidics rate, threshold, and run limit). Perform acquisition at the low flow rate setting (14 μL/min). Set the threshold level for FSC (forward scatter light) on 12,000 to eliminate noise or particles (of cellular debris) much smaller than intact cells (*see* **Note 5**). Perform acquisition of 10,000 events for each sample.
3. Place the microcentrifuge tube with the examined sample on the sample injection port (SIP), select an empty sample well in the grid, and start to collect the sample.

4. Collect events on unstained bacterial sample in PBS (pH 7.4), and on ethanol-treated sample.
5. Collect events on samples stained with individual staining (TO or PI).
6. Collect events on samples stained with double staining (TO/PI).
7. Collect scatter and fluorescence signals (pulse area measurements) of individual cells passing through the laser zone as logarithmic signals. Collect green fluorescence (TO) in the FL1 channel (533 nm ± 30 nm) and red fluorescence (PI) in the FL3 channel (>670 nm).
8. Get density plots representing FSC vs. SSC (side scatter light) with polygonal or rectilinear gate around a population of events to determine the FSC/SSC properties of the cell population (*see* **Note 6**).
9. Get density plot of FL1 vs. FL3 with quadrant gate to determine the fluorescence properties of the cell populations with double staining and display the live or dead population stain results (TO/PI) (*see* **Note 7**).
10. Dispose of stained samples and extra dye solution according to local regulations.

3.3 Correcting Fluorescence Spillover

1. Use control samples stained with individual fluorochromes to correct fluorescence spillover.
2. Get density plots of FL1 vs. FL2, FL1 vs. FL3, and FL1 vs. FL4.
3. Get density plots of FL3 vs. FL1, FL3 vs. FL2, and FL3 vs. FL4.
4. Adjust the quadrant marker position for all positive populations are cleanly contained in individual quadrants (gate lower left—LL, gate lower right—LR, gate upper left—UL, and gate upper right—UR).
5. Compare the median values of the affected channel. If the median value of the UL or LR quadrant is not equal or nearly equal to the median value of the negative population (LL), the fluorescence compensation should be applied.
6. Subtract a percentage of fluorescence signal, thereby redistributing data to lower channels on the fluorescence scale and removing the apparent fluorescence spillover. TO fluorescence is now confined to the FL1 detector, and no longer spills into FL2 or FL3. PI fluorescence is now confined to the FL3 detector, and no longer spills into FL1 or FL2.

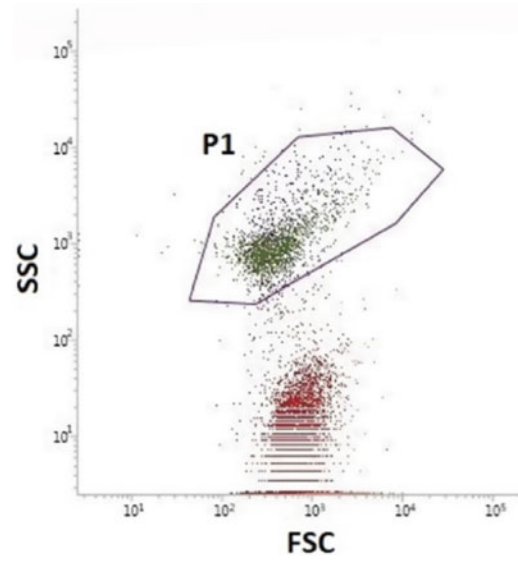

Fig. 1 FSC vs. SSC dot plot *of Bifidobacterium animalis* with gate P1 identifying the size of the population of interest. The vertical axis provides information on cell granularity; the horizontal axis indicates the cell size [7]

3.4 Data Analysis

1. Get density or dot plots analysis representing FSC vs. SSC. Plot a gate to identify the size of the cell population of interest. This allows cells to be distinguished from other particles in a sample (Fig. 1).
2. Get density plot analysis of FL1 vs. FL3 from gate with the population of interest to determine the fluorescence properties of the cell populations with double staining (TO/PI). Plot quadrant gates to identify subpopulations of interest: TO^+ PI^- cells (gate UL), TO^+ PI^+ cells (gate UR), TO^- PI^+ cells (gate LR), and TO^- PI^- cells (gate LL) (Fig. 2; *see* **Note 8**).
3. For enumeration of bacterial cells, gate other regions in the FL1 vs. FL3 plot to display live, dead, and injured cell populations. Determine the absolute count, expressed as FCU/g or mL, using the following equation:

$$N = n \times 1000 \times d$$

where n = number of events per μL;
d = dilution factor corresponding to the dilution (*see* **Note 9**).

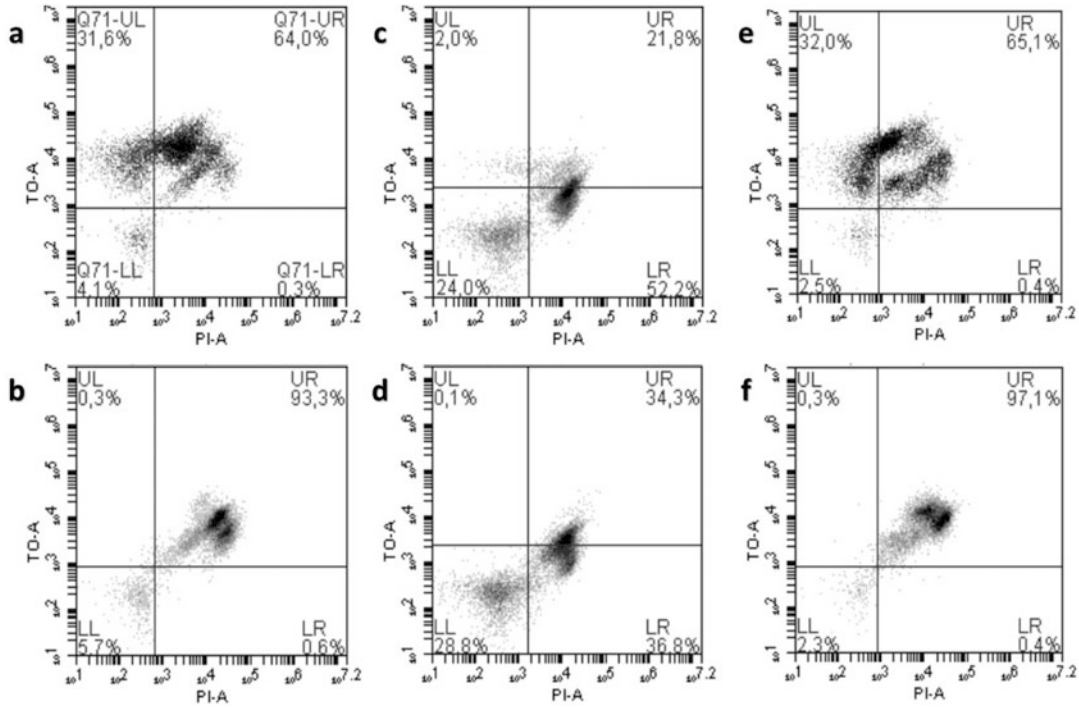

Fig. 2 Fluorescence density plot of *E. coli* UFPEDA 224 (**a**, **b**), *L. monocytogenes* ATCC 7644 (**c**, **d**) *and Salmonella* Enteritidis UFPEDA 414 (**e**, **f**) in response to staining with TO/PI after a 15-min not exposure (**a**, **c**, **e**) and exposure (**b**, **d**, **f**) to *Mentha piperita* L. essential oil (MPEO; 1.25 μL/mL) in pineapple juice at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of TO; the horizontal axis indicates the fluorescence intensity of PI. The percentages of cell populations that fell into each gate are displayed in the four edges of each plot [4]

4 Notes

1. The optical density (OD) reading of *E. coli* UFPEDA 224 [originally ATCC 25922], *Listeria monocytogenes* ATCC 7644, and *Salmonella* Enteritidis UFPEDA 414 [originally MM 6247] at 625 nm (OD625) provide 0.13 absorbance and viable counts of approximately 10^8 CFU/mL when pour-plated on BHIA [15]. Use plastic disposable UV-cuvettes with 1.5 mL capacity to adjust the inoculum concentration on a spectrophotometer.
2. Fluorochromes are sensitive to exposure to light, being necessary to handle in the dark. TO is hydrophobic, therefore maintain stock solutions in DMSO or alcohol. Prepare TO concentrated solution in concentration for proper use in the final sample volumes.

3. TO staining is adequate for analysis at 2–5 min, but it requires at least 15 min to achieve maximum intensity. PI stains very quickly, while TO enters the cells more slowly.
4. Gram-negative bacteria lipopolysaccharide (LPS) can interfere on TO uptake and other permeant dyes. 1 mM of ethylenediaminetetraacetic acid (EDTA) can be used in the staining buffer for remove the LPS.
5. Set the adequate threshold (lowest signal intensity value an event) can exclude unwanted signals, such as those from cellular debris, much smaller than intact cells [16].
6. FSC vs. SSC density plot provides information on the bacterial size and granularity and discriminate cells from cellular debris. The fluorescent signal from TO in viable cells allows their enumeration even when debris in the cell preparation contaminates a scatter gate around the cells (Fig. 1).
7. The combination of TO and PI provides a rapid and reliable method for discriminating live and dead bacteria. TO is a cell permeant DNA dye used to identify DNA-containing particles and can enter all cells, live and dead. PI is an impermeant DNA dye that only penetrates cells with damaged membranes [17].
8. TO^+ PI^- cells correspond to an intact cell membrane, and TO^+ PI^+ cells correspond to a damaged or slightly permeabilized cell membrane. TO^- PI^+ cells correspond to a permeabilized cell membrane, and TO^- PI^- cells correspond to a damaged DNA or RNA, while the cell may still be intact (Fig. 2) [18].
9. The dilution 10^5 CFU/mL was chosen as the most suitable after consecutive dilutions of the samples after the staining procedure [7].

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Chapter 3

Evaluation of Physiological Characteristics of Bacterial Cells in Foods and Water with Flow Cytometry

Jossana Pereira de Sousa Guedes and Evandro Leite de Souza

Abstract

Flow cytometry (FC) can be used to evaluate the physiological characteristics and to quantify accurately viable but nonculturable (VBNC) cells in food and water samples. The fluorescent dyes thiazole orange (TO), propidium iodide (PI), bis-1,3-dibutylbarbutiric acid (BOX), ethidium bromide (EB), and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) enable to identify cell subpopulations and investigate cell functions, such as membrane integrity, membrane potential, efflux pump, and respiratory activity.

Key words Flow cytometry, Cell damage, Viability, Nonculturable cells, Bacteria

1 Introduction

The plate count is the most widely used method to assess the live bacteria population in food and water. This classic method allows to evaluate the ability of microorganisms to reproduce and form visible colonies from an original single cell [1–3]. However, viable cell count method is a multiday process, and it does not detect viable but nonculturable (VBNC) cells [4]. The accurate determination of live, dead, and VBNC bacterial cells is important in many applications in food industry [5–7]. Stresses caused by food preservation methods can induce bacterial cells to enter a VBNC state [8–10], and some waterborne pathogens frequently exist in a VBNC state [11, 12].

VBNC cells present as an unsuccessful culture, although are not considered as dead cells because cells lose their ability to grow in culture media, but still maintain some metabolic activity [4, 13]. VBNC state can be found in different stages in response to the type and intensity of cell stress [14]. Additionally, it can be considered as an adaptive strategy under harsh environment [10]. The occurrence of VBNC foodborne and waterborne bacteria may cause fatal infections and a threat to public health due to their

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no detectability through conventional methods, besides to allow cell division and bacterial growth when favorable conditions are reestablished [14–16]. VBNC cells can be evaluated by investigation of various cell functions, such as respiration, membrane potential or integrity, and enzymatic activity [17–19].

Flow cytometry (FC) can be used to evaluate the physiological characteristics and quantify accurately VBNC bacterial cells in food and water samples with plate count-based methods [4, 20]. FC technique utilizes a combination of light scattering and emitted fluorescence by particles dispersed in a flow, allowing to evaluate various structural, physiological, and genetic conditions of bacterial cells. The specific probes and fluorescent dyes, with different cell permeability characteristics and binding to different cellular constituents, used in FC can quickly differentiate physiological functions on a large number of individual cells with a minimal sample volume [7, 17, 18].

2 Materials

1. Brain heart infusion agar (BHIA).
2. Phosphate-buffered saline (PBS; 50 mM K_2HPO_4/KH_2PO_4; pH 7.4).
3. Fluorochromes: thiazole orange (TO), propidium iodide (PI), bis-1,3-dibutylbarbutiric acid (BOX), ethidium bromide (EB), and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC).
4. Solvents: dimethyl sulfoxide (DMSO), 4 mM ethylenediaminetetraacetic acid (EDTA) solution, methanol, and absolute ethyl alcohol 99.5% (v/v).
5. 1% (w/v) glucose solution.

2.1 Inoculum Preparation

1. Centrifuge (4500 × *g*, 15 min, 4 °C) a 3-mL aliquot from a food or water sample (*see* **Note 1**).
2. Wash twice and resuspend into a tube with the same volume (3 mL) of PBS (pH 7.4) to obtain a concentrated cells suspension.
3. Adjust the optical density (OD) of the concentrated cell suspension in a spectrophotometer to provide a viable count of approximately 10^8 colony-forming units per milliliter (CFU/mL) when pour-plated on agar.
4. Use a sufficient aliquot of bacteria inoculum to desired food or water sample volume for a final viable count of 10^5–10^6 CFU/mL.

2.2 Dye Concentrated Solutions

1. Prepare dye solutions at room temperature and protected from light (*see* **Note 2**).
2. TO concentrated solution: prepare a 10 mg/mL solution in DMSO.
3. BOX concentrated solution: prepare a 6.25 mg/mL solution in methanol.
4. CTC concentrated solution: dissolve 15.152 mg in 1 mL of ultrapure water for a final concentration of 50 mM.
5. Use PI and EB ready-made water solutions with 1 and 10 mg/mL, respectively.

3 Methods

3.1 Staining Procedure

1. Centrifuge each sample (4500 × *g*, 15 min, 4 °C), wash twice, resuspend in PBS (pH 7.4), and label with the different fluorochromes.
2. Use unstained bacterial cell sample in PBS (pH 7.4) as negative control for PI, BOX, EB, and CTC staining.
3. Use ethanol-fixed cells as a positive control for PI, BOX, and EB staining, as well as a negative control for CTC staining. For a 5-mL aliquot, add 500 μL of bacteria cell suspension and 1 mL of icy saline into 3.5 mL of icy ethanol, and incubate for 30 min at 4 °C.
4. For membrane permeability: add 1 μL of TO concentrated solution (10 mg/mL in DMSO) into a microcentrifuge tube with 1 mL of the cell suspension in PBS (10 μg/mL; pH 7.4), vortex gently to mix and incubate for at least 5 and 15 min for Gram-positive and Gram-negative bacteria, respectively, in the dark at 37 °C. Add 10 μL of PI concentrated solution (1 mg/mL in water) into a microcentrifuge tube with 1 mL of the Gram-positive cell suspension in PBS (10 μg/mL; pH 7.4), vortex gently to mix and incubate for at least 5 min in the dark at 30 °C. Add 1 μL of PI concentrated solution (1 mg/mL in water) into a microcentrifuge tube with 1 mL of the Gram-negative cell suspension in PBS (1 μg/mL; pH 7.4), vortex gently to mix, and incubate for at least 15 min in the dark at 37 °C. For double staining, add 1 μL of each TO and PI concentrated solutions in the same microcentrifuge tube with 1 mL of the cell suspension in PBS (pH 7.4), vortex gently to mix and incubate for at least 5 and 15 min for Gram-positive and Gram-negative bacteria, respectively, in the dark at 30 or 37 °C.

5. For membrane potential: add 1 μL of BOX concentrated solution (6.25 mg/mL in methanol) into 12.5 mL of the Gram-positive cell suspension in PBS (0.5 μg/mL; pH 7.4), vortex gently to mix and incubate for at least 5 min in the dark at 30 °C. Add 5 μL of BOX concentrated solution (6.25 mg/mL) into 12.5 mL of the Gram-negative cell suspension in PBS (2.5 μg/mL; pH 7.4) with 4 mM EDTA, vortex gently to mix and incubate for at least 15 min in the dark at 37 °C. For double staining, add 1 or 5 μL of BOX and 12.5 μL of PI concentrated solutions in the same tube with 12.5 mL of the cell suspension in PBS (pH 7.4) or PBS with 4 mM EDTA, vortex gently to mix and incubate for at least 5 and 15 min for Gram-positive and Gram-negative bacteria, respectively, in the dark at 30 or 37 °C.
6. For efflux activity: add 1 μL of EB concentrated solution (10 mg/mL in water) into a microcentrifuge tube with 2 mL of the Gram-positive cell suspension in PBS (5 μg/mL; pH 7.4) with 1% (w/v) glucose, vortex gently to mix and incubate for at least 5 min in the dark at 30 °C. Add 1 μL of EB concentrated solution (10 mg/mL) into a microcentrifuge tube with 1 mL of the Gram-negative cell suspension in PBS (10 μg/mL; pH 7.4), vortex gently to mix and incubate for at least 15 min in the dark at 37 °C.
7. For respiratory activity: add 10 μL of CTC concentrated solution (50 mM in ultrapure water) into a microcentrifuge tube with 90 μL of the Gram-positive and Gram-negative cell suspensions in PBS (5 mM; pH 7.4) with 1% (w/v) glucose, vortex gently to mix and incubate for at least 30 min in the dark at 30 or 37 °C under stirring at 250 rpm.
8. Centrifuge (4500 × *g*, 15 min, 4 °C) cell suspension from each sample after incubation period with the fluorochromes, wash twice, and resuspend in PBS (pH 7.4).

3.2 Acquisition Settings and Data Collection

1. Select a 96-well plate and create a template. Name the plate and the samples according to the well location.
2. Define acquisition settings (fluidic rate, threshold, and run limit). Perform acquisition at the low flow rate setting (14 μL/min). Set the threshold level for FSC (forward scatter light) on 12,000 to eliminate noise or particles (of cellular debris) much smaller than intact cells (*see* **Note 3**). Perform acquisition of 10,000 events for each sample.
3. Place the microcentrifuge tube with the examined sample on the sample injection port (SIP), select an empty sample well in the grid, and start to collect the sample.
4. Collect events on unstained bacterial sample in PBS (pH 7.4) and on ethanol-treated sample.

5. Collect events on samples stained with individual staining (TO, PI, BOX, EB, or CTC).
6. Collect events on samples stained with double staining (TO/PI or BOX/PI).
7. Collect scatter and fluorescence signals (pulse area measurements) of individual cells passing through the laser zone as logarithmic signals. Collect green fluorescence (TO and BOX) in the FL1 channel (533 nm ± 30 nm) and red fluorescence (PI, EB, and CTC) in the FL3 channel (>670 nm).
8. Get density plots representing FSC vs. SSC (side scatter light) with polygonal or rectilinear gate around a population of events to determine the FSC/SSC properties of the cell population (*see* **Note 4**).
9. Get density plot of FL1 vs. FL3 with quadrant gate to determine the fluorescence properties of the cell populations with double staining (TO/PI and BOX/PI) (*see* **Notes 5–8**).
10. Get density plot of SSC vs. FL3 with polygonal or rectilinear gate around a population of events to determine the fluorescence properties of the cell populations with a single staining (EB or CTC) (*see* **Note 9**).
11. Dispose of stained samples and extra dye solution according to local regulations.

3.3 Correcting Fluorescence Spillover

1. Use control samples stained with individual fluorochromes to correct fluorescence spillover (*see* **Note 10**).
2. Get density plots of FL1 vs. FL2, FL1 vs. FL3, and FL1 vs. FL4 (*see* **Note 11**).
3. Get density plots of FL3 vs. FL1, FL3 vs. FL2, and FL3 vs. FL4 (*see* **Note 12**).
4. Adjust the quadrant marker position for all positive populations are cleanly contained in individual quadrants (gate lower left—LL, gate lower right—LR, gate upper left—UL, and gate upper right—UR).
5. Compare the median values of the affected channel. If the median value of the UL or LR quadrant is not equal or nearly equal to the median value of the negative population (LL), the fluorescence compensation should be applied.
6. Subtract a percentage of fluorescence signal, thereby redistributing data to lower channels on the fluorescence scale and removing the apparent fluorescence spillover. TO or BOX fluorescence is now confined to the FL1 detector, and no longer spills into FL2 or FL3. PI fluorescence is now confined to the FL3 detector, and no longer spills into FL1 or FL2.

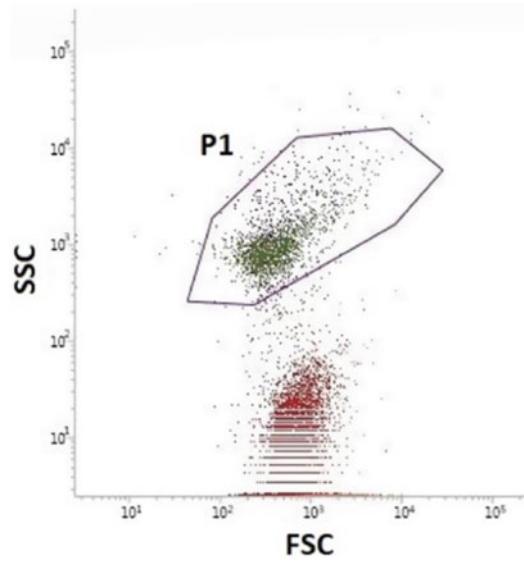

Fig. 1 FSC vs. SSC dot plot *of Bifidobacterium animalis* with gate P1 identify the size of the population of interest. The vertical axis provides information on cell granularity; the horizontal axis indicates the cell size [21]

3.4 Data Analysis

1. Get density or dot plot analysis representing FSC vs. SSC. Plot a gate to identify the size of the cell population of interest. This allows cells to be distinguished from other particles in a sample (Fig. 1).
2. FSC is measured in the plane of the beam and gives relative information on cell size. SSC is measured at 90° to the beam and provides information on cell granularity.
3. Get density plot analysis of FL1 vs. FL3 from gate with the population of interest to determine the fluorescence properties of the cell populations with double staining (TO/PI and BOX/PI). Plot quadrant gates to identify subpopulations of interest: TO^+ PI^- cells (gate UL), TO^+ PI^+ cells (gate UR), TO^- PI^+ cells (gate LR), TO^- PI^- cells (gate LL), BOX^+ PI^- cells (gate UL), BOX^+ PI^+ (gate UR), BOX^- PI^+ cells (gate LR), and BOX^- PI^- cells (gate LL) (Fig. 2).
4. Get density plot analysis of SSC vs. FL3 from gate with the population of interest to determine the fluorescence properties of the cell populations with a single staining (EB or CTC). Plot polygonal or rectilinear gates for irregularly or evenly shaped populations, respectively, to identify subpopulations of interest: EB^+ cells (right gate), EB^- cells (left gate), CTC^+ (right gate), and CTC^- (left gate) (Fig. 3).

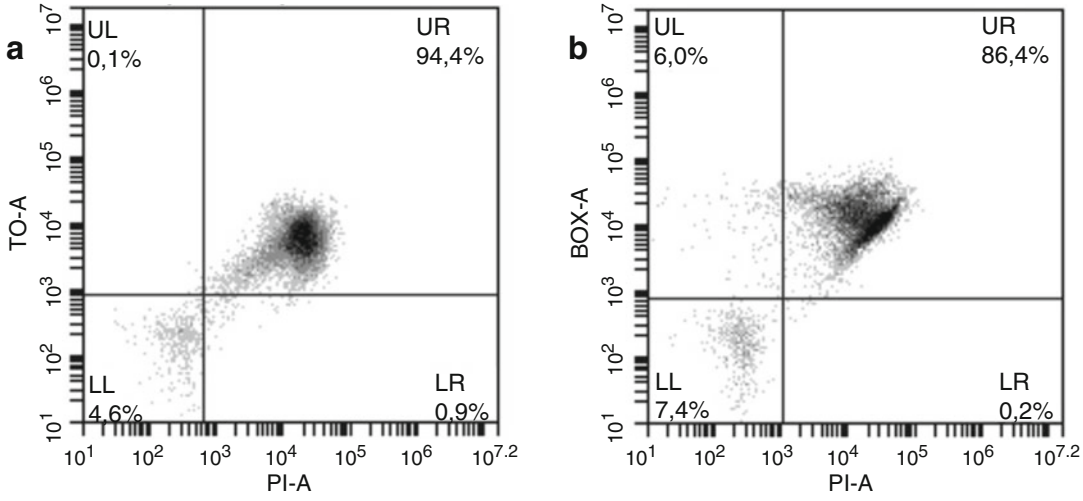

Fig. 2 Fluorescence density plot of *E. coli* UFPEDA 224 in response to staining with TO/PI (**a**) and BOX/PI (**b**) after a 15-min exposure to *Mentha piperita* L. essential oil (MPEO; 1.25 μL/mL) in pineapple juice at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of TO or BOX; the horizontal axis indicates the fluorescence intensity of PI. The percentage of cell populations that fell into each gate are displayed in the four edges of each plot [7]

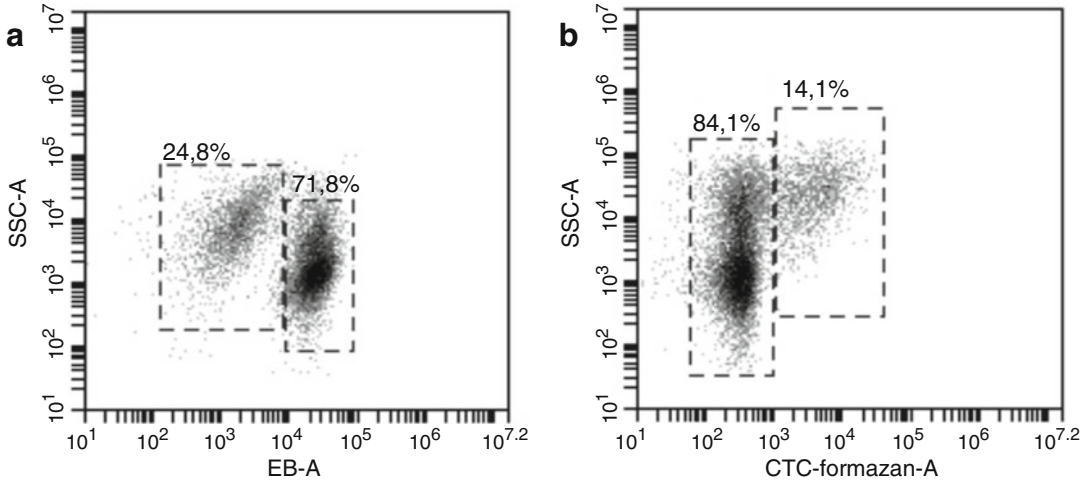

Fig. 3 Fluorescence density plots of *Escherichia coli* UFPEDA 224 in response to staining with EB (**a**) and CTC (**b**) after a 15-min exposure to *Mentha piperita* L. essential oil (MPEO; 1.25 μL/mL) in pineapple juice at 4 ± 0.5 °C. The vertical axis indicates the side-light scatter intensity; the horizontal axis indicates the fluorescence intensity of EB or CTC. The negative stain subpopulation was gated in the left rectangles; the positive stain subpopulation was gated in the right rectangles. The percentages of the cell populations that fell into each gate are shown in each plot [7]

4 Notes

1. The optical density (OD) reading of *E. coli* UFPEDA 224 [originally ATCC 25922], *Listeria monocytogenes* ATCC 7644, and *Salmonella* Enteritidis UFPEDA 414 [originally MM 6247] at 625 nm (OD625) provides 0.13 absorbance and viable counts of approximately 10^8 CFU/mL when pour-plated on BHIA [22]. Use plastic disposable UV-cuvettes with 1.5 mL capacity to adjust the inoculum concentration on a spectrophotometer.
2. Prepare concentrated solutions in concentrations for proper use in the final sample volumes. It is important to note that fluorochromes are sensitive to exposure to light, and handling in the dark is recommended.
3. Thresholds are used to exclude unwanted signals, such as those from cellular debris, much smaller than intact cells. A threshold is the lowest signal intensity value an event can have to be recorded by the FC [23, 24].
4. FSC vs. SSC density plot is the first step to discriminate cells from cellular debris based on their size and granularity. FSC is measured in the plane of the beam and gives relative information on cell size. SSC is measured at 90° to the beam and provides information on cell granularity (Fig. 1).
5. Fluorescence emitted at differing wavelengths from stained cells can be used to identify the particles in the FSC vs. SSC plot and to evaluate the bacterial cell physiological functions in the FL1 vs. FL3 plot. Besides, it allows the discrimination of cells into different sub-populations [17].
6. Use TO, PI, BOX, EB, and CTC fluorochromes to investigate the effects of different treatments on bacterial membranes and cellular functions. TO is a cell permeant DNA dye used to identify DNA-containing particles and can enter all cells, live and dead. PI is an impermeant DNA dye that only penetrates cells with damaged membranes. Depolarized cells allow the accumulation of BOX inside the cells, while polarized cells can exclude BOX. EB enters intact cell membranes, being actively pumped out of the cell, but when the efflux pump malfunctions, EB can stain the intracellular DNA in cells. Cells with an active electron transport system show accumulation of fluorescent CTC-formazan particles [25].
7. TO^+ PI^- cells correspond to an intact cell membrane, and TO^+ PI^+ cells correspond to a damaged or slightly permeabilized cell membrane. TO^- PI^+ cells correspond to a permeabilized cell membrane, and TO^- PI^- cells correspond to a damaged DNA or RNA, while the cell may still be intact (Fig. 2a) [26].

8. BOX^+ PI^- cells correspond to depolarized and nonpermeabilized cells. BOX^+ PI^+ and BOX^- PI^+ cells correspond to depolarized and permeabilized cells with different degrees of damage. BOX^- PI^- cells correspond to unstained populations of intact, polarized, and nonpermeabilized cells (Fig. 2b) [27].
9. SSC vs. FL3 determines the fluorescence properties of the cell populations with a single staining. EB^+ and CTC^+ correspond to cells with altered efflux pump activity, and non-impaired respiratory function, respectively (Fig. 3a, b) [14, 28].
10. Fluorochromes typically emit light over a broad range of wavelengths, resulting in the fluorescence signal appearing not only in the expected primary detector for that fluorochrome but in other detectors as well. This phenomenon is often called fluorescence spillover and can be a source of confusion when interpreting multiparametric data [29].
11. Most of the TO or BOX signal fluorescence is detected in FL1 (533 ± 15 BP), but there is also TO or BOX signal detected in FL2 (585 ± 20 BP) and FL3 (670 LP), so plots of data for those detectors appear to have positively fluorescent cells. No signal from TO or BOX is detected in FL4 (675 ± 12.5 BP).
12. Most of the PI signal fluorescence is detected in FL3 (670 LP), but there is also PI signal detected in FL1 (533 ± 15 BP) and FL2 (585 ± 20 BP), so plots of data for those detectors appear to have positively fluorescent cells. No signal from PI is detected in FL4 (675 ± 12.5 BP).

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Chapter 4

Detection of Sublethally Injured Cells by the Selective Medium Plating Technique

Elisa Pagán, Daniel Berdejo, Natalia Merino, Diego García-Gonzalo, and Rafael Pagán

Abstract

The cell envelope is the bacterial structure most commonly targeted by food preservation methods (heat, high hydrostatic pressure, pulsed electric fields, antimicrobials, etc.). The damage inflicted on it can be irreversible, leading to bacterial cell death, or reversible, leading to cell repair and survival if environmental conditions are suitable. The latter is known as sublethal damage. Due to its simplicity, one of the most common methods used to determine whether cells are sublethally injured at their cell envelopes is the selective medium plating technique. The method consists of plating survivors after an inimical treatment into two culture media: a nonselective one, which allows treated cells to repair sublethal damages and recover, and a selective one, in which injured cells are not capable of repairing their damages and ultimately die. Sublethally injured cells are estimated by counting the difference in the number of survivors obtained after plating treated cells in both nonselective and selective media. The most common selective recovery media used to detect damage in the cytoplasmic membrane and in the outer membrane are nutrient-rich agar with sodium chloride or bile salts added, respectively. This technique highlights the loss of both membrane integrity and functionality.

The observation of the occurrence of sublethally injured cells after inimical treatments has been proven to be key in the development of successful combined processes. Valuable synergistic effects result from combining physical technologies with antimicrobials, thereby offering great potential to improve both traditional and novel food preservation treatments.

Key words Sublethal damage, Injured cells, Cytoplasmic membrane, Outer membrane, Recovery medium, Nonselective medium, Selective medium, Sodium chloride, Bile salts

1 Introduction

Bacteria are one of the main agents responsible for food spoilage and food safety; they are therefore the main target of food preservation methods. The food industry currently applies preservation methods that rely either on the inhibition of bacterial growth or on bacterial inactivation. Methods capable of destroying bacteria have clear advantages, since they ensure food safety while extending shelf

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life. In this regard, thermal treatment is still the principal food preservation method; however, at the intensity required to assure food safety and increase shelf life, undesired changes in nutritional, functional, and sensory properties of food can occur. This limitation, together with increasing consumer demand for fresh-like food products, has encouraged the development of alternative methods for microbial inactivation, such as ionizing irradiation (IR), ultrasound under pressure (US-P), high hydrostatic pressure (HHP), and pulsed electric fields (PEF) [1]. On the other hand, chemical preservatives and natural antimicrobials (bacteriocins, essential oils, propolis, etc.) are capable of causing bacterial death, but the doses required might exceed the legal limit or might affect the quality of the foods.

One of the aspects shared by the inactivation mechanism of all these methods based on both traditional and novel technologies is the fact that cell envelopes are usually affected to a certain extent [2]. The cell envelope is made up of the cytoplasmic membrane and the cell wall, which has a completely different structure according to whether the bacterium is Gram-negative or Gram-positive (Fig. 1). In Gram-positive bacteria, the cell wall is very thick and is composed of various peptidoglycan layers and teichoic acids, which confer rigidity and physical resistance to the cell. In Gram-negative bacteria, the cell wall is thinner, but is surrounded by the outer membrane. The outer membrane differs from the common biomembrane in that its external leaflet is made of lipopolysaccharides instead of phospholipids, and it confers special resistance against the entry of certain antimicrobial compounds such as antibiotics, bile salts, and some bacteriocins. In both cell types, but especially in Gram-negative bacteria, these particular structures form a region with a distinct chemical composition [3].

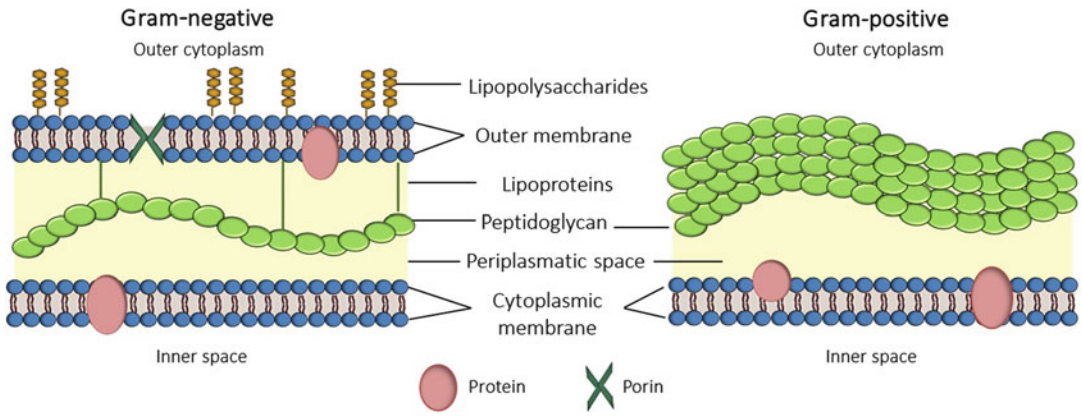

Fig. 1 Diagram of cell envelopes: Gram-negative (left) and Gram-positive (right) bacteria

The damage inflicted by inactivating agents on the cytoplasmic membrane of both Gram-positive and -negative bacteria or on the outer membrane of Gram-negative bacteria can be irreversible, leading to bacterial cell death, or reversible, leading to cell repair and survival if the environmental conditions are suitable. The latter is known as sublethal damage. For instance, PEF can cause electroporation, which involves the formation of pores on the membranes of cells and organelles. Depending on treatment intensity, PEF can induce the formation of transient or permanent pores that cause reversible or irreversible electroporation, respectively [4]. Similarly, heat [5] and HHP [6] cause reversible and irreversible permeabilization as a function of treatment conditions. In contrast, shear forces caused by US completely disrupt microbial cell envelopes, and no sublethally injured cells are detected after US treatments, which means that the mechanism of inactivation by US is an "all or nothing"-type phenomenon [7].

Several techniques have been assayed in order to study bacterial membrane damages following food preservation treatments. The most common methods are the use of fluorescent probes, electron microscopy examination, measurement of leakage of intracellular material, measurement of osmotic response, and selective medium plating technique [8, 9]. The selective medium plating technique offers several advantages: it is simple, cheap, and reproducible and does not require sophisticated or expensive equipment. Moreover, this technique allows for the differentiation of a minuscule proportion of sublethally injured cells within a large population of live and dead cells.

The selective medium plating technique consists of plating treated cells after an inimical treatment into two culture media: a nonselective one, which allows cells to repair sublethal damages and recover; and a selective one, in which injured cells are not capable of repairing their damages and ultimately die [9]. Sublethally injured cells are estimated by counting the difference in the number of survivors obtained after plating treated cells in both nonselective and selective media. The selective media most commonly used to detect damage to the cytoplasmic membrane and in the outer membrane are rich agar with sodium chloride (SC) or bile salts (BS) added, respectively. This technique highlights the loss of both membrane integrity and functionality.

The occurrence of sublethal injury after the application of a food preservation method has two consequences. First, injured cells might not be detected when specific recovery media, containing selective compounds, are used for the enumeration of survivors. Therefore, an overestimation of the treatment's lethality could occur, which might compromise food safety. Second, if repair is adequately prevented by combining the treatment with additional preservation agents (hurdles) that interfere with cellular homeostasis maintenance, the cell might not be able to survive, and the inactivation level attained might be higher [9].

In this regard, the observation of the occurrence of sublethally injured cells in their cell envelopes after lethal treatments has been proven to be key in the development of successful combined processes. The occurrence of sublethal injury not only indicates when a combination of hurdles might be successful, but it also might help us to estimate the final degree of inactivation to be reached. For instance, Somolinos et al. [10] demonstrated the occurrence of a strongly synergistic lethal effect on *Escherichia coli* by mild heat treatment combined with citral, a hydrophobic compound naturally present in certain essential oils. The synergistic effect was associated with the damages inflicted to *E. coli* cell envelopes, both to the cytoplasmic and to the outer membrane, by heat. On the other hand, Arroyo et al. [11] confirmed that the occurrence of sublethal injury in the outer membrane of *Chronobacter sakazakii* was the key for PEF to act synergistically with citral and reach more than 5 $\log_{10}$ cycles of inactivation under specific treatment conditions. Many other studies have shown similar results when combining heat, HHP, or PEF with natural antimicrobials such as essential oils [12–14] or propolis [15]. The inactivation of 5 $\log_{10}$ cycles of pathogenic microorganisms was achieved in fruit juices, thus meeting the recommendations for controlling the transmission of pathogenic microorganisms in juices given by the FDA [16]. Valuable synergistic effects have thus been observed between physical technologies and natural antimicrobials, which offer great potential to improve not only traditional heat treatments by reducing treatment intensity (and, thus, undesirable effects on food quality) but also novel treatments (PEF, HHP, natural antimicrobials, etc.) by achieving a higher degree of microbial inactivation.

In this chapter, we describe a methodology for the evaluation of the occurrence of sublethal damages on the cytoplasmic membrane of Gram-positive and -negative bacteria, as well as on the outer membrane of Gram-negative bacteria, due to its interest as a tool studying the mechanism of microbial inactivation of a specific hurdle, or for the development of successful combined processes for food preservation. In addition, we offer tips to show you how to prepare the material, follow the methods, and interpret the results.

2 Materials

2.1 Culturing Method

1. Culturing tools: micropipettes, plastic 1.5-mL tubes, petri dishes (90 mm).
2. Vortex.
3. 0.1% (w/v) peptone water (PW) solution or phosphate-buffered saline (PBS) as diluent. To prepare the solutions, the required quantities were diluted in sterile distilled water according to the manufacturer's instructions.

4. Reagents:
   (a) Bile salts (BS) (*see* **Note 1**).
   (b) Sodium chloride (SC).
5. Growth media prepared according to manufacturer instructions:
   (a) Tryptic soya broth (TSB) (or any other nutritive broth).
   (b) Nonselective medium: tryptic soya agar (TSA) (or any other nutritive agar) + 0.6% yeast extract (TSAYE).
   (c) Selective medium: TSAYE (or any other nutritive agar) + BS (TSAYE-BS) and TSAYE + SC (TSAYE-SC).

3 Methods

The assessment of the occurrence of sublethally injured cells by selective medium plating technique requires, as a previous step, the determination of the maximum noninhibitory concentration (MNIC) of the selective agents (SC or BS) to be added to the nonselective medium in order to obtain a selective one. The MNIC depends on the specific microorganism under investigation, on growth medium composition, and on environmental conditions (temperature, time, oxygen, etc.).

3.1 Determination of the Maximum Noninhibitory Concentration (MNIC) of the Selective Agent

First, selective media were prepared with TSAYE and with increasing concentrations of each solute (SC or BS) (*see* **Note 2**). In the case of SC, it is advisable to test increments of the order of 0.5%, whereas smaller increases (0.05%) are recommended for BS. In *E. coli*, for instance, the recommended ranges would be from 2% to 6% at intervals of 0.5% for SC and from 0.10% to 0.30% at intervals of 0.05% for BS. Then, aliquots of native cells from a stationary growth phase culture were plated onto the nonselective medium, as a control, and on the selective media at different SC or BS concentrations, and subsequently incubated under appropriate recovery conditions (*see* **Note 3**). For *E. coli* O157:H7 Sakai, the plates poured with the nonselective medium were incubated at 37 °C for 24 h, and those poured with the selective media for 48 h (*see* **Notes 4** and **5**). After incubation, colony-forming units (CFU) were counted. The MNIC is defined as the highest concentration that inhibits the growth of less than 20% of the cells initially inoculated in the selective medium in comparison to those grown in the nonselective medium used as a control. Figure 2 shows the percentage of cells that grew and formed a colony in the selective media, with SC (A) or BS (B), in relation to those grown in the nonselective medium. Thus, the MNIC of SC and BS were 4% and 0.25%, respectively, for *E. coli* O157:H7 Sakai.

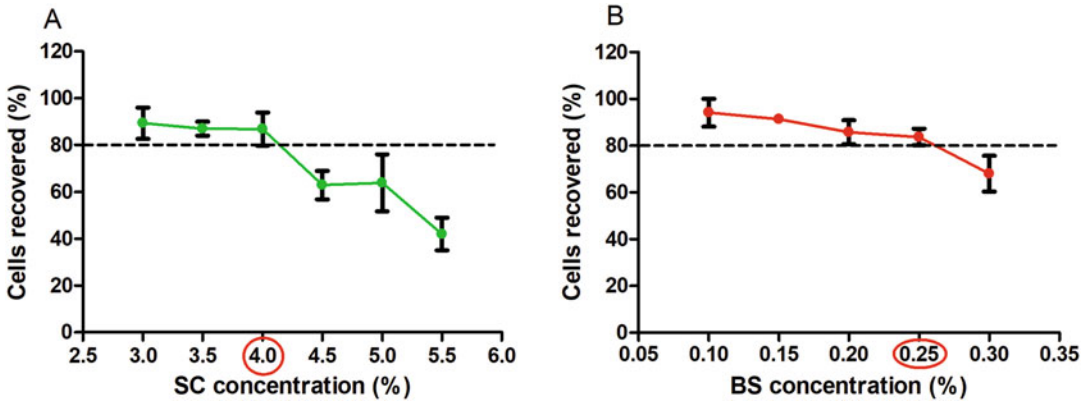

Fig. 2 Maximum noninhibitory concentration (MNIC) of SC (**a**) and BS (**b**) for *E. coli* O157:H7 Sakai

Other examples of MNIC were 5% of SC and 0.3% of BS for *Enterobacter sakazaki* [11], 3–4% of SC and 0.2–0.35% of BS for *E. coli* BJ4 or BW25113 [5, 10, 17]. For Gram-positive bacteria, only sublethal damage at the cytoplasmic membrane can be determined: for instance, by adding 6% of SC for *Listeria monocytogenes* EGD-e [17], and 7% and 15% for *Staphylococcus aureus* ATCC 13565 and ATCC 25923, respectively [18].

3.2 Selective Medium Plating Technique

Once the MNIC was known for each microorganism under specific growth media and environmental conditions, the nonselective and the selective recovery media were prepared. Then, after an inimical treatment, treated cultures were serially diluted. Subsequently, 100 μL of the stock dilution and, when necessary, of the corresponding decimal dilutions in PBS were pour-plated onto the non-selective and the selective recovery media and incubated at optimum growth temperature (Fig. 3). For *E. coli* O157:H7 Sakai, the recovery conditions were 37 °C for 24 h when recovered in the nonselective medium and 48 h when recovered in the selective one. Previous experiments showed that longer incubation times did not influence the number of surviving cells, regardless of the selective agent added.

Inactivation can be expressed as the extent of reduction in $\log_{10}$ counts (CFU) after an inimical treatment. Survival curves were obtained by plotting the decimal $\log_{10}$ fraction of survivors versus treatment time (Fig. 4). Figure 4 shows three survival curves, one for each recovery medium (nonselective and selective with SC and with BS). The results can also be represented as inactivation cycles, where the logarithmic cycles of inactivated cells at a given time are shown in different bars corresponding to the nonselective and the two selective media (Fig. 5).

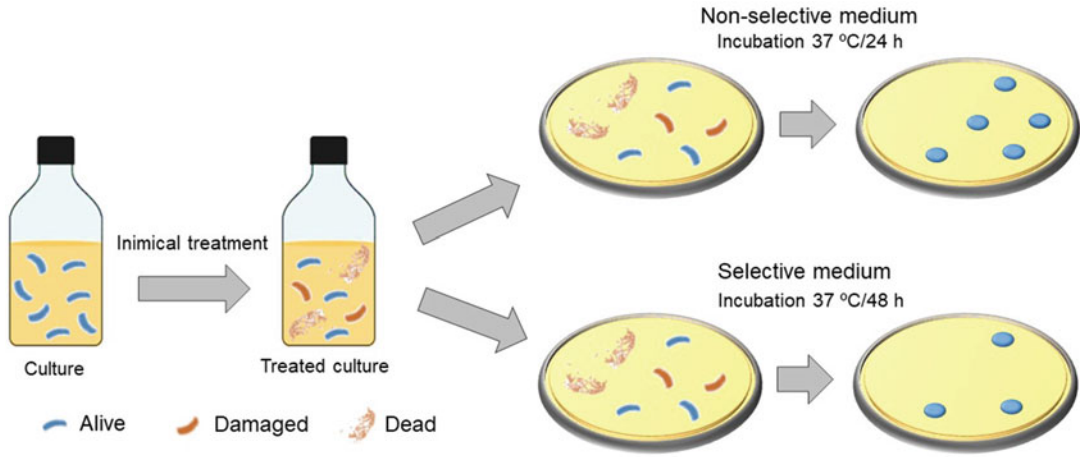

Fig. 3 Diagram of the selective medium plating technique

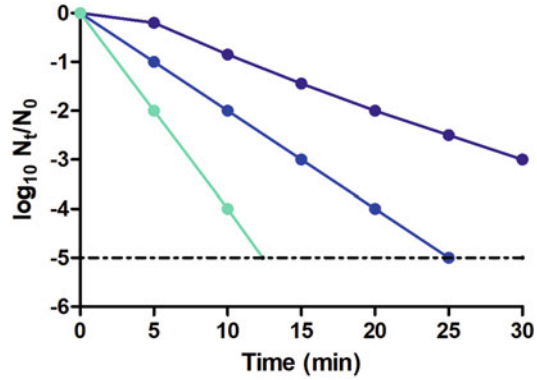

Fig. 4 Survival curves after an inimical treatment and recovery in TSAYE (●), TSAYE-SC (●), and TSAYE-BS (●). The dashed line represents the detection limit (-5 $\log_{10}$ N_t/N_0)

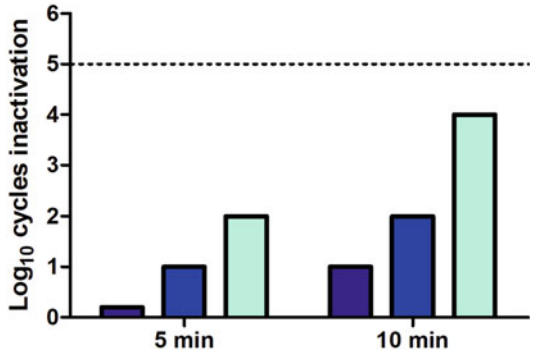

Fig. 5 Log_{10} cycles of inactivation after recovery in TSAYE (●), TSAYE-SC (●), and TSAYE-BS (●). The dashed line represents the detection limit (5 $\log_{10}$ cycles)

3.3 Interpretation of Results: Quantification of Sublethally Injured Cells

The extent of sublethal injury can be expressed as the difference between the $\log_{10}$ count (CFU) on nonselective medium and the $\log_{10}$ count on selective media. Likewise, the percentage of injured cells at each treatment interval corresponded to the following equation:

$$\%\text{Injured cells} = \left(1 - \frac{\text{CFU}/\text{mL}_{\text{selective}}}{\text{CFU}/\text{mL}_{\text{non-selective}}}\right) \times 100$$

Figure 4 shows theoretical survival curves as a result of a lethal treatment and a subsequent recovery in nonselective medium (TSAYE) and in a selective medium with sodium chloride (TSAYE-SC) and bile salts (TSAYE-BS). After a 5-min treatment, there was not a significant number of dead cells, since most cells of the initial population formed a colony in the nonselective medium, 90% of survivors were sublethally injured at their cytoplasmic membrane, and 99% of the survivors were sublethally injured on their outer membrane. Similarly, after a 10-min treatment, 90% of the initial cell population were dead, and from the 10% surviving population, 90% were sublethally injured at their cytoplasmic membrane, and 99.9% of the survivors were sublethally injured on their outer membrane. Figure 5 allows us to record the number of $\log_{10}$ cycles of inactivation, which is another interesting way of representing the results when a determined number of $\log_{10}$ cycles of inactivation is required, for instance a 5 log cycle reduction for pathogenic bacteria when pasteurizing fruit juices [16]. In our theoretical example, the 10-min treatment achieved 2 and 4 log cycles of dead cells when recovered in the selective media with SC and BS, respectively. More than 5 log cycles of inactivation were only achieved when the treatment lasted 13 min and survivors were recovered in the selective medium with BS (Fig. 4). As has been previously mentioned, the consequences of this 20-min treatment would offer a great potential for the development of combined processes: for instance, with natural antimicrobials such as certain essential oils that require the occurrence of sublethal damages that compromise the integrity and functionality of the outer membrane of Gram-negative bacteria, thus permitting the treatment to easily penetrate the cell envelopes, affect internal structure and components, and cause the death of the sublethally injured cells [5, 11].

4 Notes

1. The composition of bile salts (BS) differs among commercial suppliers. As a consequence, the MNIC should be checked whenever the batch and the supplier are changed. The BS employed in this study was Oxoid Bile Salts No. 3 (**Code:** LP0056). It consists of a specially modified fraction of bile

acid salts which is effective at less than one-third of the concentration of bile salts normally quoted. The MNIC was therefore lower than that obtained with a standardized bile extract, consisting mainly of sodium glycocholate and sodium taurocholate.

2. It is recommended to prepare each concentration in an individual bottle. For this purpose, the required amount of solute is added, then the hot agar, and, subsequently, the concentration is vigorously homogenized and sterilized. Once sterilized and prior to use, the flasks have to be shaken to avoid precipitation of the salts and to obtain a homogeneous solution.
3. After recovery in selective medium, the appearance of the colonies slightly differs from that of the colonies recovered in nonselective medium: the border of the colonies is better defined, their color is usually lighter, and their size tends to be smaller in selective than in nonselective medium.
4. The plates poured with the nonselective medium should be incubated at the optimal temperature and time for each microorganism; however, for the selective medium, the incubation time should be increased by at least 24 h. Nevertheless, it is always recommended to previously study the evolution of the number of colonies along incubation time in order to maximize the number of colonies recovered in each medium.
5. This technique can be performed using both seeding techniques: mass homogenization, with warm liquid agar, and surface spread, in which nonselective and selective agar must be prepared and solidified beforehand.

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Chapter 5

Combining Culturing Technique and Metabarcoding to Study Microbiota in the Meat Industry

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Abstract

The detection of meat microbiota along the food chain, as well as of microbiota associated with slaughterhouses and meat processing facilities, would be very useful to help researchers pinpoint sources of contamination and, thus, propose measures to prevent the presence and growth of spoilage and pathogenic microorganisms. Culture-dependent techniques have traditionally been used to determine the composition of microbiota, but in recent decades, they are being displaced by culture-independent techniques such as metabarcoding. In this study, we propose the combination of both techniques in order to obtain complete and valuable information, as well as to overcome the limitations of individual approaches.

Key words Meat industry, Spoilage bacteria, Pathogenic bacteria, Microbiota assessment, Culture-dependent techniques, Culture-independent techniques, Metabarcoding

1 Introduction

Meat is considered an important source of nutrients: particularly of protein, as well as of certain vitamins and minerals. In contrast to other foods, meat not only provides a large amount of protein (15–25%); it also supplies all essential amino acids and has no limiting amino acids. High biological value is thus ascribed to meat protein. In addition, meat is rich in several vitamins, such as riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), and pyridoxine (B_6); furthermore, it is an excellent source of zinc, selenium, phosphorus, and iron [1]. For all of these reasons, meat constitutes a vital component of human daily diet, and demand for it has increased over the last 50 years. According to the Food and Agricultural Organization [2], 335 million tons of meat was produced worldwide in 2019, with an annual per capita consumption of 43.3 kg.

Marciane Magnani (ed.), *Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-1932-2_5,

Unfortunately, its high nutritive content, high water activity (≥0.85), and moderate pH (≥4.6) make meat a highly perishable food commodity susceptible to growth of spoilage and pathogenic microorganisms [3]. Although lipid oxidation and enzymatic autolysis are involved in meat spoilage, microbial growth is the most common cause of meat quality deterioration (off-odors, off-flavors, and slime formation), leading to food waste [4]. Moreover, the presence of pathogens in meat can result in food-borne outbreaks. In 2017, 18.9% of reported strong-evidence food-borne outbreaks in the European Union were associated with meat and meat products: they are the foodstuffs most involved in outbreaks [5]. Factors influencing the microbiological quality of meat are the physiological status of the animal at slaughter, the spread of contamination during slaughtering and processing, and the conditions of storage and distribution [4]. Therefore, the determination of meat microbiota along the food chain, as well as of the microbiota associated with slaughterhouses and meat processing facilities, would be very useful to help researchers pinpoint the sources of contamination and propose necessary measures to prevent the presence and growth of microorganisms.

Culture-dependent techniques are the procedure traditionally applied to determine the composition of microbiota along the food chain. These methods rely on the isolation of microorganisms in several culture media for subsequent identification and quantification [6]. In most cases, it is necessary to use selective or differential growth media. Selective media contain inhibitory substances that limit the growth of some microorganisms, while permitting the isolation of those that can grow in their presence. Differential media, on the other hand, contain indicators that allow us to visualize the biochemical characteristics of microorganisms and, therefore, to distinguish among different types of microorganisms growing in the same media [7]. *Brilliance*™ Listeria Agar contains antimicrobial compounds, which make it a selective medium, and several indicators, which make it a differential medium. *Listeria* spp. has the enzyme β-galactosidase, which acts on the chromogenic component X-glucoside, giving rise to blue-green colonies. Moreover, *Listeria monocytogenes* has an enzyme that acts on lipase C substrate, giving rise to an opaque halo around the colony. In addition to using selective and differential growth media, it is necessary to carry out confirmatory tests: either biochemical ones, such as β-hemolysis, D-xylose, or L-rhamnose metabolism, or genomic ones, such as PCR and Sanger sequencing. Although these techniques are highly effective, they are laborious, expensive, and time-consuming, taking up to several days until results are ready. Moreover, culturing methods underestimate microbial diversity, since they are unable to detect fastidious microorganisms, microbes present in low numbers, and those microorganisms that are in a physiological viable but not culturable state [6, 8].

Considering all these limitations, culture-independent methods, such as amplicon sequencing or metabarcoding, can serve as a complementary approach. The metabarcoding technique consists of the extraction of DNA from the collected samples, and the subsequent amplification and sequencing of specific marker gene families. The 16S rRNA gene is routinely used for Archaea and Bacteria, the 18S rRNA gene for Eukaryotes, and the internal transcribed spacer (ITS) of the ribosomal gene cluster sequences for Fungi [9]. Based on nucleotide similarity (generally ≥97%), all sequences obtained are clustered into operational taxonomic units (OTUs) and then compared against databases in order to identify the microorganisms present in the microbiota [10]. Despite the fact that this culture-independent technique is often unable to distinguish beyond the genus level [11], it is one of the most popular high-throughput sequencing methods [10]. Between 2011 and 2017, metabarcoding was used in 63% of the publications that applied next-generation sequencing techniques to assess food microbiota [9].

In culture-dependent as well as in culture-independent methods, sampling is an extremely important first step in the collection of relevant microbiological information from meat products and processing facilities [12]. The diversity in types of samples might be due to variations in cell densities, cell viability, and the presence of biofilms on equipment surfaces. Hence, it is highly important to design an appropriate sampling method based on the kind of meat products and processes one is dealing with [9]. Moreover, the isolation of DNA is a critical step that can lead to misinterpretation of results. Problematic figures can either be due to sample contamination with reagents used for extraction [13], to interference with DNA from dead microbial cells or nonmicrobial DNA, or to the inhibition of biochemical reactions by different components of the matrix [14]. It is therefore of great importance to choose an appropriate extraction method according to matrix characteristics (e.g., meat species, chemical nature of surfaces), while remaining aware of methodological limitations that could have an impact on data interpretation [15, 16].

2 Materials

2.1 Sample Collection

1. Surface sample collection tool: sterile pre-moistened sponges.
2. Sampled area delimiting tool: 100 cm^2 sterile metal molds.
3. Sterile plastic bags.
4. Buffered peptone water (BPW).
5. Container with ice blocks for transporting the samples from the food industry to the laboratory (Fig. 1).

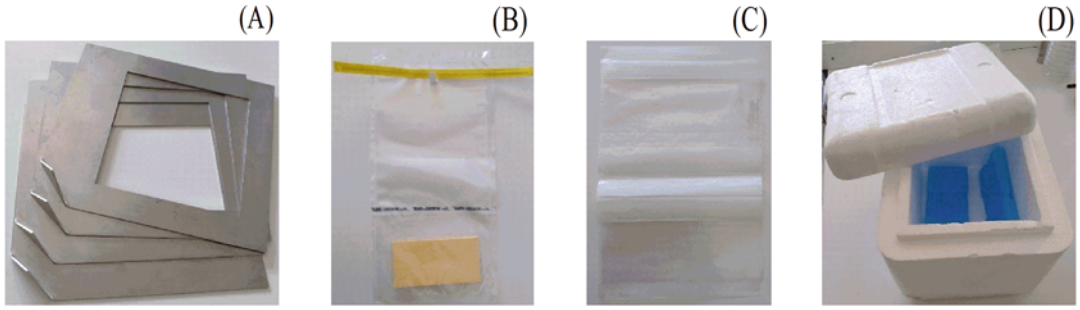

Fig. 1 Materials required for sample collection: 100 cm^2 sterile metal mold (**a**), sterile sponge (**b**), sterile plastic bag (**c**), and container with ice blocks (**d**)

2.2 Culturing Method

1. Filter bags to retain particles during sample homogenization.
2. Peristaltic homogenizer (stomacher).
3. Vortex.
4. Anaerobic jar and envelopes for those microorganisms that need a modified atmosphere to grow.
5. Culturing tools: micropipettes, plastic 1.5-mL tubes, petri dishes (90 mm), and L-shaped spreaders.
6. BPW.
7. Growth media prepared according to manufacturer instructions:
   (a) Plate Count Agar (PCA) for counting mesophilic and psychrotrophic microorganisms.
   (b) De Man Rogosa Sharpe Agar (MRS) for counting rod-shaped lactic acid bacteria, and M-17 Agar for counting spherical-shaped lactic acid bacteria.
   (c) Violet Red Bile Glucose Agar (VRBG) for counting Enterobacteriaceae.
   (d) Pseudomonas CFC/CN agar (base agar) enriched with Cephalothin, Fucidin, Cetrimide (CFC) selective supplement for counting *Pseudomonas* spp.
   (e) *Brilliance*™ Listeria Agar (formerly Oxoid Chromogenic Listeria Agar [OCLA]) enriched with OCLA selective supplement and *Brilliance*™ Listeria differential supplement for counting *Listeria* spp. and *L. monocytogenes.*
   (f) Modified Charcoal Cefoperazone Deoxycholate Agar (m-CCDA) enriched with CCDA selective supplement for counting *Campylobacter* spp.

2.3 Metabarcoding

1. Filter bags to retain particles during sample homogenization.
2. Peristaltic homogenizer (stomacher).
3. Vortex.

4. Bead beater.
5. Centrifuge and mini centrifuge.
6. Qubit™ 4 fluorometer.
7. Laboratory tools: micropipettes, plastic 1.5-mL tubes, and Qubit™ assay tubes.
8. DNA extraction kit: DNeasy™ PowerSoil™ Pro Kit.
9. DNA quantification kit: Qubit 1× dsDNA HS Assay™.
10. BPW.

3 Methods

3.1 Experimental Design

This study assessed the microbiota of a chicken hamburger meat production line in a meat-producing firm. Sampling of food and surfaces was carried out during processing. Figure 2 shows the production line design, and Table 1 shows the different sampling points as well as the food products that were being processed during sampling (*see* **Note 1**).

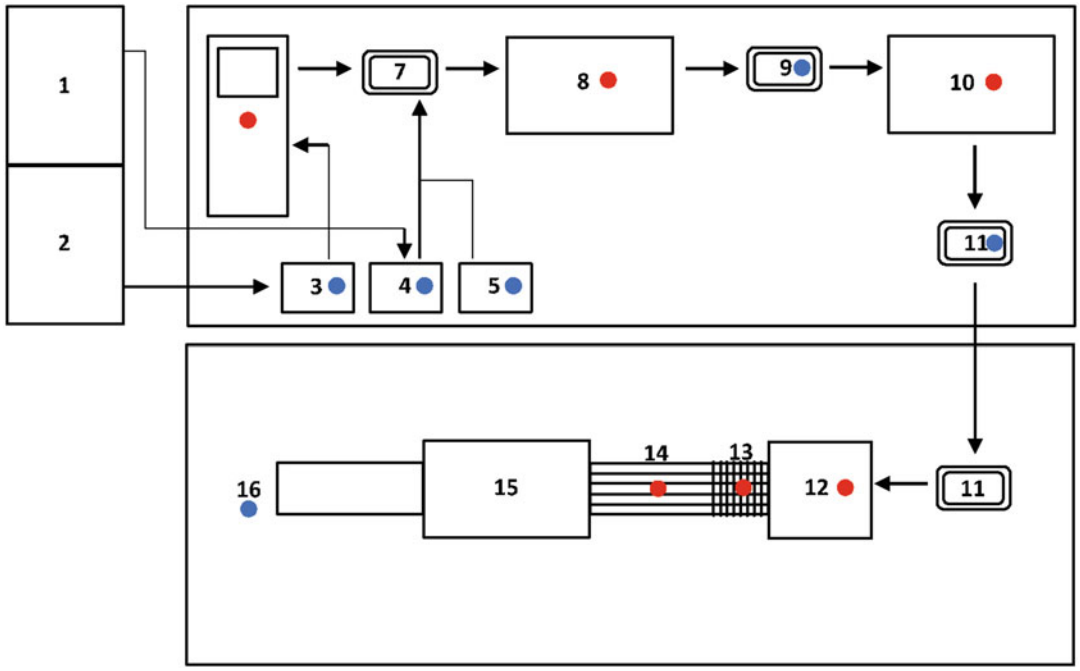

Fig. 2 Chicken hamburger production line design (1). Refrigeration chamber for vegetable raw material; (2) refrigeration chamber for meat raw material; carts containing (3) meat raw material, (4) vegetable raw material, and (5) spices; (6) formula table; (7) carts containing meat and vegetable raw material and weighed spices; (8) grinder; (9) carts containing intermediary food product after mincing; (10) kneader; (11) carts containing intermediary food product after kneading; (12) forming hopper; (13) metallic conveyor belt; (14) plastic conveyor belt; (15) packaging machine; (16) final food product. Surface sample points (●) and food sample points (●)

Table 1
Samples taken from food products and surfaces, the sampling point, and the food product being processed during sampling

Sample	Sampling point and the food product being processed
Formula table	During weighing of skin-on and skinless chickens
Grinder	Discs During the mincing of the food product chicken meatball
Kneader	Blades During the kneading of the food product chicken carrot burger meat
Forming hopper	Inner wall During the formation of the food product chicken spinach burger meat
Conveyor belt	Metallic belt at the exit of the forming hopper During the packaging of the food product chicken spinach burger meat
Meat raw material	Skin-on and skinless chickens
Vegetable raw material	Spices
Intermediary food product	Chicken with carrot after mincing and kneading
Final food product	Chicken carrot burger meat

3.2 Sample Collection

In order to follow the UNE-EN ISO 18593:2018 standard concerning horizontal methods for the sampling of surfaces in the food chain, and in view of the variety of sampling points, we used 100 cm^2 sterile metal molds and pre-moistened sponges with 20 mL of sterile BPW to collect surface samples. The sampled area depended on the subsequent study: 100 cm^2 was sampled for culturing method and 300 cm^2 for metabarcoding (*see* **Note 2**). To collect food samples, sterile plastic bags were used. Samples were transported under refrigeration conditions from the processing plant to the laboratory (<1 h), where they were immediately processed.

3.3 Culturing Method

Once in the laboratory, for surface analysis, the sponges were placed in filter bags with 100 mL of BPW and homogenized at 230 revolutions per minute (rpm) during 30 s in a peristaltic homogenizer. For food analysis, 25 g of the different food products were mixed in filter bags with 225 mL of BPW, and then homogenized at 230 rpm during 1 min. In this way, a stock dilution of each sample was obtained.

In order to assess the microbiota present in the different samples, the following microbial groups were cultured: mesophiles, psychographs, lactic acid bacteria, *Enterobacteriaceae*, *Pseudomonas* spp., *Campylobacter* spp., *Listeria* spp., and *L. monocytogenes* (*see* **Note 3**).

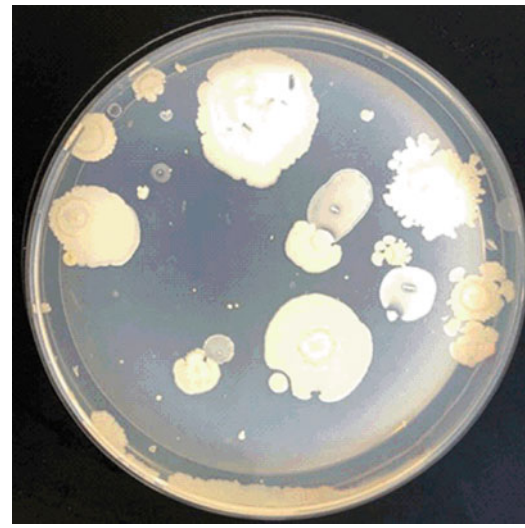

Fig. 3 Different morphologies of mesophiles on Plate Count Agar (PCA)

Mesophilic microorganisms were cultured according to the standard UNE-EN ISO 4833-1:2014. For this purpose, 1 mL or 100 μL of the stock dilution and, if necessary, of the corresponding decimal dilutions was pour-plated on PCA. After solidification of the agar, the plates were incubated in inverted position (30 °C/72 h). After the incubation period, the colonies on each plate were counted. Figure 3 shows the morphology of mesophiles in PCA.

For the culturing of **psychrotrophic microorganisms**, 100 μL of the stock dilution and, if necessary, of the corresponding decimal dilutions was spread-plated on PCA. Once the inoculum had been absorbed (10–15 min), the second layer of PCA was added to prevent the growth of *Pseudomonas* spp. (*see* **Note 4**). Once solidified, they were incubated in inverted position (7 °C/10 days). After the incubation period, the colonies on each plate were counted.

Two different growth media were used to culture **lactic acid bacteria**: one for rod-shaped bacteria (MRS), and another for spherical-shaped bacteria (M-17). Similarly, 1 mL or 100 μL of the stock dilution and, if necessary, of the corresponding decimal dilutions was pour-plated on both media. Once solidified, the plates were incubated in inverted position (30 °C/3–5 days) and under anaerobic conditions. After the incubation period, the colonies of each plate (Fig. 4) were counted.

Enterobacteriaceae culturing was carried out following the standard UNE-EN ISO 21528-2:2018. Likewise, 1 mL or 100 μL of the stock dilution and, if necessary, of the corresponding decimal dilutions was pour-plated on VRBG. Once solidified, another layer of VRBG (5–10 mL) was added to prevent widespread growth and to achieve semi-anaerobic conditions. The plates were then incubated in inverted position (37 °C/24 h), after which the colonies of each plate (Fig. 5) were counted.

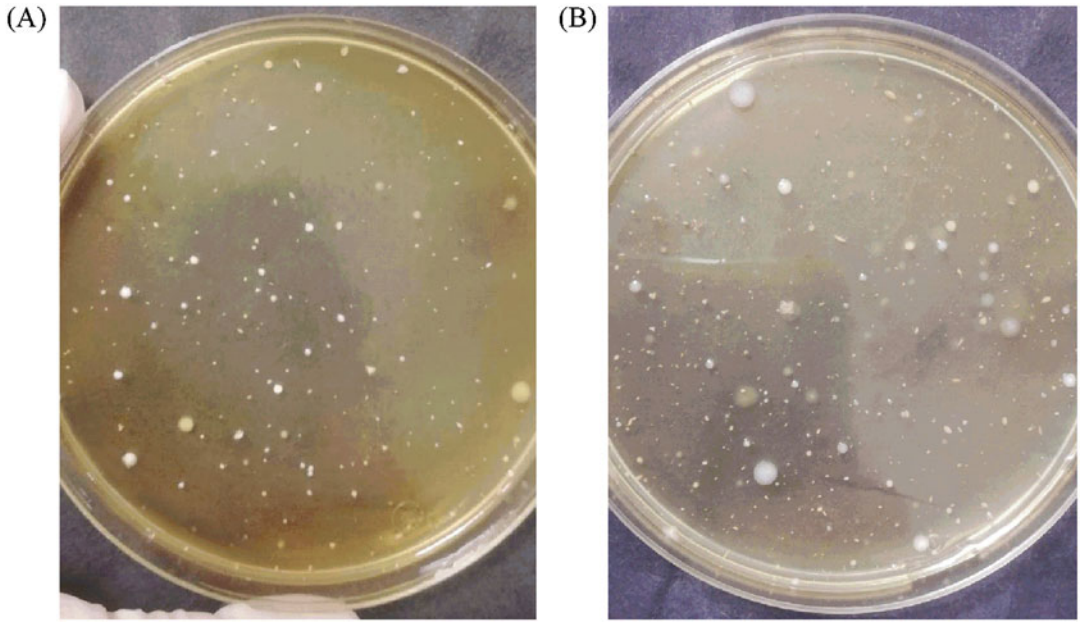

Fig. 4 Different morphologies of rod-shaped lactic acid bacteria on de Man, Rogose, Sharpe agar (MRS) (**a**) and spherical-shaped lactic acid bacteria on M-17 agar (**b**)

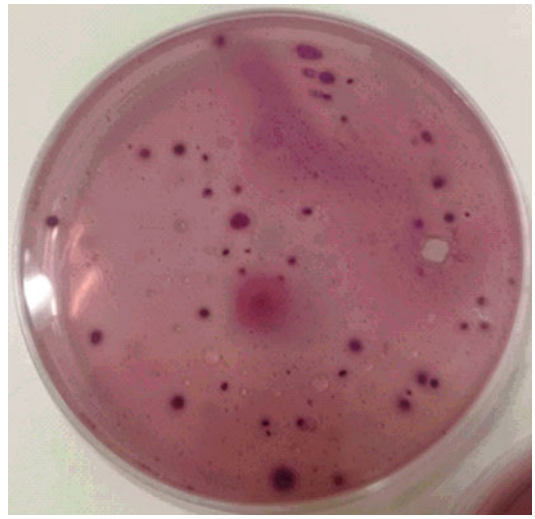

Fig. 5 Different morphologies of *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBG)

***Pseudomonas* spp**. culturing was carried out according to the standard UNE-EN ISO 13720:2011. First, 100 μL of the stock dilution and, if necessary, of the corresponding decimal dilutions was spread plated on CFC. Then they were incubated in inverted position (25 °C/24–48 h). After the incubation period, the colonies of each plate (Fig. 6) were counted.

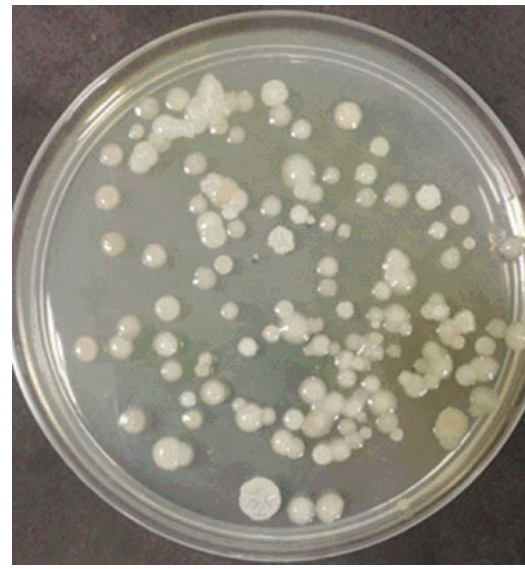

Fig. 6 Different morphologies of *Pseudomonas* spp. on CFC (base agar) enriched with Cephalothin, Fucidin, Cetrimide (CFC) supplement

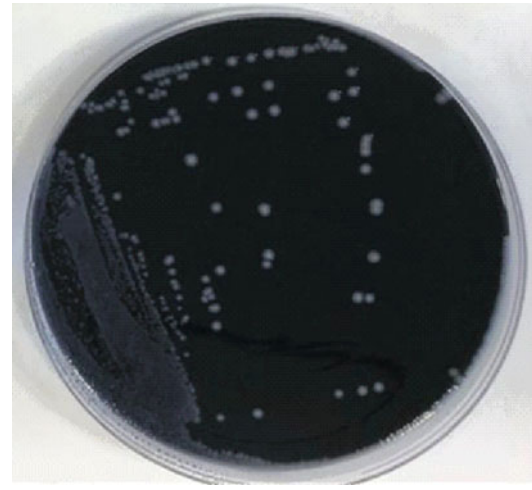

Fig. 7 *Campylobacter* spp. on Modified Charcoal Cefoperazone Deoxycholate Agar (m-CCDA)

Following the standard UNE-EN ISO 10272-2:2018 for the culturing of ***Campylobacter* spp.**, 330 μL of the stock dilution was spread-plated on m-CCDA (*see* **Note 5**). Once solidified, they were incubated in inverted position (41.5 °C/24–48 h) under anaerobic conditions. Figure 7 shows the typical morphology of *Campylobacter* spp. colonies on this culture medium.

Finally, ***Listeria* spp.** and ***L. monocytogenes*** were cultured, based on UNE-EN ISO 11290-2:2018. Likewise, 330 μL of the

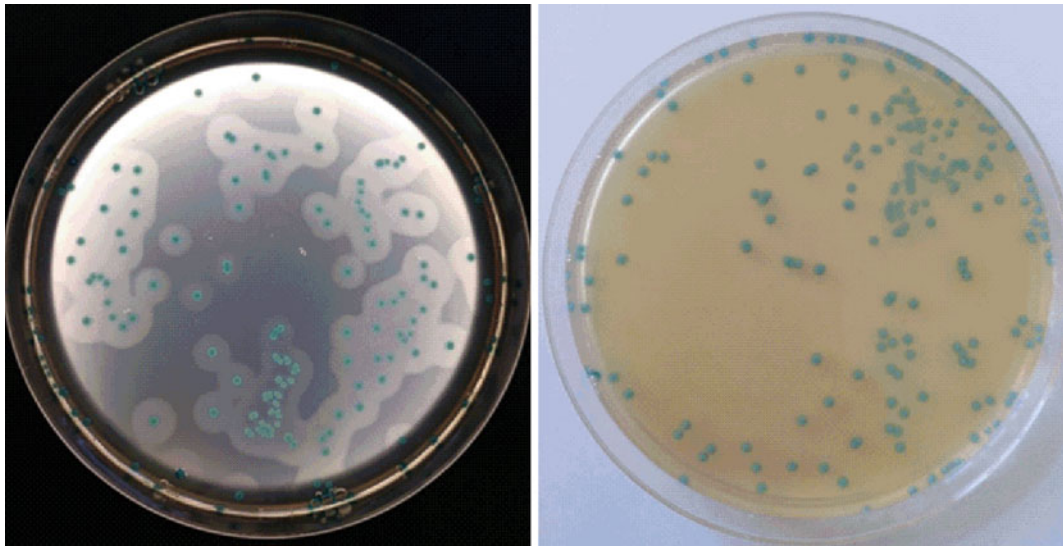

Fig. 8 *Listeria* spp. (**left**) and *Listeria monocytogenes* (**right**) on Brilliance™ Listeria agar (ALOA)

stock dilution was inoculated on *Brilliance*™ Listeria agar through surface spread (*see* **Note 5**). Once solidified, they were incubated in inverted position (37 °C/24–48 h), and after the incubation period, the colonies of each plate were counted. Figure 8 shows the typical morphology of *L. monocytogenes* on this culture medium.

Culture-dependent techniques, apart from providing information about the composition of the microbiota and their evolution along the processing line also allow researchers to isolate microorganisms with which future heat-resistance or cleaning and disinfection studies can be carried out.

3.4 Metabarcoding

Once in the laboratory, in order to recover the cells from the food matrix in BPW, the sponges and food products (25 g) were placed in filter bags with 100 mL of BPW and homogenized at 230 rpm during 15 min in a peristaltic homogenizer (*see* **Note 6**). Then, in order to recover the cells and discard suspended particles, homogenized samples were centrifuged at 3,500 RCF during 10 min at 4 °C (*see* **Note 7**). Once centrifuged, the supernatant was removed, and the pellet recovered. The DNeasy PowerSoil Pro Kit was used for DNA extraction and purification according to manufacturer protocol, the main steps of which are as follows:

1. Homogenization and cell lysis by a combination of chemical agents, which disperse the particles, and mechanical shaking, which causes the beads to collide with microbial cells and leads the cells to break open.
2. Removal of contaminating organic and inorganic matter with Inhibitor Removal Technology (IRT) reagent. This step is

Table 2
DNA concentration values and mesophile counts of certain samples

Sample	DNA concentration (ng/μL) before filtration	DNA concentration (ng/μL) after filtration	Mesophile count (CFU/mL or cm^2)
Formula table	≪	≪	8.00×10^1
Skinless chicken	2.88	440	8.55×10^4
Intermediary food product	1.39	168.8	4.59×10^4

highly important, because the presence of organic and inorganic materials may reduce DNA purity and inhibit downstream DNA applications.

3. DNA binding to the silica membrane in the column by adding a high-concentration salt solution.
4. DNA cleaning with a wash buffer and an ethanol-based wash solution.
5. Removal of residual ethanol to avoid its interference with downstream DNA applications.
6. DNA release from the silica membrane with a sterile, salt-free elution buffer (10 mM Tris) (*see* **Note 8**).

Before sending samples for metabarcoding analysis to an external laboratory, it was necessary to check whether their DNA parameters met the laboratory's requirements for sequencing: DNA amount ≥ 200 ng; DNA concentration ≥ 12 ng/μL; sample volume ≥ 20 μL; high purity (without degradation, impure or RNA contamination, extra fragments, or abnormal color). Hence, the DNA concentration of each sample was quantified according to the Qubit 1× dsDNA HS Assay Kit manufacturer protocol. Table 2 shows the DNA concentration values before and after a previous filtration, in order to assess the influence of this step, as well as mesophilic counts, in order to correlate the DNA concentration with microbial load. It is remarkable the extent to which prior filtration greatly improved DNA extraction of meat samples from "Skinless chicken" and "Intermediary food product" (*see* **Note 7**). However, samples from "Formula table" did not meet the DNA concentration requirements set by the laboratory. This does not mean that the DNA extraction process was not adequate but that the microbial load of these samples was low, and therefore the DNA concentration as well.

Finally, for characterization of the microbiota, DNA samples were sent to the external laboratory for amplification and sequencing of V3-V4 region of the bacterial 16S rRNA genes and fungal ITS2 region in Hi-seq Illumina platform paired-end 250 bp.

Table 3
Primer sequences (5′–3′) for the amplification of V3-V4 region of the bacterial 16S rRNA and fungal ITS2 region, and fragment length

Region	Fragment length	Primer	Primer sequences (5′–3′)
V3-V4	466 bp	341F	CCTAYGGGRBGCASCAG
		806R	GGACTACNNGGGTATCTAAT
ITS2	386 bp	ITS3	GCATCGATGAAGAACGCAGC
		ITS4	TCCTCCGCTTATTGATATGC

Table 3 shows primer sequences used for the amplification of both regions. Once sequenced, paired-end reads were assigned to samples based on their unique barcode, truncated by cutting off the barcode and primer sequence, and merged using the FLASH analysis tool [17]. Quality filtering of the sequences as well as detection and removal of chimera sequences was then performed according to QIIME software [18, 19]. After that, OTU clustering ($\geq$97% similarity) was carried out by UPARSE software [20], and species annotation on every taxonomic rank was performed by Mothur software [21]. Finally, alpha- and beta-diversity analyses were carried out with QIIME and R software.

Metabarcoding analysis provides information about the composition of the microbiota in each sample, the classification of the microbiota into taxonomic ranks, and the relative species abundance (i.e., proportion) within each rank or within each sample. Since all microbial DNA isolated from the sample is PCR-amplified for library preparation prior to sequencing, the initial number of copies in the sample is difficult to estimate. Additionally, alpha-diversity analysis supplies information about the complexity of species diversity within a sample, and beta-diversity analysis provides information about the differences among samples in terms of species complexity.

This technique analyzes all 16S rRNA and ITS2 copies present in the sample without discriminating whether they come from live, dormant, or dead microbial cells [8]. Therefore, complementation of results obtained by culture-independent (all microbial DNA copies present in the sample, i.e., living or dead cells) with those shown by culture-dependent technique (all microorganisms capable of forming colonies, i.e., living cells) can provide valuable information not only about the microbiota present in the samples and their progression along the food chain, but also about the microbiota that have been present at some point on the different surfaces and food products. All this information can allow us to exercise more adequate control over the contamination of meat products as a basis for the production of safe food products with prolonged shelf life.

4 Notes

1. The location of sampling points must be defined based on the study's objectives, the historical data of each industry, and after an assessment of the production line. Furthermore, depending on the aims of the study, sampling can be carried out during processing, or after the cleaning and disinfection process. It is also important to collect samples on different days and times in order to assess the variation of microbiota composition.
2. As already mentioned, the sampled area depends on the foreseen studies; for example, for the detection of microorganisms, it is recommended to sample the largest possible area (between 1000 and 3000 cm^2), while for counting microorganisms, such a large area is not necessary ($\leq$100 cm^2). Moreover, based on sampled area size, accessibility, and type of surface, several types of sampling tools can be chosen (swabs for small, inaccessible areas, and gauzes or sponges for large, accessible areas). Sampling tools can be dry, or premoistened with the diluent to improve the recovery of microorganisms. If traces of disinfectant are present on the sampling surfaces, a neutralizer (such as lecithin, saponin or polysorbate) should be added to the diluent to prevent inhibition of microbial growth. If no traces of disinfectant are expected, no neutralizers should be added, as they may have a harmful effect on bacterial cells.
3. In order to achieve a satisfactory assessment of microbial composition along the food process chain, one should familiarize oneself with the microbial groups usually associated with each food group, and one should carry out a preliminary study of the firm's facilities and handlers.
4. Superficial growth of *Pseudomonas* spp. would lead to an overestimation of the count of psychrotrophic microorganisms: thus, it is important to add a thick layer of PCA in order to limit oxygen availability for *Pseudomonas* spp., which are strict aerobes.
5. In order to increase the detection limit, 1 mL of the stock dilution needed to be inoculated. In this case, as the agar surface was not capable of absorbing much volume, 330 μL was inoculated on three different plates. At the time of the count, the colonies grown on the three plates were added up. Another option would have been to inoculate 1 mL of the stock dilution in a 140-mm diameter plate.
6. After homogenizing the sample, it is important to drain the sponge in order to extract as much sample as possible.
7. Filtration of the samples before centrifugation can prevent the presence of fat in the samples (especially in meat samples) and

can therefore improve the recovery of bacterial cells after centrifugation. Furthermore, in order to optimize the recovery of bacterial cells, the pellet can be resuspended in BPW, and the second centrifugation can be performed [22].

8. It is recommended to store the DNA at −80 °C in order to avoid its degradation.

Acknowledgments

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Chapter 6

Assessment of In Vitro Biofilms by Plate Count and Crystal Violet Staining: Is One Technique Enough?

Elisa Gascón, Natalia Merino, Elisa Pagán, Daniel Berdejo, Rafael Pagán, and Diego García-Gonzalo

Abstract

Biofilms pose a serious problem to the food industry due to their high resistance to stressing conditions, including antimicrobials and disinfectants. Therefore, it is of vital importance to have methods that allow us to determine and quantify the cells of which biofilms are composed in order to determine the effectiveness of cleaning and disinfection treatments. In this chapter, we suggest two techniques, the plate counting technique and the crystal violet staining technique, as two possible indirect methods to determine in vitro biofilm mass. To overcome individual limitations, such as the plate counting technique's disregard of the amount and localization of biomass on surfaces, or the crystal violet staining technique's failure to differentiate between living and dead cells, we propose their combined use in order to obtain complete, valuable information on the behavior of microbial biofilms.

Key words Biofilms, Food industry, Sessile cells, Microbial counts, Biomass, Plate count technique, Crystal violet, Exopolysaccharides, Extracellular matrix

1 Introduction

Most bacteria have found new ways to adapt and survive under stress conditions: one such strategy is the formation of biofilms. A biofilm is a collection of sessile microbial cells that grow in a matrix of extracellular polymeric substances (exopolysaccharides [EPS], proteins, lipids, and nucleic acids) and adhere to a surface. Biofilms can form on a wide range of biotic or abiotic surfaces, such as living tissues, industrial or drinking water pipes, medical devices, and aquatic systems, among others [1, 2]. All microorganisms, under appropriate environmental conditions, are capable of forming biofilms. However, some microorganisms are more susceptible to form biofilms than others: bacteria such as *Pseudomonas*, *Listeria*, *Enterobacter*, *Flavobacterium*, *Alcaligenes*, *Staphylococcus*, and *Bacillus* [3].

Marciane Magnani (ed.), *Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-1932-2_6,

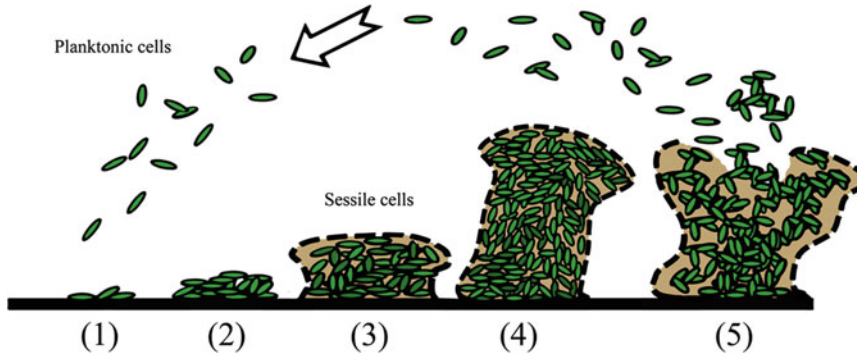

Fig. 1 Biofilm developmental stages: (1) Initial union, (2) Irreversible union, (3) Proliferation, (4) Maturation, and (5) Dispersal

The main function of biofilms is to protect internal bacterial cells from external stressing conditions, such as changes in temperature or pH, UV radiation, nutrient deprivation, or antimicrobial agents [4]. With regard to antimicrobial agents, the matrix acts as a physical barrier that reduces their spread. EPS are one of the main components that make up the extracellular matrix of biofilms. They are large molecules of neutral charge with complex structures and a range of physicochemical properties that cover a wide range of functions relevant to bacterial physiology and multicellular lifestyles [5]. It has been shown that EPS production in response to oxidative, osmotic, drying, or heat stresses can improve microbial survival [6], as well as maintain the architecture and strength of biofilms [7]. Compared with their planktonic counterparts, biofilms are 10–1000 times more resistant to various disinfectants, such as sodium hypochlorite; and antimicrobials, such as ampicillin, tetracycline, and cloxacillin [8].

The process of biofilm formation is made up of five stages [9]: initial union, irreversible union, proliferation, maturation, and dispersal (Fig. 1).

In the first stage, the biofilm begins to form when several cells are reversibly attached to the surface. This initial adhesion depends on various factors such as the physicochemical properties of the surface, pH, the amount of EPS and proteins, and genetic factors that encode the motor functions. During the second stage, the binding becomes irreversible because the interaction between the bacteria and the contact surface changes from a weak bond to a permanent bond due to a higher production of EPS. From this stage onward, it is necessary to apply a powerful cutting force or chemical breakage to eliminate the biofilm [10]. Once the bacteria have adhered to the surface, the cells begin to grow and proliferate. This growth is associated with the production of EPS, which helps to strengthen the bond between the bacteria and the substrate, as well as to stabilize the colony against environmental stresses. In the

maturation stage, the bacteria change their behavior and grow under sessile form in heterogeneous microcolonies that evolve to form an organized and complex structure. It can be flat or in the form of a mushroom, depending on the source of nutrients at its disposal. Finally, the biofilm allows the release of the bacteria in their planktonic form, thus equipped to colonize new niches and surfaces [10].

Biofilm formation causes adverse effects in several areas of human activity, including the food industry. Biofilms formed on industrial production lines lead to problems such as corrosion/damage to pipes and equipment [11], interference and blockage of food processes, and contamination of raw materials and products [10], all of which favor foodborne outbreaks [12]. In the **vegetable industry**, processes such as cutting, washing, rinsing, drying, and packaging are regarded as the main source of cross-contamination because they facilitate the entry and fixation of bacteria, thus favoring the formation of biofilms. One of the most critical points is packaging: a reported outbreak with whole melons contaminated with *Listeria monocytogenes* was due to unhealthy packaging conditions [10]. The formation of biofilms in the **dairy industry** can generate serious food safety problems and economic losses. One of the biggest problems is that microorganisms in biofilms can catalyze chemical and biological reactions, causing corrosion in metal storage tanks and pipes [13, 14]. In addition, *L. monocytogenes* biofilms are a potential source of contamination in the milking equipment of a dairy farm [15]. The **meat industry** is another major food industry that can serve as a propitious niche for the accumulation of microorganisms and the formation of biofilms. Dourou et al. [16] conducted a study to evaluate the binding, survival, and growth of *Escherichia coli* O157:H7 on stainless steel and high-density polyethylene surfaces typical of meat industry equipment. Their results showed that the binding of the bacteria depends on the type of substrate as well as on temperature. In particular, the greatest amount of fixation occurred not only during nonproduction hours in the meat manufacturing areas but also in the course of the storage period [10, 16].

To investigate and evaluate the behavior of biofilms with the purpose of ensuring food safety, it is vitally important to carry out microbiological controls designed to collect all relevant microbiological information. This will enable the assurance of food safety, and the assessment of the susceptibility of biofilms to various treatments such as antimicrobials and disinfectants. Biofilm detection methods can be classified into two types: direct and indirect (Fig. 2) [13].

The first type is based on the direct observation of microbial biofilms, which includes techniques such as electrochemical impedance spectroscopy, epifluorescence microscopy, or scanning electron microscopy, among others (Fig. 2). Direct methods, however,

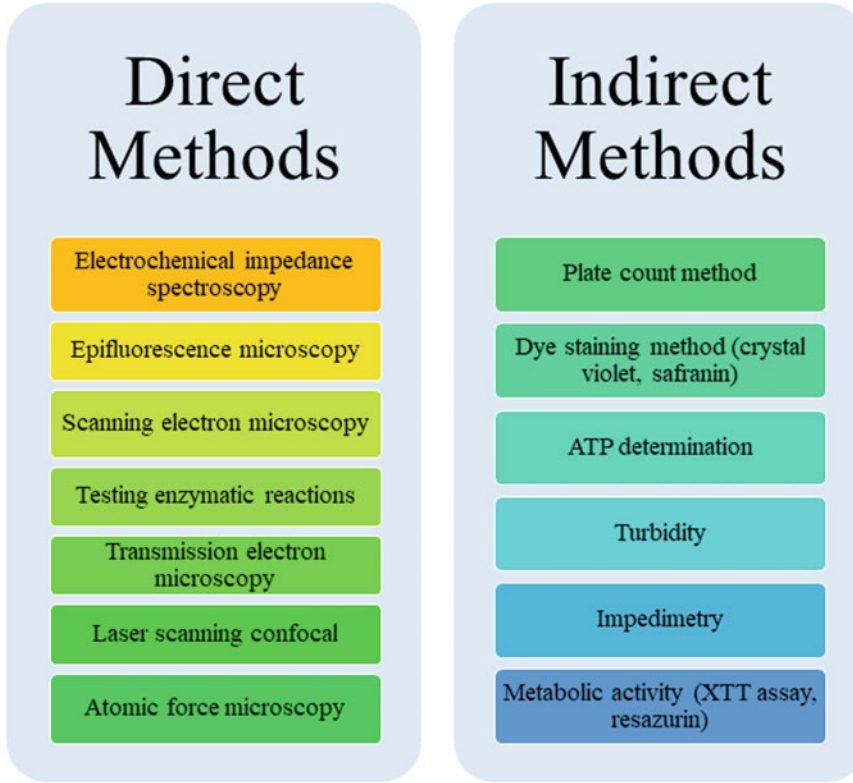

Fig. 2 Classification of the different methods used to detect biofilms on food contact surfaces

can lead to an underestimation of biofilm levels by refraining to measure thickness, and/or by overestimating the areas covered by the cells, as is the case with epifluorescence microscopy. In addition, these methods are usually difficult to implement in the food industry. On the other hand, indirect methods (such as traditional plate counting, the staining of biofilm biomass with crystal violet or safranin, ATP determination techniques, or metabolic assays) require the detachment of biofilms from the surface. These indirect methods are more appropriate for routine studies on the presence and quantification of biofilms in the food industry. The **plate count method** is one of the culture methods most widely used to estimate cell viability and physiology, as well as to determine colony-forming units (CFU) in agar media. It likewise allows us to isolate cells for future studies [17]. However, this method has two main drawbacks:

1. It does not provide information about the amount of biomass, which can lead to mechanical problems, such as pipe obstruction [18].
2. It requires a proper disaggregation of the biofilms: if several cells form a single colony, the technique underestimates the cell population.

The **dye staining method** to determine biofilm biomass offers a number of advantages: (1) versatility, since it can be applied to a wide range of different bacterial species; (2) high-throughput capability, which facilitates simultaneous testing of a number of different conditions; and (3) the possibility of quantifying the biofilm biomass and examining its distribution. Nevertheless, it also has two important disadvantages: (1) bias in the quantification of biofilm cells due to washing, and (2) it only provides the total biomass, without differentiating the physiological state of sessile cells (e.g., whether they are alive or dead) [18, 19].

In this chapter, we explain the methodology of evaluating microbial counts and biofilm mass for in vitro biofilm testing on polystyrene and stainless steel surfaces. We also provide a number of recommendations in order to avoid each of these methods' disadvantages. In addition, we propose the combination of both methods as a means of obtaining a more complete picture of a biofilm's state.

2 Materials

2.1 Quantification of Biofilms by Plate Count Technique

1. Culturing tools: micropipettes, plastic 1.5-mL tubes, and petri dishes (90 mm).
2. 24-well polystyrene and stainless steel plates.
3. Adhesive PCR Plate Seals.
4. Nutritive Agar (NA) medium for counting sessile cells of biofilms.
5. Vortex.
6. Distilled sterile water for washing.
7. 0.1% (w/v) Peptone Water (PW) solution or Phosphate-Buffered Saline (PBS) as diluent. To prepare the solutions, the required quantities were diluted in sterile distilled water according to the manufacturer's instructions.
8. PW or PBS solution with 1% (v/v) Tween 20. To prepare the solution, the corresponding volume of Tween 20 was added with a micropipette, drop by drop and very slowly. As this is a very viscous surfactant, it is recommended to use a trimmed tip (0.5–1 cm) to facilitate addition.
9. Ultrasonic bath.
10. Incubator.

2.2 Quantification of Biofilms by Crystal Violet Staining Assay

1. Laboratory tools: micropipettes, pipettes, and polystyrene macro cuvette.
2. Distilled sterile water for washing.

3. 0.1% (w/v) Crystal Violet solution. To prepare the solution, the required quantities were diluted in sterile distilled water.
4. 30% (v/v) glacial acetic acid solution. To prepare the solution, the required quantities were diluted in sterile distilled water.
5. Spectrophotometer for measuring absorbance (595 nm).

3 Methods

To form the biofilms, 24-well polystyrene and stainless steel plates with 2 mL of culture in each well were used. To avoid dehydration of the biofilms, 1 mL of sterile distilled water was added to the external wells. Biofilm formation could be studied at different temperatures and for determined time periods as a function of the microorganism investigated. It was subsequently possible to determine biofilm formation following these methods.

3.1 Quantification of the Biofilms by Plate Count Technique

This assay allows to determine the proportion of living and dead cells within the biofilm. After forming the biofilm in 24-well plates, the supernatant was removed from the wells, and the biofilms were carefully washed two or three times with 3 mL of sterile distilled water (1.5 times with respect to the initial volume of culture) to remove any remaining planktonic cells and culture medium (*see* **Note 1**). Then, 2 mL of 0.1% PW or PBS with 1% Tween 20 was added to each well and the biofilms were resuspended with the micropipette (*see* **Note 2**). Next, to facilitate the disintegration of sessile cell aggregates from the biofilms, the plates (covered with adhesive PCR plate seals) were sonicated in an ultrasonic bath for 10 min at 40 kHz (*see* **Note 3**). Depending on the microorganism, biofilm can form many or few aggregates. For this reason, it is recommended to homogenize for several seconds and then check under the microscope for the presence or absence of aggregates (*see* **Note 4**). If there are aggregates, the vortex time for serial dilution can be increased.

After sonication treatment, 100 μL was taken from samples and, if necessary, of the corresponding decimal dilutions in 0.1% PW or PBS. The extract was resuspended for 10 s in a vortex, and 100 μL thereof was inoculated into sterile petri dishes. The NA medium was immediately added for mass homogenization seeding. After solidification of the agar, the plates were incubated in an inverted position, applying the specific time and temperature conditions for each microorganism. After the incubation period, the colonies on each plate were counted. We normally look for dilution factors that allow us to work in a range of 30–300 CFU per plate to ensure that the count is made without errors. The count values can be provided as CFU/well after applying the appropriate dilution factors.

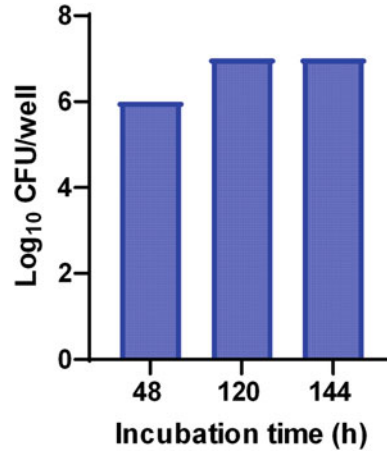

Fig. 3 Logarithmic count (CFU/well) of *Bacillus cereus* biofilms after 48 h, 120 h, and 144 h of incubation. The biofilms were formed on stainless steel plates

In Fig. 3 we can observe the example of a biofilm count at different incubation times. At 48 h, there were 10^6 CFU/well. Then, the cell population grew to a maximum of 10^7 CFU/well and remained stable until 144 h. In this case, an example of the most appropriate decimal dilution factors at 48 h would be -2, -3 and -4. This allows us to cover a wider range when counting the colonies.

3.2 Quantification of Biofilm Mass by Crystal Violet Staining Assay

Violet crystal staining assay allows for the measurement of a biofilm's total cell biomass (comprised by the extracellular matrix, living cells, and dead cells). After the formation of the biofilm on microtiter plates, the supernatant of the wells was discarded. The plates were carefully washed one or two times with sterile distilled water (*see* **Note 1**) to remove the planktonic cells that had not firmly adhered to the biofilm (*see* Subheading 3.1). They were left to dry at room temperature (20–25 °C) for at least 12 h. Next, to dye the biofilm biomass, 2 mL (a volume equal to the initial volume of culture) of 0.1% crystal violet solution was added and allowed to incubate for at least 15 min at room temperature (20–25 °C). After this period, the supernatant was removed (*see* **Note 1**), and the wells were washed two or three times with 3 mL of sterile distilled water (1.5 times the initial volume of culture) to remove the crystal violet residue. It was then left to dry at room temperature for at least 12 h. At this point, the crystal violet staining method allows us to visualize the distribution of the biofilm (Fig. 4).

In order to quantify the biomass, 2 mL of the 30% glacial acetic acid solution was added to dissolve the violet crystal biomass, and the absorbance was measured at 595 nm using a spectrophotometer. To determine the biofilm's optical density, it is important to note that the absorbance values generally lie within a linear range between 0 and 1 (*see* **Note 5**). It is advisable to only consider the

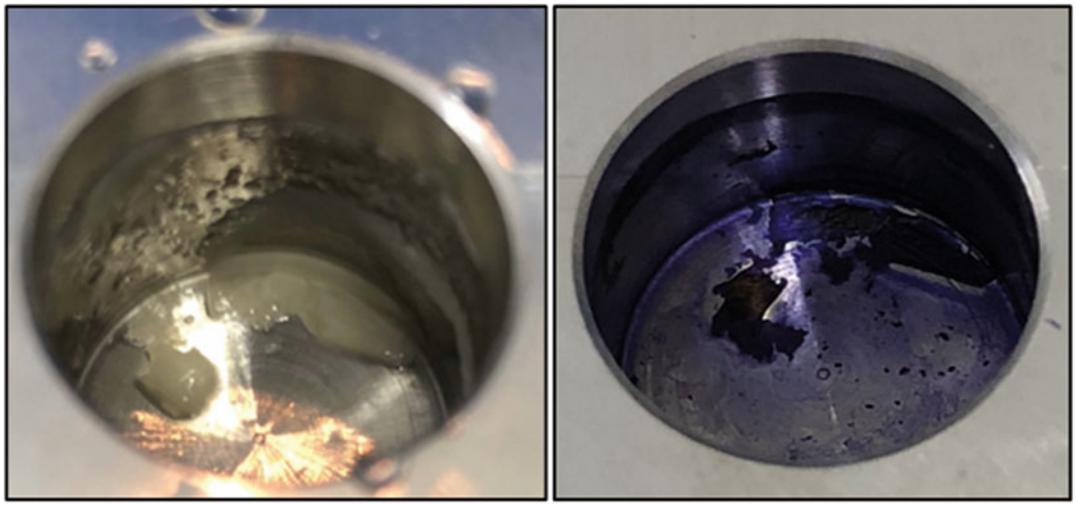

Fig. 4 Appearance and distribution of *Bacillus cereus* biofilm on a stainless steel well plate before (left) and after staining with crystal violet (right)

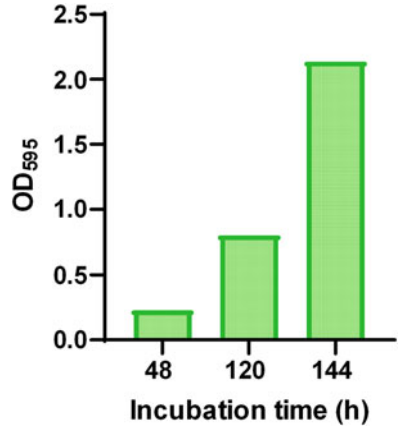

Fig. 5 Optical density (OD_{595}) of *Bacillus cereus* biofilm biomass after 48 h, 120 h, and 144 h of incubation. The biofilms were formed on stainless steel plates. The values obtained at 144 h have been multiplied by the corresponding dilution factors used to adjust to the 0–1 linear range of absorbance values

OD_{595} (Optical Density at 595 nm) values within this range. Dilutions can be made with the same solution of glacial acetic acid (30%). It is recommended to apply dilution factors such as 1/2, 1/4, or 1/8 or, if necessary, to apply a higher factor to ensure that the OD_{595} values of each sample lie within that 0–1 range, applying its corresponding dilution factor in the final results.

However, it should be noted that this microbial suspension will not allow us to perform plate counts, because the method practically destroys the biofilm's sessile cells.

In Fig. 5, we can observe an example of the OD_{595} of the biomass of biofilms at different times. The biomass increases

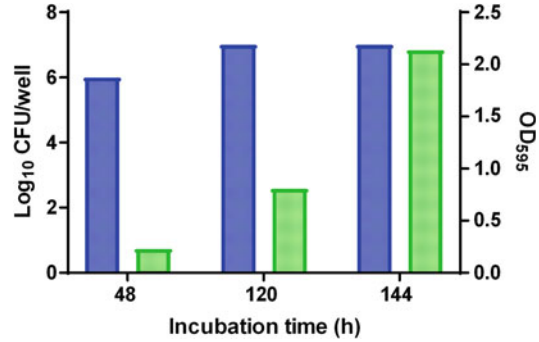

Fig. 6 Logarithmic count (CFU/well) (▬) and optical density (OD_{595}) (▬) of *Bacillus cereus* biofilm biomass at 48 h, 120 h and 144 h. The biofilms were incubated in stainless steel plates. The values of OD_{595} obtained at 144 h have been multiplied by the corresponding dilution factors used to adjust to the 0–1 linear range of absorbance values

along the entire incubation time. At 120 h, the biomass has increased to three times more than at 48 h; and at 144 h the biomass is more than double that observed at 120 h.

If we combine the results from microbial counts and crystal violet staining (Fig. 6), we can see that in the interval of 120–144 h, although the bacterial counts remain constant at 10^7 CFU/well, there was an increase in biomass due to the production of EPS, the main component of the extracellular matrix.

In conclusion, the plate count method is a traditional approach that allows us to know a biofilm's CFU per well, and thus, its food safety implications. It can be complemented with the quantification of biomass by the crystal violet staining method to visualize the hotspots of biofilm formation and provide a measurement of the amount of biofilm. This allows us to correlate the number of colonies with extracellular matrix production (EPS, proteins, lipids, and nucleic acids), thereby providing us with additional information about the biofilm's behavior. In addition, a greater increase of EPS can improve the degree of protection of the cells. This would have negative implications on food safety, since the effectiveness of cleaning and disinfection processes would be reduced. By combining both methods, we can overcome the disadvantages of each individual method and gain a better understanding of biofilms.

4 Notes

1. The supernatant can be removed in various ways depending on the biofilm's shape, firmness, and adhesion. The plate can be turned over and gently tapped, removing all the supernatant at once. Alternatively, it can be removed with a pipette or micropipette. For washing, it is recommended to use the

micropipette. It is highly important to do this slowly, drop by drop, taking care not to break the biofilm. The volume of sterile distilled water depends on the methodology used to form the biofilm. In case a different methodology is used, the volume of sterile distilled water required for the washings should be proportional to the initial volume of culture. The number of washes depends on the strength of the biofilm: if it is weak, one wash is recommended; if it is strong, two or three washes might be necessary.

2. Tween 20 is a surfactant used to assist in the breakdown of sessile cells from biofilms when applying ultrasound treatment.
3. It is highly important to control the bath temperature, which should not be too high (<30 °C) to avoid cell inactivation. On the other hand, one should also make sure that the parafilm is well attached to the plate to avoid possible contamination and water ingress during treatment.
4. To observe the aggregation of cells, another suggestion is to do a count in the Thoma cell counting chamber under the microscope, and then to compare the results obtained with the plate count.
5. The linear range of absorbance values depends on equipment and on laboratory conditions. Situations vary from one laboratory to another. Therefore, it is recommended to make a standard line to determine the most appropriate linear range for each situation.

Acknowledgments

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Chapter 7

Evolution Assays for the Isolation of Mutant Bacteria Resistant to Natural Antimicrobials

Daniel Berdejo, Elisa Pagán, Natalia Merino, Diego García-Gonzalo, and Rafael Pagán

Abstract

Natural antimicrobial compounds have been proposed as a promising alternative to current preservation treatments for minimally processed foods. However, the currently required doses are too high, which leads to sensory alteration due to their strong organoleptic properties. For this reason, further research is still needed regarding their mode of action in order to optimize their antibacterial properties. In this regard, it has become useful to deliberately obtain resistant mutant strains in order to study the underlying mechanisms of antimicrobial resistance. Two different evolution assay protocols have been designed for the obtention of mutant strains with increased resistance against natural antimicrobials: cyclic exposure to prolonged sub-inhibitory doses and cyclic exposure to short lethal treatments. The phenotypic and genotypic characterization of the evolved strains will provide knowledge about cellular response and resistance mechanisms against antimicrobial compounds, which will help to optimize their use as preservatives in the food industry or as cleaning and disinfection treatments.

Key words Antimicrobial resistance, Mutagenesis, Evolution assay, Natural antimicrobial compounds, Essential oils, Individual constituents, Bacteria, Whole-genome sequencing

1 Introduction

Thermal inactivation is the main technology used in the industry as a preservation method to ensure food safety and stability. However, new consumer trends have encouraged the search for preservation methods that manage to maintain the nutritional and sensory properties of food while ensuring microbial safety and stability. In this regard, natural antimicrobial compounds such as essential oils (EOs) and their individual constituents (ICs) have been proposed as a promising alternative to current preservation treatments for minimally processed foods [1, 2]. These natural compounds have been extensively studied and have been shown to possess excellent antimicrobial properties against food-related pathogens

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[3, 4]. Moreover, most of them are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. However, the doses required to use them as a single method of food preservation are too high, since their strong organoleptic properties might cause sensory alteration [5, 6]. One of the devised solutions is to apply them in combination with other antimicrobial compounds or food preservation technologies to achieve synergistic lethal effects [7], thereby reducing treatment dose and intensity while avoiding the alteration of treated food [8, 9]. Nevertheless, the design of effective combined treatments for microbial control in foods also requires a thorough understanding of their mechanisms of action on bacteria: for this reason, further research is still needed in order to optimize their antibacterial properties.

In recent years, several investigations have focused on the study of microbial genotypic resistance with the purpose of obtaining a better understanding of the mechanisms of cellular response to antimicrobials. Antibiotic resistance studies are an outstanding example thereof [10]. In fact, once the success derived from studying resistant mutant strains was observed, several authors devised laboratory evolution assays designed to obtain resistant strains that would allow for more in-depth studies [11]. The first evolution assays were based on the cyclic exposure of bacterial cell populations to prolonged sub-inhibitory doses [12]. The obtention of mutant strains thus became a useful tool for the study of resistance to antibiotics. Nevertheless, this technique was initially discarded in favor of the study of bacterial behavior against natural antimicrobials, due to the antioxidant properties of the latter [13], which reduced mutagenic frequency and would therefore prevent the occurrence of mutant strains [14]. However, recent studies have reported the emergence of resistant strains to natural antimicrobials: against ICs (carvacrol, citral, and limonene oxide) in *Escherichia coli* [15], *Staphylococcus aureus* [16], *Listeria monocytogenes* [17], and *Salmonella* Typhimurium [18], and against EOs (*Citrus sinensis*) in *S. aureus* [19] by cyclic exposure to prolonged sub-inhibitory doses. In addition, whole-genome sequencing of those strains allowed to identify the mutations responsible for the increased resistance [16–20].

Evolution assays were adapted to lethal treatments in order to investigate resistant strains obtained from survivors to high antibiotic concentrations [21]. This technique was likewise employed in other research areas such as food preservation, and new protocols were designed with the purpose of obtaining resistant mutant strains from the tails of survival curves after the application of lethal treatments. In this way, resistant strains were also obtained under physical food preservation technologies, such as heat [22] and high hydrostatic pressure [23]. Evolution assays have thus also been adapted to the isolation of resistant mutant strains following the application of lethal doses of natural antimicrobial compounds [18].

In this chapter we describe the methodology required to perform evolution assays with natural antimicrobials by two different protocols: (a) by cyclic exposure to prolonged sub-inhibitory doses and (b) by cyclic exposure to short lethal treatments. Our aim is to explain how to obtain and characterize mutant resistant strains against natural antimicrobial compounds. The phenotypic and genotypic study of these strains will allow for a better understanding of the mechanisms of bacterial resistance and, consequently, lead to a more profound knowledge of the mechanisms of antimicrobial action displayed by natural antimicrobial compounds. This information might lead to the design of new and more effective food preservation strategies in the industry.

2 Materials

2.1 Evolution Assay by Cyclic Exposure to Prolonged Sub-inhibitory Doses

1. Cryovial of the bacterial strain to study.
2. Culturing tools: micropipettes, pipette tips, plastic 1.5-mL tubes, petri dishes (90 mm), inoculation loops, and L-shaped spreaders.
3. Growth media: tryptic soya agar and broth supplemented with 0.6% yeast extract (or any other nutritive agar and broth).
4. Natural antimicrobial compound (essential oils, individual constituents, natural extracts, etc.).
5. Phosphate-buffered saline (PBS).
6. Glass test tubes and caps.
7. Vortex.
8. Incubator with orbital shaker.
9. Cryovials.

2.2 Evolution Assay by Cyclic Exposure to Short Lethal Treatments

1. Cryovial of the bacterial strain to study.
2. Culturing tools: micropipettes, pipette tips, plastic 1.5-mL tubes, petri dishes (90 mm), inoculation loops, and L-shaped spreaders.
3. Growth media: tryptic soya agar and broth supplemented with 0.6% yeast extract (or any other nutritive agar and broth).
4. Natural antimicrobial compound (essential oils, individual constituents, natural extracts, etc.).
5. Phosphate-buffered saline (PBS).
6. Glass test tubes, glass flasks (250 mL), and caps.
7. Vortex.
8. Centrifuge.
9. Incubator with orbital shaker.
10. Cryovials.

3 Methods

Prior to either of the two evolution assay protocols, it is necessary to obtain a working bacterial culture of the wild-type strain (WT) from which evolution assays will be triggered, as well as to determine the minimum inhibitory concentration (MIC) of the antimicrobial that is being tested. MIC is established as the lowest concentration of the antimicrobial compound capable of inhibiting bacterial growth. The MIC value will be used later on to perform the evolution assays.

The following **steps 1–4** to obtain the **initial working bacterial culture** are common to both evolution protocols:

1. From a cryovial of WT, inoculate and streak over the agar plates surface with an inoculation loop to obtain individual colonies (*see* **Note 1**).
2. Incubate the agar plates for 24 h at 37 °C (*see* **Note 2**).
3. Inoculate a single colony in 5 mL of growth broth in test tube (*see* **Note 3**).
4. Incubate overnight at 37 °C and 130 rpm until a stationary phase culture is obtained (*see* **Note 4**).

It is then necessary to carry out **MIC determination** of the tested natural antimicrobial:

5. Prepare 5 mL test tubes of growth broth with increasing concentrations of the natural antimicrobial, and shake vigorously by vortex (*see* **Note 5**).
6. Inoculate the test tubes with a stationary phase culture at an initial concentration of 1×10^5 CFU/mL (colony-forming units/mL).
7. Prepare positive control tubes with 5 mL of growth broth inoculated at 1×10^5 CFU/mL without antimicrobial, and negative control tubes with 5 mL of growth broth non-inoculated with the natural antimicrobial.
8. Incubate all the test tubes for 24 h at 37 °C and 130 rpm.
9. Observe the turbidity of the growth broth. If the growth medium is cloudy, this means that bacteria have grown, whereas no turbidity indicates that the concentration of the antimicrobial is sufficient to inhibit bacterial growth (*see* **Note 6**).
10. The lowest concentration that has inhibited bacterial growth is established as the MIC.

Use 0.5× of the MIC to carry out the evolution assay by cyclic exposure to prolonged sub-inhibitory doses, and 2× the MIC to conduct the evolution assay by cyclic exposure to short lethal treatments. Figure 1 shows the scheme of the two protocols of evolution assays designed to obtain mutant strains resistant to natural antimicrobial compounds.

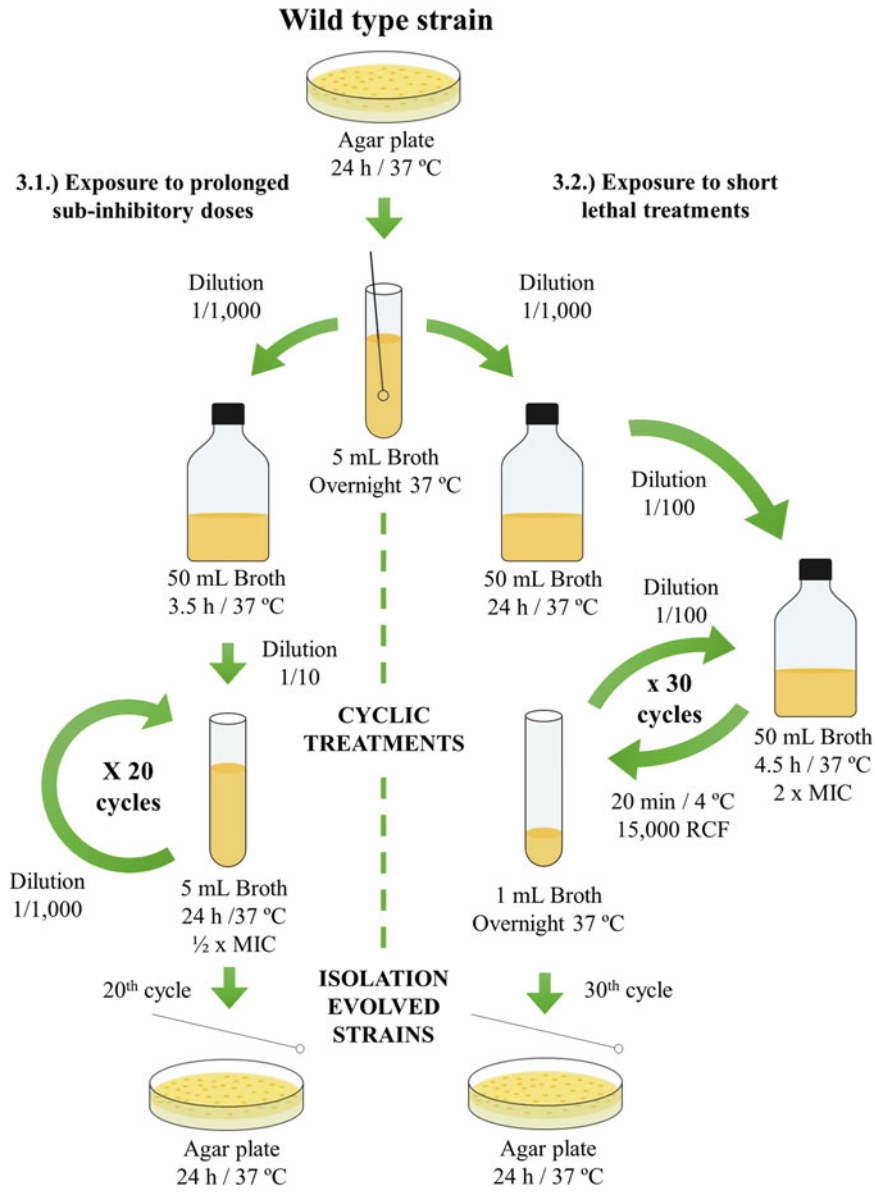

Fig. 1 Diagram of evolution assay (3.1) by cyclic exposure to prolonged sub-inhibitory doses and (3.2) by cyclic exposure to short lethal treatments

3.1 Evolution Assay by Cyclic Exposure to Prolonged Sub-inhibitory Doses

This protocol is based on the application of constant stress to the bacterial population at low concentration. The aim is to allow the occurrence of mutations in the bacterial population that improve its growth fitness in the presence of the antimicrobial agent. Thus, if such mutations occur, the agent's selective presence will facilitate the emergence of such strains in contrast with the WT as well as with other mutants whose mutation is not related to resistance to the agent, thereby allowing the isolation of strains that are resistant to the selected antimicrobial compound (Fig. 2).

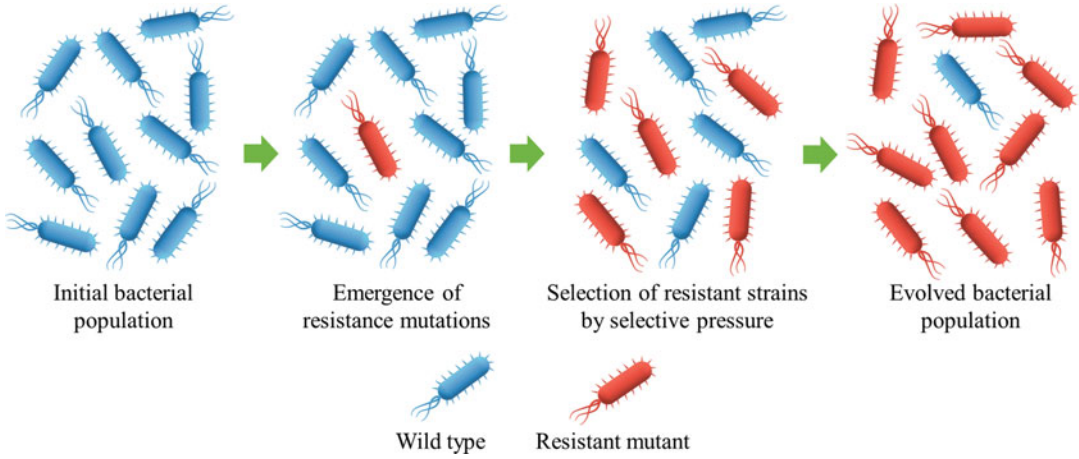

Fig. 2 Selection of resistant mutant strains by cyclic exposure to prolonged sub-inhibitory doses

To carry out the evolution assay by cyclic exposure to prolonged sub-inhibitory doses, the following steps should be taken (continue from **step 4**):

5. Dilute working bacterial culture of WT 1:1,000 into 50 mL growth broth and incubate for 3.5–4 h at 37 °C and 130 rpm until an exponential phase culture is obtained (*see* **Note 7**).
6. Once grown, inoculate 5 mL growth broth in test tubes at an initial concentration of 10^6 CFU/mL in the presence of 0.5× of the MIC of the antimicrobial compound.
7. Incubate for 24 h at 37 °C and 130 rpm until stationary phase is reached (*see* **Note 4**).
8. Return to **step 6**. After 20 cycles, continue with **step 9** (*see* **Note 8**).
9. After the 20th cycle, dilute the bacterial culture in PBS, and inoculate and spread on agar plates to obtain individual colonies.
10. Incubate the agar plates for 24 h at 37 °C (*see* **Note 2**).
11. After the incubation on agar plates, select several colonies and store them in cryovials (*see* **Note 9**).

3.2 Evolution Assay by Cyclic Exposure to Short Lethal Treatments

This protocol is based on the application of short cyclic treatments to the bacterial population at high concentration. Its goal is to provoke the emergence of mutations associated with resistance to lethal treatments of the antimicrobial agent. Evolved bacteria that have suffered mutations implying increased resistance will survive lethal treatments based on the resistance of the WT. In this way, the lethal treatments applied will inactivate the most sensitive cells, while sparing the mutant strains that are resistant to antimicrobial compounds (Fig. 3).

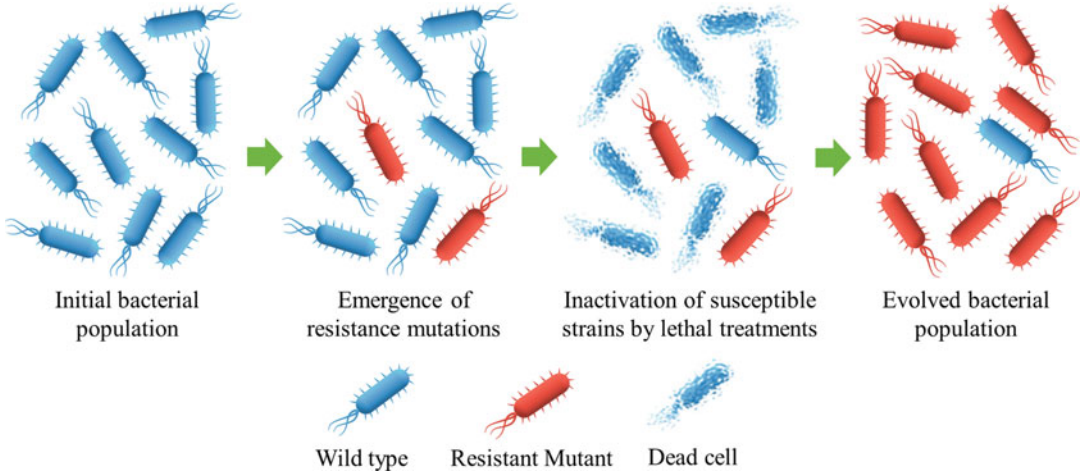

Fig. 3 Selection of resistant mutant strains by cyclic exposure to short lethal treatments

To carry out the evolution assay by cyclic exposure to short lethal treatments, the following steps should be taken (continue from **step 4**):

5. Dilute working bacterial culture of WT 1:1,000 into 50 mL growth broth and incubate for 24 h at 37 °C and 130 rpm until a stationary phase culture is obtained (*see* **Note 4**).
6. Once incubated, dilute stationary phase culture 1:100 (initial concentration of 10^7 CFU/mL) in glass flasks containing 50 mL growth broth in presence of 2× the MIC of the antimicrobial compound.
7. Apply the treatment while maintaining the flask at 37 °C for 4.5 h (*see* **Note 10**).
8. Centrifuge the treated cells for 20 min at 15,000 RCF and 4 °C, wash twice with fresh growth broth, and resuspend in 1 mL of growth broth in test tube.
12. Incubate test tubes overnight at 37 °C and 130 rpm until stationary phase is reached (*see* **Note 4**).
13. Return to **step 6**. After 30 cycles, continue with **step 14** (*see* **Note 8**).
14. After the 30th cycle, dilute the bacterial culture in PBS, and inoculate and spread on agar plates to obtain individual colonies.
15. Incubate the agar plates for 24 h at 37 °C (*see* **Note 2**).
16. After the incubation on agar plates, select several evolved colonies and store them in cryovials (*see* **Note 9**).

Finally, the isolated strains obtained either by cyclic exposure to prolonged sub-inhibitory doses or by short lethal treatments must

be phenotypically and genotypically characterized. The WT must be used as control to evaluate the resistance of the evolved strains, and, likewise, in order to carry out the comparisons among the genomes with the purpose of finding the mutations that have occurred during evolution assays responsible for the increased resistance.

On the one hand, it is recommended to first evaluate the resistance of the evolved strains against the natural antimicrobial used in the evolution assay at both bacteriostatic and bactericidal concentrations. For this purpose, MIC and minimum bactericidal concentration (MBC) can be determined, survival curves to lethal treatments can be obtained, and results can be compared with the WT. These methodologies are explained by Berdejo et al. [16]. Increased resistance may also occur against other natural antimicrobials not used in the evolution assay [15]; thus the same methodology can be used to test other antimicrobials. According to recent results in evolved strains, an antibiotic susceptibility test should be performed due to the fact that increased cross-resistance to antibiotics has been observed [18], and these compounds probably have similar mechanisms of action. In addition, resistant strains isolated by natural antimicrobials have also demonstrated increased resistance to other food preservation technologies such as heat or pulsed electric fields [15]. Such results will provide more information on the behavior of the evolved strains, as well as on the direct resistance and cross-resistance which will have emerged in the evolution assays between natural antimicrobials, antibiotics, and food preservation technologies.

On the other hand, whole-genome sequencing (WGS) of WT and of the evolved resistant strains, followed by comparison between them, will allow a determination of the genetic modifications that cause the increase in resistance [16, 18]. These results will thus provide knowledge regarding cellular response and resistance mechanisms (cellular targets, repair systems, etc.) against the antimicrobial compound, which might help to optimize their use as preservatives in the food industry or as cleaning and disinfection treatments.

4 Notes

1. For evolution studies it is recommended to always use the same cryovial, or original strain, to avoid the occurrence and accumulation of random mutations in the WT, which make it more difficult to study genotypic resistance.
2. Incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.

3. Check the size and shape of the colonies, and verify the homogeneity in the agar plate to avoid microbial contamination.
4. To obtain a stationary phase culture, incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
5. The concentration range should be adjusted based on the natural antimicrobial's bacteriostatic activity and on the resistance of the bacteria under study. Based on our experience, the range for ICs with high antimicrobial activity, such as carvacrol or thymol, or EOs, such as oregano or thyme EO, lies between 50 and 300 μL/L (with intervals of 50 μL/L). The range used for other less active compounds such as limonene oxide or citrus EOs, such as orange or lemon EO, is from 500 to 2,000 μL/L (with intervals of 100 μL/L).
6. To obtain an objective measurement, it is recommended to read the optical density at 595 nm (OD_{595}). 10% of the OD_{595} value of the positive control has been established as the lower limit to consider that a bacterial strain was grown [11].
7. To obtain an exponential phase culture, incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
8. Depending on the mutation frequency of the bacteria, the natural antimicrobial used, and treatment conditions, the evolution assay should be prolonged until increased resistance is observed. For this reason, it is recommended to perform an antimicrobial resistance test every three cycles to detect the emergence of resistant strains.
9. The population may be genotypically heterogeneous depending on the number of evolutionary cycles: for this reason, it is recommended to evaluate several colonies in order to select the one that is most resistant.
10. Time and temperature of the lethal treatment can be modified according to the susceptibility of the WT to the antimicrobial used. This treatment's design seeks to inactivate a large part of the bacterial population (>5 $\log_{10}$ cycles of reduction), while nevertheless allowing for the recovery of surviving cells before proceeding to the evolution assay.

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Chapter 8

Preparing Yeast Suspension Through Serial Dilution for Enumeration

Cíntia Lacerda Ramos and Karina Teixeira Magalhães-Guedes

Abstract

Yeasts are essential to obtain a variety of fermented foods and beverages, including bread, cheese, wine, and others. This group of microorganisms may also be associated with food spoilage. To better understand and control the food quality, yeast enumeration is an important step. However, yeasts may be present in a high population, which is challenging to perform direct counting from samples. Thus, serial dilution should be performed before yeast enumeration. This method consists of a consecutive ten times dilution of food sample to reach a value of colonies that can be easily counted in the plates.

Key words Foodborne yeasts, Food associated yeasts, Diluent solutions, Ten times dilution, Yeast enumeration

1 Introduction

The large and diverse group of food-associated yeasts includes several hundred species. They are an essential agent acting favorably in fermented foods and beverages. However, these microorganisms can attack many foods due to their relatively varied environmental requirements. Although most yeasts are aerobes, they are tolerant of a wide range of pH, ranging from pH 2 to above pH 9. Furthermore, their growth temperature range (10–35 °C) is also vast. Only some species can grow below or above this range. Regarding moisture, foodborne molds require relatively low values. In general, most species can grow at a water activity (aw) of 0.85 or less, although yeasts generally require a higher water activity.

Yeasts may grow in food and cause deterioration and decomposition. They can spoilage any type of food and crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. They also grow in processed foods and food mixtures. The dilution plating method is commonly used to detect spoilage yeasts in foods [1–4].

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Quantitative assessment of microorganisms can be challenging due to their abundance, exponential proliferation capacity, species diversity within a population, and specific physiological needs. Further, the four-phase nature in which bacteria and yeast replicate (lag, log, stationary, and death) increases this challenge. An accurate estimation of the microbial population is essential for successful identification, isolation, cultivation, and characterization [1–3, 5]. Hence, microbiologists have employed serial dilution and various plating methods for over a century to quantify bacterial reliably and yeast load in clinical, industrial, pharmaceutical, and academic laboratory environments. This methodology was first described in 1883 by the German scientist and physician Robert Koch, who published his work on infectious disease-causing agents. Known as the father of modern bacteriology, Kock and the techniques first described by him have become a standard for microbial enumeration and cultivation worldwide [6].

The isolation, enumeration, and identification of yeasts from foods and beverages follow the same principles and strategies that are used for yeasts in general. These involve sequential operations of the sample's rinsing or maceration, dilution, and enumeration of yeast cells. Estimation of yeast population may be performed by agar plating, most probable number, membrane filtration, microscopic, or electronic methods. Then, the isolates are purified and identified to genus, species, or strain level [1, 4].

Procedures of homogenization for yeast isolation may consist of manually shaking or mixing the sample with a known volume of diluent using a blender, orbital shaker, or peristaltic agitator (Stomacher), after grinding if necessary. Diluents commonly used comprise distilled water, saline, phosphate buffer, and the most common 0.1% (w/v) peptone water. Contact time ranges from less than 1 to several minutes (generally 5–10 min). Based on the assumption that the separation of yeast cells from natural habitats requires much rougher treatment of samples, a series of ecological surveys were conducted on the yeast population of different fruits. The overall results indicate that pre-isolation treatments based on vigorous shaking, percolation with an excess of water, and the sonication of samples, allowed the recovery of a higher number of colonies forming units and species [1]. Furthermore, as Fleet [7] stated, "the assumption that maceration is an ecologically sound prelude to microbiological analysis requires more rigorous scrutiny, especially since it is already known that extracts of vegetables, herbs, and spices are toxic to some microorganisms."

Sterile peptone water (0.1% w/v) is the recommended diluent for preparing samples to be plated on general-purpose enumeration media [8]. However, given the diversity of intrinsic food characteristics and yeast biodiversity, there is no ideal diluent. It is always necessary to consider the nature of the food, and the species sought to select the diluent. Furthermore, it should not be forgotten that

the most crucial objective is to recover spoilage yeasts. Although it is necessary to standardize this operation, it is recognized by the International Commission on Food Mycology that specific protocols are not yet available, either concerning the type of food or sample contact time [1], which makes it difficult to compare results from different laboratories. According to Fleet [7], based on an international collaborative study under the auspices of the above-mentioned Commission, it is possible to conclude that apart from diluent composition and timing between dilution and plating, other factors such as the stage of the cell life cycle, cell stress before dilution, degree of cell clumping and aggregation, shear forces during shaking, presence of contaminating metal ions, pH, and temperature could all have an impact on the survival of the yeast cells during a dilution.

2 Materials

1. Prepare the diluents: Peptone water 0.1% (w/v), or saline solution (0.85% NaCl w/v), or peptone salt solution (0.85% NaCl w/v and 0.1% w/v) (*see* **Note 1**).
2. Food sample.
3. Instruments for homogenization such as wrist-action shaker, peristaltic agitator (e.g., Stomacher), magnetic stirrer, orbital shaker, or a blender (*see* **Note 2**).
4. Prepare sterilized tubes containing 9 mL of diluent solution and pipettes for serial dilution (*see* **Note 3**).

3 Methods

1. Dilute the food sample 10× in diluent solution (e.g., saline solution or peptone water or peptone salt solution) (*see* **Note 1**): 25 g (solid sample) or 25 mL (liquid sample) and add to the flask containing 225 mL of diluent solution (*see* **Note 4**). Consider this step as the first dilution (10^{-1}).
2. Homogenize the samples for 1–5 min by using, e.g., wrist-action shaker, peristaltic agitator, magnetic stirrer, orbital shaker, or a blender (*see* **Note 2**).
3. Transfer aseptically 1 mL from the food sample (10^{-1}) into a tube containing 9 mL of diluent solution. In this tube, the sample is diluted 100× (10^{-2}) (*see* **Note 3**).
4. Homogenize suspension in the tube and, aseptically, transfer 1 mL of the homogenized sample to a novel tube containing 9 mL of diluent solution. This tube contains sample diluted at 1000× (10^{-3}).

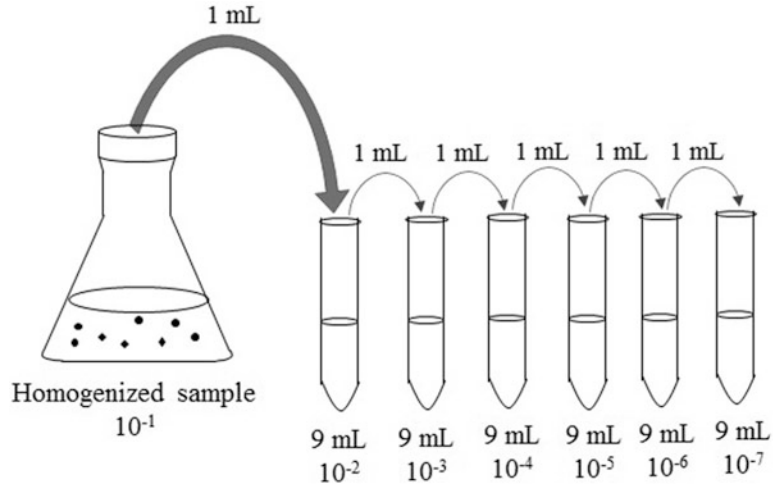

Fig. 1 Diagram demonstrating serial dilution of food samples for yeast enumeration

5. Repeat **step 2** many times as necessary, depending on the food sample and yeast population present in the samples. Generally, samples diluted until 10^{-7} are satisfactory. Figure 1 illustrates the serial dilution preparation.
6. Diluted samples are ready to be spread onto the plates for enumeration of yeast populations. Chapter 10 describes the different methods for yeast enumerations in foods.

4 Notes

1. Several diluents may be employed for the enumeration of yeasts in foods. Generally, distilled water is not recommended due to osmotic shock effects. Saline solution (0.85% w/v NaCl), peptone water 0.1% (w/v), or a combination of peptone salt solution 0.85% NaCl (w/v) and 0.1% (w/v) are commonly recommended for analysis of foods. Sterile milk may be used as diluents in the analysis of dairy products. Diluents with a lower water activity (by adding 20–30% of glucose, or 18–26% of glycerol) may be employed to enumerate osmotolerant yeast, such as *Zygosaccharomyces rouxii*. Surfactants such as Tween 80 (0.01–0.05%) may be included in the diluent to enhance cell clumps separation. Furthermore, commercial standard diluents are available and may be used.
2. Low-tech methods of homogenization have also been reported as the use of sterile gloves to hand-squeeze aseptically collected grapes for *S. cerevisiae* isolation [9].

3. Ten dilutions may also be applied using different volume-scale such as adding 100 μL of samples into microtubes containing 900 μL of diluent solution.
4. In case of keeping samples to be further analyzed, it is essential to add 20% glycerol in the diluent and store the samples at −20 °C to maintain microbiota viability.

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Chapter 9

Standardizing Suspension of Yeast for Inoculation in Food Fermentations

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Abstract

The standardization of the inoculum is essential for the success of all fermentative process and depends mainly on maximizing the activity of the microorganism to improve the productivity and final yield. Different methods can be used to estimate and standardize the inoculum, such as the growth curve method, in which the combination of absorbance measurements and viable cell count or dry weight analyses reflect the microbial population in the sample. Another method is the measurement of cell density, in which the McFarland equivalence turbidity standards are compared with the inoculum turbidimetry with a standard scale that estimates the inoculum concentration. This chapter describes methods of standardization and preservation of the microbial inoculum.

Key words Inoculum, Standardization, Fermentation process, Growth curve, McFarland

1 Introduction

The use of yeasts in the production of food and beverages improved over the years. Since the twentieth century, the utilization of yeast has become a standard practice in industrial fermentation [1]. The inoculum is defined as a suspension of microorganisms sufficiently concentrate that is added to start the fermentation itself. In industrial fermentation applications, the quality and quantity of inoculum play an essential role in achieving the fermentation rate and final product yield. For most fermentations, the inoculum volume, taking into account a freshly prepared cell culture, is typically between 3% and 10% of the total volume of the substrate to be fermented [2].

The inoculum preparation starts standardizing cell suspension and, once the desired cell concentration is reached, the inoculum must be added to the bioreactor. The inoculum concentration

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depends on the desired metabolites produced by yeast, the available concentration of nutrients for growth, growth conditions (temperature, pH, oxygen availability), and what is the expectation of yield and productivity [3].

Prior knowledge of the growth rate of the microorganism is essential for different fermentation processes. In general, before inoculation, it is necessary to know the microorganism growth curve, which is characterized by three main phases: the lag, exponential, and stationary phases [4]. These phases can be determined through its growth curve that allows estimating the density at which point a cell culture should be before being transferred to a new medium. Therefore, the build of a growth curve is a method used to standardize the inoculum for food, which can be performed by combining methods that indirectly quantify cell concentration, such as measuring absorbance, and methods with direct quantification, such as viable cell counting and dry weight [5, 6].

The standardization of yeast suspension for inoculation in food can be performed by cell density as well. McFarland Equivalence Turbidity Standards can be used to approximate the concentration of cells in a suspension visually. For visual comparison, the turbidity of the pattern and the yeast suspension must have the same dispersion in the light, so that the approximate yeast population in the cell suspension is calculated [7]. In specific fermentative processes, the inoculum standardization can be carried out with the help of the Neubauer chamber, counting the number of yeast cells per milliliter (*see* Chapter 11).

Yeast cell cultures are generally easy to store, maintain, and cultivate, resulting in inoculum with a large number of cells. Microorganisms used in industrial processes must be adequately preserved as a pure culture. Through different techniques, it is possible to maintain all the characteristics of the microbial cell and, thus, whenever new production is started, the quality of the final product is also kept. Therefore, inoculum preservation methods are essential for laboratories, industrial applications, and biotechnology and related areas [8].

This chapter includes methods for standardizing yeast cell suspension for inoculation in foods, such as growth curve employing cell absorbance [9] and cell dry weight [10, 11], McFarland equivalence turbidity [12], and methods for inoculum preservation, such as deep freezing at −70 °C [13, 14], lyophilization [15, 16], and refrigerated storage [17].

2 Materials

2.1 Growth Curve

1. Sterile Erlenmeyer.
2. Pipettes.
3. Shaking incubator.

4. Sterile tubes.
5. Laboratory incubator with the temperature set at the temperature specified by the test to be performed.
6. pH meter.

2.1.1 Culture Media

YEPD (yeast extract peptone dextrose) Broth (g/L): Yeast extract (10), peptone (20), glucose (20).

Malt Extract Broth (g/L): Malt extract (17), mycological peptone (3), final pH 5.4 ± 0.2.

Sabouraud Dextrose Broth (g/L): Mycological peptone (10), dextrose (20), final pH 5.6 ± 0.2.

Potato Dextrose Broth (g/L): Potatoes, infusion from (200), dextrose (20), final pH 5.1 ± 0.2.

2.1.2 Spectrophotometer

1. Necessary equipment and appropriate techniques for preparation of sample and dilution (*see* Chapter 8).
2. Equipment for plating samples (*see* Chapter 10).
3. Spectrophotometer.
4. Pipettes.
5. Cuvette.

2.1.3 Dry Weight

1. Centrifuge.
2. Vacuum oven at 60 °C.
3. Desiccator.
4. Analytical balance.
5. Sterile tubes.

2.2 McFarland Equivalence Turbidity

1. Equipment for plating samples (*see* Chapter 10).
2. Loop sterilization device.
3. Inoculating loop, swabs, or transfer pipettes.
4. Sterile tube.
5. Saline or broth.
6. Vortex mixer.
7. Light source.

2.3 Preservation of Inocula

1. Eppendorf or similar tubes.
2. Glycerol (80%).
3. Freezer at −70 °C.

2.3.1 Freezer Freezing at −70 °C

2.3.2 Lyophilization

1. Skimmed milk (or another cryoprotectant).
2. Ampoules borosilicate glass.
3. Sterile Pasteur pipettes.
4. Blowtorch.
5. Freezer at −70 °C.
6. Freeze dryer.

2.3.3 Refrigerated Storage

1. Culture media with agar (add 20 g/L).
2. Sterile tubes.
3. Inoculating loop.
4. Mineral oil.
5. Sterile distilled water.

3 Methods

3.1 Growth Curve

1. Prepare the broth for yeast growth (300 mL) (*see* **Note 1**).
2. Inoculate 5 mL of broth media in sterile tubes.
3. Add stock yeast cultures (*see* **Note 2**).
4. Place in a shaking incubator.
5. Incubate overnight at 30 °C.
6. Add the 200 mL of broth media in a sterile Erlenmeyer.
7. Inoculate 1% (v/v) of yeast pre-inoculum (*see* **Note 3**).
8. Incubate at 30 °C.

3.1.1 Spectrophotometer

1. Collected, aseptically in laminar flow cabinet, 2 mL of culture broth for absorbance readings and plating; at pre-established time intervals (*see* **Note 4**).
2. Place 1 mL of the culture broth in the Cuvette (*see* **Note 5**).
3. Perform absorbance readings (Abs) on a spectrophotometer (*see* **Note 6**).
4. Make the respective dilutions of the yeast suspension (*see* Chapter 8) (*see* **Note 7**).
5. Perform plating using the spreading technique (*see* Chapter 10).
6. The readings and plating will be taken until the yeast growth reaches the stationary phase (*see* **Note 8**).
7. Incubation of 30 °C.
8. Perform logarithmic transformations for values of viable cell count (*see* **Note 9**).

9. Make a linear regression curve, plot log CFU/mL versus time (h).
10. From the graph, identify the exponential phase of growth. Using two-time points within the exponential phase of growth and corresponding cell numbers.
11. Calculate the mean generation time from the equation:

$$X = 2^n \times X_0$$

where.
X_o = initial concentration of cells.
X = concentration of cells after time t.
n = number of generations.

12. Standardize the inoculum according to the need.

3.1.2 Dry Weight

1. Collected, aseptically in laminar flow cabinet, 10 mL of culture broth for absorbance readings and dry weight analyses; at pre-established time intervals (*see* **Note 10**).
2. Centrifuge yeast suspension at 10,000 × *g* for 15 min.
3. Discard the supernatant (*see* **Note 11**).
4. Dry the cells in a vacuum oven at 60 °C for 24 h (*see* **Note 12**).
5. Place in a desiccator for 30 min.
6. Weigh on an analytical scale.
7. Construct a calibration curve, plotting the absorbance values representing the corresponding cell density and dry weight in a graph, obtaining a linear regression equation (*see* **Note 13**), this equation was used to estimate the cell mass to be used as an inoculum.

3.2 McFarland Equivalence Turbidity

1. Plating the inoculum using the spreading technique (*see* Chapter 10).
2. Incubation for 24 h at 30 °C.
3. Sterilize the inoculating loop (*see* **Note 14**).
4. Prepare the inoculum by suspending five distinct colonies, ±1 mm in diameter in 5 mL of sterile distilled water (*see* **Note 15**).
5. Evenly suspend the inoculum on a vortex mixer for 15 s.
6. Invert the McFarland Equivalence Turbidity Standard gently to suspend the polystyrene microparticles entirely.
7. Visually compare the turbidity of an actively growing broth culture or a yeast suspension prepared from an 18–24 h culture to the appropriate McFarland Standard.

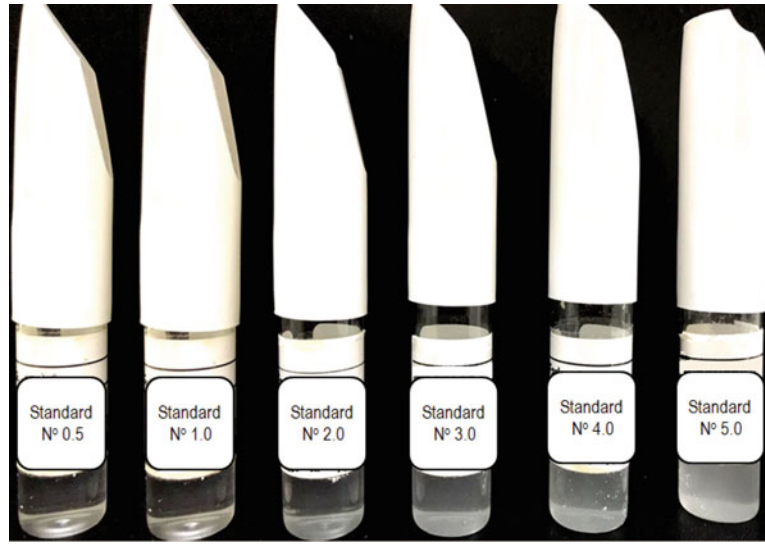

Fig. 1 Standard McFarland scale. Standard No. 0.5: Approximate Cell Density 1.5×10^8/mL; No. 1.0: Approximately 3.0×10^8/mL; No. 2.0: Approximately 6.0×10^8/mL; No. 3.0: Approximately 9.0×10^8/mL; No. 4.0: Approximately 1.2×10^9/mL; No. 5.0: Approximately 1.5×10^9/mL

8. For visual comparison, use adequate light or read the tubes against the white card with contrasting black lines (*see* **Note 16**).
9. Equal obliteration or distortion indicates a turbidity match (Fig. 1).
10. After standardization, use the yeast suspension (*see* Subheading 4) to inoculate in the food (*see* **Note 17**).

3.3 Preservation of Inoculum

3.3.1 Freezer Freezing at −70 °C

1. Cultivate samples in culture broth (*see* Subheading 2.1.1) (*see* **Note 1**).
2. Incubate with shaking at 30 °C overnight.
3. Add 0.8 mL of the yeast cultures in Eppendorf or similar tubes (*see* **Note 18**).
4. Add 0.2 mL of 80% glycerol (*see* **Note 19**).
5. Place the tubes in a freezer at −70 °C (*see* **Note 20**).

3.3.2 Lyophilization

1. Cultivate samples in medium broth (*see* Subheading 2.1.1) (*see* **Note 1**).
2. Incubate with shaking at 30 °C for 48 h.
3. Mix equal volumes of the inoculum in culture medium and skimmed milk (*see* **Note 21**).
4. Using sterile Pasteur pipettes, inoculate about six drops (0.2 mL) of the suspension into the ampoules.

5. Constrict the ampoule with the aid of a torch, to facilitate vacuum closure after freeze-drying.
6. Freeze samples at −70 °C freezer overnight.
7. Lyophilize the samples in the freeze dryer for 6 h (*see* **Note 22**).
8. Vacuum seal with a blowtorch.
9. Remove the ampoules from the freeze dryer and store (*see* **Note 23**).

3.3.3 Refrigerated Storage

1. Pour the culture media agar into sterile petri dishes and slanted tubes.
2. With inoculating loop, spread the sample in the slanted tubes and petri dishes.
3. Incubation at 30 °C for 48 h.
4. For maintenance on agar, store at refrigeration temperature (4–8 °C).
5. For maintenance in mineral oil, submerge the slant agar surface in mineral oil, store at refrigeration temperature (4–8 °C).
6. For storage in distilled water, with the inoculation loop, remove 10 colonies of yeasts from petri dishes and suspend in sterile distilled water, seal, and store (*see* **Note 24**).

4 Notes

1. For all the media mentioned in Subheading 2.1.1, add the components to sterile distilled or deionized water and autoclave 15 min at 121 °C, adjust the pH. If you are going to use another media check how to make and sterilize.
2. The yeasts can be freeze-dried, frozen, dried, or another way (*see* Subheading 3.3). Therefore, the inoculated quantity can be in g or mL. In this stage, the inoculated quantity is not standardized; the reactivation of the inoculum to be viable in the construction of the growth curve is important. Be careful about the possibility of using inoculum of improper age.
3. The initial concentration of inoculum should be similar to the study of the growth of yeasts in culture medium with the reality that occurs in food, which has a low initial microbial load.
4. Sample collection intervals depend on the concentration of the inoculum, usually in the first hours, in which the microorganism is in the lag phase of growth, samples are taken over a longer time, as the absorption increases gradually and the microorganism enters the log phase, samples are taken in short periods (15–30 min, for example), to have several points for the construction of the growth curve.

5. The glass cuvette and quartz cuvette are indicated for the analysis of liquid samples, and the glass cuvettes are indicated for when working in a visible region, while the quartz cuvettes are for the ultraviolet region with wavelength below 340 nm. Disposable plastic cuvettes are often used in rapid tests where speed is more important than high accuracy, but with the inconvenience of being used only once.
6. The wavelength depends on the color of the culture medium broth used, usually for media yellow use 580–595 nm, orange use 595–650 nm, generally for yeast suspensions use 540, 600, or 640 nm.
7. Dilutions are essential to be able to correlate the count of cell numbers with the absorbance, at the beginning of the growth curve, few dilutions are necessary; however, when yeast enters the exponential phase, the number of dilutions must always increase, to obtain plates with the number of countable colonies.
8. The stationary phase is reached when the absorbance readings on the spectrophotometer start to have constant values, with small variations.
9. Logarithmic transformations are done to mitigate the variation between the analyzed data and equalize the differences between the data.
10. Relatively large samples are needed for the measurements to be meaningful. This means that it is not possible to follow the growth of a microbial population from its initial masses, being necessary that the mass reaches a critical level.
11. Washing the cells before drying can cause loss of material.
12. The cell suspension pellet, free of supernatant, should be placed in a container of known weight, and weighing should be done until there is no change in weight (constant).
13. The yeast suspension tubes should be of similar diameter as the McFarland Equivalence Turbidity Standard.
14. Sterilize the entire wire tip by passing it at an angle through the flame of a gas burner until the entire length of the wire becomes orange from the heat to ensure absolute sterilization, including the shaft to remove any dust or possible contaminants. Cool the wire tip in the petri dish lid before obtaining the inoculum or touch the middle edge of the dish to avoid killing the cells and spreading the culture.
15. Touch only a single growth area with the inoculation wire to obtain the inoculum. Never drag the loop or needle across the surface and be careful not to dig into the solid medium.
16. Using instruments which use alternative light sources, such as scattered light, has not been validated.

17. The minimum inoculum concentration must be sufficient for the satisfactory development of the fermentation process of the target food.
18. Use screw-capped tubes with a volume of 1.5 mL or more, which are suitable to stock yeast in triplicate.
19. The final concentration of the glycerol obtained is 16% [13]. The proportion of glycerol and culture medium can also change. For example, you can add 50% yeast suspension and 50% glycerol, in this case, the glycerol solution concentration must be changed.
20. At every 6 months interval, the samples can be subculture to verify cell viability. For this, a scrape of the middle surface, still frozen, can be removed and transferred to a plate with culture medium agar, incubating it at 30 °C for 48 h.
21. Use 20% skimmed milk as a cryoprotectant, substances that protect cell structures during the period of freezing, thawing, and dehydration. Other cryoprotectant can be used such as glucose, trehalose, chitosan, etc.
22. Immediately after removing from the freezer, the ampoules must be placed in the freeze dryer in operation. The samples must not have a drop in temperature or thaw.
23. It can be stored in cardboard boxes at room temperature. Every 6 months, revitalize each sample in culture broth to check viability.
24. Storage in distilled water can be done at room temperature. For cell viability tests, the inoculum must be transferred to new culture medium every 3 months for the method of storage in distilled water and mineral oil, and every 30 days for maintenance on agar.

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Chapter 10

Enumerating Yeast in Foods and Water Using the Spread Plating Technique

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Abstract

Yeasts arouse great interest from the scientific community both for the application in the biotechnology field and for the ability of this microorganism to cause various degrees of food deterioration, being one of the main concerns of food hygiene and food safety. Microbiological examination of foods is predominantly based on culturing techniques to detect and enumerate living microorganisms. Each of these tests follows differentiated procedures, which, in turn, depend on the target microorganism. One of these techniques is the spread plate, which consist of adding a known volume of food sample on a petri dish containing a specific solidified, which will be covered in more detail in this chapter.

Key words Standard plate counts, Solid culture medium, Yeast detection, Yeast enumeration, Spoilage yeasts

1 Introduction

Yeasts are extremely important when considering biotechnological and industry applications. They are used for a long time in different industrial sectors and contribute to the production of various fermented foods, alcoholic beverages, bread making, dairy products, meat, cereal-based foods, and others. The probiotic activity observed in some yeast is another novel property that is attracting increasing interest [1, 2]. On the other hand, yeasts can also cause various degrees of deterioration and in foods and beverages, with major economic loss [3].

Yeast spoilage is favored in products with low pH, generally 5.5 or lower, products with high sugar (40–70%) or high salt (5–15% NaCl) content and by the presence of organic acids and other easily metabolized carbon sources. Yeast spoilage is often manifested by growth on the surface of products such as cheeses and meats. In addition, they are responsible fermentation of sugars in liquid

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and fluid products, such as fruit juices (concentrated or not), soft drinks, honey, jams and preserves, salad dressings, soy sauce, sugar syrups as well as cider and wines, causing formation of off-flavor compounds, loss of texture, gas production, and package swelling and shrinkage [3, 4]. Thus, the enumeration of yeast is one of the most significant criteria for the control of hygiene and for the prevention of deterioration in food and beverages [5, 6].

The isolation, enumeration, and identification of yeasts from foods and water follow the same principles and steps involved in the characterization of microorganisms, in general. These involve the sequential operations of rinsing or maceration of the sample, dilution of the suspension, enumeration of the yeast cells in suspension by agar plating, purification of isolates, and identification of isolates to either genus, species or strain level [7].

Yeasts in foods can be enumerated using the standard plate count method, and the results are expressed in number of colony-forming units per g or per mL (CFU/g or CFU/mL). Yeast enumeration tests use a variety of culture media, whose formulation varies according to the tests they are intended for. In general, the choice of culture medium that favors the growth of yeasts and inhibits the growth of bacteria and filamentous fungi is of great importance for the stages of isolation and identification [8].

The spread plate technique has advantages in relation of the pour plate technique (*see* Chapter 10), such as flexibility in handling, less harmful temperature effects to microorganisms, and easy to enumerate and to select colonies [9]. In this way, the sample is carefully applied over the solidified nutrient medium and spread using the glass or plastic rod. The use of the spread plate technique in food microbiology is reliable and widely used. In this chapter, an overview of methods for yeast enumeration using the spread plate technique is provided.

2 Materials

1. Prepare all solutions (media and reagents) using distilled, deionized, or of equivalent quality water.
2. Storage should be done in flasks made of inert materials, such as neutral glass or polyethylene.
3. Most of the culture media are sterilized by moist heat. However, there are heat-sensitive media components that should be sterilized by filtration.
4. Sterilization time depends on the size of load and containers. Excessive autoclaving time should be avoided to prevent Maillard reaction degradation and breakdown of medium constituents.

5. Add heat labile supplements with aseptic precautions to the cooled medium (45 °C) [10].
6. Equipment and supplies required to perform the spread plate must be controlled carefully to produce accurate yeasts counts.

2.1 Basic Equipment for Preparing Culture Media and Enumerating Yeasts [11]

1. Autoclave.
2. Sterile micropipettes (in a sort range of volumes).
3. Sterile pipette tips.
4. Glass or plastic petri dishes, sterile.
5. Bottles, flasks, and tubes, for heating and storage of culture media, and to prepare dilutions.
6. Vortex mixer.
7. Stomacher®.
8. Spreaders, made of glass or plastic, and ethyl alcohol for flame sterilization.
9. Cryovials.
10. Freezer storage boxes.
11. Bunsen burner.
12. Laboratory incubator, with temperature range able to set to the cultivation conditions.
13. pH meter.

2.2 Diluents [12] (See Notes 1 and 2)

1. Peptone water (PW) (0.1% m/v): Peptone 1 g, distilled or deionized water 1 L. Dissolve the peptone in the water, adjust pH to 7.0 ± 0.2 and sterilize at 121 °C for 15 min, 1 atm in an autoclave.
2. Butterfield's phosphate buffer (PB) (0.1 M, pH 7.0): Stock solution: Monopotassium phosphate (KH_2PO_4) 34 g.
3. Saline Solution (NaCl 0.85%): Sodium chloride (NaCl) 8.5 g, distilled water 1 L.

2.3 Culture Media (See Note 3)

Different culture media can be used for the specific yeast count in food and beverage, they can be classified according to Table 1. Their composition, pH that the media must be to be used, and directions to prepare are presented below.

2.3.1 Basal Media

Malt extract (MEA) agar (g/L): Malt extract (20), glucose (20), peptone (1), agar (15). Final pH 5.6 ± 0.2.

Potato dextrose (PDA) agar (g/L): Potato starch or potato extract (from 200 g potato infusion), dextrose (20), agar (15). Final pH 5.6 ± 0.2.

Yeast extract peptone dextrose (YPD) agar (g/L): Yeast extract (10), peptone (20), agar (15). Final pH 7.0 ± 0.2.

Table 1
Different culture media for yeast enumeration in food and beverages

Classe	Medium	Purpose	References
Basal	Malt extract (MEA) agar Potato dextrose (PDA) agar Peptone yeast extract (YPD) agar Sabouraud-glucose (SGA) agar Tryptone-glucose-yeast extract (TGY) agar Universal medium for yeasts (YM) agar	For the isolation and enumeration of yeasts from foods the use of general-purpose media which allow the recovery of all kinds of yeast	[13, 14, 15, 16]
Acidified	Acidified media can be made from one of the basal means above	Adjust the pH to 3.5, whose pH, inhibits bacterial growth. Acidification must be done before pouring the agar medium with an appropriate amount of HCl (0.1 N), sulfuric acid (0.1 N), or organic acids 10% (tartaric acid, lactic, citric) may be used as well	[17]
Biostatic agents and antibiotics	Basal medium plus, chloramphenicol, pimaricin, or chlortetracycline	Is suggested the use of two different antibiotics each in 100 mg/L concentration. Inhibits the growth of Gram-positive and Gram-negative bacteria	[15, 18, 19]
	Basal medium supplemented with dyes	Auramine-O (25 μ/m), gentian violet (5 μ/mL) and malachite green (1 μ/m) inhibit completely the growth of various fungal species.	[20]
	Dichloran Rose Bengal Chloramphenicol (DRBC) agar	The advantage of this medium is that the spreading growth of mold colonies is restricted. In addition, contain chloramphenicol for the inhibition of bacteria	[21–24]
	Oxytetracycline glucose yeast extract (OGYE) agar	Enumeration and cultivation of yeasts and fungi from foods	[25]
Selective	Dichloran 18% glycerol (DG18) agar	The final concentration of glycerol in the medium, 18%, reduces the value of a_w in the medium from 0.999 to 0.95 and favors the recovery of xerophilic yeasts such as *Zygosaccharomyces rouxii*	[26–28]
	Malt extract agar (MEA) with 30% glucose	Used to recover xerophilic yeasts from concentrated products	[15]
	Tryptone yeast extract agar (TGY) with 10% glucose	Is most suitable for enumerating *Z. rouxii* in a wide range of reduced aw foods	[14, 29]
	Basal medium (MEA or TGY) supplemented with 0.5% acetic acid	Spoilage yeast capable to grow in low acid and/or preservative-containing foods	[30]
	MYGP Copper Agar	Used for isolation and cultivation of wild yeasts in the brewing industry	[31]

(continued)

Table 1
(continued)

Classe	Medium	Purpose	References
Differential	Lysine agar	Useful medium to enumerate non-*Saccharomyces* yeasts when they are present in foods or beverages along with *Saccharomyces* species	[32, 33]
	Molybdate Agar	Isolation and differentiation of a variety of yeasts	[34, 35]
	Wallerstein Laboratory Nutrient Agar (WL)	Allows differentiation between commercial *Saccharomyces cerevisiae* from wild	[36, 37]
	CHROMagar Candida	Differentiation between *Candida* species	[38, 39]
	Dekkera/Brettanomyces Differential Medium (DBDM) agar	Able to efficiently recover *Dekkera/Brettanomyces* sp. from wine-related environments	[40]

Source: Adapted [45]

Sabouraud-glucose (SGA) agar (g/L): Glucose (20), peptone (1), agar (15). Final pH 7.0 ± 0.2.

Tryptone-glucose-yeast extract (TGY) agar (g/L): Tryptone (5), glucose (100), yeast extract (5), agar (15). Final pH 7.0 ± 0.2.

Universal medium for yeasts (YM) agar: Yeast extract (3), malt extract (5), peptone (10), glucose (20), agar (15). Final pH 7.0 ± 0.2.

2.3.2 Acidified Media

Acidified media can be made from one of the basal means above. Acidification must be done before pouring the agar medium with an appropriate amount of HCl (0.1 M), sulfuric acid (0.1 M) or 10% organic acids (tartaric acid, lactic, citric) (*see* **Note 4**).

2.3.3 Biostatic Agents and Antibiotics

Basal medium supplemented with chloramphenicol, chlortetracycline, or pimaricin 100 mg/L.

Basal medium supplemented with dyes Auramine-O Auramine (25 μ/m), gentian violet (5 μ/mL), and malachite green (1 μ/m) (*see* **Note 5**).

Dichloran Rose Bengal chloramphenicol (DRBC) agar (g/L): Glucose (10), bacteriological peptone (5), potassium phosphate monobasic (1), magnesium sulfate heptahydrate (0.5), chloramphenicol (0.1), rose Bengal (5% sol., w/v) (0.5 mL), dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol (1.0 mL), agar (15). Final pH should be 5.6 (*see* **Note 6**).

Oxytetracycline glucose yeast extract (OGYE) agar (g/L): Yeast extract (5), glucose (20), biotin (0.0001), oxytetracycline (0.1), agar (15). Final pH should be 7.0 ± 0.2 (*see* **Note 7**).

2.3.4 Selective Media

Dichloran 18% glycerol (DG18) agar (g/L): Peptone (5), glucose (10), potassium dihydrogen phosphate (KH_2PO_4) (1), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (0.5), dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol (1.0 mL), chloramphenicol (0.1), agar (15), glycerol (220). Final pH 5.6 ± 0.2 (*see* **Note 8**).

Malt extract (MEA) agar with 40% glucose (g/L): Malt extract (12), yeast extract (3), glucose (400), agar (15). Final pH 5.5 ± 0.2 (*see* **Note 9**).

Basal medium (MEA or TGY) supplemented with 0.5% acetic acid (g/L): Malt extract, powdered (20), glucose (20), peptone (1), agar (15). Final pH 3.8 ± 0.2.

MYGP copper agar (g/L): Malt extract (3), yeast extract (3), glucose (10), peptone (5), agar (15), Tween 80 (10 mL), $CuSO_4$ 1.95% (w/v) aqueous stock solution. Final pH 6.2 ± 0.2.

2.3.5 Differential Media

Chapter 11 shows some examples of differential culture media that allow characterizing different yeast colonies such as Lysine agar, Molybdate Agar, Wallerstein Laboratory Nutrient, and CHROMagar Candida (*see* **Notes 10**, **11**, and **12**).

Dekkera/Brettanomyces differential medium (DBDM) agar (g/L): Yeast nitrogen base 6.7 g, ethanol 6% v/v, cycloheximide 0.01 g, *p*-coumaric acid 0.1 g, bromocresol green 0.022 g, agar 20 g. Distilled water 1 L. Final pH 5.4 ± 0.2.

3 Method

3.1 Diluents (See Note 13)

3.1.1 Peptone Water (PW) (0.1% m/v)

1. Dissolve the peptone in the water.
2. Adjust pH 7.0 ± 0.2.
3. Sterilize at 121 °C for 15 min.

3.1.2 Butterfield's Phosphate Buffer (PB) (0.1 M, pH 7.0)

1. Stock solution: Dissolve the monopotassium phosphate in 500 mL of water.
2. Adjust the pH to 7.2 with 1 N sodium hydroxide (NaOH) solution (about 175 mL).
3. Dilute the stock solution to 1 L.
4. Sterilize at 121 °C for 15 min.
5. Leave to cool at room temperature.
6. Store in refrigerator.

3.1.3 Saline Solution (NaCl 0.85%)

Sterilize at 121 °C for 15 min.

3.2 Culture Media Preparation

3.2.1 Basal Media

Malt extract (MEA) agar, peptone yeast extract agar (YPD), Sabouraud-glucose agar (SGA), and Universal medium for yeasts (YM) agar:

1. Mix ingredients steam to dissolve agar.
2. Sterilize for 15 min at 121 °C.

Potato dextrose (PDA) agar:

1. Boil 200 g sliced, unpeeled potatoes in 1 L distilled water for 30 min.
2. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).
3. Mix in other ingredients and boil to dissolve.
4. Autoclave for 15 min at 121 °C.

Tryptone-glucose-yeast extract (TGY) agar (g/L):

1. Sterilize at 121 °C for 10 min (prolonged heating will cause medium browning).

3.2.2 Acidified Media

1. Adjusting the pH of the culture medium to 3.5 to 4-0 before pouring it into petri dishes.
2. Weigh the agar separately and dissolve it in 200 mL of distilled water.
3. Mix the other reagents in distilled water (350 mL) and adjust the pH.
4. Complete the item 3 volume with water to 800 mL.
5. Sterilize the two portions separately.
6. Join the two portions before pouring into the petri dishes.

3.2.3 Biostatic Agents and Antibiotics

Chloramphenicol is heat stable and can be added with other ingredients before autoclaving, hence its use is more convenient.

Antibiotics

1. It is suggested the use of two different antibiotics, each one in 100 mg/L concentration.
2. Prepare chloramphenicol stock solution: 0.1 g chloramphenicol in 40 mL distilled water.
3. Add this solution to 960 mL medium mixture before autoclaving (*see* **Note 14**).
4. Prepare chlortetracycline stock solution: 0.5 g of the antibiotic in 100 mL distilled water.
5. Sterilize by sterile filtration.

6. Use 10 mL of this solution for each 990 mL of autoclaved, cooled to 40–50 °C medium.
7. Refrigerate in the dark and re-use remaining antibiotic stock solutions for up to a month.
8. Stock solutions should be brought to room temperature before adding to cooled medium [41].

Biostatic Agents

Stock solutions of the dyes are prepared using distilled water. Because of the relatively low solubility of some dyes in water, they can be first dissolved in a minimal amount of 95% ethanol and then made to volume with distilled water.

DRBC Agar

Sterilize by autoclaving at 115 °C for 15 min.

OGYE Agar

1. Sterilize by autoclaving at 115 °C for 15 min.
2. Cool to 50 °C.
3. Aseptically add 0.1 g of the antimicrobial agent, oxytetracycline, to the medium at 50 °C. Mix well.

3.2.4 Selective Media

DG18 Agar

1. Mix the items and steam to dissolve agar.
2. Bring the volume to 1 L with distilled water.
3. Add 220 g glycerol (analytical reagent grade).
4. Sterilize by autoclaving at 121 °C for 15 min.

MEA Agar with 40% Glucose

1. Dissolve malt extract and agar in water by heating in a double saucepan.
2. Reduce heat to avoid caramelization and add glucose.
3. Stir until dissolved.
4. Sterilize by autoclaving at 115 °C for 15 min.

MEA or TGY Supplemented with 0.5% Acetic Acid

1. Sterilize by autoclaving at 115 °C for 15 min.
2. After cooling to 50 °C, add 5 mL glacial acetic acid per liter of medium.
3. Pour into plates immediately since the medium cannot be re-heated.

MYGP Copper Agar

1. Dissolve and mix all the reagents except $CuSO_4$.
2. Sterilize by autoclaving at 115 °C for 15 min.
3. Autoclave separately $CuSO_4$ 1.95% (w/v) aqueous stock solution.
4. Added $CuSO_4$ stock solution to this medium immediately before pouring the plates to give a final $CuSO_4$ concentration of 195 ppm.

3.2.5 Differential Media

DBDM Agar

1. Suspend 20 g agar in 500 mL distilled water.
2. Sterilize by autoclaving at 115 °C for 15 min.
3. All components should be sterilized by membrane filtration.
4. Sterile filter solution through an 0.45-μm absolute membrane into a previously sterilized container.
5. Mix the sterile-filtered nutrients with the sterilized agar just prior to pouring the plates.

3.3 Samples Preparation (See Note 15) [42, 43]

1. Homogenize the sample following the procedures described in Chapter 8 (*see* **Note 16**).
2. Aseptically remove the portions to be used for microbiological analysis.
3. Analytical units for general quantification of yeasts consists of 25 g or 25 mL of the sample, although alternative quantities can be used (10 g or 10 mL) (*see* **Note 17**).
4. To proceed with the analysis, the analytical unit must be diluted and homogenized with a suitable diluent, to allow inoculation into or onto culture media.
5. Liquid foods: transfer the analytical unit directly to tubes or flasks containing the amount of diluent necessary for a 1:10 dilution.
6. Solid or concentrated liquid foods:
   (a) Transfer the analytical unit to a sterile homogenization flask or bag.
   (b) Add to the sample the amount of diluent necessary to obtain a 1:10 dilution.
   (c) Homogenize the analytical unit with the diluent (*see* **Note 18**).

3.4 Serial Dilutions

After homogenization, prepare a series of decimal dilutions of the sample based on the estimated concentration of yeast in the sample [12, 43].

1. The primary homogenate of a food sample is generally prepared in a 1:10 ratio (10^{-1}), obtained by adding m grams or milliliters of the sample to $9 \times m$ (milliliters) of diluent (Fig. 1).
2. From the initial dilution (10^{-1}), make the other desired dilutions.
3. Select dilutions for the spread plate method so that the total number of colonies on the plate will be between 15 and 150 (*see* Subheading 3.6).

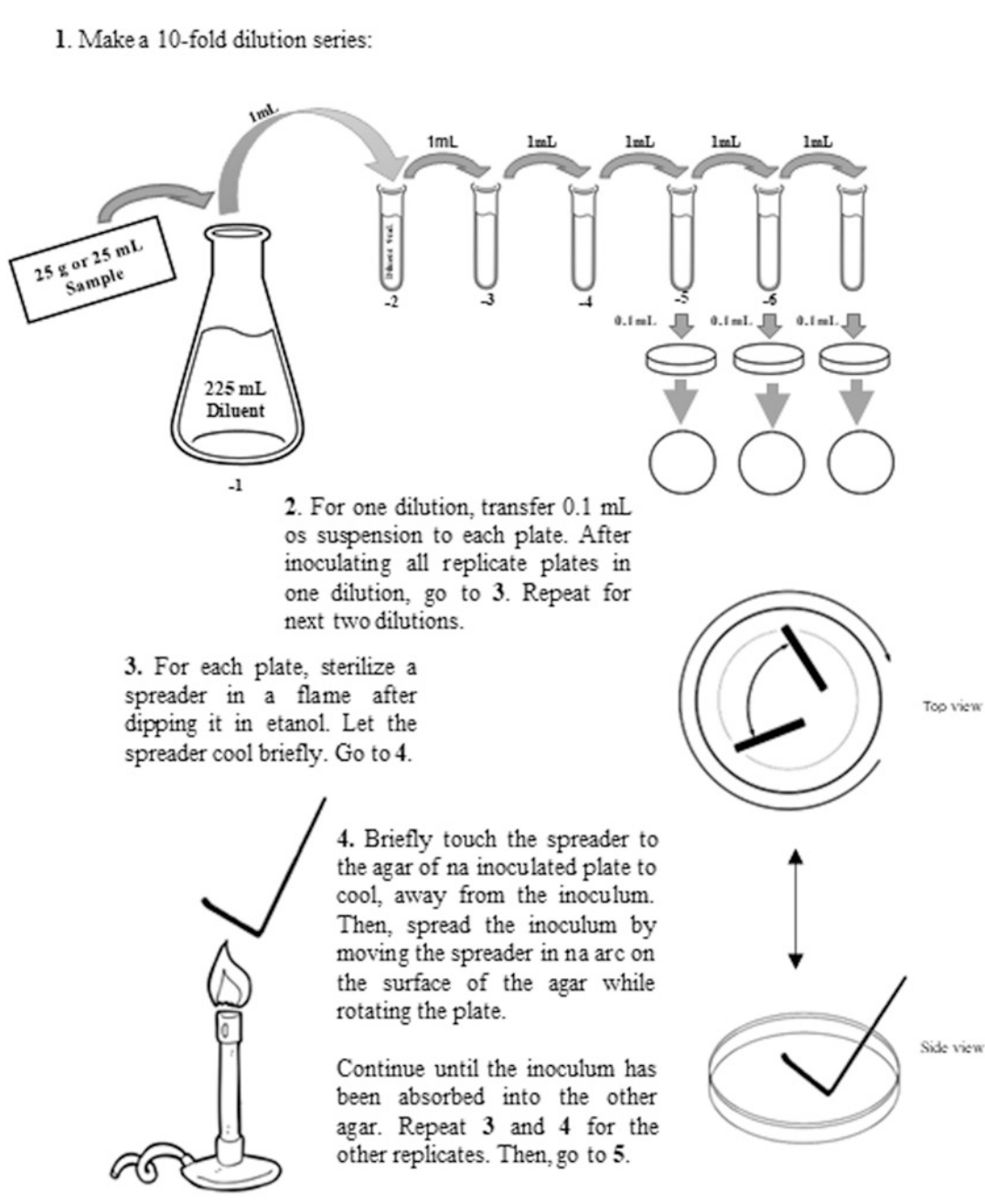

Fig. 1 Spread plate technique procedure for yeast enumeration

3.5 Enumeration [43, 44]

The basic procedure in the spreading technique consists of inoculating (0.1 mL) of the homogenized sample (and its dilutions) on to the agar plates already solidified and spread inoculum with a sterile glass or plastic spreader (Drigalski), followed by incubation of the plates until visible growth occurs (Fig. 1) (*see* **Note 19**).

1. Select three or more dilutions of the sample to be inoculated.
2. Using a pipette with a maximum holding capacity of 1 mL (and 0.1 mL graduation markings).
3. Inoculate 0.1 mL of each dilution onto the surface of previously prepared plates.
4. Verify whether the identification of the plate corresponds to the sample and dilution that are being inoculated and whether the plate contains the correct culture medium.
5. Work in a laminar flow cabinet or in the proximity of the flame of a Bunsen burner.
6. Carefully spread the inoculum onto the entire surface of the medium as fast as possible, using glass or plastic spreader (Drigalski), and continue until all excess liquid is absorbed.
7. Utilize a different spreader for each plate or, alternatively, flame sterilize the spreader after each plate, starting with the greatest dilution plate and going to the smallest dilution plates.
8. Let the agar dry for a few minutes, put the plates together.
9. Incubate them upside down. Incubate them inverted.
10. Let plates remain undisturbed until counting.

3.6 Incubation (See Notes 20–22) [4]

The incubation temperature for yeast enumeration varies from 25 to 28 °C, when the growth can be observed between 24 h and 120 h. For enumeration of mesophilic yeasts, it is recommended to incubate the plates at 28 °C for 72 h using basal media. The time × temperature relationship of incubation must also be considered according to the origin of the sample. Whether these samples are cold or chilled, colony formation should be evaluated at 15 °C for 7–10 days of incubation. Selective and differential media (Table 1) can delay the appearance of colonies, and growth should then be observed for 48 h.

3.7 Counting the Colonies and Calculating the Results [12]

1. At the end of the incubation period, examine plates for uniformity of colonies and lack of contamination.
2. For counting the colonies and calculating the results, select plates with 15–150 colonies (*see* **Note 23**).
3. Calculate the number of cells per g or mL of original culture (CFU/g or CFU/mL) using the Eq. 1.

$$\frac{\text{CFU}}{\text{g}} \text{ or } \frac{\text{CFU}}{\text{mL}} = n \times \frac{1}{\text{sample volume}} \times \text{D.F.} \quad (1)$$

where.

n: average of colonies in the plate's replicates for the same dilution factor.

sample volume: the volume, in mL, taken from the respective dilution to spread plating.

D.F.: dilution factor is the reciprocal of the chosen dilution used to count the colonies number.

4. As an example, the number of colonies resulting from a triplicate of a 10^{-6} dilution is:

   Plate 1: 30 colonies.
   Plate 2: 28 colonies.
   Plate 3: 32 colonies.
   $n = 30$ colonies.
   These arose from 0.1 mL of a 10^{-6} dilution, so D.F. is $\frac{1}{10^{-6}} = 10^6$.

$$\frac{\text{CFU}}{\text{g}} \text{ or } \frac{\text{CFU}}{\text{mL}} = n \times \frac{1}{\text{sample volume}} \times \text{D.F.} = 30 \times \frac{1}{0.1} \times 10^6 = 3.0 \times 10^7$$

3.8 Morphological Characterization [15, 45]

Due to morphological differences between the colonies of yeast species, the following factors should be included in a comprehensive colony description (Fig. 2).

Shape: Circular or irregular.

Size: Large (5 mm), moderate (2–5 mm), or small (2 mm).

Surface: Whether glistening or dull. Concentric, radial tripes, radial valleys, or granulated.

Edges: Plain, filiform, undulate, folded, serrated, lobate, or filamentous.

Side view: Convex, umbonate, crateriform, crateriform folded on center, convex and folded, concave, pseudomycellium, folded and flattened, flat or raised. Figure 3 shows colonies of some yeast species in YPD agar.

3.9 Purification and Maintenance of Yeast Culture [15, 45]

After the yeast enumeration process, it is necessary to purify and keep cells in pure culture before and after confirming your identity. Cultures must be pure for sequence analysis, growth tests and morphological examination.

1. For purification, streak the selected colonies onto the surface of suitable medium such as glucose-peptone-yeast extract agar or YM agar.

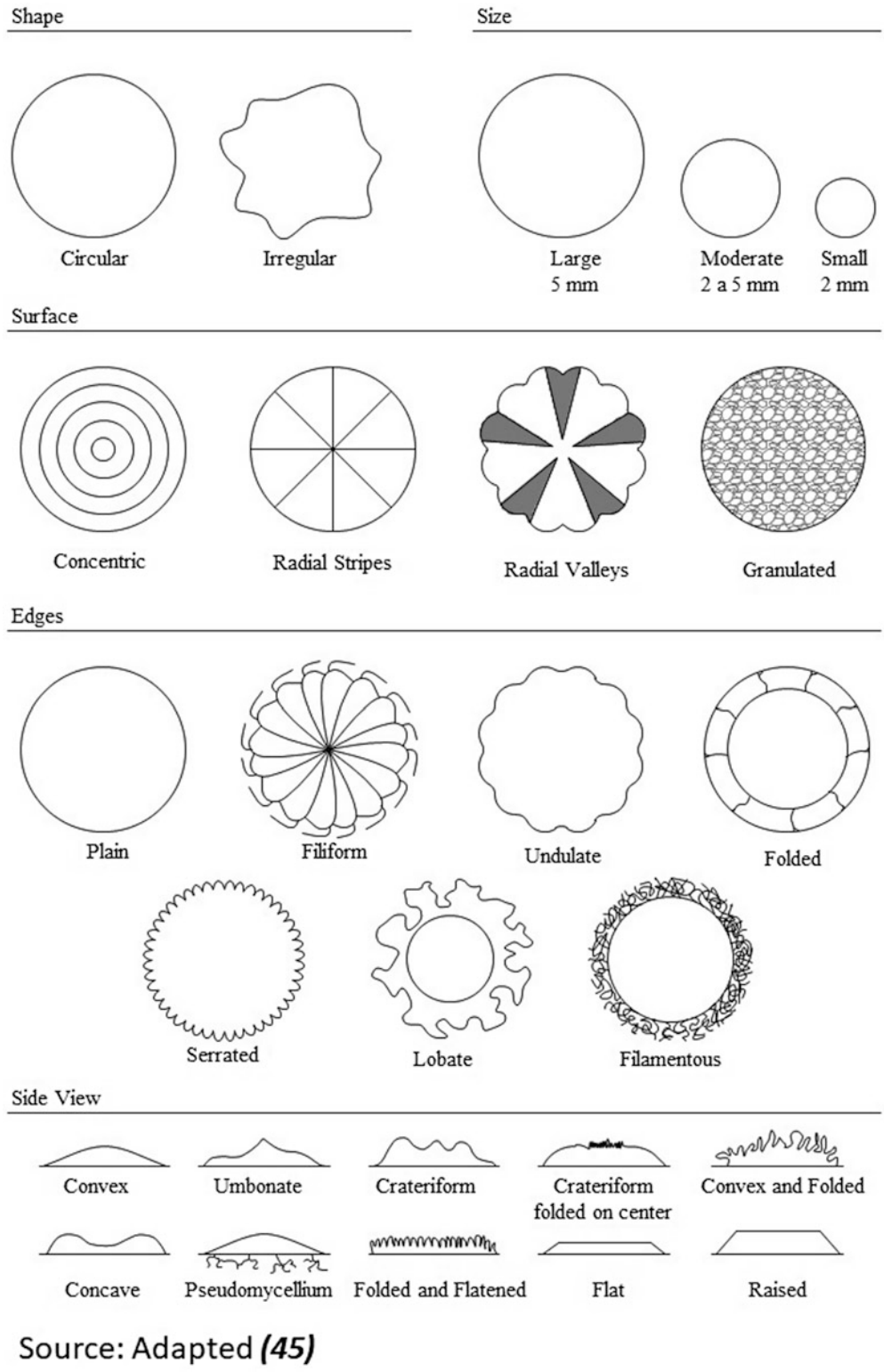

Fig. 2 Main morphologies of yeast colonies

Fig. 3 Morphology of colonies from different yeasts grown in YPD agar. (**a**) *Wickerhamomyces anomalus*; (**b**) *Kazachstania exigua*; (**c**) *Kodamaea ohmeri*; (**d**) *Yarrowia lipolytica*; (**e**) *Candida rugosa*; (**f**) *Schwanniomyces vanrijiae*; (**g**) *Candida ethanolica*; (**h**) *Pichia guilliermondii*; (**i**) *Saturnispora* sp.; (**j**) *Cyberlindnera saturnus*; (**k**) *Candida tropicalis*; (**l**) *Candida parapsilosis*; (**m**) *Schizosaccharomyces pombe*; (**n**) *Torulaspora delbrueckii*; (**o**) *Saccharomyces cerevisiae*; (**p**) *Hanseniaspora uvarum*

2. Incubate the plates at 28 °C for 24 ± 3 h.
3. Single, well-separated colonies of each form are selected and streaked again; twice is generally sufficient to obtain pure cultures, but it may be necessary to streak colonies several times.

Considering the completed isolation and purification steps, it becomes necessary to store the pure cultures. The maintenance of cultures can be performed in solid medium, liquid medium, freezing, or lyophilization (*see* Chapter 9 for more details).

4 Notes

1. It is important to use a diluent containing enough solute to minimize osmotic shock to fungal cells in high-sugar or high-salt foods when serial dilutions are made prior to plating.
2. In some cases, to favor the separation of cell aggregates, the use of surfactant agents such as polysorbate (Tween 80) (0.05% v/v) is recommended.
3. Heat the mixture in suitable containers (borosilicate glass or stainless steel) until ingredients are in solution and the agar is melted completely. The volume of agar and the type of container used should be such that no part of the contents will be more than 2.5 cm from the glass or from the surface of the agar.
4. Like most yeasts, they give strong growth under acid conditions (pH 3.0–7.0), with limiting values being around pH 1.5–2.5 and pH 8.0–8.5, at least for *Saccharomyces cerevisiae*.
5. An appropriate amount of each dye solution is added preferably to MEA before autoclaving. The final dye concentrations in culture media ranged from 0.25 to 5.000 ppm (0.25–5.000 μg/mL) depending on the dye assayed.
6. The advantage of this medium is that the spreading growth of mold colonies is restricted, allowing more accurate colony counts on crowded plates. Plates with this medium must be incubated in the dark to prevent formation of photo-induced inhibitors. However, some yeast and mold strains may be inhibited completely by rose Bengal if the medium is exposed to light. Stock solutions of rose Bengal and dichloran do not require sterilization and are stable for long periods. Caution: Chloramphenicol is toxic; skin contact should be avoided. To examine foods with a water activity greater than 0.95.
7. OGYE agar: The medium loses its bacteriostatic effect if incubated at temperatures greater than 25 °C.
8. Ideal media for foods with a water activity less than or equal to 0.95. In addition to chloramphenicol and dicloran, also contains glycerol, which reduces the water activity of the medium.
9. Some species are osmophilic (e.g., *Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygosaccharomyces rouxii*), diagnostic plating can include use of a high osmotic medium supplemented with 40% glucose. If these media are used, the sample diluent should be high osmotic as well, e.g., 0.1% peptone water with 40% glucose.
10. Lysine agar is a particularly useful medium to enumerate non-*Saccharomyces* yeasts when they are present in foods or beverages along with *Saccharomyces* species. It exploits the fact that

most *Saccharomyces* species cannot utilize lysine as a nitrogen source and will not form colonies on this medium. However, some *Saccharomyces* (e.g., *S. unisporus*, *S. kluyveri*), occasionally found in food ecosystems, can utilize lysine, and grow on this agar [33]. Generally, agar plating media are incubated at 25–30 °C for 2–7 days, after which colonies are examined.

11. Reliable counts for brewers' yeast are obtained with the medium at pH 5.5. Adjustment to pH 6.5 facilitates the counting of bakers' and distillers' yeasts. The time and temperature of incubation will vary according to the materials tested and the organisms sought. Temperatures of 25 °C are used for brewing materials and 30 °C for baker's yeasts.
12. Chromogenic medium must be stored in the dark.
13. Late plating of diluted samples should be avoided. Depending on the diluent used and the yeast in the diluted sample, the number of viable cells can be reduced by up to 30% of the initial population after a period of contact between the sample and the diluent.
14. When both chloramphenicol and chlortetracycline are used, add 20 mL of the above chloramphenicol stock solution to 970 mL medium before autoclaving.
15. The analytical unit is the amount of material withdrawn from a sample to be subjected to one or more tests. The number of analytical units that should be withdrawn and the amount of material of each analytical unit depend on the number and types of tests that will be performed on the same sample.
16. Withdrawing the analytical unit(s), the content of the sample should be well homogenized to ensure that the portion to be removed will be representative for the material as a whole.
17. Larger sample sizes increase reproducibility and lower variance compared with small samples.
18. Homogenization can be achieved by manual agitation, shaking the flask in an inverted position 25 times (concentrated liquids, soluble powders), agitation in a peristaltic homogenizer (Stomacher®) for 1–2 min (soft foods, pasty foods, ground or minced foods, poorly soluble powders) or in a blender (hard foods).
19. Spread plates, rather than pour plates, are recommended for both isolation and enumeration because the increased aeration of surface growth favors recovery and subsequent growth of the yeast cells.
20. In the absence of laboratory incubator, room temperature may be an alternative, however the required time should be extended to 7 days.

21. Incubation at 30 °C for 48–72 h for Lysine agar, WL Agar, molybdate agar, and at 37 °C for 48 h in CHROMagar Candida. Colonies are counted after 12 days in DBDM agar incubation at 25 °C.
22. Psychrophilic species have been isolated from refrigerated condiments, so isolation plates of these food products may need to be incubated at 5–10 °C for several weeks if low-temperature spoilage is occurring.
23. If all plates from dilutions tested show no colonies, report the CFU/g or CFU/mL as <1 times the lowest dilution.

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Chapter 11

Enumerating Distinct Yeast in the Same Food Sample

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Abstract

The enumeration of yeasts in food is widely performed in the field of microbiology. When more than one yeast species coexists in a food, it is interesting to enumerate and differentiate them, for which the use of selective and differential culture media can be used, making it possible to observe the growth, texture, color, shape, and size of the colonies. Another method that can also be used is the microscopy visualization of cells. This chapter aims to show different culture media that allow characterizing different yeast colonies and different techniques for observing cells under the microscope, in samples of food and beverages (which for convenience in this chapter we will call food from now on).

Key words Differential media, CHROMagar, Selective media, Optical microscopy

1 Introduction

The enumeration and differentiation of yeasts in food is a fundamental parameter that gets an idea of the degree of the presence and role of each species present in different situations such as fermented food, food contamination, food quality, and the influence of yeast metabolism in the final composition of the food. In addition, these parameters allow detecting spoilage yeasts, which are non-desired in the food and affect the organoleptic characteristics and quality of the final product.

Traditionally, there is a great interest in yeast identification at genus and species levels, which is accomplished based on tests for phenotypic characteristics (morphology plus physiological and biochemical tests) [1]. Currently, identification using methods that apply molecular techniques is the most used because it is faster and more reliable. However, basic phenotypic information is necessary to understand how yeast survives and grows in the food, as well as to differentiate them, and this information is achieved through

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simple techniques, such as plating in selective and differential media and the observation of cells morphotypes under a microscope [2].

The selective and differential culture media are based on several factors. They can provide different sources of carbon, nitrogen, or contain concentrations of compounds that can select and differentiate yeasts according to the size, texture, color, and shape of the colonies. Another sort of medium is the chromogenic one, based on the enzymatic interaction with a chromogenic substrate, in which the different yeast species grow with a characteristic color in the colony, due to compounds with different absorbance that are released when the chromogens are degraded by enzymes specific to each yeast [3]. Other methods can be used to guarantee the efficiency of yeast differentiation, such as microscopic aspects of the colony and cell morphologies, with the verification of the presence and type of spores, presence of hyphae, production of filaments, shape, and other structures.

The use of an optical microscope allows enumerating and differencing yeast species by the shape and size of their cells, as well as by the size and number of their reproductive structures (spore formation, budding, or binary fission) [3]. Microscope counting and observation are carried out mainly by using wet mount slides and by Neubauer chamber [4]. The combination of the use of selective and differential media and microscopy visualization allows distinguishing different species of yeasts that coexist in the same food sample, and it is a widely applicable practice. In this chapter, methods for enumerating different yeasts in the same food sample will be discussed; a summary can be viewed in Fig. 1.

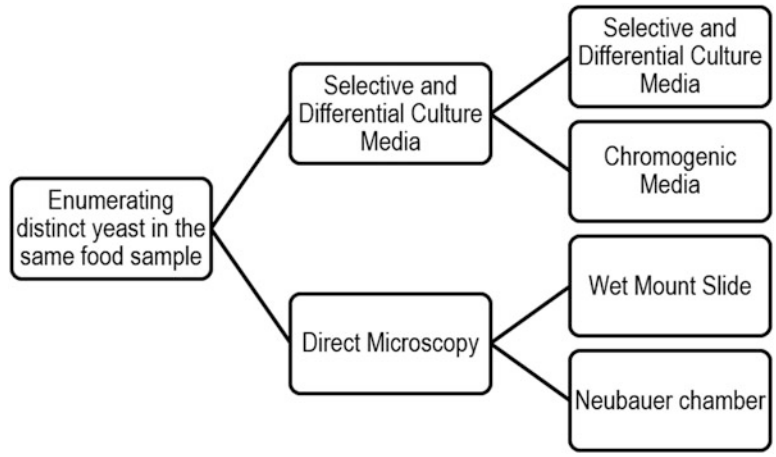

Fig. 1 Methods used for enumeration and differentiation of yeasts present in the same food sample

2 Materials

2.1 Selective and Differential Culture Media

1. Basic equipment and appropriate techniques for preparation of sample and dilution (*see* Chapter 8).
2. Equipment for plating samples (*see* Chapter 10).
3. Incubator (30 °C and 37 °C).
4. pH meter.

2.1.1 Medium (See Note 1)

Lysine agar composition (g/L, except for potassium lactate): glucose (44.5), potassium dihydrogen phosphate (1.78), magnesium sulfate (0.89), calcium chloride fused (0.178), sodium chloride (0.089), adenine (0.0018), DL-methionine (0.0009), L-histidine (0.0009), DL-tryptophan (0.0009), boric acid (0.000009), zinc sulfate (0.000036), ammonium molybdate (0.000018), manganese sulfate (0.000036), ferrous sulfate (0.00022), lysine (1), inositol (0.02), calcium pantothenate (0.002), aneurine (0.0004), pyridoxine (0.0004), p-aminobenzoic acid (0.0002), nicotinic acid (0.0004), riboflavin (0.0002), biotin (0.000002), folic acid (0.000001), potassium lactate (10 mL), agar (17.8), distilled water for 1 L of final volume.

Molybdate Agar composition (g/L): proteose-peptone (10), sucrose (40), phosphomolybdic acid (1.9), agar (15), distilled water for 1 L of the final volume.

Wallerstein Laboratory Nutrient Agar composition (g/L): bromocresol green (0.022), calcium chloride (0.125), casein enzymic hydrolysate or peptone (5), dextrose (50), ferric chloride (0.0025), magnesium sulfate (0.125), manganese sulfate (0.0025), monopotassium phosphate (0.55), potassium chloride (0.425), yeast extract (4), agar (20), distilled water for 1 L of the final volume.

CHROMagar Candida composition (g/L): peptone (10.2), chromogenic mix (5-bromo-4-chloro-3-indolyl/N-acetyl-b-D-glucosaminide and 5-bromo-6-chloro-3-indolyl phosphate p-toluidine salt) (22), agar (15), distilled water for 1 L of final volume.

2.2 Direct Microscopy

1. Optical microscope.
2. Slides or Neubauer chamber and 24 × 24 mm coverslips.
3. Filter paper.
4. Pipette.
5. Inoculating loop.
6. Immersion oil (if necessary).

Dye solution composition: methylene blue (1 g); distilled water (10 mL), sodium citrate, dihydrate (2 g).

3 Methods

3.1 Selective and Differential Culture Media

1. Add the components to sterile distilled/deionized water (*see* **Note 2**).
2. Gently heat and bring to boiling (*see* **Note 3**).
3. Mix thoroughly and adjust the pH (*see* **Note 4**).
4. Autoclavable media (Molybdate Agar and WL agar): Autoclave 15 min at 121 °C.
5. No autoclavable media (Lysine agar and CHROMagar Candida): Swirling or stirring regularly to prevent overheating.
6. Cool to 45–50 °C.
7. Pour into sterile petri dishes (*see* **Note 5**).
8. Make the respective dilutions of the food samples if necessary (*see* Chapter 8) (*see* **Note 6**).
9. Perform plating using the spreading technique (*see* Chapter 10).
10. Incubation (*see* **Note 7**).
11. Count the number of colonies and classify the different yeasts according to the reactions in the respective medium.

3.1.1 Lysine Agar

Many yeast species can grow in a liquid synthetic medium containing L-lysine as the main nitrogen source. On this medium pitching, yeasts are suppressed; the *Saccharomyces* yeasts are not supposed to grow on this media. Lysine agar is considered an excellent selective medium for estimating wild yeast contamination in commercial samples of beer, baker, wines, and other yeasts, as well as in large-scale fermentation and cell propagation [5]. Recent studies have used the Lysine medium to differentiate yeasts [6, 7, 8]. The characteristics of yeasts that grow in the Lysine agar are described in Table 1.

3.1.2 Molybdate Agar

In this medium, different species of yeast give rise to different colored colonies in the presence of molybdate, providing a count of different yeasts present in the same food sample [9]. An advantage of using this culture medium is that the growth of filamentous fungi can be completely inhibited with the addition of propionate [10] facilitating the differentiation and isolation of yeast colonies. The different yeasts according to the reactions on Molybdate agar are shown in Table 2.

3.1.3 Wallerstein Laboratory Nutrient Agar (WL)

The WL Agar differential medium contains 0.1% bromocresol green, which allows general discrimination among some yeast species based on colony color, size, and morphology [12]. The WL medium was initially developed to monitor the yeast community during industrial fermentation processes and is widely used to

Table 1
Appearance of yeasts according to growth on Lysine agar

Colony size	Species	Filaments	Other properties
0.6 mm	*Candida mycoderma*	Lobed, narrow fringe of closely filaments at the periphery	Semi-transparent border
1.5–2.0 mm	*Candida tropicalis*		Spiky, blastospore formation
2.5–3 mm	*Trichosporon cutaneum*	Long filaments	Irregular thickened center
1.5–3.0 mm	*Candida krusei*		Strongly scalloped margins
0.5 mm	*Torulopsis candida*	Very small branched filaments (fringe-like)	Colonies were often lobed
0.4 mm	*Rhodotorula mucilaginosa*	No filaments	Pink with a characteristic ovoid shape

Source: Adapted [5]

Table 2
Properties of yeast colonies on Molybdate agar

Macro colonial pigment	Species	Other properties
No extracellular opacity		
White, opaque, or colorless translucent	*Torulopsis glabrata*	No pseudo mycelia
	Cryptococcus neoformans	Mucoid colony
	Candida krusei	
	Candida mycoderma	Slow glucose fermentation
	Candida zeylanoides	
	Candida lipolytica	
	Candida brumptii	
	Candida rugosa	
	Trichosporon capitatum	Forms arthrospores
	Debaryomyces sp.	Forms ascospores
Green	*Candida curvata*	
	Candida parapsilosis	
	Candida albicans	Forms chlamydospores
	Candida catenulata	
Blue	*Candida pulcherrima*	Forms pulcherrima (ovoid to ellipsoidal in shape) cells
Increased extracellular opacity		
Green	*Candida tropicalis*	
	Saccharomyces sp.	Forms ascospore
Blue	*Candida macedoniensis*	
	Candida guilliermondii	
	Candida robusta	

Source: Adapted [11]

Table 3
Characteristics of yeasts according to their growth on WL agar

Macro colonial pigment	Species	Surface	Consistency
Green to cream-colored	*Saccharomyces* sp.	Knoblike, smooth, opaque	Creamy
Intense-green	*Hanseniaspora uvarum*	Flat, smooth, opaque	Buttery
Cream	*Zygosaccharomyces bailii*	Small, elevated dome, smooth	Creamy
Cream-less colonies with a hint of green	*Torulaspora delbrueckii*	Knoblike, smooth, opaque	Creamy
Bright green	*Saccharomycodes ludwigi*	Knoblike, convex, smooth, opaque	Creamy
Intense green	*Schizosaccharomyces pombe*	Small, smooth, opaque	Buttery
Red	*Rhodutorula* sp.	Knoblike, convex smooth, mucoid	Buttery
Cream with hint of red, red-brown from bottom	*Metschnikowia pulcherrima*	Small, convex	Floury
Gray-green with hint of blue	*Pichia membranefaciens*	Elevated, convex, wrinkled	Floury
Cream to blue-gray (blue after 8 days)	*Pichia anomala*	Flat, smooth	Creamy
Cream, appears after 8 days	*Brettanomyces intermedius*	Small, elevated to a dome, smooth	Creamy

Source: Adapted [15]

monitor yeast growth dynamics by enumeration of viable cells [6, 8, 13, 14]. Aspects of yeast colonies grown on WL agar can be seen in Table 3 and Fig. 2.

3.1.4 CHROMagar Candida

CHROMagar Candida can be used for the isolation and differentiation of *Candida albicans*, *C. krusei*, and *C. tropicalis*, yeasts that may be present in food (*C. albicans* present as a contaminant). The chromogenic substrates confirm yeast hexosaminidase and alkaline phosphatase activity, so the yeast cells were differentiated according to the color and the morphology of colonies on CHROMagar Candida [16]. The characteristics of yeasts grown on the CHROMagar Candida are described in Table 4.

3.2 Direct Microscopy

3.2.1 Wet Mount Slides

1. Make the dye solution (*see* **Note 8**).
2. If you want to analyze culture in a solid medium, place a drop of the dye solution on the slide (with an inoculating loop). This procedure is unnecessary if the sample is liquid. In this case, mix the dye solution with an equal volume of suspension containing yeasts, place a drop of the mixture on a slide and proceed to **step 5**.

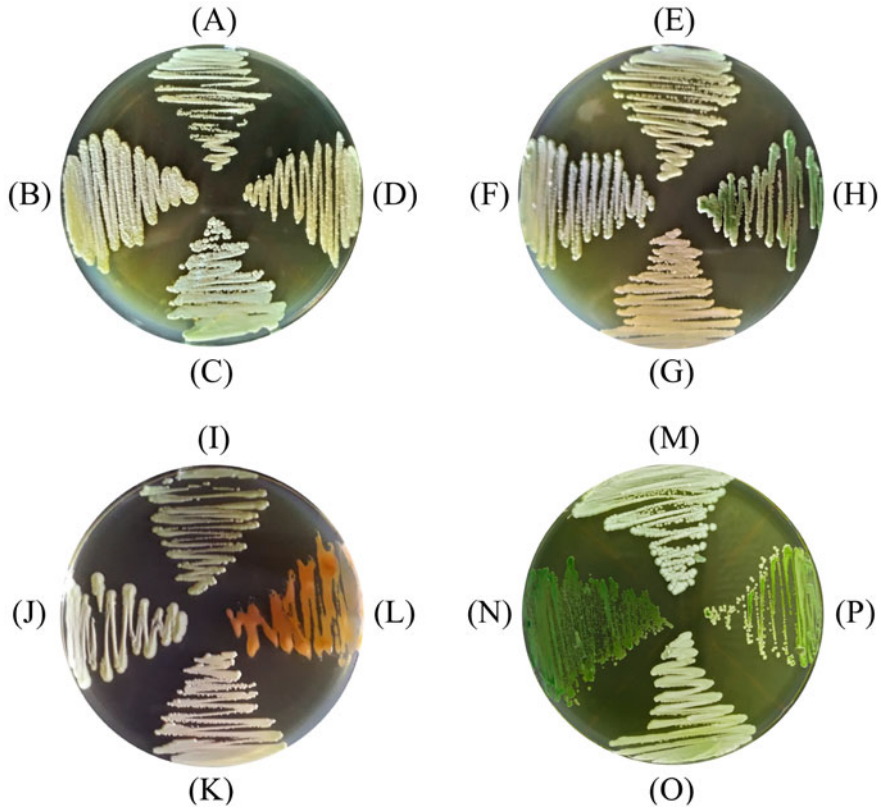

Fig. 2 Yeast colony morphology on WL agar (**a**) *Cyberlindnera saturnus*; (**b**) *Schwanniomyces vanrijiae*; (**c**) *Wickerhamomyces anomalus*; (**d**) *Yarrowia lipolytica*; (**e**) *Saturnispora* sp.; (**f**) *Candida tropicalis*; (**g**) *Candida rugosa*; (**h**) *Kazachstania exigua*; (**i**) *Torulaspora delbrueckii*; (**j**) *Pichia anomala*; (**k**) *Pichia membranifaciens*; (**l**) *Rhodotorula mucilaginosa*; (**m**) *Saccharomyces cerevisiae*; (**n**) *Hanseniaspora uvarum*; (**o**) *Zygosaccharomyces bailii*; (**p**) *Schizosaccharomyces pombe*

3. Sterilize the inoculating loop, let it cool, touch the top of a colony.
4. Mix the cells on the slide with the dye drop.
5. Place the coverslip over the drop (*see* **Note 9**).
6. Take to the optical microscope (*see* **Note 10**).
7. Examine approximately 1000 cells (*see* **Note 11**).
8. Consider viability as the percentage of unstained cells (*see* **Note 12**).
9. For differentiation, carefully analyze the cell morphology.

3.2.2 Neubauer Chamber

1. Mix 100 μL sample with the same volume as the methylene blue solution (*see* **Note 13**).
2. Homogenize the mixture by shaking and let it stand for 1 min.
3. Place the coverslip over the Neubauer chamber (*see* **Note 14**).

Table 4
Visual and microscopic aspects of yeasts grown on CHROMagar Candida

Macro colonial pigment	Species	Other properties
Apple green	*Candida albicans*	Chlamydospores; abundant pseudo and true hyphae
Dull blue, to purple color with pale pink edges	*Candida tropicalis*	Abundant pseudo hyphae with blastoconidia
White to pale pink	*Candida parapsilosis*	Clusters of blastospores
Pale pink colonies	*Candida krusei*	Large, flat, spreading, matt surfaces, pseudo mycelium
White large glossy pale pink to violet	*Candida glabrata*	No pseudo hyphae
Pink to purple	*Candida guilliermondii*	Small with Pseudo hyphae and blastospores
Pink gray purple	*Candida lusitaniae*	Branched pseudo hyphae present
White to light pink	*Candida famata*	No pseudo hyphae
Dark green	*Trichosporon* spp.	
Pink to off white cream	*Candida kefyr*	
Dark pink/violet	*Saccharomyces* sp.	Very small, pinpoint colonies

Source: Adapted [16, 17]

4. Homogenize again and collect an aliquot of the solution (sample diluted in Methylene Blue).
5. Fill the camera using a micropipette (*see* **Note 15**).
6. Wait 2 min for the cells to settle in the chamber.
7. Perform the count on the four quadrilaterals located on the sides and the one in the middle of the chamber (*see* **Note 16**) (Fig. 3).
8. Calculate the cell concentration (Cel/mL) from the equation:

$$\text{Cel/mL} = \sum \text{cells} \times 25 \times \text{FD} \times 10^4/N.$$

where

Σ cells: the sum of the number of viable (or total) cells counted in the *n* grid (*see* **Note 17**).

25: total number of chamber squares.

FD: dilution factor (*see* **Note 18**).

10^4: chamber constant (*see* **Note 19**).

N: number of squares counted among the 25 available (5 recommended, **step 7**) [4].

Yeast cells have about 5 μm diameter, and most important features can be seen in a light microscope, it is a quick practice that allows enumerating and differentiating yeasts, observing the

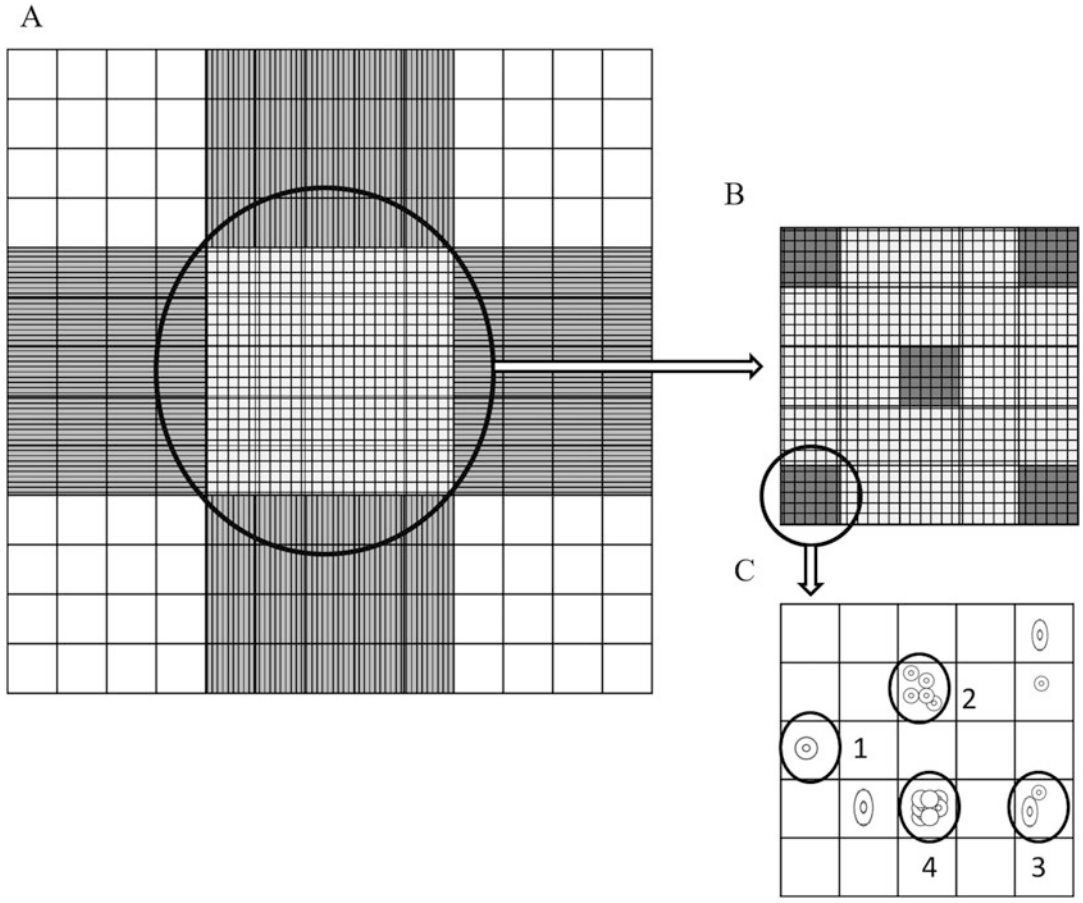

Fig. 3 Schematic drawing of the Neubauer chamber. (**a**) Details of the central quadrant; (**b**) Details of the 25 squares of the central quadrant used for yeast counting, the extreme four ones and the central squares (dark gray) are suggested as sampling during the count; (**c**) Details of one of the quadrants to be counted, 1: single-cell; 2: cluster with cells easily distinguishable by their nuclei and cytoplasm; 3: yeast budding; 4: cluster, whose cells are difficult to distinguish from each other

physiological state of the cells, evidence of contamination, and the presence of yeasts of interest in food [18]. The visual features most used to differentiate yeast in microscopic observations are shown in Table 5.

4 Notes

1. Ideal media for detecting and enumerating yeasts should suppress the bacterial and filamentous fungi growth, so antibiotics and some antifungal compounds (chloramphenicol, rose Bengal, dichloran, propionate, and others) can be added to the yeast media to inhibit bacterial and filamentous fungi presence.

Table 5
Yeast cell morphology by microscope observation

Yeast species	Shape of the cell	Sexual structure	Other properties
Brettanomyces bruxellensis	Ovoid; ellipsoidal; cylindrical to elongate	Multilateral budding	Pseudo hyphae abundantly
Candida zemplinina	Ellipsoid to elongated multilateral	Budding	Cells occur singly or in pairs
Kloeckera apiculata	Apiculate; ovoid; elongate	Bipolar budding	Cells occur singly or in pairs
Lachancea thermotolerans	Spherical to ellipsoidal	Multilateral Budding	Spherical ascospores 1–4 spores per ascus
Metschnikowia pulcherrima	Globose to ellipsoid	Multilateral Budding	Acicular to filiform Ascospore (sphaeropedunculate) 1–2 spores per peduncle
Candida stellata	Ovoid to elongate	Multilateral Budding	Spherical ascospores (1 per ascus)
Saccharomyces cerevisiae	Globose; ovoid; elongate	Multilateral Budding	Globose to short ellipsoidal ascospores 1–4 spores per ascus
Saccharomyces ludwigii	Lemon shaped, elongated with a swelling in the middle	Bipolar budding	Spherical and smooth ascospores 4 or 2 spores per ascus
Schizosaccharomyces pombe	Globose; ellipsoidal; cylindrical	Fission	Globose to ellipsoidal ascospores 2–4 spores per ascus
Torulaspora delbrueckii	Spherical to ellipsoidal	Multilateral Budding	1–4 spores per ascus Conjugation tubes
Wickerhamomyces anomalus	Spherical to elongate	Multilateral Budding	Hat-shaped ascospores (1–4 per ascus), Asci are deliquescent

Source: Adapted [19]

2. For the Lysine agar: first, dissolve the potassium lactate in distilled water and, then, add the other ingredients. For the Molybdate agar: first, it is necessary to make a phosphomolybdic acid solution that will not go into the autoclave: Add 12.5 g of P_2O_5'20MoO_3 (phospho-12-molybdic acid) to 100 mL of sterile distilled/deionized water. All other components of the base agar must be autoclaved before adding 15 mL of phosphomolybdic acid solution in 985 mL of the cooled sterile base.
3. Heating can be done in a steam flow using an autoclave with temperature not exceeding 100 °C, or in a microwave oven (set the microwave oven power to 60% for a safer operation). After the initial boil, remove the medium from the autoclave or the oven, rock gently with your hands (use thermal gloves), and

then return to the autoclave or oven. Repeat bursts of heating until the complete fusion of the agar (visibly large bubbles will replace the foam).

4. Adjust pH final before pouring into petri dishes to 4.8 ± 0.2 (Lysine agar and CHROMagar Candida); 5.3 ± 0.2 (Molybdate agar); Adjust pH before autoclaving to 6.5 ± 0.1 (WL agar).
5. To remove surface moisture of prepared medium drying at 37 °C, store the prepared medium at 2–8 °C. The chromogenic medium must be stored in the dark.
6. In the Lysine agar, the number of cells in the inoculum is essential, small numbers of cells (approximately 100 to 1000) still grow to a limited extent on the medium. When the number of yeast cells exceeds 10,000, a count of the colonies in the medium provides a direct measure of the yeasts count.
7. Incubation at 30 °C for 48–72 h for Lysine agar, WL agar, Molybdate agar, and at 37 °C for 48 h in CHROMagar Candida.
8. Dissolve the methylene blue in distilled water. Add sodium citrate (dihydrate) and stir until completely dissolved. Filter with a filter paper and add 100 mL of distilled water to the filtered. Other dyes can be used, for example, trypan blue and violet methylene.
9. Take care not to press the material biological, and this can make visualization difficult or damaged cells.
10. For microscopic yeast count, daughter cells less than half the size of the parent cell are not counted. The concentration of cells to be analyzed should be in the range of 40–60 cells per microscope field. Make the respective dilutions if necessary.
11. Place the preparation under the microscope and focus with the lowest magnifying lens first, using the coarser adjustment and then use the fine adjustment. Move successively to the highest magnification, use the 100× objective lens, if necessary, place on top of the area to observe a drop of immersion oil, and adjust the focus. Do not use immersion oil with any other objective lens.
12. The methylene blue dye (vital dye) stains nonviable cells in blue. To precisely evaluate the cell viability, it is recommended to perform plate counting since the methylene blue method is based on the presence of specific enzymes, and these enzymes may be present in cells that do not reproduce so fast or even some non-viable cells not dead indeed. For a better and reliable result, the counting of cells stained with methylene blue must be performed after 60 s so that the dye is absorbed, and up to 10 min after contact to avoid cell toxicity.

13. If necessary, dilute the sample. Ideally, between 20 and 60 cells per chamber quadrant favor the counting process. The dilution can be done with the dye solution itself or previously in sterile distilled water. Take the dilution in account to do the math.
14. For the fixation moisten the two lateral channels existing among the counting area in the chamber (quadrilateral).
15. Place the pipette or capillary close to the space between the Neubauer chamber and the coverslip and fill the entire area corresponding to the chamber's quadrilateral.
16. Perform the count with 400× magnification, counting the cells (total and viable) present in 5 of the 25 squares, in the central quadrant of the chamber (Fig. 3a), the four quadrilaterals located on the sides of the chamber and from the middle one (Fig. 3b). Adopt the technique of always counting in one direction, observing the existence of cells located in the line that divides the smaller squares. Viable cells in the sample show little or no staining, while nonviable cells are stained in blue.
17. Enumerate cells with a very visible nucleus (Fig. 3c): count isolated cells as one cell (1); counting cluster made up of cells easily distinguishable by their nuclei and cytoplasm as groups of isolated cells and counting each cell (2); daughter cells less than half of the size of the parent cell are not counted (3), cluster, whose cells are difficult to distinguish from each other, should be counted as a single group (4).
18. Example of calculated dilution factor (FD): 100 μL of sample added with 900 μL of sterile distilled water (tenfold dilution), then 100 μL of this dilution is mixed with 100 μL of dye solution (twofold dilution), obtaining a dilution factor equal to 20.
19. The value of the chamber constant refers to the inverse of the central quadrant volume used for counting; the quadrant volume is 0.1 mm^3, which is equivalent to 0.0001 mL (10^{-4} mL).

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Chapter 12

Detection and Quantification of Yeast Species in Food Samples for Quality Control

Cíntia Lacerda Ramos and Karina Teixeira Magalhães-Guedes

Abstract

Yeasts grow in a high range of environmental conditions, which make them potent contaminants of foods and beverages. Thus, detect and quantify yeast species are essential for quality control of the foods. The isolation, enumeration, and identification of yeasts from foods and beverages follow the same principles and strategies that are used for yeasts in general. Different approaches have been used to enumerate yeast species, including traditional methods based on physiological characteristics, molecular characterization, and protein profile evaluation of microorganisms.

Key words Physiological identification, Specific pair primers, PCR, qPCR, MALDI-TOF, Yeast spoilage, Culture-independent method

1 Introduction

Foods and beverages are spoiled when they are no longer pleasing the consumer's eyes and taste. Generally, they have an undesired odor, appearance, taste, or texture, or combinations of these defects. Microbial spoilage, especially by yeast, can occur at any stage throughout the production chain from the raw material, harvest, during handling and processing, to the final packaged product. The consequences of a spoilage outbreak can be impacting [1–3] and include:

- Economic loss due to wasted product or product with inferior quality and value.
- Losses with product disposal.
- Costly litigation and forensic investigation to determine cause, liability, and compensation, in the event of a massive outbreak.
- Adverse impact on company reputation and brand image.
- Effect on human health, causing pathologies.

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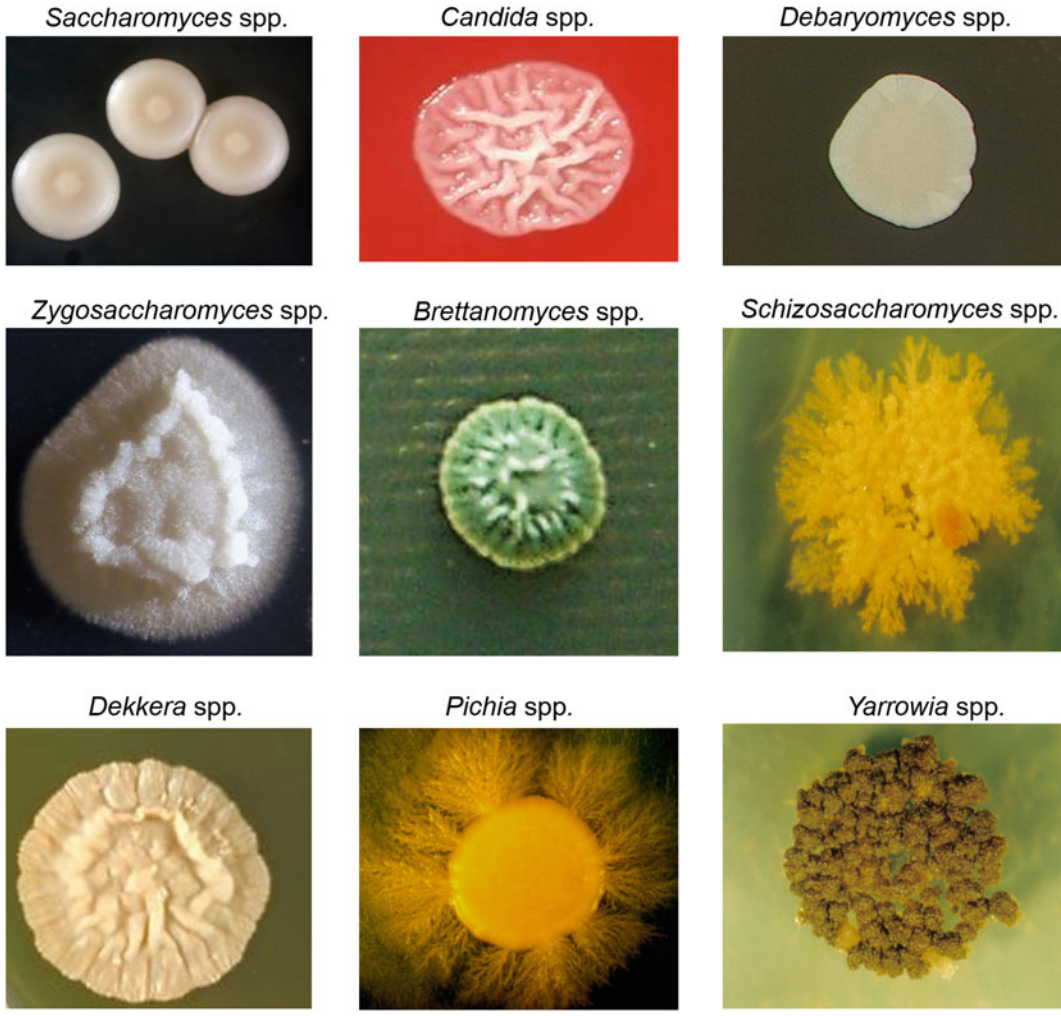

Fig. 1 Colony morphology of the main yeast genera associated with spoilage under different growth conditions

Although bacteria and molds (filamentous fungi) are the most frequently perceived organisms of spoilage, yeast damage is also significant. Generally, food containing high acid concentration (low pH), high sugar or salt contents, products preserved with weak acids, and long-term frozen products are prone to spoilage by yeasts. While a great diversity of yeast species can be isolated from foods and beverages, only a few yeast genera are frequently associated with spoilage outbreaks (Fig. 1). Food and beverage spoilage by yeasts is well documented in the scientific literature, and it is addressed from ancient times to recent times [1–8].

Spoilage is a result of yeast growth in the product, and a vast array of metabolites are formed. In this way, the characteristics of the food, such as chemical, physical, and sensory parameters, are changed. Carbon dioxide (gas) production by yeasts causes the

containers of packaged products to swell and eventually explode as a consequence of the internal pressures increase, which can be as high as 5 atm. As yeasts grow on the surfaces of solid products, they become visually evident as individual colonies, or as a film of dull, dry, or slimy biomass. In liquid products, yeast growth may occur as a film of biomass that floats on the surface of the product or as sediments of cells within the product. These visual signs of spoilage are usually accompanied by the development of off-odors and off-flavors in the product [2, 9]. These defects become noticeable when yeast growth reaches approximately 10^5 cells or colony-forming units (CFU) per gram or milliliter and are evident at 10^7 CFU/g or mL.

The ubiquitous occurrence of yeasts in the environment ensures that most foods become contaminated with yeasts during different stages in the production chain. The main classes of food that suffer spoilage by yeast are as follows [2]:

Meat products. Freshly slaughtered beef, lamb, pork, poultry, and seafood meats harbor low populations (10^1 to 10^3 CFU/g) of yeasts that generally represent about 5–10% of the total microbiota. Yeasts grow during the storage of meats at 5 °C.

Dairy products. Yeasts are found in raw and pasteurized milk at 10^1 to 10^3 CFU/mL. However, their growth is limited under refrigeration, as psychotropic bacteria quickly overgrow them. Nevertheless, milk is an adequate substrate for their growth, and in the absence of bacterial competition, they reach populations of 10^8 to 10^9 CFU/mL. Fermented dairy products are prone to spoilage by yeasts due to their higher acidity, which restricts bacterial competition. Yogurts undergo gaseous, fermentative spoilage, usually from yeast contaminants that originate from raw materials, such as fruits, and from ineffective cleaning and sanitation of processing equipment.

Vegetable products. Vegetables are more prone to spoilage by bacteria than by yeasts. It is due to the proteinaceous nature and neutral pH of vegetable tissues. However, some reports about yeast species causing problems in tomatoes have been described. Generally, yeast spoilage is more frequently related to fermented vegetables, such as the pink discoloration of sauerkraut.

Fruit products. Yeasts are present as part of the surface microbiota of healthy, undamaged fruits at populations of 10^1 to 10^3 CFU/cm^2. By damaging the structural integrity of fruits, their sugary and acidic tissues are exposed, which are excellent substrates for fermentative yeast growth, causing eventual product spoilage. Fermentative spoilage yeast may also spoil processed fruit products, such as fruit juices, juice, and pulp concentrates, canned fruits, dried fruits, glazed fruits, ready-to-

eat fruit slices, and fruit salads. Fruit and fruit products are ingredients used in the elaboration of other commodities such as yogurts, jams, syrups, pies, cakes, and several beverages, and they can be a primary source of yeast contamination and spoilage of these products.

Nonalcoholic beverages. Carbonated and noncarbonated soft drinks, energy beverages, sports drinks, and various health beverages are typical nonalcoholic beverages. These beverages are characterized by the presence of approximately 10% carbohydrates as well as having ingredients that include fruit juices, flavors, organic acids (e.g., citric, malic, lactic, and acetic acids), vegetable extracts, colorants, antioxidants, vitamins, and carbonation. Their sugary, acid (pH 3.0–3.5) and relatively oxygen-free conditions make them highly susceptible to fermentative yeast spoilage.

Alcoholic beverages. These products present another specialized habitat for yeast spoilage. Ethanol tolerance and the ability to grow at low pH values are the fundamental properties that enable yeasts to grow and spoil these products selectively. Bottled beers that have been appropriately processed by filtration and/or heat pasteurization rarely undergo spoilage. However, wines are more likely to spoilage.

Bakery products. Although yeasts are primarily responsible for the fermentative production of bakery products (e.g., the baker's yeast *Saccharomyces cerevisiae*), they can also cause spoilage of these products. During storage, bread can develop alcoholic, fruity, and acetone-like off-flavors, and some may show visible yeast growth as white, chalky, or other colored spots.

Products with high contents of sugar or salt. Foods containing high concentrations (40–70%) of sugar such as sugar cane, sugar syrups, molasses, honey, malt extract, fruit juice concentrates, jams, jellies, confectionery products, and dried fruits are prone to yeast spoilage.

Prevention of yeast spoilage requires good manufacturing practices, including the hygienic practices, the application of the principles of Hazard Analysis Critical Control Point (HACCP) analysis, or other quality management programs. Prevention and minimization of contamination are, therefore, essential requirements in the management of yeast spoilage. Raw materials and ingredients used in food and beverage processing should be free of yeast contamination through the adoption of appropriate specifications. Effective cleaning and sanitation of equipment and process lines to eliminate yeast contamination is another essential requirement. Routine monitoring of end products to ensure that they conform to appropriate specifications is usually part of an overall strategy to manage yeast spoilage [1, 2, 4–6].

Generally, the ecological principles associated with the occurrence and growth of microorganisms in foods, are also applied to yeast spoilage of foods. For a better understanding and management of food and beverage spoilage by yeasts, a systematic process of investigation and knowledge acquisition is required, and this information includes isolation, enumeration, and taxonomic identification of the species and strains responsible for the spoilage [1–8]. These involve the sequential operations, also called as culture methods. Start by rinsing or maceration of samples, followed by dilution and enumeration of yeast cells [4, 10]. Over recent decades, the classical methods for microbiology and physiology studies have been, in part, replaced by sophisticated methods such as polymerase chain reaction (PCR)–based DNA techniques, fluorescent microscopy, and cytometry flow. These methods are known as uncultured methods [5–7].

The most frequent procedure for the enumeration of yeast is viable plate counting. In this method, samples are serially diluted and plated onto a suitable growth medium. The diluted samples may be spread onto the surface of agar plates, or mixed with molten agar, poured into plates, and allowed to solidify. Then, the plates are incubated under proper conditions that permit yeast reproduction so that colonies develop and can be observed without the aid of a microscope. It is assumed that each yeast colony arises from an individual cell that has undergone cell division. By counting the number of colonies and considering the dilution factor, the population of yeasts in the original sample can be determined. Specific yeasts can be tolerant or intolerant of various conditions or chemical compounds. Also, they may be able to use specific carbon or nitrogen sources. These properties can be used to obtain information about the possible identity of an unknown yeast, by observing yeast growth on plates containing various additions and different conditions. The information gained from the differential plating can be associated with information on the yeast morphology observed through microscopy, to reach a tentative identification, or at least to eliminate some possibilities [4]. Although the use of a combination of physiological tests is laborious and time-consuming, it is traditionally employed for yeast identification in food samples. For this reason, a protocol is described in this chapter.

A simple method for yeast enumeration is the direct yeast cell counting. Direct microscopic counts are performed by adding a known volume of the sample over a specific area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Counting chambers, such as the Petroff-Hauser and Levy counting chambers, are commonly used to perform the direct counting because they are produced with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area and convert the numbers observed directly to volume makes the direct

enumeration procedure relatively straightforward. Direct counting procedures are rapid and used to assess the sanitation level of a food product [4]. This method may be used to count total yeast in the samples; however, it is not possible to distinguish among different yeast species.

Another method used for the quantification of yeasts is carried out by cultivation in liquid medium and characterization of the produced cell biomass. Cell mass may be determined by weighing whole cells; biomass can be correlated with cell numbers by reference to a standard curve. Wet weight or dry weight of yeast may be used for the estimation of cell numbers. Microbial biomass is estimated by measuring constant biochemical components of microbial cells, such as protein, adenosine triphosphate (ATP), lipopolysaccharides, peptidoglycan, and chlorophyll. Biomass can also be estimated by measured turbidity that can then be correlated with cell numbers by reference to a standard curve [4]. These methodologies are employed as indirect ways of yeasts' quantification in food samples; however, as mentioned for direct microscopic counts, these tools do not allow to distinguish different yeast species in a sample.

The use of molecular DNA-based methods of yeast detection and enumeration has increased in recent years due to their specificity, rapid identification, and independence of the metabolic state of the cell. Individual yeast species may be quickly determined from comparing the nucleotide sequence of domains 1 and 2 (D1/D2) of the LSU rRNA gene or internal transcribed spacer (ITS) obtained by sequencing analysis with those deposited in databases of DNA sequences [11, 12]. Some laboratories perform DNA sequencing or have contracts with companies that perform it for them. However, this is not the case for many laboratories; thus, other molecular methods can be employed for yeast identification. Species-specific primer pairs, random amplified polymorphic DNA (RAPD), repetitive element palindromic (rep-PCR), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs), pulsed-field gel electrophoresis (PFGE), and others are some examples of alternative methods. The use of species-specific primer pairs is a simple method and may be useful to identify a target and known species as the case of some spoilage yeast species [13–17]. The protocol of this technique is detailed in this chapter. In practice, it consists of a PCR, followed by separation of amplicons by gel electrophoresis and verification of the band presence, which characterizes the target species. However, the inclusion of several species-specific primer pairs in the same PCR may provide an unclear profile of bands.

A technique that has been successfully employed for microbial identification, including yeast species, is the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). This method is simple and rapid and consists of the extraction of abundant structural proteins such as ribosomal proteins

from microbial colonies. The ionizing laser vaporizes these extracted proteins with mass-to-charge ratio (m/z) peaks with varying intensities, generating mass spectra. The obtained spectra for each isolate are compared with those available in a reference database for microbial identification. Unknown yeasts can be identified by matching their spectrum to the most similar spectrum in the database. The MALDI-TOF software provides scores that allow us to identify microbial genus and species with accuracy. This method has been used for yeast identification [18–20], and a protocol is proposed in this chapter.

Fast and efficient techniques can be used to detect spoilage yeasts in food by uncultured methods. Fluorescence microscopy procedure for quantitation of viable and nonviable yeasts in beverages and other liquid samples eliminates the need for incubation time, thus reducing the analytical time required. Total yeasts can be counted; however, different yeast species in the same sample are not detected. An advantage of this technique is that living and dead yeast cells can be differentiated [5].

Flow cytometry (FCM) is a technique used to detect and measure the physical and chemical characteristics of a population of microbial cells (bacteria and yeast). In this process, a suspension of cells (bacteria and yeast) is injected into the flow cytometer equipment. The sample is focused to ideally flow one cell at a time through a laser beam, where the light scattered is characteristic to the cells and their components. Microbial cells are often labeled with fluorescent markers, so light is absorbed and then emitted in a band of wavelengths. High number of microbial cells (bacteria and yeast) can be quickly examined, and the data gathered are processed by a computer. Specific fluorescent markers for yeast species may be used, allowing to evaluate specific species [6].

The real-time PCR is well established internationally as a method in brewing microbiology, primarily concerning the detection of beverages spoilage bacteria/yeast [21, 22]. Large wine and brewing companies, the central laboratories of beverages groups, and commercial service laboratories use real-time PCRs for the detection and identification of beverages spoilage bacteria/yeast and, to some extent, also for wild yeast as well as wine/brewing yeast. Real-time PCR provides a rapid and reliable means for identifying and differentiating *Saccharomyces* and non-*Saccharomyces* wine/brewing species. Real-time PCR can be used to identify single unknown yeast strains at the species level. It can also serve as a tool for finding trace contaminations in mixed populations at concentrations of one contaminating cell in 1000 culture yeast cells (e.g., one cell of *Saccharomyces cerevisiae* in 1000 cells of *Saccharomyces pastorianus* ssp. *carlsbergensis*). Identifying the correct species to which a wine/brewing yeast strain belongs can rapidly be carried out [7]. Due to its high applicability for yeast species detection from food samples, a protocol of this technique is described in detail below.

2 Materials

2.1 Yeast Culture

For yeasts counting from food samples, different media may be used depending on the investigation interest. Chapter 11 shows some examples of selective and differential media frequently employed for quality analysis in food samples such as MYGP Copper Agar and Lysine medium, both used for wild yeast growth in the brewing industry; DG18 agar (dichloran 18% glycerol agar) used for osmophilic and xerophilic yeasts growth in food samples; WL nutrient agar used for wild yeasts from brewing and fermentation process (medium at pH 5.5 is employed for brewers' yeast while at pH 6.5 for bakers' and distillers' yeasts); and DBDM (*Dekkera-Brettanomyces* differential medium) described by [23] is specially used for counting of the contaminants yeast (*Dekkera* spp. and *Brettanomyces* spp.) in the fermentation process. Prepare and sterilize the culture medium according to the manufacturer's instructions (*see* **Note 1**).

2.2 Identification by Physiological Characterization (See Note 2)

1. Sterilize distilled water for yeast suspension preparation.
2. Prepare a stock solution of each carbon and nitrogen source at 1 M (e.g., maltose, glucose, sucrose, inulin, raffinose, melibiose, galactose, lactose, trehalose, melezitose, methyl-α-D-glucoside, soluble starch, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylose, L-arabinose, D-ribose, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, Myo-inositol, DL-lactate, succinate, citrate, D-gluconate, D-glucosamine, N-acetyl-D-glucosamine, hexadecane, 2-keto-D-gluconate, 5-keto-D-gluconate, saccharate, xylitol, L-arabinitol, arbutin, propane 1,2-diol, butane 2,3-diol, cadaverine, creatinine, L-lysine, ethylamine, nitrate, nitrite) and sterilize (*see* **Note 3**).
3. Prepare and sterilize plates containing base medium for carbon (YNB) and nitrogen (YCB) source assimilation tests according to the manufacturer's instruction (*see* **Note 4**). Add carbon and nitrogen sources to the plates at a final concentration of 0.025 M (*see* **Note 5**).
4. Prepare and sterilize plates containing a basal medium, such as YPD (yeast extract 1.0%, peptone 2.0%, dextrose 2.0%, agar 1.5%) or MEA (malt extract 2.0%; dextrose 2.0%, peptone 0.6%, agar 1.5%).
5. Prepare and sterilize Fermentation Medium (FM): peptone soya 0.75%, yeast extract 0.5%, bromothymol blue 0.04%. Add FM and the carbon sources at a final concentration of 0.025 M (e.g., maltose, glucose, sucrose, inulin, raffinose, melibiose, galactose, lactose, trehalose, melezitose, starch, D-xylose, methyl-α-D-glucoside) into the tubes containing Durham tubes (*see* **Note 3**).

6. Sterilize the "stamp" by autoclaving at 121 °C for 20 min. This "stamp" allows inoculating 21 yeast isolates at the same time onto the plate, as shown in Fig. 2.

2.3 Identification by Specific Primer Pairs by PCR

1. Commercial kits for DNA extraction (*see* **Note 6**).
2. Commercial kits for PCR.
3. Specific pair primers. Table 1 shows some specific pair primers described for the identification of different yeasts regarding quality interest in food samples (*see* **Note 7**).

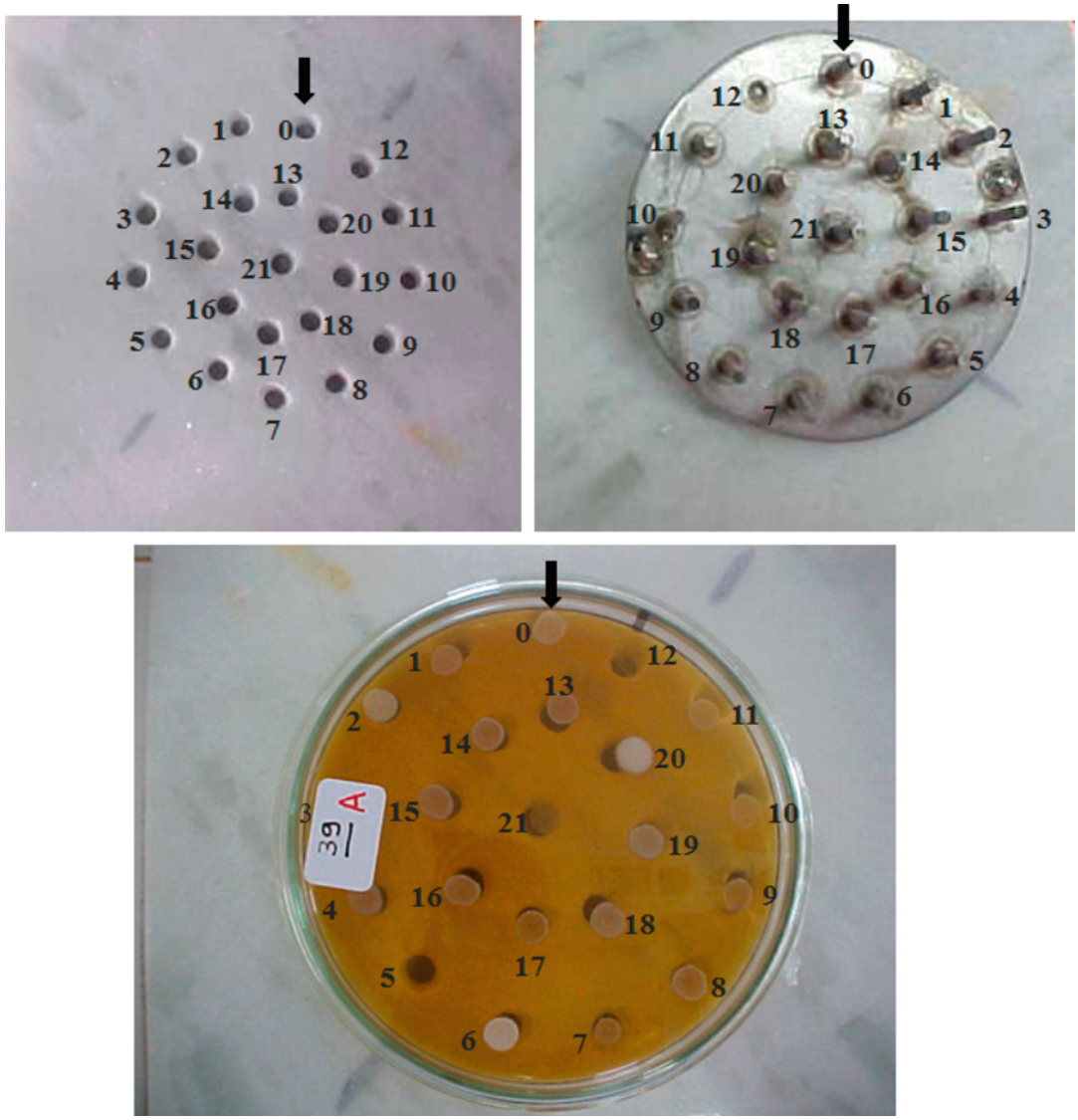

Fig. 2 "Stamp" used to inoculate multiple yeasts at the same time onto plates for yeast identification by physiological characterization method

4. Thermal cycler, gel electrophoresis equipment, loading buffer, agarose, and DNA marker (molecular weight standard with the bands ranging from 100 bp and longer).
5. Prepare stock buffer solution 50× TAE: 242 g/L Tris base, 57.1 mL glacial acetic acid, 20 mL of 0.5 M EDTA (pH 7.5). The buffer solution for use is 1×, dilute it before use.
6. Ethidium bromide (stock solution 1 mg/mL) or other DNA intercalating dye (SYBR Safe or SYBR Green).
7. Ultraviolet transilluminator system and Polaroid camera.

2.4 Identification by MALDI-TOF

1. Protein extraction: deionized water, absolute ethanol, formic acid 70%, acetonitrile, and matrix solution α-cyano-4-hydroxycinnamic acid (CHCA, Fluka®).
2. Analysis: 96-well MALDI flex plates (Bruker® Daltonics), MALDI-TOF microflex LT spectrometer (Bruker Daltonics®), and the automatic MALDI Biotyper system.

2.5 Independent Culture Method: Identification and Quantification by qPCR

1. Commercial kits for extraction of total DNA from food samples.
2. Commercial kits for qPCRs.
3. Specific pair primers. Table 1 shows some specific pair primers described for identification and quantification of different yeasts regarding quality interest in food samples.
4. Real-time PCR thermal cyclers and software system.

3 Methods

3.1 Yeast Culture

1. For yeast counting, inoculate the prepared samples (as described in Chapter 8) in the specific media (according to investigation interest) and incubate for 48–72 h at 30 °C (*see* **Note 8**). Representatives of each morphotype (the square root of the total is recommended) may be submitted to the evaluations described below (biochemical or specific primer pairs or MALDI-TOF) to confirm the species. For calculation of yeast population, *see* Chapter 10.

3.2 Identification by Physiological Characterization

1. Transfer each colony to sterilized microtubes containing 1.0 mL of sterile distilled water. Then, incubate the tubes at 30 °C for 24 h for reserves exhaustion. After incubation, standardize yeast inoculum at 620 nm for OD of 1.0.
2. Fermentation assay: Inoculate 150 μL of inoculum into the tubes containing MF added to the carbon source. Incubate until 21 days, however, evaluate at 7 days intervals. The media showing yellow color are positive.

Table 1
Primers used for yeast identification by PCR using specific primer pairs and qPCR

Microorganism	Primers	Fragment size (pb)	Annealing temperature (°C)	References
Pair specific primer				
Brettanomyces anomalus/B. Bruxellensis	DB90F 5′ GAYACTAGAGAGAGRRGGARGGC 3′ DB394R 5′ ACGAGGAACGGGCCGCT 3′	305	65	[13]
Debaryomyces hansenii	DhPadF 5′ GCGACTATGAACAGGTTTCCAACGA 3′ DhPadR 5′ CCTTCAATGTAACATCAGCGGCCC 3′	400	67	[15]
Saccharomyces bayanus	LgHOF 5′ TGGAAAGTCTACGAGAACAAGCC 3′ LgHOR 5′ CCTCTATGTGAAGTCCGTATACTG 3′	700	55	[17]
S. cerevisiae	ScHOF 5′ GTTAGATCCCAGGCGTAGAACAG 3′ ScHOR 5′ GCGAGTACTGGACCAAATCTTATG 3′	400	61	[17]
S. pastorianus	ScHOF 5′ GTTAGATCCCAGGCGTAGAACAG3′ ScHOF 5′ GCGAGTACTGGACCAAATCTTATG 3′	300	61	[17]
Trigonopsis cantarellii	CAN-F 5′ CGGTCCTTGCATATGTTCCT 3′ CAN-92 5′ GGTAGCTGATCACCGAAAGC3′	290	67.5	[16]
qPCR				
Brettanomyces bruxellensis	BbF1 5′ CACGAGGGTGTTTTCTTCAAAG 3′ BbR1 5′ AAATTACAACTCGGTTGCCC 3′	243	66	[22]
Candida californica	CcF3 5′ CATTAACAAGCTCGACCTCAGATC 3′ CcR3 5′ CACTGTGCTCCGCAGAGACTC 3′	187	64.5	[22]
Candida zeylanoides	CZ-5f 5′ CGATGAGATGCCCAATTCCA 3′ CZ-3bw 5′ GAAGGGAACGCAAAATACCAA 3′	191	58	[21]
Curvibasidium pallidicorallinum	CpR1f 5′ GCCGAGTTTTGTAAAGTAGAAGCT 3′ CpR3 5′ TCCAACAGACTTGTATGCGGTC 3′	243	67	[22]
Dekkera bruxellensis	DBRUXF 5′ GGATGGGTGCACCTGGTTTACAC 3′ DBRUXR 5′ GAAGGGCCACATTCACGAACCCCG 3′	79	69	[24]

(continued)

Table 1
(continued)

Microorganism	Primers	Fragment size (pb)	Annealing temperature (°C)	References
Hanseniaspora uvarum	HU-5fw 5′ GGCGAGGGATACCTTTTCTCTG 3′ HU-3bw 5′ GAGGCGAGTGCATGCAA 3′	172	59	[21]
Metschnikowia species	Mt8F 5′ TTCCTCACCCTCGTAAGACTACC 3′ Mt8R 5′ CGGACCCAAATCTCTTCAAATT 3′	155	66	[22]
Metschnikowia pulcherrima	MP-5fw 5′ CAACGCCCTCATCCCAGA 3′ MP-3bw 5′ AGTGTCTGCTTGCAAGCC 3′	253	60	[21]
Meyerozyma guilliermondii/caribbica	MeF3 5′ GAGATCAGACTCGATATTTTGTGAG 3′ MeR5 5′ GTCTAGGCAGGCAGCATCAAC 3′	156	64	[22]
Pichia angusta	PANG-5fw 5′ GTGTCCATTTCCGTGTAAGA 3′ PANG-3bw 5′ AGCCCACCCACAAG 3′	175	56	[21]
Pichia anomala	PA-5fw 5′ ACGTCATAGAGGGTGAGAAT 3′ PA-3bw 5′ AAACACCAAGTCTGATCTAATG 3′	197	57	[21]
Pichia kluyveri	PK-5fw 5′ AGTCTCGGGTTAGACGT 3′ PK-3bw 5′ GCTTTTCATCTTTCCTTCACA 3′	169	55	[21]
Rhodotorula mucilaginosa	RM-5fw 5′ GCGCTTTGTGATACATTTTC 3′ RM-3bw 5′ CCATTATCCATCCCGGAAAA 3′	169	54	[21]
Saccharomyces cerevisiae	SC-5fw 5′ AGGAGTGCGGTTCTTTCTAAAG 3′ SC-3bw 5′ TGAAATGCGAGATTCCCCCA 3′	215	59	[21]
S. cerevisiae/bayanus	ScF1 5′ GCGTCTAGGCGAACAATGTTCTTA 3′ ScR1 5′ CCCCAAAGTTGCCCTCTCCA 3′	199	67	[22]
Torulaspora delbrueckii	TD-5fw 5′ GTGGCGAGGATCCCAG 3′ TD-3bw 5′ CTATCGGTCTCTCGCAA 3′	186	58	[21]
Williopsis saturnus	WS-5fw 5′ GGGTGTCCAGTGCTTTG 3′ WS-3bw 5′ CCCAAGAAGGGAAGATAATCAC 3′	199	56	[21]

3. Assimilation assay: Add 300 μL of each yeast into the "stamp" wells (Fig. 2) and stamp onto the plates containing the different carbon and nitrogen sources. Incubate at 30 °C for 48–72 h. Apparent growth is considered positive (*see* **Note 9**).
4. Growth temperature assay: Inoculate the yeasts (using the "stamp" according to that described in **step 3 for Assimilation assay**) onto basal media (YPD or MEA) and incubate at different temperatures (e.g., 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C).
5. For yeast species identification, compare the obtained results with those described by [25, 26]. An online tool may be used by simply adding the data to the online form available in CBS Database (http://www.wi.knaw.nl/Collections/BiolomicsID.aspx?IdentScenario=Yeast2011ID).

3.3 Identification by Specific Primer Pairs by PCR (See Note 10)

1. Extract DNA from each yeast isolate by using a commercial kit according to manufactures' instruction or using the heating method (*see* **Note 6**).
2. Prepare the PCRs using a PCR kit according to manufacturer protocol. Use specific pair primers (Table 1) depending on investigation interest.
3. Using a thermal cycler, perform the amplification according to conditions described in Table 1.
4. Using agarose gels 0.8–1.5% (*see* **Note 11**), the amplification products are separated by electrophoresis apparatus using buffer TAE 1×. Then, the gel is stained using a DNA intercalating dye (e.g., Ethidium Bromide, SYBR Safe, SYBR Green). DNA fragments are visualized by UV transillumination system, and images are captured and stored using a polaroid camera and specific software. Use a molecular weight standard to compare the amplicons fragments (*see* **Note 12**).

3.4 Identification by MALDI-TOF (See Note 13)

1. Add portions of yeast colonies into microtubes containing 300 μL of deionized water, vortex for 30 s and then add 900 μL of absolute ethanol. Mix again for 30 s and centrifuge for 2 min at 10,000 × *g*.
2. Remove the supernatant and add 50 μL of formic acid 70%, and 50 μL of acetonitrile to the pellet. Vortex for 30 s and centrifuge for 2 min at 10,000 × *g*.
3. Remove the supernatant. The obtained precipitate contains yeast proteins to be evaluated by MALDI-TOF.
4. Add the obtained precipitate to the 96-well MALDI flex plates (Bruker Daltonics®) containing 1 μL of matrix solution α-cyano-4-hydroxycinnamic acid (CHCA), wait for complete

evaporation of the liquid, and then insert the plates into the equipment for analysis (*see* **Note 14**).

5. Before the analyses, perform an external calibration of MALDI-TOF MS using a standard bacterial protein test (provided by Bruker Daltonics®).
6. Perform the analysis in triplicate, using the MALDI-TOF microflex LT spectrometer (Bruker Daltonics®). Use the automatic MALDI Biotyper system for yeast identification (*see* **Note 15**).

3.5 Independent Culture Method: Identification and Quantification by qPCR

1. Extract DNA from food samples by using a commercial kit according to manufactures' instruction (*see* **Note 16**).
2. Prepare the PCRs using a PCR kit according to manufacture protocol. Use specific pair primers (Table 1) depending on investigation interest.
3. Using a thermocycler, perform the amplification according to conditions described in Table 1 (*see* **Note 17**).
4. Standard curves: For identification and quantification of yeast specie by qPCR method, a standard curve from samples containing a known number of yeast cells needs be performed. Thus, identified yeast species (the subject of investigation) are cultivated in YPD or MEA at 30 °C for 24 h. Then, the cells are counted using a Neubauer chamber. The DNA from the known yeast populations is extracted using commercial kits and serially diluted (1:10), e.g., from 10^8 to 10^7 down to 10 cell/mL. Each point on the calibration curve is measured in triplicate (*see* **Note 18**).
5. For results analysis, some parameters need be considered: percentage of efficiency, R^2 for standard curves, and slope (*see* **Note 19**). The data are analyzed by the software provided by the qPCR equipment by comparing the quantification obtained from food samples and standard curve samples.

4 Notes

1. Most of the media employed for yeast cultivation are available in commercial form.
2. There are commercial kits available in the market for yeast identification. Otherwise, it is possible to perform batch tests in the laboratory. In this chapter, several tests are described; however, there are additional tests that can also be performed for more reliable identification as described by [25, 26].

3. Use all sources of carbon and nitrogen that are available in the laboratory. A higher number of tests will provide more reliable results. The carbon and nitrogen solutions are filter-sterilized.
4. YNB (Yeast Nitrogen Base) is used for carbon source evaluation, and YCB (Yeast Carbon Base) is used for nitrogen source evaluation. Both are available in commercial form.
5. Carbon and nitrogen sources are added after medium sterilization when pouring into the plate at a temperature around 45 °C.
6. There are several commercial kits available for DNA extraction from yeast isolates. Perform the DNA extraction according to manufactures' instruction. Otherwise, the genomic DNA from several yeast species (e.g., *Saccharomyces* spp., *Pichia* spp., *Candida* spp., and others) may be extracted by heating at 95 °C in ultra-pure water solution. Check quantity and quality using, for instance, NanoDrop (Thermo Scientific®) and Bioanalyzer 2100 (Agilent®) or similar. The DNA may be stored at −20 °C for further use.
7. Table 1 shows the same examples of specific pair primers for conventional PCR and qPCR described in the literature. However, it is essential to evaluate and validate it before the test. Novel primers may be designed according to investigation interest.
8. The temperature of incubation may vary according to the yeast and food sample analyzed.
9. Mark the yeast "zero" on the plates, as shown in Fig. 2. Furthermore, note the yeast order. Use a basal medium plate, such as YPD or MEA, as a positive control.
10. Species-specific primer pairs tool for yeast species identification is valid when used for known species is the subject of the search. Otherwise, for unknown species, the sequence analysis of rDNA regions, such as the 18S, the D1/D2 domains of the 26S, and the internal transcribed spacer (ITS1-5.8S rRNA-ITS2 region) may be indicated.
11. The concentration of agarose in the gel can vary according to the expected size of the PCR product. For a lower molecular weight of PCR products, use a higher concentration of agarose.
12. It is crucial to perform the PCR using positive and negative controls, which means to use DNA from known yeasts.
13. The MALDI-TOF protocol is based on Bruker® equipment. For other equipment, it is essential to follow the manufacturer's instructions.

14. Other matrix solutions and extraction methods may be used for microbial identification by MALDI-TOF, as described by [27].
15. The organism databases are the key components for the microbial identification of commercial MALDI platforms. The manufacturers have continually updated them with the discovery of new microbial species and annotations. For microbial identification, follow the standard identification scores provided by the Bruker Biotyper® software: ID score < 1.7 indicates not reliable identification "not reliable ID"; ID score ≥ 1.7 and < 2.0 indicate a reliable identification and probable genus-level ID "Genus level ID"; and ID score ≥ 2.0 indicates a reliable identification and secure genus level "Genus level ID and probable Species-level."
16. There are several commercial kits available for DNA extraction from different samples. Perform the DNA extraction according to manufactures' instruction or with some adaptations depending on the food matrix. Check quantity and quality using, for instance, NanoDrop (Thermo Scientific®) and Bioanalyzer 2100 (Agilent®) or similar. The isolated DNA may be stored at −20 °C for further use.
17. Following the cycling temperatures, it is increased by 1 °C every 5 s from 50 °C to 99 °C to obtain the melting curve. Perform all analyses in triplicate. For an excellent resolution, the DNA concentration in the samples must be limited to 50 ng per analysis, except for standard curves.
18. It is essential to repeat the standard curve every run with the food samples for accurate comparison. In the case of many food samples to be analyzed, the standard curve may be performed in a separate run. However, add at least samples from three points of the standard curve in the run with the DNA from food samples.
19. Consider acceptable values ranging from 85 to 110% (1.85–2.1) for percentage of efficiency; desired $R^2 \geq 0.99$, and slope ranging from −3.6 to −3.3.

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Chapter 13

Evaluation of Yeast Inoculated in Parallel to the Autochthonous Microbiota in Food Samples

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Abstract

Fermentation is one of the oldest and most economical methods of producing and preserving food. For a greater standardization of the fermentative processes, new cultures are selected to reduce the fermentation time, increase the microbiological quality of the food, and alter and standardize the sensory attributes. Therefore, starter cultures are a practice performed to improve quality and add value to food and beverage fermented. The combination of traditional and molecular techniques is widely used to monitor and quantify the yeast starter population. This chapter will cover some techniques used to evaluate and quantify the inoculation strain and the microbiota involved during the food and beverage fermentation.

Key words Starter yeasts, qPCR, PFGE, Fermented food, MALDI-TOF

1 Introduction

Fermentation is one of the oldest and most economical methods of producing and preserving food and beverage. The preparation of these fermented foods and beverages was in an artisan way and without any knowledge of the microorganisms' role (bacteria, yeast, and filaments fungi) involved [1]. Methods for the fermentation of meat [2], coffee beans [3], cocoa beans [4–7], wine [8, 9], yogurt [10], kefir [11–13], cheese [14], alcoholic beverage [15, 16], Kombucha [17, 18] have been described.

Starter cultures are live microorganisms that develop through the fermentation of a particular substrate present in the medium, bringing some benefits to the product generated, such as adding organoleptic characteristics, better product stability, reducing processing time, and others. The *Saccharomyces cerevisiae* is an example of yeast commonly used as a starter culture, and these strains are employed in main industrial and laboratory processes; different commercial strains of *S. cerevisiae* were used and showed differences

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in the production of various fermented products as wine [8], cocoa [19], coffee [20], sugar cane spirit (cachaça) [21, 22] and others.

However, several other species non-*Saccharomyces* are also used. In wine, the yeasts *Wicherhamomyces anomalus, Torulaspora delbrueckii, Meyerozyma guilliermondii, Kazachstania aerobia, and K. servazzii* can be inoculated [9]. Giafardini and Zullo [23] used the *Candida diddensiae*, *C. adriatica*, and *W. anomalus* species to improve the production in the fermented foods of table olives. *Zygosaccharomyces rouxii* and *M. guilliermondii* are used as a starter culture for soy sauce fermentation [24].

Several studies using selected yeasts isolated from coffee have been successfully carried out. The inoculation of a starter culture was shown to improve coffee flavor and aroma, reduce processing time and drying time, and increase the product's economic value [25, 26]. *S. cerevisiae* and *T. delbrueckii*, isolated from natural coffee fermentative, improved the beverage quality, and some attributes have been found through it, such as caramel, chocolate, herbaceous materials, yellow fruits, and almonds [25]. The authors used both qPCR and DGGE to analyze quantitively and qualitative yeast populations.

S. cerevisiae, *T. delbrueckii*, *Pichia kluyveri*, *Hanseniaspora uvarum* were inoculated to enhance cocoa fermentation and improved chocolate taste [5, 6, 19]. The methods for yeast enumeration and identification were plate counting, DGGE, qPCR. In some food fermentation, yeast is inoculated as a monoculture, while in others as a mixed cocktail containing yeasts and bacteria like, for example, the Kefir [13], Kombucha [18], Cocoa [4], nondairy beverage [27], among others.

The food industries routinely use starter culture; therefore, a great deal of research is needed to study how the starter culture develops in food, whether it is present throughout the fermentation process, and in what quantity. Different methods can be used, always trying to evaluate the yeasts inoculated in the food to characterize its behavior in the fermentation process.

The methods described in the following sections are used to evaluate and monitor yeast strains in the laboratory and industrial processes. Some of them are used in a unique way or the combination of more than one method. The currently available and validated methods for the determination of yeasts in foods include 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM) [28, 29], slide culture technique [30], pulse field gel electrophoresis (PFGE) [31, 32], quantitative polymerase chain reaction (qPCR) (Table 1) [34, 35], and matrix-assisted desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Fig. 1) [46–48]. This chapter will cover the use of starter cultures in fermented foods and techniques to assess the wild and inoculum population during the fermentation process.

Table 1
Specific primer used for yeast

Target yeast group	Primer	Sequence (5′—3′)	Size (bp)	References
Universal yeast	YEASTF	GAGTCGAGTTGTTTGGGAATGC TCTCTTTCCAAAGTTCTTTTCATC TTT	124	[33]
Brettanomyces bruxellensis	DB RUXF	GGATGGGTGCACCTGGTTTACAC GAAGGGCCACA TTCACGAACCCCG	79	[36]
Kluveromyces marxianus	*	TCCTCGACAGTAATGATAA AGCACTCAATTCATCGTA	140	[37]
Debaromyces anomalus	*	GAGCAGACTGAGAAGTTC CGACCATAGAAGAGTGAG	100	[37]
Kazachtania turicensis	*	GTTGCATGGCAATCAAAA CGAAGACGCTCAAGAATA	101	[37]
Saccharomyces cerevisiae	*	CGACAACAAATTGCTGAA CTCTCGAACATAACTCTGTA	147	[37]
Pichia kluyveri	PK-5fw	AGTCTCGGGTTAGACGT GCTTTTCATCTTTCCTTCACA	169	[38]
Hanseniaspora uvarum	HU-5fw	GGCGAGGATACCTTTTCTCTG GAGGCGAGTGCATGCAA	172	[38]
S. cerevisiae	SC-5fw	AGGAGTGCGGTTCTTTGTAAAG	215	[38]
		TGAAATGCGAGATTCCCCT		
P. anomala	Anom	GTTAAAACCTTTAACCAATA AAATGACGCTCAAACA	nd	[39]
P. guilliermondii	Guill	CAAAACCACATTTAATTATTT AAATGACGCTCAAACA	nd	[39]
P. kluyverii	Kluy	CACCAAACACCTAAAAT AAATGACGCTCAAACA	nd	[39]
Candida albicans	Calb-F	CYGGCTCUGTCTATG1TYC GTCTARGCTGGCAGTATCG	411	[40]
C. glabrata	Cgla-F	CYGGCTCUGTCTATGTTYC TAACACTCTACACCGAGGCG	398	[40]
Clavispora lusitaniae	Clus-F	CCTGCGGGAAHGTAATTG UACGCCAGCGTCCTAGAAT	442	[40]
Issatchenkia orientalis	Iori-F	CAGGUGGAGTCTGTGTGGA TCTGGCCCTGGCTATAACACC	416	[40]
Trichosporon asahii	Tasa-F	AATCCCGTGCTTGATACGAC GRGRAGTCACATTCCTAC	319	[40]
Torulaspora delbrueckii	Tods L2	CAAAGTCATCCAAGCCAGC TTCTCAAACAATCATGTTTGGTAG	nd	[41]

(continued)

Table 1
(continued)

Target yeast group	Primer	Sequence (5′—3′)	Size (bp)	References
I. orientalis	Isa 1	GTTTGAGCGTCGTTTCCATC AGCTCCGACGCTCTTTACAC	nd	[41]
Metschnikowia pulcherrima	MPL3	CTCTCAAACCTCCGGTTTG GATATGCTTAAGTTCAGCGGG	nd	[41]
Kazachstania slooffiae	KSact-f	CAAACCGCTGCCCAATCTTC GCTTCAAGACCCAAGACGGA	131	[42]
Zygosaccharomyces bailii	ZBF	CATGGTGTTTTGCGCC CGTCCGCCACGAAGTGGTAGA	122	[43]
Brettanomyces species	BRET	GTTCACACAATCCCCTCGATCAAC TGCCAACTGCCGAATGTTCTC	108	[44]
Yarrowia species	YAL	ACGCATCTGATCCCTACCAAGG CATCCTGTCGCTCTTCCAGGTT	106	[44]
Meyerozyma guilliermondii	MeF	GAGATCAGACTCGATATTTTGTGAG GTCTAGGCAGGCAGCATCAAC	156	[45]

Asterisk primer name not set, *nd* not described

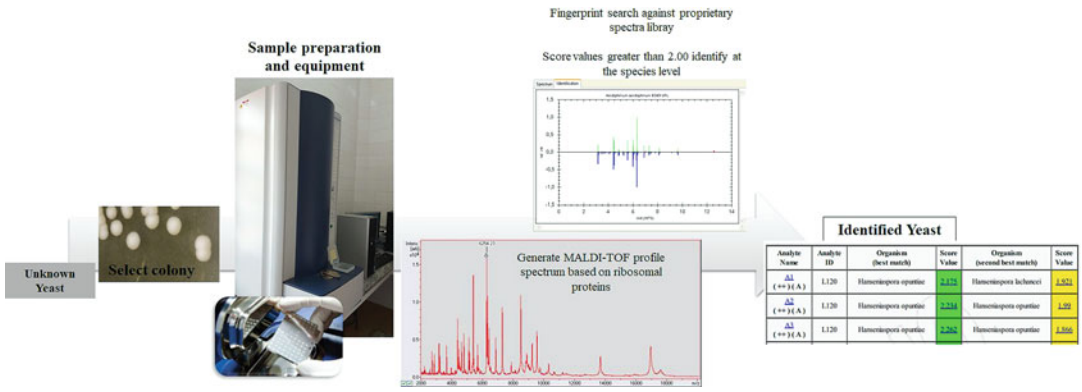

Fig. 1 Steps for identification by MALDI-TOF

2 Materials

The following materials are needed to evaluate yeast starter according to the technique used.

2.1 Cell Viability

1. Methylene blue (1 g).
2. Distilled water (10 mL).
3. Sodium citrate, dihydrate (2 g).
4. Neubauer chamber.

2.2 3M™ Petrifilm™ Rapid Yeast and Mold Count

1. 0.1% peptone water sterile: Dissolve 0.1 g/L of peptone in water e and proceed with sterilization. This solution is used for samples suspension cells.
2. 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM).
3. Pipette.
4. Incubator (28 °C).

2.3 Slide Culture Technique

1. YPD agar (g/L): Yeast extract (10), peptone (20), glucose (20), agar (20).
2. Sterile petri dishes.
3. Sterile dissecting knife.
4. Sterile microscopy slide.
5. Sterile coverslip.
6. Wetting chamber.
7. Calcofluor White dye (5.0 mg/mL).
8. Microscopy.

2.4 Pulse Field Gel Electrophoresis (PFGE)

1. Lysing enzymes.
2. CPES buffer.
3. CPE buffer.
4. Proteinase K.
5. Ethylenediamine tetraacetic acid (EDTA).
6. TE buffer.
7. Agarose for PFGE.
8. TAFE buffer.

2.5 Quantitative Polymerase Chain Reaction (qPCR)

1. Rotor for qPCR analysis.
2. Rotor-Gene PCR SYBR Green Mix Master 2× (kit contains taq polymerase, dNTP, buffer).
3. Species-specific primer (forward and reverse).
4. Talc-free gloves.

5. Pipettes and tips of 0.1, 10, 100 μL.
6. 200-μL microtubes.
7. Quantified template DNA (extracted DNA from the inoculum and sample DNA separately).

2.6 Matrix-Assisted Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF)

1. Ultrapure water.
2. Formic acid.
3. Ethanol.
4. Acetonitrile.
5. Trifluoroacetic acid (TFA).
6. Alpha-Cyano-4-hydroxycinnamic acid (CHCA).
7. Eppendorf, pipette and tip.

3 Methods

Many industrial processes need to determine the proportion of living cells in yeast cell material through microscopic observation with methylene blue dye [49]. The counting of viable cells is done in a Neubauer chamber (*see* Chapter 11).

3.1 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM)

1. Aseptically prepare a 1:10 dilution of each test portion (*see* **Note 1**).
2. Prepare tenfold serial dilutions in 0.1% peptone water (*see* **Note 2**).
3. Place the 3M Petrifilm RYM Plate on a flat, level surface.
4. Lift the top of the film and dispense 1 mL of each dilution onto the center of the bottom film of each plate.
5. Roll the film down onto the sample.
6. Place the 3M Petrifilm Flat Spreader on the center of the plate of the spreader to distribute the sample evenly (*see* **Note 3**).
7. Remove the spreader and leave the plate undisturbed for at least 1 min to permit the gel to form.
8. Incubate the 3M Petrifilm RYM Count Plates at 28 °C in a horizontal position with the clear side up in stacks of no more than 40.
9. Enumerate plates after 48 h of incubation (*see* **Note 4**).
10. Analyze colonies morphology (*see* **Note 5**).
11. The circular growth area is approximately 30 cm^2, plates containing greater than 150 colonies can be either estimated (*see* **Note 6**).
12. Food samples may occasionally show interference on the 3M Petrifilm RYM Count Plates (*see* **Note 7**).

13. If required, colonies may be isolated for further identification by direct microscopy or biochemical analysis, lift the top film, and pick the gel's colony.

3.2 Slide Culture Technique

1. Add the components of YPD agar to sterile distilled/deionized water and autoclave for 15 min at 121 °C.
2. Cool to 45–50 °C.
3. Add the Calcofluor White dye (*see* **Note 8**).
4. Pour into sterile petri dishes (*see* **Note 9**).
5. Cut the YPD agar block (~20 × 20 mm) using a sterile dissecting knife.
6. Placed in a sterile microscopy slide.
7. Make the respective dilutions of the yeast suspension (*see* Chapter 8).
8. Inoculate 20 μL of cell suspensions over the YPD block (*see* **Note 10**).
9. Covered the cells with a 24 × 24 mm sterile coverslip.
10. Placed in a wetting chamber, containing 100 μL of sterile.
11. Incubation at 25 °C (16–24 h) (*see* **Note 11**).
12. Observed under a light microscope (micro-colonies and single cells) (*see* **Note 12**).

3.3 PFGE Analysis

1. Grow yeast for 48 h.
2. Transfer some colonies using sterile tips (the tip of the colony tip) to microtubes containing 80 μL of a Lysing-enzymes solution (*see* **Note 13**).
3. Mix gently with a tip.
4. Prepare the plug agarose: 75 mg of agarose to 6.25 mL of CPE buffer (*see* **Note 14**).
5. Mix 80 μL of the plug agarose in the microtubes containing the cells with the enzyme (*see* **Note 15**).
6. Mix gently, avoiding the formation of bubbles, and quickly apply the mixture to the wells (*see* **Note 16**).
7. Then transfer the plugs to microtubes (identified) containing 0.5 mL of CPE buffer.
8. Incubate 4 h at room temperature.
9. Afterwar, remove CPE buffer solution from the microtubes and add 0.5 mL of solution 3 with proteinase K (*see* **Note 17**).
10. Keep overnight in a water bath at 50 °C.
11. Prepare a solution of EDTA (186.1 g EDTA (disodium ethylenediaminetetraacetate•$2H_2O$) and 800 mL distilled water (*see* **Note 18**).

12. The next day, if the run is not processed, remove solution 3 with proteinase K solution and add 1 mL of 0.5 M EDTA.
13. Store in a refrigerator.
14. Following the run, start washing the plugs with 0.5 mL TE buffer (Tris–HCl 10 mM, EDTA 1 mM pH 8.0): 3 times at 50 °C for 20 min and 4 or 5 times at room temperature for 15 min.
15. Preparation of the gel: (1) Weigh 1.1% agarose and dilute in 170 mL of 1× TAFE buffer (*see* **Note 19**). Reserve about 5 mL of agarose to weld the wells; (2) After solidifying, remove the comb and place the plugs in the wells. Solder the wells with the remaining agarose in a 65 °C bath; (3) Then place the gel in the tub (CHEF) containing 0.5× TAFE buffer and program the device.
16. Race conditions: *Block 1*—All chromosomes migrate together, release the plug for the gel (initial pulse = 5 s, press second and third buttons simultaneously; final pulse = 5 s, press third and fourth buttons simultaneously; running time = 1 h, run time marks in h; volts/com = 6 (volts); and chain do not move); *Block 2*—The smallest chromosomes migrate (initial pulse = 60 s; final pulse = 60 s; running time = 8 h; and volts/cm = 6); *Block 3*—The largest chromosomes migrate (initial pulse = 100 s; final pulse = 100 s; running time = 12 h; and volts/cm = 6).

3.4 qPCR Analysis

1. Place the equipment block in the freezer the previous day to keep the samples refrigerated during the assembly of the reaction.
2. Reactions prepared with a final volume of 20–25 μL, and the calculation of the number of reactions (*see* **Note 20**).
3. Reaction preparation: Each reaction was composed of 12.5 μL of Rotor-Gene PCR SYBR Green Mix Master 2×, 0.8 μM of each primer (forward and reverse), 1 μL of DNA template (standardized at 50 ng), and the volume is made up to 25 μL with ultra-pure water. Example of the number of reactions:

   Samples + 6 standard curve points × triplicate = 33 reactions

   (a) 1 reaction = 12.5 μL of Rotor-Gene.
   (b) 33 reactions = *X*.
   (c) *X* = 412.5 μL of Rotor-Gene (*see* **Note 21**).
4. Standard curve: For standard curves, yeast species should be grown on YPD agar at 28 °C for 24 h. The cells are counted using a Neubauer chamber. DNA is extracted using the QIAamp DNA Mini Kit and serially diluted (1:10) from 10^8 to 10^3 cells/mL (*see* **Note 22**).

5. After the ready mix, distribute 24 μL in Eppendorf for analysis and then add 1 μL of template DNA to each Eppendorf.
6. Take the samples for analysis on the equipment.

3.5 MALDI-TOF Analysis

1. Preparation of the matrix: weigh 0.01 g in 1 mL of organic solution (*see* **Note 23**). Weigh the CHCA (0.01 g in 1 mL) in the Eppendorf, add the organic solution, vortex, and centrifuge for 1 min at 12,000 rpm (keep the matrix in the dark, refrigerated for up to 15 days).
2. Isolated should be growing for 18–21 h.
3. Transfer a small amount of yeast colony with a sterile toothpick to the Eppendorf.
4. Add 6 μL of 2 5% formic acid (2.5 mL of formic acid for 7.5 mL ultra-pure water).
5. Mix with a vortex mixer for 1 min.
6. Place 0.6 μL of the suspension obtained earlier (**step 4**) on the MALDI-TOF plate (specific to the equipment) (*see* **Note 24**).
7. Add 1 μL of the matrix to each well and mix carefully not to mix the samples.
8. Wait for the plate to dry completely before placing it in the equipment for analysis.

4 Notes

1. Dairy products: Pipet 11 mL or weigh 11 g of sample into 99 mL sterile 0.1% peptone water. Shake 25 times to homogenize. All other foods: Weigh out 25 g of a sample from test portion into a sterile stomacher bag and dilute with 225 mL of 0.1% peptone water; shake at high speed to homogenize.
2. Use appropriate sterile diluents: Butterfield's phosphate buffer (ISO 5541-1), Buffered Peptone Water (ISO), 0.1% peptone water, peptone salt diluent, saline solution (0.85–0.90%), bisulfite-free Letheen broth, or distilled water. Do not use diluents containing citrate, bisulfite, or thiosulfate with 3M Petrifilm RYM Plates; they can inhibit growth. If citrate buffer is indicated in the standard procedure, substitute with 0.1% peptone water, warmed to 40–45 °C.
3. Spread the inoculum over the entire 3M Petrifilm RYM Count Plate growth area before the gel is formed. Do not slide the spreader across the film.
4. To enhance interpretation is allowed for an additional 12 h of incubation time. If a 60-h time-point for interpretation is not convenient, extending the incubation time to 72 h is an acceptable alternative.

5. Yeast colonies appear raised and small with defined edges. Colonies may appear pink/tan to blue/green in color. Mold colonies appear flat with a dark center and diffused edges. Colonies may appear blue/green to variable upon prolonged incubation.
6. Estimation can only be done by counting the number of colonies in one or more representative squares and determining the average number per square. The average number can be multiplied by 30 to determine the estimated count per plate. If a more accurate count is required, the sample will need to be retested at higher dilutions; report the final results in colony-forming units/gram (UFC/g).
7. Example: (a) Food with a uniform blue background color (usually seen in organisms used in cultivated products). These should not be counted (b) intense blue stains (usually seen with spices or granulated products).
8. Calcofluor White dye from a sterile water stock solution of 5.0 mg/mL at a final concentration of 2.5 μg/mL.
9. Pour 12.5 mL of YPD agar into a sterile 90-mm petri dishes and allow to solidify. The medium should not be too thin to guarantee the availability of nutrients and reduce the possibility of dehydration, important overtime incubation times (16–24 h). On the other hand, it should not be very thick to allow focusing with 100× objective
10. Cell density must be high enough to facilitate counting of micro-colonies without overlap, after a long incubation time (16–24 h).
11. A minimum incubation time of 16 h is necessary to assess the viability of the yeast accurately, and the incubation should not exceed 18 h; longer incubation times may result in underestimating the percentage of viable cells.
12. Observe cells by phase-contrast or by epifluorescence microscopy using an epifluorescence microscope; the cells that gave rise to a micro-colony (four cells or more) are deemed as viable. Single (unbudded), double, or triple cells are considered as non-viable.
13. 7 mg of the enzyme (lysing enzyme) for 1 mL of CPES buffer (citric acid 0.210, Na_2HPO_4 0.426, EDTA-Na_2 0.186, sorbitol 5.630, and dithiothreitol 0.020 g per 25 mL). The CPES buffer must be filtered and stored in the refrigerator.
14. CPE buffer (for 200 mL add citric acid 1.68 g, Na_2HPO_4 3.41 g, and EDTA-Na_2 1.49 g). The buffer must be filtered and stored in the refrigerator. Prepare in a falcon tube and melt agarose in a microwave bath. The falcon tube goes into a Becker with water.

15. Before using the holder, make sure it has gone through the cleaning process (cleaned with 10% hydrogen peroxide and rinsed with sterile distilled water). After applying the plugs, you can put them in the refrigerator for half an hour to solidify more quickly.
16. Agarose should be added at a temperature of 65 °C, so before adding it, keep it in a water bath at this temperature.
17. Solution 3 (for 200 mL add tris 0.24 g, Lauryl sodium sulfate 2.0 g, EDTA-Na_2 33.5 g) Adjust pH to 9.0 with NaOH. The buffer must be filtered and stored in the refrigerator. Before use, take time before dissolving the SDS.
18. For the preparation of a solution of EDTA: Stir the EDTA into distilled water, adjust the pH to 8.0 with NaOH solution, if you use solid NaOH pellets, you will need 18–20 g of NaOH. Add the last of the NaOH slowly so that you do not overshoot the pH. You may wish to switch from solid NaOH to a solution toward the end for more precise control. The EDTA slowly goes into the solution as the pH nears 8.0. Dilute the solution to 1 L with distilled water and filter the solution through a 0.5-micron filter. Dispense into containers as needed and sterilize in an autoclave.
19. Buffer TAFE 20× (g/L Tris-base 24, EDTA-Na_2 2.9, glacial acetic acid 5 mL). Autoclave and store in the refrigerator. Dilute in distilled water when using. Comments: Leave the tub already cooling with water (3 L) with a hose passing in a cold bath at approximately 3 °C since the previous day and add the buffer just before the race to cool down too.
20. The calculation of the number of reactions to be made must be carried out carefully. To prepare the mix, calculate the number of reactions and multiply by the volume of the reagent. Always run the standard curve with the samples and perform the entire analysis in triplicate.
21. Mix all reagents (except for template DNA) in a larger Eppendorf and then distribute the volume in the rest of the Eppendorf.
22. The highest cell concentration of the standard curve should be two to three log/cell more than the inoculated. Each point on the calibration curve is measured in triplicate.
23. Organic solution: 33.3% ethanol, 33.3% acetonitrile, 33.3% TFA 10% for 20 mL organic solution. Then you must measure 6.66 mL of ethanol, 6.66 mL of acetonitrile, and 0.66 mL of TFA and make up to 6 mL.
24. Place the yeast solution carefully in each well of the plate, perform the analysis in triplicate, and do not let the samples mix; after adding all the samples, wait for the solution to evaporate.

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Chapter 14

Double-Layer Plaque Assay Technique for Enumeration of Virus Surrogates

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Abstract

Double-layer plaque assay technique (DLA) can be used to enumerate, isolate, and detect bacteriophages in food, beverage, and water samples. DLA technique allows phage contact with host bacterium in an environment containing two layers of agar. The phage infects the host bacterium, and translucent or clear zones (plaques) appear containing phage particles and lysed bacteria. Enumeration of phages requires a short time of analysis. It is a simple and efficient method applicable to various bacteriophages. In this chapter, we used MS2 Human Norovirus surrogate as an example.

Key words Bacteriophages, Enumeration, Host bacterium, Overlay technique, Plaque assay

1 Introduction

Foodborne viruses are classified as enteric viruses and replicate in the host gastrointestinal tract. However, the difficulty in assessing the survival of Human Noroviruses (NoVs) in food is the non-replication of NoVs without using host cell culture, requiring a complex experimental approach for manipulation in microbiological research [1].

Viral substitutes as bacteriophages are often used in research due to the complexity of the cell culture systems necessary to propagate viruses that infect humans [1]. Substitutes are typically selected due to morphological and physiological characteristics similar to the pathogens of interest. They should be equivalent to or slightly more resistant to treatments than the target organism. Moreover, they should be nonpathogenic and present similar growth and survival behavior [2]. In this sense, substitutes (surrogates) have been used to estimate the behavior of foodborne pathogenic enteric viruses in foods and water.

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The double-layer plaque assay technique is the classical method used in phage research for enumeration, isolation, and detection of bacteriophages. It is also used to isolate mutants and new phages and characterize the plaque's morphology (size of the plaque, presence/absence of a halo, and clear versus turbid lysis) [3, 4].

This technique is also known as double agar overlay plaque assay, double layer, soft agar overlay, or double agar layer [4]. The technique involves phage suspensions grown with host bacterium in a dilute molten agar (overlay or top agar) dispersed onto a solid medium (underlay or bottom agar) [5]. The top agar is commonly referred to as soft agar and contains the same medium of bottom agar, however, with a lower agar concentration. The bottom agar is used for bacterial growth containing 1–1.5% agar [6]. Phages can be directly inoculated on top of the second layer and dried or mixed with the host bacterium and the soft agar [7].

During the incubation period (optimal temperature and time for bacterial growth), the host bacterium forms a lawn on the solid medium, except when infectious phage particles inhibit the growth or lyse the cells, resulting in a translucent or clear zone (visible to the naked eye), termed a plaque. Each plaque represents a single phage particle in the original sample. Sufficient progeny phages from each infected bacterial cell are needed for plaque expansion, allowing localized infection, lysis, or altered bacteria growth [4]. However, some phage plaques are difficult to distinguish because of the nature of the phage, small size, or incomplete lysis. In this case, to facilitate plaque formation, the agarose can be used in lower concentration (0.2%) to replace the agar [8], and divalent ions ($CaCl_2$ and $MgCl_2$) can be added to allow phage adsorption to the bacterial receptor [9].

Enumeration of phages by the double-layer plaque assay technique is an efficient and simple method, applicable to many bacteriophages and can be implemented with minimal costs [7]. However, it may show high variability. Therefore, the optimization of each phage-host can be time-consuming. Moreover, cross contamination with other bacteria and other phages and changes in the host's growth behavior can significantly impact the results [10]. This chapter describes the application of the double-layer plaque assay technique for enumeration of MS2, a NoV surrogate.

2 Materials

1. Culture tools: Variable or fixed-volume micropipettes (*see* **Note 1**), sterile pipette tips, sterile plastic10- to 20-mL tubes and petri dishes (90 mm).
2. Growth media: Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB), and Bacteriological agar.

3. Equipment: Water bath or heating block maintaining at 40 ± 1.0 °C, incubator stabilized at 36 ± 1.0 °C for growth of microorganisms, autoclave at 121 °C, and vortex.

2.1 Single-Layer Plaque or Bottom Agar

1. Prepare the TSA medium according to the manufacturer's instructions and sterilize it in an autoclave at 121 °C for 15 min.
2. When the medium cools down to 50 ± 1.0 °C, dispense ~20 mL of medium per petri dish. Keep the petri dishes in the safety cabinet until solidification (*see* **Note 2**).
3. If necessary, the petri dishes can be stored at 4 ± 1.0 °C for up to 7 days. Before using the petri dishes containing TSA stored, let them dry to avoid possible interference of condensed water (*see* **Note 3**).

2.2 Double-Layer Plaque or Top Agar

1. Prepare the TSB medium according to the manufacturer's instructions, with the addition of bacteriological agar to a concentration of 0.5%.
2. Warm the medium (TSB + agar of 0.5%; soft agar) to melt the agar (agar melting temperature range from 85 °C to 95 °C).
3. Sterilize the medium (soft agar) in an autoclave at 121 °C for 15 min.
4. Dispense ~5 mL of medium (soft agar) into sterile plastic tubes.
5. If needed, store them at 4 °C up to 7 days. Before use, stored tubes containing soft agar should be warmed (*see* **Note 4**).

2.3 Trypticase Soy Broth

1. Prepare the TSB medium according to the manufacturer's instructions and sterilize it in an autoclave at 121 °C for 15 min.
2. Distribute volumes previously fixed (9 mL or 900 μL) in sterile plastic tubes or sterile Eppendorf.

2.4 Overnight Host Bacteria Stock Cultures

1. If petri dishes containing TSA were stored at 4 °C, let them dry before the experiment (*see* **Note 3**).
2. Inoculate the loopful of *E. coli* C3000 (or equivalent bacteriophage host) directly from the stock culture (*see* **Note 5**) in petri dishes containing TSA.
3. Overnight incubate the petri dishes at 36 ± 1.0 °C.
4. Transfer a single colony to 9 mL of TSB and overnight incubate at 36 ± 1.0 °C.
5. Harvest cells by centrifugation (5000 × *g*, 4 °C for 15 min) and adjust the inoculum to the estimated level (approximately 8 log CFU/mL).

3 Methods

3.1 Double Agar Overlay Plaque Assay (Fig. 1)

1. Identify the petri dishes containing TSA using the number of the corresponding dilution to be inoculate (e.g., −1 to −9).
2. Add the overnight culture of *E. coli* C3000 (or equivalent bacteriophage host) in 50 mL of medium (1:50; soft agar) at 40 ± 1.0 °C. Mix and pour the contents (~5 mL) over the surface of TSA (*see* **Notes 4**, **6** and 7).
3. Wait for the solidification of layers for 30 min (*see* **Note 8**).
4. Separate ten sterile Eppendorf and add 900 μL of TSB (or equivalent diluent) in each tube and identify them with sequential numbers corresponding to the dilutions (e.g., −1 to −9).
5. Add 100-μL of MS2 stock bacteriophage (or another bacteriophage) to the first Eppendorf, vortex it, change the pipette tip and transfer 100 μL to the second Eppendorf in the series (*see* **Note 9**).
6. Inoculate 100 μL of each dilution on the surface of TSA (full dish).
7. Incubate the petri dishes at 36 ± 1.0 °C overnight (*see* **Note 10**).
8. Count the plates formed in the TSA in contrast to white light.

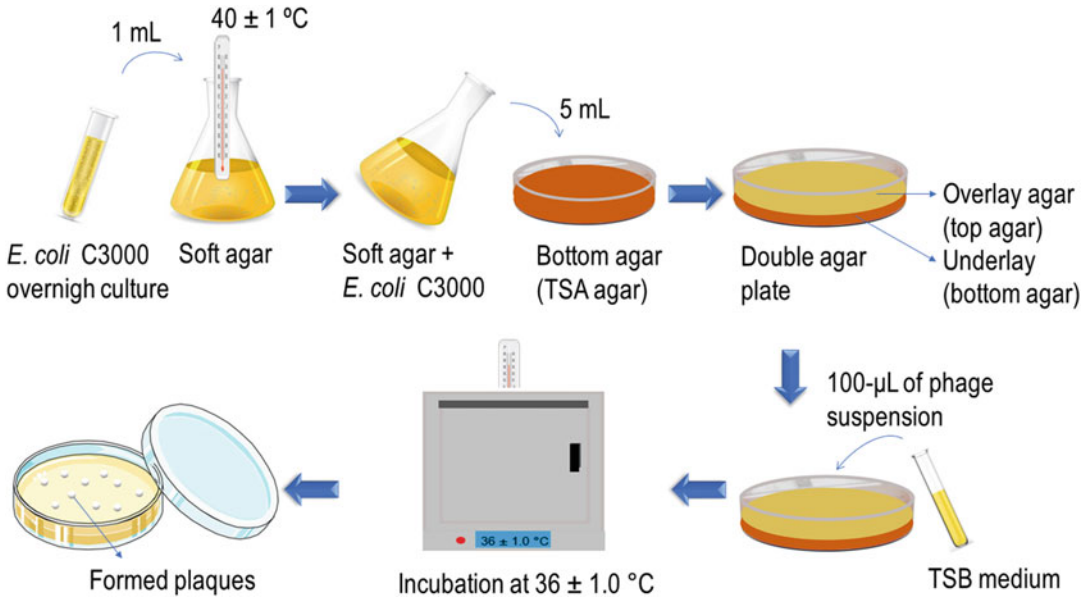

Fig. 1 Double agar overlay plaque assay

4 Data Analysis and Calculations

1. Count the number of plaque-forming units (PFU) in plaque counts within the desired range of 30–300 PFU.
2. Use the following equation for determine the titer of the original phage preparation:

$$\text{Number of plaques} \times 10 \times \text{reciprocal of counted dilution} = \text{PFU/mL}$$

5 Notes

1. To avoid cross-contamination, micropipettes should be cleaned before use and periodically calibrated.
2. To avoid condensation of water on the petri dishes, the agar must be dried in the biological safety cabinet, with sterile air flowing directly above the agar.
3. Stored TSA petri dishes should be partially uncovered in a safety cabinet for 10–15 min to reduce condensation before inoculation.
4. Before use, soft agar stored at 4 °C up to 7 days in tubes should be warm to 40 ± 1.0 °C in a water bath or heating block. The soft agar must be completely melted to avoid crystalline areas in the overlayer that difficult to enumerate the PFU.
5. Stock cultures of the host strain should be maintained at −80 °C, and used to prepare the working cultures for the analysis.
6. The temperature of 40 ± 1.0 °C needs to be strictly controlled to maintain the host viability.
7. 5 mL of soft agar is sufficient for the overlayer onto the solid TSA in one petri dish.
8. The petri dishes must always be opened in the biological safety cabinet.
9. Sufficient mixing is achieved with a low setting in a vortex for ~30 s. Long mixing times are not recommended for phage suspensions.
10. *E. coli* C3000 can grow fast; therefore, plaques may be visible after few hours of incubation.

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Chapter 15

Detection of Protozoan Parasites on Leafy Greens Using Multiplex PCR

Minji Kim and Karen Shapiro

Abstract

Protozoan pathogen contamination of leafy green presents a health risk for fresh produce consumers. This chapter describes a simple multiplex PCR (mPCR) assay for simultaneous detection of *Cryptosporidium* spp., *Giardia* spp., *Cyclospora cayetanensis*, and *Toxoplasma gondii* on leafy greens. The nested mPCR assay provides a rapid, inexpensive, and simple approach for simultaneous detection of protozoan pathogens on fresh produce.

Key words Multiplex polymerase chain reaction (mPCR), *Cryptosporidium*, *Giardia*, *Toxoplasma*, *Cyclospora*, Sequence confirmation, Leafy greens, Detection, Vegetables, Fresh produce

1 Introduction

Protozoan pathogen contamination on leafy greens is of growing importance due to their capacity to cause illnesses in consumers of fresh produce [1–5]. Four key foodborne protozoan pathogens, including *Cryptosporidium* spp., *Giardia* spp., *Cyclospora cayetanensis*, and *Toxoplasma gondii* are prevalent protozoan parasites that can cause foodborne illnesses [6]. *C. cayetanensis* and *Cryptosporidium* spp. are currently considered the most important protozoan pathogens, based on the number of disease outbreaks in people that have been associated with contaminated fresh produce [7]. A multistate outbreak of cyclosporiasis reported in the United States (2018) was linked to read-to-eat salad mix sold at fastfood restaurants [8]. Unlike C. *cayetanensis* which is a human pathogen, zoonotic transmission of *Cryptosporidium* spp. can occur with oocysts (environmental stage of the parasite) originating from either human or animal feces. Numerous gastrointestinal disease outbreaks have been attributed to produce contaminated with *Cryptosporidium* spp. [9]. The genus *Cryptosporidium* has many species, including *C. hominis* (human-specific) and *C. parvum*

Marciane Magnani (ed.), *Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-1932-2_15,

(zoonotic) [10]. Within the genus *Giardia, G. duodenalis* is most often implicated in human infections, with Assemblages A and B most commonly implicated (also referred to as *G. lamblia* or *G. intestinalis*) [11]. Like *Cryptosporidium* spp., *Giardia* spp. can be shed by many animal hosts as well as humans. This protozoan can also cause gastrointestinal disease in infected people, and contamination of produce has been documented widely, including in leafy greens [4]. Infection with the zoonotic protozoan *T. gondii* is widespread in humans worldwide, and consumption of raw fruits and vegetables is a significant risk factor for acquiring *T. gondii* [12]. An outbreak of toxoplasmosis in Brazil was associated with the consumption of leafy greens in 2009 [13].

Despite the risk of exposure to protozoan parasites through consumption of fresh produce, standardized detection methods are only currently available for *Cryptosporidium* spp. and *Giardia* spp. using immunomagnetic separation (IMS) and direct fluorescent antibody (DFA) assay [14]. However, these microscopy methods rely on immunoassays that are costly, require additional expertise, and lack molecular confirmation that is essential for accurate risk assessment. The need for efficient and affordable methods for detection of *Cryptosporidium* spp., *Giardia* spp., *T. gondii*, and *C. cayetanensis* is exemplified by field studies that have demonstrated widespread presence of these pathogens in leafy greens. Here, we describe a simple molecular approach using multiplex polymerase chain reaction (mPCR) that can simultaneously detect and differentiate the presence of these four protozoan pathogens in leafy greens [15]. Systematic laboratory spiking experiments using a spinach as a model leafy green demonstrated that the lowest limits of detection using the mPCR assay were 1–10 (oo)cysts per g of spinach when 10 g sample processed. The mPCR assay provides a rapid (<24 h), inexpensive ($10 USD/sample), and simple approach for simultaneous detection of protozoan pathogens on leafy greens. Further analyses for discriminating the viability or infectivity of parasites when detected on produce will enable more accurate determination of health risk to consumers, which is currently a critical need for establishing efficient assays [16, 17].

2 Materials

2.1 Leafy Green Processing (Washing)

1. Weighing dish.
2. 18-oz Whirl-pak bag (*see* **Note 1**).
3. 50-mL and 15-mL conical tubes.
4. 500-mL glass or plastic bottle.
5. Motorized serological pipette controller and pipette tips.
6. Pipettes and filtered pipette tips (*see* **Note 2**).

7. 50-mL syringe and 0.45-μm pore-sized syringe filter.
8. Tween 80.
9. Filter-sterilized 0.1% Tween 80: Transfer 3 mL of Tween 80 to a 50-mL conical tube using a serological pipette. Make up to 30 mL with ultrapure water (for example, Milli-Q water) to make 10% Tween 80 solution. Swirl the tube manually until viscous Tween 80 is dissolved. Filter the 10% Tween 80 solution through a 0.45-μm pore-size syringe filter. Transfer 5 mL of filter-sterilized 10% Tween 80 to a 500-mL bottle. Make up to 500 mL with ultrapure water to make 0.1% Tween 80.
10. 1.5-mL conical bottom screw cap microcentrifuge tubes that can withstand temperatures from −196 °C to +100 °C (*see* **Note 3**).

2.2 Nucleic Acid Extraction

Extraction of protozoan DNA from leafy green wash pellets is performed using the QIAGEN DNeasy Blood and Tissue Kit.

1. DNeasy Blood and Tissue Kit (QIAGEN, cat. no. 69504).
2. Vortex mixer.
3. Dry heating block (*see* **Note 4**).
4. Microcentrifuge (capable of attaining 20,000 × *g*).
5. Pipette and pipette tips with barrier filter.
6. Liquid nitrogen and a benchtop dewar (*see* **Note 5**).
7. Hot plate and boiling water (>2 L) in a 4-L container (*see* **Note 6**).
8. Floating microtube rack and 12-inch forceps (*see* **Note 7**).
9. Proteinase K (*see* **Note 8**).
10. Ethanol (96–100%), molecular grade.
11. Nuclease-free water.

2.3 Polymerase Chain Reaction

1. Thermal cycler.
2. Mini centrifuge.
3. PCR reagents including 10× PCR buffer and AmpliTaq® polymerase, and dNTP mix (*see* **Note 9**).
4. Bovine serum album (BSA), molecular biology grade aqueous solution (*see* **Note 10**).
5. Nuclease-free water.
6. Forward and reverse primers (Table 1).
7. 0.2-mL PCR tubes and a 96-well PCR tube rack.

Table 1
Primer sets used in nested multiplex PCR (mPCR) for simultaneous detection of *Cryptosporidium*, *Giardia*, *Toxoplasma gondii*, and *Cyclospora cayetanensis*

Protozoa	Target gene	Primer	Direction	Nucleotide sequence (5′ – 3′)	Amplicon size (bp)	Reference
External reaction						
Cryptosporidium *T. gondii* *C. cayetanensis*	18S[a]	m18SeF	Forward	CGGGTAACGGGGAA TTAGGG	751–779	[15]
		m18SeR	Reverse	TCAGCCTTGCGACCA TACTC		
Giardia.	GDH[b]	GDHeF	Forward	TCAACGTYAAYCGYGG YTTCCGT	455	[18]
		GDHiR	Reverse	GTTRTCCTTGCACATC TCC		
(Alternative) *Giardia*	18S	g18SeF	Forward	AAGTGTGG TGCAGACGGACTC	497	[19]
		g18SeR	Reverse	CTGCTGCCGTCC TTGGATGT		
Internal reaction						
Cryptosporidium	18S	m18ScryF	Forward	TGGAATGAGTTAAGTA TAAACCCCT	543	[15]
		m18ScryR	Reverse	GCTGAAGGAG TAAGGAACAACC		
T. gondii	18S	m18StoxF	Forward	GGTGTGCACTTGG TGAATTCTA	405	[20]
		m18StoxR	Reverse	TGCAGGAGAAG TCAAGCATGA		
C. cayetanensis	18S	m18ScycF	Forward	TCGTGGTCA TCCGGCCTT	359	[15]
		m18ScycR	Reverse	TCGTC TTCAAACCCCCTAC TG		
Giardia.	GDH	GDHiF	Forward	CAGTACAACTCYGCTC TCGG	432	[18]
		GDHiR	Reverse	GTTRTCCTTGCACATC TCC		
(Alternative) *Giardia*	18S	g18SiF	Forward	CATCCGGTCGATCC TGCC	292	[21]
		g18SiR	Reverse	AGTCGAACCCTGA TTCTCCGCCAGG		[19]

[a]18S small subunit (ssu) ribosomal RNA (rRNA) gene
[b]GDH: glutamate dehydrogenase gene

3 Methods

3.1 Recovery of Protozoan Parasites from Leafy Greens

1. Weigh desired amount (e.g., 10–250 g) of leafy greens on weighing dishes (*see* **Note 1**).
2. Transfer each measured batch of leafy greens to an 18-oz Whirl-Pak bag (*see* **Note 1**).
3. Add 100 mL of 0.1% Tween 80 to the Whirl-Pak bag (*see* **Note 11**). Remove excess air out of the bag, fold the opening four times and close it by folding the wire inside.
4. Manually wash the leafy greens in the bag by externally hand-rubbing the bag back and forth for 2 min (*see* **Note 12**).
5. After manual agitation, transfer wash solution from the Whirl-pak bag to 50-mL conical centrifuge tubes using a motorized serological pipette. Two 50-mL conical tubes will need to be processed for each sample to accommodate the total 100 mL washing solution.
6. Centrifuge the conical tubes at 900 × *g* for 15 min at 4 °C. After centrifugation, gently aspirate supernatant to retain approximately 5 mL in each 50-mL conical tube using a serological pipette (*see* **Note 13**).
7. Combine the two 5-mL concentrated sample containing pelleted debris and liquid (herein referred to as pellets) from the same leafy green sample into a single 15-mL conical tube. Centrifuge again at 900 × *g* for 15 min at 4 °C.
8. Gently remove supernatant to retain 1 mL in the 15-mL conical tube using a serological pipette.
9. Transfer the 1 mL pellets to screw cap microcentrifuge tubes for nucleic acid extraction. Centrifuge 1 mL samples at 16,000 × *g* (or maximum speed of benchtop microcentrifuge) for 5 min and remove supernatant using a pipette to retain a 100 μL final pellet volume in the microcentrifuge tube (*see* **Note 14**). At this step, the sample can be frozen if needed or can proceed to extraction immediately.

3.2 Nucleic Acid Extraction

1. Preheat the heating block to 56 °C. Prepare a liquid nitrogen dewar and a large beaker of boiling water.
2. Start with 100 μL of samples in microcentrifuge tubes (wash pellet). Add 180 μL Buffer ATL provided in the QIAGEN DNeasy Blood and Tissue Kit to the sample. Include one extraction negative control containing only 180 μL Buffer ATL to ensure there is no cross contamination between samples during DNA extraction. Mix thoroughly by vortexing or pipetting.

3. Arrange tubes in a floating microtube rack and gently place in liquid nitrogen for 4 min. Immediately transfer the rack to boiling water for 4 min (*see* **Note 15**). Remove the rack and let cool for 2 min before opening the tubes.
4. Add 40 μL Proteinase K. Mix thoroughly by vortexing for 10 s at maximum speed.
5. Place sample tubes in a dry heating block and incubate overnight at 56 °C (*see* **Note 16**).
6. Following overnight incubation, remove samples from the dry heating block and increase the dry heating block temperature to 70 °C.
7. Add 200 μL Buffer AL to samples and vortex for 10 s. Incubate samples in the dry block incubator at 70 °C for 10 min.
8. Remove samples from the dry heating block and add 200 μL ethanol (96–100%) to samples. Mix thoroughly by vortexing for 10 s. Turn on the dry heating block temperature to 95 °C for later use in **step 11**.
9. Transfer the sample mixture into a DNeasy Mini spin column placed in a 2-mL collection tube. Centrifuge for 2 min at 9000 × *g* (≥6000 × *g*). Discard the flow-through and the collection tube bottom. Place the spin column in a new 2-mL collection tube.
10. Add 500 μL Buffer AW1. Centrifuge for 2 min at 9000 × *g* (≥6000 × *g*). Discard the flow-through and the collection tube bottom. Place the spin column in a new 2-mL collection tube (*see* **Note 17**).
11. Add 500 μL Buffer AW2. Centrifuge for 4 min at 14,000 × *g* (20,000 × *g*). In the meantime, make 1:10 AE buffer and nuclease-free water mixture (e.g., mix 130 μL Buffer AE with 1300 μL nuclease-free water) in a 2-mL microcentrifuge tube. Heat the mixture to 95 °C in the dry heating block.
12. After centrifugation in **step 11** is done, discard the flow-through and the collection tube bottom. Place the spin column in a new 2-mL collection tube.
13. Centrifuge for 1 min at 14,000 × *g* (20,000 × *g*). Discard the flow-through and collection tube bottom (*see* **Note 18**).
14. Place a spin column to a new 1.5-mL microcentrifuge tube. Add 50 μL 1:10 AE buffer preheated to 95 °C directly onto the center of the spin column membrane. Incubate for 5 min at room temperature with a cap closed.
15. Centrifuge for 2 min at 9000 × *g* (≥6000 × *g*). Check to ensure liquid filtered through to the microcentrifuge tube. Remove and discard the spin filter column. Store eluted DNA at 4 °C for up to 48 h or freeze at −20 °C.

3.3 Multiplex Polymerase Chain Reaction (mPCR)

Here we describe the procedure of a multiplex PCR (mPCR) assay for simultaneous detection of *Cryptosporidium* spp., *Giardia* spp., *T. gondii*, and *C. cayetanensis* in an external PCR followed by parasite differentiation via internal (nested) specific PCR assays [15]. PCR experiments should be performed in a clean environment such as a PCR workstation to minimize the risk of sample contamination.

1. Disinfect PCR workstation (or equivalent clean counter space for DNA-free work) using 10% bleach and ultrapure water (*see* **Note 19**).
2. Remove PCR reagents (PCR buffer, dNTP mix, primer sets and 10% BSA) from the freezer and place on ice to thaw reagents while keeping them chilled.
3. Inside a PCR workstation, prepare an external PCR master mix cocktail in a microcentrifuge tube according to Table 1. Reaction volume can be scaled up appropriately depending on the number of samples. Include the number of PCR-negative control (also referred to as no-template control (NTC)) and PCR-positive controls in the reaction volume (*see* **Note 20**). Two sets of forward and reverse primers (m18SeF-m18SeR and GDHeF-GDHeR) are used in the external reaction (Table 2) (*see* **Note 21**). Mix reagents thoroughly by vortexing or pipetting.
4. Dispense 45 μL external master mix into 0.2-mL PCR tubes.
5. Ideally, use separate workspaces for master mix preparation and DNA addition to avoid contamination of PCR reagents. Transfer PCR tubes with master mix to another clean workspace. Add 5 μL template DNA to the PCR tubes. Mix thoroughly by pipetting up and down several times.
6. Briefly spin PCR tubes using a mini centrifuge to move all liquid on the PCR tube wall to the bottom.
7. Place PCR tubes in a thermal cycler and start the cycling program as detailed in Table 3 (*see* **Note 22**).
8. Once the external PCR is done, remove PCR tubes from the thermal cycler and put on ice or store at 4 °C (for up to 48 h) until used in the internal reactions.
9. For nested PCR assays, prepare internal PCR master mix cocktail in four separate microcentrifuge tubes (one for each protozoan pathogen target) according to Table 2 (*see* **Note 23**). Preparing the master mix should be done in a DNA-free workspace.
10. Dispense 48 μL internal master mix into PCR tubes.
11. Add 2 μL external reaction amplicon to the PCR tube (*see* **Note 24**).

Table 2
PCR mixture

Component External reaction	50-μL reaction	Final concentration	Component Internal reaction	50-μL reaction	Final concentration
10× PCR buffer	5.0 μL	1×	10× PCR buffer	5.0 μL	1×
dNTP mix (10 mM)	1.0 μL	400 μM of each dNTP	dNTP mix (10 mM)	1.0 μL	400 μM of each dNTP
m18S forward primer (20 μM)	0.5 μL	0.2 μM	Pathogen-specific forward primer (50 μM)	0.5 μL	0.2 μM
m18S reverse primer (20 μM)	0.5 μL	0.2 μM	Pathogen-specific reverse primer (50 μM)	0.5 μL	0.2 μM
GDH forward primer (20 μM)	0.5 μL	0.2 μM			
GDH reverse primer (20 μM)	0.5 μL	0.2 μM			
BSA (10%)	1.6 μL	3.2 μg/μL	BSA (10%)	1.6 μL	3.2 μg/μL
Taq polymerase (5 U/μL)	0.3 μL	1.5 U	*Taq* polymerase (5 U/μL)	0.3 μL	1.5 U
Nuclease-free water	35.1 μL	–	Nuclease-free water	39.1 μL	–
Subtotal	45.0 μL		Subtotal	48.0 μL	
Template DNA (added at **step 5**)	5.0 μL		**Template DNA** (added at **step 11**)	2.0 μL	
Total	50.0 μL		Total	50.0 μL	

Table 3
PCR thermal cycle conditions

			3-step cycling (35 cycles)				
Reaction	Step	Initial denaturation	Denature	Anneal	Extend	Final extension	Hold
External	Temp	94 °C	95 °C	58 °C	72 °C	72 °C	4 °C
	Time	3 min	40 s	40 s	90 s	4 min	∞
Internal	Temp	94 °C	95 °C	59 °C[a]	72 °C	72 °C	4 °C
	Time	3 min	40 s	40 s	90 s	4 min	∞

[a]Use 60 °C for *Cryptosporidium* internal reaction

12. Place the PCR tubes in the thermal cycler and start the cycling program as detailed in Table 3 (*see* **Note 25**).
13. After the internal PCR reaction is completed, store PCR amplicons at 4 °C for up to 48 h or freeze at −20 °C until used.

14. Analyze the presence of the nested amplification products by running the PCR amplicons and a DNA ladder in a 2% agarose gel containing 1× RedSafe (e.g., 1 g of agarose powder in 50 mL of 1× TBE buffer and 2.5 μL RedSafe (20,000×) (*see* **Note 26**) at 100 v for 1 h or until samples move 70–80% downward of the gel.
15. Visualize the PCR product using an ultraviolet transilluminator. Use the DNA ladder to infer the size of amplification products in your samples and/or compare the band size to the target band size in the corresponding positive control as described in Table 1.
16. Purify internal PCR products from the gel using a gel purification kit (*see* **Note 27**) for sequence confirmation for conclusive molecular identification (*see* **Note 28**). Store remaining PCR products at −20 °C.

4 Notes

1. The amount of leafy greens to be analyzed can vary depending on available space and numbers of samples that need to be processed. The mPCR assay was originally validated using a relatively small volume of spinach (10 g) due to limited space available for conducting studies on organisms considered as biosafety level 2 pathogens. Most investigations suggest using larger amounts ranging from 25 to 250 g (summarized in [15]) to represent a typical consumed meal. For 10 g of leafy greens, 18-oz Whirl-pak bags are adequate, but the size of the Whirl-pak bags should increase to hold leafy green sample volume as sample portion increases.
2. Use DNase-, RNase-free filtered barrier pipette tips ideal for molecular biology applications.
3. For the freeze-thaw cycle using liquid nitrogen and boiling water in nucleic acid extraction, use screw cap, conical bottom, microcentrifuge tubes that can withstand temperatures from 196 °C to 100 °C to prevent accidental pop-up of flip-top tubes in boiling water. Avoid using skirted bottom microcentrifuge tubes, as the seams can leak during rapid temperature change in the freeze-thaw cycle.
4. Dry heating block (also referred as to dry bath, block heaters, or dry block incubator) that can hold 2-mL microcentrifuge tubes (Fisher Scientific cat. no. 88-860-022, or equivalent).
5. Use a benchtop cryogenic dewar that can withstand temperature of −196 °C for safe short-term transport and storage of liquid nitrogen. Wear safety goggles, a face shield, lab coat, insulated gloves when handling liquid nitrogen. Any unused

liquid nitrogen remaining in a dewar should be allowed to evaporate. Do not dispose of liquid nitrogen into a sink.

6. To prevent rapid temperature cooling of boiling water when frozen samples are placed during the freeze/thaw procedure, use a large volume of boiling water (>2 L).
7. Use a floating microtube rack and 12-inch forceps or similar tool to grasp the floating microtube rack for gentle placement and removal from liquid nitrogen and boiling water. Do not touch liquid nitrogen with bare skin or disposable gloves, or place tools (e.g., forceps) in contact with liquid nitrogen.
8. Additional proteinase K may be needed to add 40 μL per sample as the proteinase K provided in the QIAGEN kit is intended for use at 20 μL per sample. Proteinase K can be purchased separately (QIAGEN, cat. no. 19133, or equivalent).
9. AmpliTaq® DNA polymerase with 10× PCR Buffer I (15 mM $MgCl_2$) (Applied biosystems, cat. no. N8080152, or equivalent) and 10 mM dNTP Mix consisting dATP, dCTP, dGTP, and dTTP (Invitrogen, cat. no. 18427088, or equivalent).
10. Nuclease-free bovine serum albumin (BSA) 10% aqueous solution (Sigma-Aldrich, cat. no. 126615, or equivalent). BSA can be sub-aliquoted to 1.5-mL microcentrifuge tubes and stored at −20 °C.
11. Increase volume of 0.1% Tween 80 to sufficiently submerge leafy greens in the Whirl-pak bag accordingly when the amount of sample increases.
12. Previous work has demonstrated that manual leaf washing yielded higher recoveries and more consistent detection of protozoan parasites as compared with stomacher processing [15]. However, if mechanical blending is preferred due to practical reasons, leafy greens in **step 2** can be mechanically agitated using a device such as a stomacher as follows:
    (a) Place leafy green samples in one side of 24-oz Whirl-pak filter bag. Add 40 mL of 0.1% Tween 80 to the Whirl-Pak filter bag. Note that the amount of Tween 80 may increase as the amount of sample per bag increases.
    (b) To prevent potential leaking during the agitation, place the Whirl-Pak bag inside a bigger sized resealable bag (e.g., ziploc bag). With the opening of the Whirl-Pak bag with leafy green unsealed, slide the bag along the machine door to remove air out of the bag.
    (c) Close the door and operate the beating against the bag at the highest speed for 2 min. We used Smasher™ Blender/Homogenizer (bioMérieux) with FAST mode (620 strokes/min) [15]. The agitation setting can vary between stomacher machines.

(d) After the cycle is done, take out the eluant from the opposite side of filter bag where sample is located and transfer it to a 50-mL conical tube.

(e) Wash the filter with additional 40 mL of 0.1% Tween 80 by applying Tween 80 to the filter using a motorized serological pipette. Transfer the rinse solution to a new 50-mL conical tube. Proceed to **step 13**. Note that Tween 80 volume may increase when the sample amount increases. Multiple 50-mL conical centrifuge tubes can be replaced with one large volume centrifuge tube (e.g., 250-mL centrifuge tube, Corning, cat. no. 430776, or equivalent).

13. Use a motorized serological pipette controller with the lowest speed option (either by adjusting the aspirating speed or gently pressing the control switch). Gently aspirate eluant from the top surface without touching the pellet. Retain approximately 5 mL based on the graduated line in the conical tube.
14. The optimal volume that can be processed for DNA extraction in the spin column of QIAGEN DNeasy Blood and Tissue kit is 100 μL. Samples for DNA extraction can be stored at −20 °C at this step. If concentrated pellet volume cannot be reduced to 100 μL due to large pellet size, then up to 200 μL of partial sample can be placed in the spin column for DNA extraction.
15. This freeze–thaw cycle will rupture (oo)cyst walls prior to the remaining process of DNA extraction. Previous experiments demonstrated that increasing freeze–thaw cycles do not increase parasite DNA detection [22].
16. The QIAGEN DNeasy Blood and Tissue Handbook indicates that samples can be incubated with proteinase K at 56 °C until they are completely lysed. Depending on sample types, the treatment time can vary (to expedite processing time, this step could be shortened if validated for a desired vegetable matrix).
17. Add the appropriate amount of ethanol (96–100%) as indicated on the Buffer AW1 and Buffer AW2 bottles before the first use to make a working concentration.
18. As residual ethanol on the spin column may interfere with subsequent reactions, additional 1 min of centrifugation step is included to ensure complete removal of ethanol from the spin column. Often, no residual ethanol (or liquid) is visible in the collection tube after the last centrifugation.
19. PCR workstations and micropipettes should be thoroughly disinfected using 10% household bleach and ultraviolet (UV) before use. Because of the nested design of the PCR assay, DNA amplification is particularly sensitive and cross

contamination can occur if proper separation of DNA from reagents is not maintained, or if trace DNA material is present on surfaces or equipment. Thoroughly apply 10% bleach to all surfaces, ideally allow for 30 min of contact time, followed by ultrapure water (or 70% alcohol) to remove residual bleach. Preferably, preparation of PCR master mix and addition of DNA template into the master mix should be done in separate PCR workstations to reduce cross-contamination among samples.

20. Nuclease-free water is used instead of DNA template for PCR-negative controls. PCR-positive controls consist of target parasite DNA. We typically use extracted DNA from 1000 (oo) cysts stock solutions for positive controls.
21. This nested mPCR assay was designed by using a primer set (m18S) that simultaneously amplifies a target region of the 18S ribosomal RNA (rRNA) gene of *C. parvum*, *T. gondii*, and *C. cayetanensis* in the external reaction (Table 2). The m18S primer set is then multiplexed with a primer set targeting the glutamate dehydrogenase (GDH) gene of *Giardia* in one external PCR. When the mPCR assay (m18S—GDH) was applied on spiked spinach leaves, *Giardia* was detected in as low as 5 cysts/g spinach (in 10 g) [15]. Note that other *Giardia* primer sets targeting the 18S rRNA gene [19] can perform better for other matrices when multiplexed with the m18S primer set (unpublished data).
22. Initial denaturation temperature and time may vary depending on PCR reagents in use.
23. To discriminate the four parasites via internal nested reaction, four pathogen-specific internal primer sets are separately used in the nested reaction.
24. While 5 μL template DNA (genomic DNA) is used for external reaction, 2 μL of external amplicon is used as DNA template for internal reaction.
25. PCR thermal cycle conditions for the internal reactions were similar to those of the external reaction except for the annealing temperature, which was increased to 59 °C for *Giardia*, *T. gondii*, and *C. cayetanensis* and to 60 °C for *Cryptosporidium*.
26. Use safe alternatives to highly mutagenic ethidium bromide (EtBr) such as RedSafe (Bulldog Bio, cat. no. 21141, or equivalent) for detecting nucleic acid in agarose gel. RedSafe can be added in agarose solution before it is solidified. RedSafe can be also added to the running buffer to avoid depletion of RedSafe in the bottom portion of the gel during gel electrophoresis.

27. Use QIAquick gel extraction kit (QIAGEN cat. no. 28704, or equivalent).
28. Because unknown environmental organisms can also yield amplicons consistent in size with positive controls, it is recommended that sequence confirmation of suspect positives be always performed for conclusive molecular identification. The identity of DNA amplicons can be confirmed via Sanger sequencing.

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Chapter 16

Viability of *Trypanosoma cruzi* in Food and Beverages

Rodrigo Labello Barbosa and Karen Signori Pereira

Abstract

Chagas' disease can occur via oral transmission through food and beverages contaminated with *Trypanosoma cruzi*. This chapter aims to present an analytical methodology for the direct detection of the viability of *T. cruzi* in food matrices, from evaluations of survival (in vitro), as well as of infectivity, pathogenicity, and virulence (in vivo). The methodology has already shown important results in previous studies, and new perspectives can be pointed out when considering the complex biological cycle of *T. cruzi* and its current relevance as a foodborne parasite.

Key words Chagas' disease, Foodborne, Oral transmission, Sieving, Survival, Virulence

1 Introduction

Trypanosoma cruzi [Chagas, 1909] [1] is a hemoflagellate protozoan that currently belongs to the Excavata Supergroup, Euglenozoa Group, Kinetoplastea Subgroup [2], and is grouped into discrete typing units (DTU), according to ecological, epidemiological, and clinical criteria [3].

T. cruzi is the etiologic agent of Chagas' disease, which has a complex life cycle, and includes an intermediate host (hematophagous Hemiptera), a definitive host (*Homo sapiens*), and reservoir mammals in the maintenance of domestic and peridomestic cycles, and the wild [4, 5].

Chagas' disease is an anthropozoonosis classified as an infectious and neglected disease, which must be understood from the parasite–host–environment interaction, and presents the acute, chronic, and indeterminate phases [4, 6].

The worldwide prevalence has made it a global health topic in recent years. The numbers are variable, but in the Americas alone, it is estimated that about six to seven million individuals are infected, and there are approximately 75 million people at risk of infection in the world [7, 8].

Marciane Magnani (ed.), *Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-1932-2_16,

The vector-borne is the main form of transmission of human Chagas' disease. However, the oral route is also a primary transmission mechanism, especially in the wild cycle. In humans, it can occur sporadically and circumstantially and is not classified as accidental [5, 6].

Oral transmission through food contaminated with *T. cruzi* is associated with the consumption of fruit juices, raw milk, vegetables, water, game meat, mammalian blood, or exotic habits in the Americas. In general, acute Chagas' disease (ACD) related to foodborne outbreaks can present severe symptoms and fatality, given the limited availability of drugs for treatment [9–11]. Given the relevance in public health and its economic impacts, *T. cruzi* is among the 10 most important foodborne parasites [12].

Currently, factors such as the opening of new food-consuming markets without certification of parasitological quality must be identified because they may contribute to the intensification of Chagas' disease as an important trend for the coming years without stricter regulations [13, 14].

Fundamentally, despite methodological advances and legislation aiming at surveillance [15], investigations of ACD foodborne outbreaks are based on clinical and laboratory diagnosis of the hosts with correlation of possibly contaminated food intake, and eco-epidemiological evidence [16, 17].

Thus, the objective proposed here is to present an analytical methodology for the direct detection of the viability of *T. cruzi* in food matrices, from evaluations of survival (in vitro), as well as infectivity, pathogenicity, and virulence (in vivo).

The methodology was developed between 2007 and 2008, has already yielded important results in specific contexts and previous academic studies. It requires investments in adequate infrastructure, knowledge, and specialized services, being one of the outputs spanning Parasitology and Food Science and Technology, in a multidisciplinary view.

When contemplating in vitro and in vivo stages, both can be indicative of failures in Good Manufacturing Practices (GMP) that result in contamination along the production chain, as well as risks to human health.

It is important to note that the protocol herein provided are the minimum necessary for the feasibility of the proposed methodology, so that an attentive professional can find an inclusive and consistent reference in this work. It supports, directs the experimental design (Fig. 1), and can be adapted to other studies of *T. cruzi* or other foodborne or waterborne parasites, and food safety, if appropriate.

In addition, the methodology has the potential to be applied in clarifying outbreaks of foodborne Chagas' disease as well as for quality control and surveillance, jointly with risk analysis applied to food safety and advances in legislation.

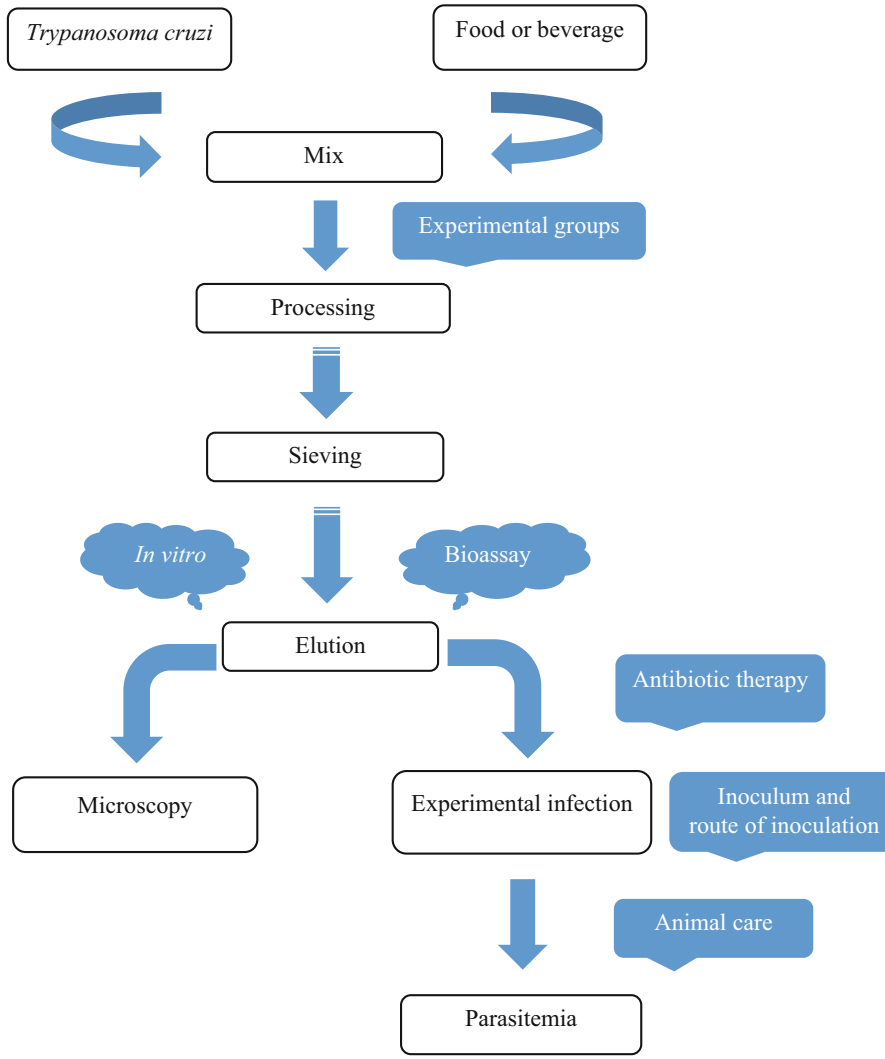

Fig. 1 Simplified flowchart of methodology for the viability of *T. cruzi* in food and beverages

Its scope stimulates the development of science, technology, and innovation, as it serves as a basis and can adapt to the objectives and needs of different laboratories, research groups, or other institutions.

It is also necessary to consider that the specific approach of parasitology in food and beverages is still recent. Therefore, new perspectives can be highlighted when considering the complex biological cycle of *T. cruzi* and its current relevance as a foodborne parasite.

2 Materials

2.1 Trypanosoma cruzi

In general, *T. cruzi* is maintained in the laboratory in vivo through successive experimental infections in triatomine vectors (*see* **Note 1**) or laboratory animals (*see* **Note 2**) or in vitro in acellular culture media (*see* **Note 3**) or cells (*see* **Note 4**), allowing observation of the developmental stage at a given moment in the parasitic life cycle. The storage of blood, culture, and vector forms (*see* **Note 5**) can occur by cryopreservation (*see* **Note 6**) for prolonged periods, with preservation of viability [18–21].

2.2 Food Matrix

Preferably use in natura food or beverages or in the usual mode (*see* **Note 7**), as commonly available to the consumer [19, 20, 22–24].

2.3 Sieving System

A vertical column in which layers of filter medium are inserted between membranes (stationary phase), in a sterile environment. The column can be made up of different materials (*see* **Note 8**), of varying volumes. The filter medium can consist of different shapes, materials, and sizes (*see* **Note 9**). Membranes can consist of fabrics or other porous materials [19, 20, 25].

2.4 Eluent

Sterile elution solution (*see* **Note 10**) is recommended to assist the process of sieving of the parasites in food matrices [19, 20, 25].

2.5 Microscope

The sieving product or elution product (eluate) can be analyzed using a microscope (*see* **Note 11**) for in vitro visualization of *T. cruzi* survival [18–21].

2.6 Experimental Host

Laboratory animals (*see* **Note 12**) are used for in vivo analysis of infectivity, pathogenicity, and virulence of *T. cruzi* [18–20, 26–29].

2.7 Antibiotic

The use of antibiotics or another suitable active agent (*see* **Note 13**) is indicated as a prophylactic measure for experimental hosts [19, 20, 25].

3 Methods

3.1 Trypanosoma cruzi

Select the *T. cruzi* strain, and the developmental stage of interest (*see* **Note 14**). Then, select the required concentration of the parasite, and perform dilution, if necessary. It is recommended to perform isolation, identification, and genetic characterization of *T. cruzi* [18–21].

3.2 Food Matrix

Select the food or beverage of interest. If processing for transport or storage is required (*see* **Note 15**), maintain the identity and quality standards of the matrix before handling (*see* **Note 16**).

Perform homogenization. Then, if necessary, distribute in aliquots. Measuring the potential for hydrogen (pH) of the food matrix is recommended [19, 20, 22–24].

3.3 Experimental Contamination of the Food Matrix

Mix *T. cruzi* and the food matrix, in pre-established proportions or using a dilution factor (DF), if convenient (*see* **Note 17**). Perform homogenization [19, 20].

3.4 Experimental Groups

The experimental design must contain negative control, positive control, and test groups (*see* **Note 18**). In addition, depending on the proposed aim, the plan may contain or be called method white (reagent white or method blank), fortified white (spike white), and/or fortified sample (fortified matrix or spike matrix), for instance [19, 20].

3.5 Processing of the Food Matrix

Select the appropriate processing (*see* **Note 19**) to which the experimental groups will be submitted in relation to the proposed objective [19, 20, 22–24].

3.6 Sieving

Complex food matrices can be subjected to sieving, a process in which particles of varying dimensions can be separated into fractions. The food matrix experimentally contaminated with the parasite is added to the sieving system. If necessary, activate the column with eluent prior to adding food matrix. Depending on the food matrix, the type of processing used, and the required volume of the eluate, there may be a need to apply pressure (*see* **Note 20**) to the sieving system, using the Pascal Principle, e.g., the eluate obtained is collected in an appropriate container (*see* **Note 21**). Sieving allows the isolation and direct visualization of live parasites in complex food matrices, as well as the inoculation of the product obtained by different in vivo routes of inoculation. Depending on the characteristics of the food matrix and processing, the sieving step may not be needed. Alternatively, other types of technologies or parasites may be added or applied to the methodology, according to the intended objective [19, 20, 25].

3.7 Elution

When necessary, it occurs by adding eluent to the sieving column. Elution can occur under pressure. The eluate can be collected together with the sieving product or later, in separate containers. Eluate can be used in other analytical methodologies. It is convenient that the pressure be applied in only one of the steps, without changing the sieving system (*see* **Note 22**) [19, 20, 25].

3.8 Microscopy

Eluate can be analyzed under optical microscopy, without staining, by direct visualization of *T. cruzi*. It is recommended to keep the slides in a humid chambre. When studying epimastigotes or trypomastigotes, the characteristic movement of the flagellum is visualized, when the parasite survives after processing. Qualitative or

quantitative assessment of parasite survival can be performed (*see* **Note 23**). The visualization of live parasites at this step indicates that there is survival, but does not discard the need for the in vivo stage for analysis of infectivity, pathogenicity, and virulence. Live parasites in in vitro tests are evidence and viability markers. It is recommended that non-visualization of parasites in vitro is reported as an inconclusive result or undetected parasite. Visual recording of results under microscopy is recommended. Alternatively, other types of microscopy or technologies may be applied to the methodology [18–21].

3.9 Inoculum and Route of Inoculation

Select the volume (*see* **Note 24**) of the inoculum (*see* **Note 25**), and the route of inoculation (*see* **Note 26**), according to the proposed objective, and the ethical considerations for the model animal used. To assess the virulence of *T. cruzi*, it is recommended that all the eluate (total volume) be used in the bioassay [18–20, 26, 27, 29].

3.10 Experimental Infection

Select the appropriate animal model for the aim of the bioassay, considering the parasite–host–environment interaction. Attention should be paid to the hosts' susceptibility and resistance to Chagas' disease. Genetic constitution, immune system, age group, sex, and weight must be considered (*see* **Note 27**). Other experimental characteristics and conditions should be specified (*see* **Note 28**). Define the number of animals per experimental group, respecting the principles of the 3R's in Laboratory Animal Science [18–20, 26–29].

3.11 Antibiotic Therapy

The administration of antibiotic or another active ingredient that has no effect on the parasite of interest is indicated as prophylaxis to experimental infection, also considering the route of inoculation, and the type of inoculum (*see* **Note 29**). An evaluation prior to the choice of the antibiotic is recommended, according to the manufacturer's instructions for use [19, 20, 25].

3.12 Parasitemia

The presence or absence of the trypomastigote in the phase of the biological cycle that occurs in the blood circulation of the vertebrate host, as a laboratory diagnosis, can also be observed under microscopy, following the same recommendations. Blood collection (*see* **Note 30**) must be carried out in accordance with ethical considerations for the animal model used. It is convenient that the result of the parasite search when absent in the blood is reported as undetected. It is recommended to define the period of observation of the host (*see* **Note 31**), as well as the period for carrying out the laboratory examination and experimental outcome, according to the objective (*see* **Note 32**) [18–21, 26, 27].

3.13 Animal Care

Animals must be kept under controlled sanitary conditions throughout the experiment. In the end, euthanasia and/or disposal

of infected animals or carcasses must be followed according to standards established in Laboratory Animal Science [18–20, 26, 27, 29].

3.14 Statistical Analysis

The experimental design should include adequate statistical analysis, when convenient and applicable [18–20].

3.15 Biosafety, Ethics, and Bioethics

If there is a transfer of *T. cruzi* from biological reference collections, presentation of certification of transfer of genetic heritage between institutions is recommended as well as following specific procedures or legislation, when appropriate. All steps of the handling and disposal of *T. cruzi* must follow biosafety standards of level 2. The use of personal and collective protection equipment must occur in all stages (*see* **Note 33**). There should be serological monitoring for Chagas' disease of collaborators who manipulate infectious forms of the parasite, as a preventive healthcare. In Brazil, it requires compulsory notification. Given the current scientific and technological scenario, certain procedures still need to be performed on animal models. In vivo experimental studies provide microscopic and macroscopic evidence. However, the development of alternative methods should be encouraged whenever possible. The handling of animal models must be in accordance with standards in Laboratory Animal Science and present legislation, with approval and certification from institutional Animal Ethics Committee. In Brazil, all procedures must follow Law 11,794/2008. Considerations about biosafety, ethics, and bioethics are based on the current context, in order to guide future advances [6, 18, 26, 27, 29, 30].

3.16 Quality of Analytical Results

Apply the scientific method at all stages and promote the elimination of factors that may influence the results, when carrying out all procedures under controlled laboratory conditions, in compliance, as a guarantee of results, in order to avoid false or mistaken interpretations [31].

4 Notes

1. For example, *Panstrongylus* sp., *Rhodnius* sp., or *Triatoma* sp.
2. Mainly *Mus* sp. mice.
3. Such as Liver Infusion Tryptose (LIT) or McNeal, Novy and Nicolle (NNN).
4. Example: macrophages or fibroblasts.
5. Example: amastigotes, epimastigotes, metacyclic trypomastigotes, or blood trypomastigotes.
6. Such as glycerin 10% v/v, and liquid nitrogen at −196 °C.
7. Mainly solid or liquid form.

8. For instance, plastic.
9. Example: metal, plastic, or glass spheres.
10. Such as 0.15 M sodium chloride solution.
11. For example, optical microscope.
12. Mainly *Mus* sp. mice too.
13. Example: a substance with a broad spectrum, and low cost.
14. Example: blood or metacyclic trypomastigote.
15. Such as a thermal processing.
16. For instance, thawing at room temperature.
17. Example: 1:2 or DF = 3.
18. Negative control should be food or beverage without *T. cruzi* or parasite free; positive control: *T. cruzi*, in determined concentrations if applicable; test groups: e.g., experimentally contaminated food matrix or eluate.
19. Such as incubation period and temperature.
20. Example: with a syringe plunger.
21. For example, a flask or tube.
22. Do not replace the plunger or piston.
23. It should be between slide and cover slip for counting in Neubauer chamber.
24. Example: microliters.
25. Choose an experimental group in this step such as, for example, test group.
26. Example: intraperitoneal, gavage, or oral.
27. For example: inbred mice, immunodeficient, adult, male, and average weight of 30 g.
28. Considerations: water and chow ad libitum or fasting, use of substances, environmental enrichment, pregnancy, or marking to identify animals.
29. Example: in immunodeficient hosts, via intraperitoneal and/or testing fresh food or in natura beverages.
30. For instance, the volume of five microliters.
31. Example, for 40 or 60 days after infection (d.a.i).
32. As for example, investigation of the day of onset of parasitemia, the day of mortality after experimental infection or euthanasia for injury research.
33. Example: biosafety cabinet.

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Chapter 17

Detection of *Giardia* Cysts and *Cryptosporidium* Oocysts in Edible Shellfish: Choosing a Target

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Abstract

Bivalve mollusks are filter-feeding animals that are often consumed raw or partially cooked. They can harbor a wide variety of microorganisms such as the pathogenic protozoa *Giardia* and *Cryptosporidium*. Both these pathogens are well-known causative agents of diarrhea in humans and have been associated with several water and foodborne outbreaks around the world. Their infective stages, cysts and oocysts, respectively, can remain on the gills and other organs of shellfish, posing a potential threat to human health. There is no standard protocol or valid ISO for the detection of cysts and oocysts from shelled mollusks. The aim of this chapter is to describe the main methods used to detect *Giardia* cysts and *Cryptosporidium* oocysts from shellfish, based on techniques adapted from clinical and environmental parasitology, as well as molecular procedures. The monitoring of these foodborne protozoa in bivalve mollusks is of great relevance to public health, contributing to knowledge of contamination in one of the main food products derived from aquaculture. Indeed, it also reflects the quality of the environmental health surrounding its cultivation, highlighting another important aspect related to global environmental epidemiology.

Key words *Cryptosporidium*, *Giardia*, Protocol, Shellfish

1 Introduction

Shelled mollusks, also known as shellfish, are among the most important animals derived from aquaculture destined for human consumption. The most recent The State of World Fisheries and Aquaculture census revealed that almost 18 million tons of mollusks were produced worldwide, representing 56.3% of the production of marine and coastal aquaculture [1].

Despite its importance as a source of food and income, for decades freshwater and marine bivalve mollusks have been used as "sentinels" of environmental pollution, as they are sedentary filter-feeding species and may therefore be indicators of the sanitary

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quality of the surrounding cultivation areas, which are also sometimes used for human recreational purposes [2–5].

Another important public health aspect is infectious outbreaks linked to the consumption of bivalve mollusks, as the tissues of the animals may harbor a wide variety of pathogenic microorganisms, posing a risk to health as they are often consumed raw or with minimal cooking. Moreover, the risk of infection can increase when the animals are sold without the cleaning or purification procedures—especially UV depuration—applied by the mariculture industry [6–10].

The contamination of bivalve mollusks by pathogenic protozoa has only attracted global attention in the last 25 years, with *Giardia* and *Cryptosporidium* being the most commonly detected protozoa in different edible shellfish species, or in those of no commercial interest [11–13].

These parasites are of significant importance to human health as they are recognized agents of diarrheal diseases, and their risks are often neglected [14]. In addition, both are recognized as important foodborne agents in other different food matrices, such as salads, milk, juices, and meat [15–17].

Until now, few giardiasis outbreaks have been related to shellfish consumption, and none have been identified as caused by *Cryptosporidium* [16, 18]. Although there is no apparent relationship between shellfish vehicles and outbreaks of giardiasis or cryptosporidiosis, several factors should be considered: (1) the lack of a system for the reporting of foodborne diseases in many countries, which leads to under-estimation or underreporting of infections; (2) the unavailability of the original food matrix suspected of or responsible for originating the outbreak, for further analysis; (3) the extended incubation period exhibited by both protozoa (1–2 weeks), and the difficulty in performing the retrospective association between the ingestion of bivalves and the appearance of clinical signs or symptoms [13, 16]. Indeed, some biological aspects of both protozoa must be taken into consideration, which reinforces the importance of monitoring these bivalve mollusks. *Giardia* and *Cryptosporidium* are ubiquitous in aquatic environments, and their infective stages (cysts and oocysts, respectively) are immediately released as infectious upon excretion [19]. Also, the infectious dose required to establish an infection is low for both, meaning that along with the high number of (oo)cysts excreted, they can spread easily and pose a great risk to public health [20].

It is also important to highlight that cysts and oocysts exhibit considerable longevity in coastal environments, as they can withstand great variety in temperature and salinity and remain viable outside their hosts in aqueous environments for several months to a year in seawater [21–23]. There is still no correlation between microbiological fecal indicators and pathogenic protozoa in mollusk flesh or in waters where they are cultivated and, unlike other microorganisms, they are not inactivated or quickly removed from the

environment [5, 22, 24]. Finally, both protozoa can remain in the bivalve tissues, even after depuration procedures [4, 6, 8, 10, 24].

1.1 Overview of Strategies for the Detection of Cryptosporidium Oocysts and Giardia Cysts in Shellfish

No standard validated method for the detection of *Cryptosporidium* and *Giardia* in shellfish is available, making comparison difficult, as each study utilized one or more types of bivalve mollusk, and different analytical methods [13, 25–27]. Another important factor that makes detection complex relates to the transit of the protozoa through the shellfish, which can vary, being concentrated in different animal tissues [28–30]. Thus, prior to detection, it would be reasonable to consider which tissue or other compound will be chosen for further analysis, and also to consider the most edible relevant species destined for human consumption from each specific geographical location [5, 8, 13].

Overall, gills and the digestive tract are frequently employed for this purpose, with tissue homogenates [11, 24, 31, 32] or washings mainly used to concentrate the protozoa [8, 33, 34]. Other strategies have previously been employed to detect the protozoa, with hemolymph extracted from the adductor muscle [30, 35, 36], inner-shell water (intravalvular liquid) [4, 8, 37] or the pooled whole mollusk [26, 38] also used.

Several studies have adopted individual shellfish (whole flesh) as their analytical material [39, 40] or have taken specific parts or organs of animals separately as their samples [12, 34, 41]. However, it should be remembered that the analysis of pooled shellfish or organs, while increasing detection rates, may substantially diminish costs, being considered a more representative sample and facilitating the assessment of foodborne protozoa risk associated with shellfish consumption in low-income food-deficit countries or those with lower financial budgets.

Despite the use of the bivalve (tissue or whole flesh) target, there is a concern that for successful isolation of both protozoa through the shellfish, the protocol applied must obey a minimum of three major steps: *concentration*: successive centrifugations, coarse-sieving, or usage of pepsin digestion solution; *purification*: flotation or immunomagnetic separation (IMS) using magnetic beads coated with anti-*Cryptosporidium* and anti-*Giardia*; *detection method of cysts and oocysts*: microscopy visualization—preferably through the use of direct immunofluorescence, using specific monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) against epitopes of cysts and oocysts—considered the gold standard, and molecular techniques such as PCR [25, 26, 30]. PCR protocols are also used, but present some difficulties, such as the removal of the inhibitors from the matrices. The advantage of this technique is that it allows the source of contamination to be tracked—which may be particularly important in foodborne outbreaks—through the identification of *Cryptosporidium* species and genotypes and *Giardia duodenalis* genetic groups [13, 27].

2 Materials

2.1 Pre-Sampling Harvesting

Prepare all solutions with correct molarity or concentration using ultrapure water at room temperature. After preparation, store solutions at 4 °C (*see* **Note 1**).

2.2 Reagents

1. Elution solution: Prepare 1 L of Tween 80 (0.1%) (*see* **Note 2**).
2. Sterile PBS solution (0.04 M). Adjust pH to 7.2.
3. Diethyl ether (*see* **Note 3**).

2.3 Materials

1. Petri dishes.
2. Sterilized clam knife.
3. Scalpel and scalpel blades.
4. Tissue homogenizer.
5. Centrifuge and micro centrifuge tubes (15 mL and 1 mL, respectively).
6. Sample mixer (RK Dynal®) or similar.
7. Pasteur pipette.
8. Tweezers.

2.4 Sample Collection

1. Samples must be collected using suitable tools. Immediately transport shellfish to laboratory in clean plastic bags and suitable refrigerated containers.
2. Samples must be kept under refrigerated conditions until processing.

3 Methods

3.1 Protocol 1: Detection of Protozoa through Liquid Materials from Mollusks

3.1.1 Bivalve Opening

Open each animal with suitable tools looking for the umbo (the oldest part of the shell; the junction that connects both shells) (Fig. 1a); section the adductor muscles of the bivalve to facilitate opening (Fig. 1b) (*see* **Note 4**).

3.1.2 Sample Processing

Internal Content

1. Each sample represents a pool of one dozen oysters (Fig. 1c), with the gill sets and inner-shell water (intravalvular liquid) removed from each animal.
2. Aspirate all the inner-shell water content of the animals with a Pasteur pipette (Fig. 1d) and place in clean and decontaminated centrifuge tubes (*see* **Notes 5** and **6**).

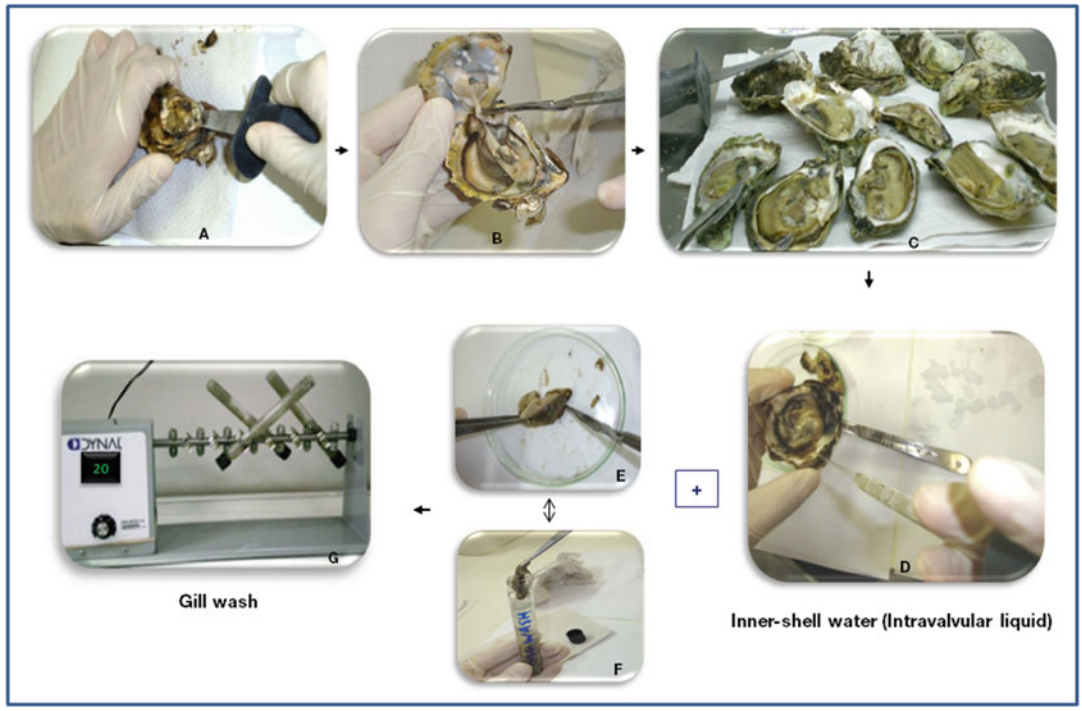

Fig. 1 Shellfish processing: analysis of inner-shell water and gill wash. (**a**) Opening of the umbo (junction that connects both shells); (**b**) Section of the adductor muscles of the bivalve to facilitate opening; (**c**) Each sample represents a pool of one dozen oysters; (**d**) Aspiration of all the inner-shell water content of the animals; (**e**) Extirpation the entire set of gills from each animal; (**f**) Sets of gills on glass tube; and (**g**) Tubes (corresponding to the set of gills from 12 animals) in sample mixer

3. Sieve the liquid content of all the tubes. After this step, centrifuge the liquid (1050 × *g* for 10 min) (*see* **Note 7**).
4. Remove all supernatants and maintain a volume of 2 mL of sediment in each tube. Complete the tube with ultrapure water and centrifuge again under the same conditions.
5. Remove the supernatant and transfer the sediment into properly identified micro tubes. Keep all tubes at 4 °C until the purification process using IMS.

Gill Collection and Processing

1. After opening the bivalve (*see* Subheading 3.1.1), excise the entire set of gills from each animal with the aid of a scalpel and tweezers (Fig. 1e) (*see* **Note 8**).
2. Place four sets of gills on each glass tube (Leighton tubes may be used) (Fig. 1f). Next, add about 2 mL of elution solution to the tube and gently shake manually so that the liquid meets the gills.

3. After removing the fourth gill set, complete the tube with Tween 80 (0.1%) elution solution until all gill sets are submerged.
4. Place the three tubes (corresponding to the set of gills from 12 animals) in the sample mixer (IMS rotor may be used) and leave to homogenize for 1 h at 20 RPM (Fig. 1g).
5. After this step, remove each tube from the rotor and vortex for 15 s.
6. Open each glass tube separately and remove each set of gills individually, placing each one in a sieve over a beaker. Gently, wash the gills with 3 mL of elution solution while sieving. Aspirate all the sieved liquid and transfer to 15-mL centrifuge tubes.
7. Collect the gill washing liquid from the empty glass tubes and place in 15-mL centrifuge tubes.
8. Add 5 mL of elution solution to the glass tube (empty) and mix by vortexing for 10 s. Aspirate the liquid and add to the centrifuge tubes.
9. Centrifuge all tubes at 1.050 × *g* for 10 min (*see* **Note 7**).
10. Remove all supernatants and complete with ultrapure water and centrifuge again under the same conditions.
11. Remove supernatant and transfer sediment into properly identified micro tubes. Maintain all tubes at 4 °C until the purification process using IMS.

3.2 Protocol 2: Detection of Protozoa through Homogenized Tissue Materials from Mollusks

Proceed as described in Subheading 3.1.1.

3.2.1 Bivalves Opening

3.2.2 Sample Processing

Gill and Gastrointestinal Tract Removal

1. After opening the bivalve, excise the entire set of gills and gastrointestinal tracts from each animal with the aid of a scalpel and tweezers and transfer them to petri dishes.
2. With the aid of a tweezer, transfer all the sets of both tissues (separately) to tissue homogenizer.
3. Add elution solution containing Tween 80 (0.1%) and distilled water (2:1).
4. Homogenize the tissues until they become a liquid solution.
5. Transfer the solution to glass centrifuge tubes.
6. Add 4 mL of refrigerated diethyl ether (in order to remove lipids) to each tube.

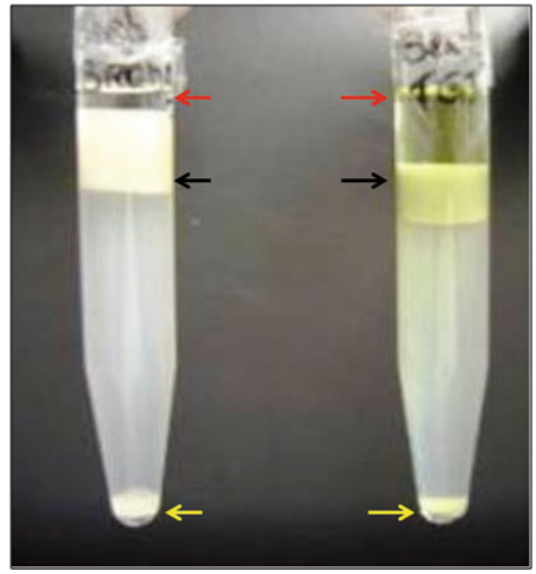

Fig. 2 Analysis of homogenized tissues. Tissues subjected to centrifugation with ether-PBS: yellow arrows: represent the sediments analyzed by IFA, gills (left tube) and gastrointestinal tract (right tube); black arrows: mucus in tissues; red arrows: amount of lipids present in tissues

7. Cover each tube and wrap the edges with cotton.
8. Shake each tube vigorously for 30 s.
9. Complete centrifuge tube with sterile PBS solution (0.04 M; pH 7.2).
10. Centrifuge at 1.250 × *g* for 5 min. After this, three phases will be produced (Fig. 2).
11. Remove the supernatant and the remaining tissue and lipids with the aid of wooden and cotton toothpicks (Fig. 2).
12. Transfer the sediment to micro tubes.
13. Maintain all tubes at 4 °C until purification using IMS.

3.3 Purification Using Immunomagnetic Separation (IMS)

1. For all pellets, proceed to immunomagnetic separation phase in accordance with reference method 1623.1 [42] or ISO 15553 [43] (*see* **Note 9**).
2. After the IMS procedure, the final volume will be 100 µL.
3. Separate 50 µL per slide (the volume to be used for immunofluorescence assay) and the remaining 50 µL for PCR (polymerase chain reaction). In this case, the total number of oocysts/cysts will be the total number of (oo)cysts visualized on the slide multiplied by 2.

3.4 Detection of Protozoa by Direct Immunofluorescence-Assay

1. Immunofluorescence assay (IFA) must be processed according to the manufacturer's instructions. The only change is in the volume placed in the slide well (50 μL), with the rest utilized in PCR (*see* **Notes 10** and **11**).
2. Keep slides incubated in a humid chamber. After drying, fixing, and staining, the entire smear in each well must be examined at 400× or 600× magnification using an epifluorescence microscope. DAPI and DIC should be applied as per USEPA 1623.1 protocol [42].

3.5 Detection of Protozoa by Molecular Methods

1. Use the 50 μL remaining from the IMS procedure to extract the DNA (*see* **Note 12**).
2. After DNA extraction, amplify the DNA by nested-PCR protocols (*see* **Note 13**).

If the molecular analyses of the samples are positive, the use of two or three genes is encouraged to determine the genotype present.

4 Notes

1. Shellfish farming producers recommend the consumption of animals within 5 days of harvest or purchase. However, from our personal experience, the animals should be processed within 48 h, as even in areas of high microbiological quality, specimens spoil quickly, generating a pungent smell (bad odor), and bacterial proliferation.
2. Add 100 μL of Anti Foam A to the elution solution. Use the magnetic stirrer to homogenize the solution until the reagents are completely dissolved.
3. Must be stored at 4 °C prior to use.
4. Use individual protection equipment before starting: coat, gloves, and safety goggles, as oysters may be harvested from areas impacted by sewage.
5. Rinse all centrifuge tubes and Pasteur pipettes with Tween 80 (0.1%) prior to the experiments to reduce the likelihood of parasite attachment. The use of glass materials is preferable as adhesion of cysts and oocysts is greater with plastic, reducing the possible loss of protozoa.
6. Take care not to suck grease or fragments from the shell into the animals, as this may interfere with visualization and the IMS process.
7. All tubes containing inner-shell water and gill wash liquid may be also centrifuged following the recommendations of the last version of the USEPA method (1623.1) for liquid materials (1500 × *g* for 15 min) [42].

8. Avoid moving material from the gastrointestinal tract (hepatopancreas) to the glass tube, as well as the mantle (the layer on top of all the other organs).
9. Consider using thermic rather than acid dissociation [44]. It is important to perform this step twice (80 °C for 10 min) as shellfish are rich in mucous tissue and lipids.
10. Use only IFA commercial kits recommended by validated methods, as per the standard procedures established for water samples:
    (a) MeriFluor® *Cryptosporidium/Giardia*, Meridian Diagnostics Cincinnati, OH.
    (b) Aqua-Glo™ G/C Direct FL, Waterborne, Inc. New Orleans, LA.
    (c) Crypt-a-Glo™ and Giardi-a-Glo™, Waterborne, Inc. New Orleans, LA.
    (d) EasyStain™C&G, BTF Pty Limited, Sydney, Australia.
11. Gastrointestinal tract homogenate analysis by IFA may be more difficult than gill homogenate examinations, due to the presence of thick layers on the slides. Therefore, (oo)cysts may not be detected due to masking [32, 34].
12. For DNA extraction, use commercial kits. Freezing–thawing cycles may also be used for *Cryptosporidium*. The number of cycles employed in the extraction process is critical, with a greater number of cycles potentially leading to DNA degradation [45].
13. For nested PCR protocols, consider the genes described in Table 1 for each pathogenic protozoan. In the event that nested PCR second reactions are positive, proceed to sample purification and then to gene sequencing.

Table 1
Most commonly used locus for the amplification of *Giardia* and *Cryptosporidium* genes in environmental samples

Protozoan	Locus	Reference
Giardia	18S rRNA	[46, 47]
	ß-giardin	[48, 49]
	Tpi	[50]
	Gdh	[51]
Cryptosporidium	18S SSU rRNA[a]	[52, 53]

[a]It is important to proceed to the nested PCR protocol for locus *gp60* to confirm the sample contamination of *Cryptosporidium parvum* or *Cryptosporidium hominis* genotypes

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Chapter 18

Protocol for the Detection of *Toxoplasma gondii* Oocysts in Water Samples

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Abstract

Toxoplasma gondii has emerged as an important etiology in waterborne protozoa outbreaks, this organism has an evolutionary form, known as oocysts, capable of maintaining viability for a long time in the environment and water. The diagnosis of this protozoan in water samples is difficult, mainly because of variability in the water physical parameters which makes the development of a standard technique difficult. Some advances in methodologies have been described for the diagnosis of *T. gondii* oocysts in water; nevertheless, there are yet no official and/or commercial kits for this diagnostic. Here we describe a method to be applied in water matrices to concentrate, purify, and detect *T. gondii* oocysts, and this protocol aims to be simple to establish in common laboratories with basic molecular biology equipment (DNA extraction and PCR).

Key words Waterborne protozoa, Methods, Environmental protozoology, Water testing

1 Introduction

Toxoplasma gondii is a zoonotic parasitic protozoan, capable of infecting humans and other homeothermic animals. Several forms of transmission occur in the toxoplasmosis cycle, among them, waterborne transmission emerges as an important transmission route capable of causing large-scale outbreaks [1, 2].

Felids, especially domestic cats, are the *T. gondii* definitive hosts, so in them parasite sexual development occurs, this infection culminates in oocyst production, which are shed through feces into the environment. Under temperature and humidity suitable conditions, oocysts become infectious and can be carried to watersheds [3]. *T. gondii* oocysts are highly resistant to environmental conditions and can remain viable for months to years in the environment; this evolutionary form is also resistant to the main disinfectants

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widely used, including those based on chlorine widely used in water treatment [4, 5].

Among waterborne diseases, several etiologies are known [6]. Among protozoa, *Cryptosporidium*, *Giardia*, and *T. gondii* are the main etiologies in protozoa waterborne outbreaks [7, 8]. Toxoplasmosis outbreaks have historically been described as restricted and commonly related to community food transmission mainly through the ingestion of tissue cysts; however, since 2000, waterborne transmission has been described more frequently [1–9].

The diagnostic of *T. gondii* in water matrices, mainly in raw and treated water, is essential for the characterization of occurrence and distribution of this protozoa in different locations, from this knowledge parameters based in risk assessment can be estimated to provide a better control of toxoplasmosis dissemination [10]. In epidemic situations, specifically when a water source is considered suspected in an outbreak, a positive outcome in a water test can confirm the evaluated source as the outbreak cause. Nevertheless, *T. gondii* oocyst detection in water samples is complex as the known methods lack sensitivity and specificity [10, 11].

The main factor that negatively impact the diagnostic techniques applied to water testing is the diversity of the physical-chemical characteristics of water in the environment, especially turbidity; this provide a great variation in the performance of the methods used for diagnosis [12, 13].

Different protocols for *T. gondii* diagnostic in water samples are known, from simple techniques whose are mainly applied to direct diagnostic in fecal samples from definitive hosts to techniques more adapted to inherent necessities of methods applied to environmental investigations [14]. Between the most modern techniques stand out the immunomagnetic separation efficiency, the purification of concentrated samples, and the diagnostic/quantification ability of qPCR; however, there is still no commercial availability of kits for wide use in routine diagnostic [15, 16, 27]. Specifically with respect to *T. gondii*, morphological identification methods are not able to distinguish *T. gondii* oocysts from at least four other coccid species (*Hammondia hammondi*, *H. heydorni*, *Neospora caninum*, and *Besnoitia*); therefore, the simple direct transposition of methods routinely used in hosts may not be suitable for environmental matrices [3, 17].

Considering the absence of commercial kits that include these new diagnostic advances, the methods used as a routine to investigate the presence of *T. gondii* oocysts in water samples still consist of sample concentration by filtration, elution with surfactant solution (Tween® 80 0.1%), later centrifugal concentration of the eluted material, with or without a fluctuation purification step in

a dense solution (sucrose, cesium chloride) [18–20]. For visualization and identification of parasitic forms by microscopy, UV light microscopy is superior when compared with brightfield microscopy since coccid oocysts present a characteristic autofluorescence when exposed to this wavelength [20, 21].

This technique, based on concentration by filtration, surfactant elution and microscopic visualization, was adapted from methodologies applied to diagnosis and monitoring of *Cryptosporidium* and *Giardia*. The different methodologies applied to *T. gondii* oocyst diagnosis and monitoring in water samples lacks efficiency, especially when compared to the reported efficiencies of methods applied to other protozoa such as *Cryptosporidium* and *Giardia*, for these ones worldwide standardized and validated methods are available [22]. Filter cartridges eluted by agitation indicated for *Cryptosporidium* and *Giardia*, when applied for concentration of *T. gondii* oocysts in high-turbidity samples, present a recovery efficiency 10 times lower than the recommended [10].

Since the popularization of molecular techniques is applied to diagnosis, detection by PCR started to be commonly applied as it has a greater sensitivity and specificity and can differentiate *T. gondii* from other coccids. However, the relative resilience of oocysts to methods generally applied to cell disruption, in addition to the known presence of PCR inhibitors in environmental and fecal samples, represents a challenge for obtaining pure DNA [23, 24]. In order to overcome the occurrence of inhibition on genetic material amplifications, studies have demonstrated a greater robustness of the loop-mediated isothermal amplification (LAMP) technique, which is not inhibited by the main inhibitors present in the environment; however, it is not yet a widely known and used technique like PCR [25, 26].

Therefore, to avoid occurrence of false negatives due to analytical inadequacy, an extensive method validation must be performed, as well as an internal amplification control (IAC) must be employed in the PCR. Methods used to monitor the presence of pathogens in water and food for human consumption must be thoroughly tested, and the extent of the influence of pre-analytical factors must be known since these data provide greater reliability of the obtained results [22].

Molecular diagnostic techniques such as conventional PCR and LAMP do not allow oocyst quantification within a sample, so techniques that allow quantification such as qPCR must be validated for a better understanding of the occurrence and contamination dimension [10, 27–29].

2 Materials

1. Distilled water
2. Tween 80 (Polysorbate 80) 0.1% (V/V).
3. Vacuum pump.
4. Silicone tubes.
5. Kitassate 4 L.
6. Filtration system for 47 mm diameter membrane.
7. 47 mm Mixed cellulose esters (MCE) membranes, maximum porosity of 5 μm.
8. Disposable petri dishes.
9. Calibrated loop.
10. Disposable Pasteur pipettes.
11. Conical tubes for centrifugation (50 mL).
12. Centrifuge with rotor for conical tubes.
13. Crystal sugar.
14. Densimeter.
15. Phenol.
16. Aqueous sucrose solution (sp.g. 1208 g/L).
17. Microtubes.
18. Brightfield microscope.
    Optional: Phase-contrast, differential interference contrast (DIC), UV light microscope (330–380 nm excitation and 400 nm barrier).
19. DNA Extraction kit (including all materials required from the manufacturer).
20. General PCR reagents and equipment.
21. General DNA electrophoresis reagents and equipment.

3 Methods

3.1 Indication

This protocol is indicated for the concentration, purification, and detection of *T. gondii* oocysts in raw, treated, spring, and ground water samples.

3.2 Sampling

When the water is piped, sampling can occur directly from the tap. If it is not, sampling can occur directly in the watershed or fountain, in this case the sampler must be cautious to not revolve the soil/sediment. The minimum water volume to be processed by this technique is described below:

1. Raw water: at least 3 L.
2. Treated water: 100 L.
3. Spring and groundwater: 100 L.

3.3 Concentration

A high oocyst dilution is expected in water matrices, this can drive to a low oocyst concentration on collected sample, so a concentration step aims to concentrate the oocysts to a low volume for detection; as there is not a concentration technique able to concentrate only *T. gondii* oocysts, it is important to take into account that other samples constituents (e.g., organic/inorganic matter, soil, etc.) will be also concentrated (*see* **Note 1**).

3.4 Filtration

1. Using the silicone tubes, plug the vacuum pump to the kitassate.
2. Connect the filter system to the kitassate, ensure that the system is well placed to not lose vacuum pressure during filtration.
3. Place the membrane in the designed space of the filter system and lock with the filter system clamp.
4. Turn on the vacuum pump.
5. Rinse the whole filter system with Tween® 80 (0.1%).
6. Add sample in the filter system cup and continue adding the sample during the filtration.
   (a) Whenever the filtration speed dramatically decreases, change the membrane, as the filter membrane pores have saturated (the frequency of the membrane change will depend on the turbidity of the water sample).
   (b) If the membrane has saturated and some sample is still in the filtration cup, use a disposable pipette to remove the sample until the membrane appears dry.
   (c) Be careful with the vacuum pump temperature; long period filtration can cause a pump overheat.
   (d) Be careful with the filtrate volume and kitassate capacity; whenever the kitassate reaches the capacity, remove the vacuum pressure, remove the filtration system and drain out the kitassate.
7. Place used membranes, with the concentrated matter, in a disposable petri dish and add Tween 80 0.1% until it is covered.
   (a) If membrane filtration and membrane elution are not performed in the same place, the membranes can be packaged in a plastic bag with Tween 80 0.1% and kept under refrigeration (4 °C).

3.5 Membrane Elution and Centrifuge Concentration

1. With the loop part of a calibrated loop, perform a smooth membrane scraping in different directions during 20 min in each membrane.
   (a) If membranes are placed in a plastic bag, carefully remove the membrane and place them in a disposable petri dish with Tween 80 0.1% for scraping, and save the liquid.
2. After scrapping, transfer the Tween 80 0.1% solution from the petri dish to a centrifuge conical tube.
   (a) If membranes are placed in a plastic bag with Tween 80 0.1%, also transfer the plastic bag liquid to the centrifuge conical tube.
3. Centrifuge the tubes (2100 × *g*/10 min).
4. Carefully remove the supernatant using a disposable Pasteur pipette. Leave a minimum quantity of supernatant for pellet resuspension. Resuspend the pellet using the Pasteur pipette.
   (a) If more than one tube is necessary to fit all the liquid generated in the elution, transfer all the resuspended pellets from the same sample to a single conical tube.
5. Centrifuge the tubes (2100 × *g*/10 min).
6. Carefully remove the supernatant using a disposable Pasteur pipette. Leave a minimum quantity of supernatant for pellet resuspension.
7. When the final sediment is greater than 500 μL, purification must be performed before detection.

3.6 Purification

During the membrane elution and centrifuge–concentration step, various organisms and environmental compounds were concentrated in addition to the oocysts, so when a high pellet volume is obtained in concentration, the purification step is necessary to remove as many components as possible that could interfere with the detection phase.

1. Resuspend the concentrated sample (0.5–5 mL) in 40 mL of sucrose solution (g.sp. = 1.208 g/mL) in a 50-mL conical tube.
   (a) *T. gondii* oocyst density is 1.11 ~ 1.14 g/mL.
2. Centrifuge at 1250 × *g* for 10 min.
3. Collect 5 mL of the superficial meniscus using a Pasteur pipette, and transfer it to a new 50-mL conical centrifuge tube.
4. Add 40 mL of distilled water and mix vigorously.
5. Centrifuge at 2100 × *g* for 10 min.
6. Carefully discard the supernatant using a Pasteur pipette and resuspend the formed sediment with 0.5–1.5 mL of distilled water (uses the sufficient volume necessary to resuspend the sediment and form a homogeneous appearance mixture).

7. Place the purified material in a sterile microtube and store under refrigeration (4 °C) until detection.
   (a) Detection steps must be performed within 48 h.

3.7 Detection

In the detection step, there is a direct parasite diagnostic, through evaluation of microscopic morphological structure, or through PCR DNA amplification.

3.7.1 Microscopic Detection (See **Note 2**)

1. Wet-mount 20 μL of the concentrate/purified on slide and coverslip.
2. Screen the slide in brightfield microscopy, phase contrast or UV light microscopy with 330–380 nm excitation and 400 nm barrier in the 40× objective.
3. When positive, the sample will present oocysts of approximately 12 μm, when not sporulated, characterized by a spherical shape with a modulated interior, and when sporulated, a spherical to elliptical shape containing two sporocysts with four sporozoites inside (*see* **Note 3**).

3.7.2 PCR Detection

To obtain more accurate results in the polymerase chain reaction (PCR), it is suggested to extract DNA by means of commercial kits.

DNA Extraction

The most efficient DNA extraction and purification are those indicated for extracting nucleic acids from faces and environmental samples (e.g., soil, water, soil, and plants; water, for drinking water, raw water).

A method validation should be carried out to assure that the kit protocol is able to extract DNA from *T. gondii* oocysts.

PCR Targeting the rep529

A PCR targeting the non-codified 529 bp repeated sequence from *T. gondii* genome based on primers tox4 and tox5 described by Homan et al. (2000) is indicated.

1. Assembly reaction components (Table 1) in an identified PCR microtube (200 μL) or in each PCR microplate well.
2. Allocate microtubes in thermocycler. Check reagents' manufacturer's instructions regarding PCR cycle. A cycle example is described in Table 2.

After PCR amplification, run an electrophoresis for DNA detection as follows:

1. Prepare 1.5% (w/v) agarose gel with TBE or TAE buffers.
2. Run the electrophoresis with DNA dye and loading buffer of preference following the manufacturer's instructions.
3. Expose the gel to indicated wavelength of the DNA dye used, a sample is considered positive when a DNA band of 529 bp is observed (*see* **Note 3**).

Table 1
Components, concentrations, and volumes for assembly of PCR targeting the 529 bp repeated fragment of *Toxoplasma gondii* genome

Component	Concentration of use	Volume (μL)
Ultrapure water	–	8.25
$MgCl_2$	50 mM	1.25
PCR *buffer*	10×	2.50
dNTP[a]	10 mM	0.50
Primer Tox4	20 pmol/μL	1.00
Primer Tox5	20 pmol/μL	1.00
Taq DNA polymerase	5 U/μL	0.25
DNA (sample)	–	2.00

[a]dNTP = deoxynucleotide triphosphates

Table 2
Example of cycle conditions for a PCR targeting the 529 bp repeated fragment of *Toxoplasma gondii* genome

Steps	Temperature (°C)	Duration	Cycles
Initial denaturation	94	300	1
Denaturation	95	30	35
Annealing	63	30	
Extension	72	60	
Final extension	72	300	1

4 Notes

1. High turbidity levels negatively affect technique efficiency.
2. *T. gondii* oocysts are microscopically indistinguishable from those of *Neospora* and *Hammondia* genus. Oocysts of coccids when exposed to UV light emit fluorescence in blue color, facilitating their visualization in complex samples. Due to the difficulty of microscopy detection mainly caused by low final volume of the sample analyzed, presence of confounding structures and the non-differentiation between *Neospora* and *Hammondia* and *T. gondii* oocysts, concomitant use of molecular methods is recommended.
3. In outbreak situations, laboratory tests results should be evaluated together with epidemiological analysis, as a negative test does not exclude the possibility of *T. gondii* oocysts presence, so *T. gondii* diagnostic techniques applied to environmental

samples can present low sensitivity among other causes due to the difficulty in collecting and/or concentrating large volumes. Time elapsed between the first clinical signs, case notification, and sample collection should always be accounted in outbreak situations.

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Chapter 19

Detection of *Toxoplasma gondii* in Milk and Cheese

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Abstract

The *Toxoplasma gondii* is a parasite of great importance in public health that presents three biological forms of bradyzoites in tissue cysts, sporozoites in sporulated oocysts, and tachyzoites, a form of rapid multiplication found during the acute phase in blood and milk. Considering the importance of milk as a food, and its participation as a transmission patterns in outbreaks of toxoplasmosis, it is necessary to standardize a technique for recovery of *T. gondii* in this matrix. This protocol describes a detection technique for *T. gondii* tachyzoites and oocysts in milk and cheese samples.

Key words Recovery, Centrifugation, Food, Cow

1 Introduction

Toxoplasma gondii is a mandatory intracellular parasite of cosmopolitan distribution, which causes toxoplasmosis. Vertical transmission can occur when the pregnant woman acquires a primary infection during pregnancy, tachyzoites cross the placental barrier and reach the fetus. The horizontal transmission, in turn, of the agent can occur in several ways, through the ingestion of oocysts containing sporozoites and cysts containing bradyzoites and tachyzoites. The oocysts are eliminated in the feces of the definitive hosts (felids) in noninfective and nonsporulated form, under favorable conditions of temperature, oxygenation, and humidity, undergoing sporulation, contaminating sand, soil, water, and vegetables. Cysts, in turn, are found in tissues, can be ingested through the consumption of raw or undercooked meat. Other routes of transmission include the intake of milk, cheese, and blood transfusion, the form of tachyzoites being found in these foods [1–4].

Milk is a white, opaque liquid secretion produced by mammary glands of females of the lactating mammal class, with nutritional properties and suitable for human consumption. Milk is considered

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raw when it does not receive heat above 40 °C or other type of decontaminating treatment. Cheese is a solid food produced from the coagulation of milk from cows, goats, sheep, buffaloes, and other mammals. The goat milk [5], woman milk [6], and rats milk [7] have already been reported in the literature as important routes of transmission of toxoplasmosis, the goat being already described as a cause of outbreaks of the disease in humans [8, 9]. Transmission by cow's milk has not been very well elucidated; however, previous cross-sectional studies have detected the presence of the parasite's DNA in this milk [10, 11]. An outbreak of toxoplasmosis in Brazil had cow cheese as a suspect food, but the authors believed it to be post-milking contamination [12].

Given the importance of these foods in the transmission of toxoplasmosis, this document aims to describe the protocol for the detection of *T. gondii* tachyzoites and oocysts in milk and cheese samples. This protocol was based on articles by Costa et al. [12] and Ferreira Neto et al. [5].

2 Materials

2.1 For Detection of *T. gondii* in Milk

1. Hydrophilic cotton to remove fat from milk.
2. Isothermal box for storage of samples during transport.
3. Bench centrifuge for 50-mL tubes.
4. Sterile collection bottle.
5. Artificial ice for transportation.
6. Gloves: individual protection equipment.
7. Microtube (1.5 mL): storage of processed sample.
8. Conical bottom centrifuge tubes (50 mL).
9. Pasteur pipette: for pipetting samples throughout the process.

2.2 For Detection of *T. gondii* in Cheese

1. Gauze: used in sample filtration.
2. Tweezers: to assist in the understanding of cheese samples and their cutting.
3. Scalpel: to perform sample slicing.
4. Scale.
5. First-time plastic packaging: sample homogenization container.
6. Gloves: individual protection equipment.
7. Pasteur pipette: for pipetting samples throughout the process.
8. Tween 80 solution (0.1%): neutral detergent used to wash the sample (1 mL of Tween 80 is used for each 100 mL of distilled water).

9. Conical bottom centrifuge tubes (50 mL).
10. Centrifuge.
11. Microtube (1.5 mL): storage of processed sample.

3 Methods

3.1 Milk Processing

1. Perform the antisepsis of the lactating female's teats using the pre-dipping technique [13], so that there is no contamination of the sample by dirt present on the ceiling.
2. After pre-dipping, discard the first three milk jets from the teats, by manual milking, collect 50 mL milk per animal.
3. The milk should be collected with the aid of a sterile weak sealed and refrigerated and transport to the laboratory, send them in sealed seals (*see* **Note 1**) and refrigerated (3–7 °C), with artificial ice, in isothermal boxes at a temperature that stays between 3 and 7 °C (*see* **Notes 2** and **3**).
4. Transfer the sample to a conical bottom centrifuge tube (50 mL), and centrifuge at 1000 × *g* for 10 min (*see* **Notes 4** and **5**).
5. Gently remove the fat layer from the supernatant using cotton wool (*see* **Note 6**) and complete the conical bottom centrifuge tube until the volume of 50 mL is complete (this step must be performed three times).
6. The pellet formed in the previous step must be stored in 1.5-mL microtubes at 4 °C until DNA extraction.
7. DNA extraction can be performed by commercial kit, following the manufacturer's recommendations.
8. For detection of *T. gondii* DNA, PCR primers for the repeated region of 529 base pairs (pb) are recommended [14].

3.2 Cheese Processing

1. With the aid of tweezers and scalpels, cut the cheese into slices and in a first-use bag place 25 g of the sample taken from various points (surface and depth).
2. Add 225 mL of 0.1% Tween 80 to the plastic bag and manually soak the sample for 2 min.
3. Filter the suspension in double gauze and transfer to 50-mL conical bottom centrifuge tubes.
4. Centrifuge at 2100 × *g* for 10 min, carefully discarding the supernatant with the aid of the Pasteur pipette until the minimum volume necessary to resuspend the pellet remains.
5. Aliquot the sediment in a microtube and store at 4 °C until detection.

6. DNA extraction can be performed by commercial kit, following the manufacturer's recommendations.
7. For the detection of *T. gondii* DNA, PCR primers for the repeated region of 529 base pairs (pb) are recommended [14].

4 Notes

1. It is understood by sealed bottle: one where there is no contact between internal content and external content.
2. Milk, even when refrigerated, must be processed within 48 hours after collection, so that there is no excess microbial growth.
3. All processing, both for milk and cheese, is carried out with the purpose of concentrating the sample's DNA and also reducing elements that may interfere negatively in the extraction process, DNA purification or in the polymerase chain reaction (PCR).
4. The low rotation is so that only possible existing oocysts/tachyzoites go to the bottom of the container and not the fat.
5. The dilution of oocysts in the environmental matrix can lead to an oocyst concentration below the detection limit, the objective of this step is to concentrate a larger sample volume so that the detection techniques are efficient even in low concentration.
6. The presence of fat in the extracted is not desirable, as its presence can inhibit the amplification of DNA.

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Chapter 20

Detection of *Toxoplasma Gondii* in Meat

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Abstract

Meat is a food derived from animal tissue which serves for human consumption when it is considered safe to them. Pathogens present in this tissue could cause a health risk. Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii* which affects all homeothermic animals and has their meat as the main mode of transmission. To ensure the consumer's biosecurity the diagnosis is essential to establish effective prevention services. Due to the low number of cysts in muscles and the nonexistence of specific macroscopic lesions, the accuracy of detection needs to be improved. The peptic digestion of meat is used to concentrate and purify *T. gondii* cysts with the aim of increasing the sensibility of detection. For detection, bioassay in mice is considered the gold standard for parasite isolation from meat because of its high accuracy. In association of both techniques, the molecular analysis allows the increasing of sensibility, specificity, and to reach genotypic information that contributes to epidemiological evaluations. An accurate diagnosis is important to the establishment of efficient control and preventive services against toxoplasmosis. In this chapter, materials and methods for diagnosis and isolation of *T. gondii* in meat are presented by use of peptic digestion, bioassay in mice, and polymerase chain reaction (PCR).

Key words Diagnosis, Toxoplasmosis, Foodborne, Public health, Cyst

1 Introduction

Meats are defined as: all animal tissues judged as edible and applied to human consumption if they are considered safe to consume [1]. The security in meat consumption is established in the absence of toxic effects and pathogens. However, due to the large and complex production chain and the obtention by consumers of unverified products, meat is one of the major modes of transmission in foodborne diseases [2, 3]. The foodborne pathogens are divided into viruses, bacteria, fungus, helminths, and protozoa. Among foodborne protozoa, *T. gondii* is the most transmitted by meat. This mode of transmission is responsible for 50–64% of the parasite transmission [3].

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Toxoplasmosis is a zoonosis that affects all homeothermic animals. The etiological agent, *T. gondii* presents oocysts (environmental form) and cysts (tissue form) as transmission forms [4, 5]. Meat is the mode of transmission that most reach humans. This fact may occur because of a lack of good practices in production and because of unsafe eating habits which are from cultural or low educational levels [3, 5, 6]. Independently of the disseminated transmission cause, the diagnosis of the parasite in meat ensures the reduction of risk infection because it allows the establishment of good control and prevention services [7].

Cysts of *T. gondii* are mainly described in the brain, heart, masseter, and diaphragm of the intermediate hosts. The animal species more affected by cysts are sheep, swine, poultry, and cattle respectively [7, 8]. Even with this knowledge, the diagnosis of cysts in food is not easy to get. In 100 g of meat, one cyst of *T. gondii* is expected with a maximum diameter of 100 μm, a fact that makes impossible macroscopic identification [4, 9, 10]. These factors reduce the possibility of parasite identification, making the diagnosis difficult.

To reduce diagnosis errors, techniques that improve the sensibility of detection are proposed. Peptic digestion is a protein digestion technique that aims to concentrate and purify the parasite in the muscle [11]. With the product of peptic digestion, it is possible to execute molecular tests as PCR with more accuracy than execute PCR directly from DNA extraction of muscle. In this case, beyond the organic matter which can reduce DNA quality, the maximum tissue amount used for good DNA extraction is low (~200 mg). The molecular technique generally presents high specificity which increases the trustable diagnosis of the parasite [12]. These techniques also permit genotype identification of *T. gondii* that are essential to reach a good epidemiologic investigation and relate mode of transmission to infected people. The genotyping may also help in the understanding of pathogenicity and virulence of parasite strain.

The bioassay is the gold standard for *T. gondii* isolation. This technique is based on challenging experimental animal models susceptible to the parasite [11]. It is described as expensive and difficult to apply due to the high technical knowledge demanded, but it is highly accurate to cysts diagnosis in meat [8, 13]. Preceded by peptic digestion, in the technique the animals are challenged and clinical signs and parasitic forms can be observed after some days passed the challenge. The association among peptic digestion, bioassay, and molecular analysis by PCR forms an accurate method to diagnose, isolation, and genotyping of *T. gondii* in meat.

2 Materials

2.1 Obtaining and Transporting Samples

1. Isothermal box.
2. Plastic packaging for first use.

2.2 Peptic Digestion

1. Conical bottom centrifuge tubes (50 mL).
2. Distilled water.
3. Gauze.
4. Gloves.
5. Hand blender.
6. Magnetic shaker with heating or benchtop shaker incubator with heating.
7. Pepsin 1:10,000 (100 g).
8. pH meter.
9. Scalpel (sterile).

2.3 Bioassay in Swiss Mice

1. Binocular optical microscope up to 1000× magnification.
2. Coverslip (24 × 24 mm).
3. Distilled water.
4. Gloves.
5. Infrastructure for *vivarium* activities.
6. Laminar flow hood.
7. Needle (25 × 8 or 25 × 7 mm).
8. Optical microscope slide.
9. Penicillin (injectable).
10. Streptomycin (injectable).
11. Syringe (3 mL).

2.4 Molecular Analysis

1. Agarose P.A.
2. Automatic pipettes (1000, 200, and 10 μL).
3. Centrifuge for microtubes.
4. Centrifuge for PCR microplate.
5. Deoxyribonucleotides (100 mM) (A, T, C, G).
6. Distilled water.
7. Electric digital power supply for electrophoresis.
8. Gloves.
9. Horizontal electrophoresis chamber.
10. Laminar flow hood.

11. Microtube (1.5 mL, 200 μL).
12. PCR microplate.
13. Specific oligonucleotides (forward and reverse primers).
14. Taq Polymerase Platinum 5 U/μL enzyme (along with specific buffer and $MgCl_2$).
15. Thermal cycler.
16. Ultraviolet light ($\lambda = 254$ at 300 nm).

3 Methods

3.1 Meat Obtention

1. Get at least 50 g of meat from homeothermic animals which have been consumed by individuals related to infection (*see* **Note 1**).
2. Put the obtained meat in its inviolate package or in a primary plastic package and seal with scotch tape or other way that permits effective sealing (*see* **Note 2**).
3. Identify the samples by type of beef cutting, animal species, manufacture date, sampling date, and sampling local.
4. To transport, the obtained meat must be sent (*see* **Note 3**) in sealed packages, refrigerated in an isothermal box which maintains the temperature of 3 and 7 °C.

3.2 Concentration

To *T. gondii* cysts rupture and muscular digestion, the Peptic Digestion Technique adapted from Dubey [11] is described hereafter.

3.2.1 Peptic Digestion: Adapted from Dubey [11]

1. Execute the remotion of the tissue associated with muscle with sterile scalpel.
2. Weigh 50 g of meat and cut into pieces of 1–2 cm with a sterile scalpel.
3. In a beaker (1000 mL) covered by aluminum paper, crush the meat pieces by a hand blender for 15 s.
4. Add 125 mL of saline sterile solution (0.85%) (*see* **Note 4**) and mix with a hand blender in high speed for 30 s.
5. Wash the tip of hand blender with 125 mL of saline and add the product of wash in the crushed tissue.
6. Add 250 mL in pepsin solution (*see* **Note 5**) to 37 °C.
7. Incubate the mixture in magnetic shaker with heating to 37 °C for 60 min in moderated speed.
8. Filter the mixture in gauze (2 sheets).
9. Transfer the volume to conical centrifuge tube (50 mL).
10. Centrifuge in 1200 × *g* for 10 min.

11. Discard the supernatant and suspend the pellet in 20 mL of PBS (pH 7.2) (*see* **Note 6**).
12. Add 15 mL of sodium bicarbonate solution (1.2%; pH 8.3) (*see* **Note 7**).
13. Centrifuge in 1200 × *g* for 10 min.
14. Discard the supernatant and suspend the pellet in 5 mL of sterile saline solution (0.85%) (*see* **Notes 4** and **8**).

3.3 Detection

The *T. gondii* detection technique proposed to verify the parasite in meat is Bioassay in Swiss mice and polymerase chain reaction (PCR). Both can be used independently for detection; however, for diagnosis improvement, the conjugated execution is recommended. For independently detection, the procedure described ahead will continue from **step 14** from the Subheading 3.2.1 of this chapter. For conjugated execution, bioassay is done first and then the PCR, using the product of **step 8** from the Subheading 3.3.1.

3.3.1 Bioassay in Swiss Mice (see **Note 8**): Adapted from Dubey [11] (see **Note 9**)

1. Add 1000 IU of penicillin and 100 μg/mL of streptomycin to the peptic digestion product.
2. Heat the peptic digestion product with antibiotic in sterile saline solution (0.85%) at 37 °C (*see* **Note 4**).
3. Inoculate 1 mL in 3 Swiss mice intraperitoneal inoculation.
4. Observe the animals twice a day for 42 days (*see* **Note 10**); Notice behavior changes and clinical signs (e.g., apathy, photophobia, paresis, piloerection, bloating, and dry stools).
5. After animal death or euthanasia, harvest the peritoneal exudates.
6. Apply a exudate drop in an optical microscope slide and cover with coverslip (24 × 24 mm); observe by optical microscope in 400× magnification.
7. The named "positive exudate" results from the observation of the presence of tachyzoite forms of *T. gondii* (*see* **Note 11**).
8. In case of negative peritoneal exudate, harvest the mouse's brain from the same exudate observed.
9. Make a tiny transversal cut in the middle portion of brain (thickness ≤ 1 mm), place it in an optical microscope slide and press against the cut using a coverslip (24 × 50 mm).
10. Observe by optical microscope in 100× magnification to search for *T. gondii* cysts.
11. In case of "positive brain": observation of *T. gondii* cysts in the brain cut (*see* **Note 12**).

12. In case of negative brain, macerate the brain in 2 mL de salina using a needle 40 × 12 mm and syringe (3 mL).
13. Make an intraperitoneal inoculation in a Swiss mouse with 0.5 mL of brain macerate.
14. Observe the mouse twice a day for 3 months to verify health condition and clinical signs.
15. At the end of the period, make the animals euthanasia.
16. Harvest the mouse brain.
17. Search for *T. gondii* cysts as described at **steps 8** and **9** of this protocol.

Positive bioassay: *Observation of T. gondii tachyzoites in exudate or cyst(s) in the brain. Cyst(s) observation in brains of mice from the first and second inoculation is considered positive too.*

3.3.2 Molecular Analysis

The peptic digestion product (*see* Subheading 3.2.1) or the brain macerate of challenged mice in bioassay (*see* Subheading 3.3.1) should be submitted to DNA extraction. To prepare the peptic digestion product for molecular analysis, the following procedure must be done:

1. Centrifuge the peptic digestion product at 10,000 × *g* for 1 min.
2. Discard the supernatant and add 0.5 mL of ultrapure water.
3. Centrifuge at 10,000 × *g* for 1 min.
4. Discard the supernatant and add 0.3 mL of ultrapure water.
5. Store the sample in a 1.5-mL microtube at −20 °C until DNA extraction and purification.

DNA Extraction and Purification

For this procedure, the use of specific commercial kits to the specific type of sample is recommended because they are standardized and ensure a good reproducibility. The peptic digestion product can be considered a corporeal fluid, and the brain macerate can be considered a corporeal tissue. There are other options to DNA obtention as phenol-chloroform protocol [14] although this option can present more variability in efficiency due to differences in procedures from lab to lab.

Polymerase Chain Reaction (PCR)

For qualitative detection of *T. gondii*, PCR primers of the repeated region of 529 bases pairs (bp) are recommended. Primers (*see* **Note 13**) were described for Homan et al. [15].

1. Group the components (*see* **Note 14**) of PCR in microtube or microplate of 200 μL.
2. Centrifuge (spin) the reactions in a centrifuge for PCR microplate.

3. Place the reactions in the thermal cycler.
4. Program (*see* **Note 14**) the PCR cycles applied to the target sequence of 529 bp.
5. To verify the PCR products, execute horizontal electrophoresis in agarose gel and visualize the DNA by ultraviolet light ($\lambda = 254$ a 300 nm).

Positive PCR: *Observation of DNA band in agarose gel at 529 bp height. The visualization must not have DNA bands in negative control or ambiguous DNA bands in samples.*

4 Notes

1. In case of nonexistence of meat from this occasion, harvest at least 50 g of meat that have been consumed in the local of the infection occurrence, and with the same precedence of the meat consumed in the suspect period of individuals infection by *T. gondii*.
2. Seal package means the internal content does not have contact to the external content.

   If the original packages of meat are already violated, put the meat in its package in a primary plastic package and seal as recommended.
3. The material must arrive in the lab up to 48 h after harvest.
4. Saline solution preparation (0.85%)

Reagent	Concentration	Mass/volume
NaCl	P.A.	8.5 g
Distilled water	–	1000 mL[a]

[a]Enough quantity to

5. Pepsin solution preparation

Reagent	Concentration	Mass/volume
Pepsin	1:10,000 (100 g)	1.3 g
NaCl	P.A.	2.5 g
HCl	P.A.	3.5 mL
Distilled water	–	250 mL[a]

[a]Enough quantity to

6. Phosphate-buffered saline (PBS) preparation 10×[] (pH 7.2)

Reagent	Concentration	Mass/volume
NaCl	P.A.	90.0 g
Na_2HPO_4	P.A.	10.9 g
NaH_2PO_4	P.A.	3.2 g
Distilled water	–	1000 mL[a]
pH = 7.2		

[a]Enough quantity to

7. Sodium bicarbonate solution preparation (1.2%; pH 8.3)

Reagent	Concentration	Mass/volume
$NaHCO_3$	P.A.	0.18 g
Distilled water	–	15 mL[a]
pH = 8.3		

[a]Enough quantity to

8. Storage at 4 °C. At least 3 mL for bioassay technique and 250 μL for molecular assay.
9. This technique can be done just with an authorization from Ethics Committee on the Use of Animals and under a veterinarian supervision.
10. If animals present an excess suffering and debility, they must be euthanized before the 42 days period.
11. The *T. gondii* tachyzoites present a stick form with apical narrowing with 3 × 6 μm dimension and high motility.
12. The cysts have a 5–50 μm diameter and content stick forms inside its wall with 2 × 6–8 μm dimension.
13. Primers forward and reverse of the non-coding fragment with 529 bp for PCR.

Gene	Primer	Sequence	Reference
Non-coding fragment 529 bp	Tox4	5′-CGCTGCAGGGAGGA AGACGAAAGTTG-3′	[15]
	Tox5	5′-CGCTGCAGACACA GTGCATCTGGATT-3′	

bp = bases pair

14. Components, concentrations, and the program of PCR must be adapted for each lab routine. And the reagents utilized must be based on the procedure described for primers which, in this chapter, has been in accordance with Homan et al. [15]

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Chapter 21

Detection of *Toxoplasma Gondii* and *Cyclospora Cayetanensis* in Oysters

Minji Kim and Karen Shapiro

Abstract

Protozoan parasites constitute a neglected group of foodborne pathogens for which few validated detection methods are available in shellfish. This chapter describes a nested PCR assay for the detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* that was validated in different tissues of oysters. The assay can consistently amplify DNA from as few as 5–10 oocysts in hemolymph or whole-tissue homogenates.

Key words Polymerase chain reaction (PCR), *Toxoplasma*, *Cyclospora*, Sequencing, Shellfish, Detection, Mollusks, Bivalves

1 Introduction

When the risk of acquiring foodborne illness is considered per weight of food type consumed, seafood is by far the most hazardous [1]. Foodborne disease outbreaks from ingestion of pathogenic organisms in shellfish constitute significant health risks to consumers [2]. Terrestrial biologic pollutants can contaminate shellfish through overland runoff carrying fecal pathogens from land to sea. Bivalves are filter feeders that filter large volumes of water across their gills and concentrate organic matter and particles including pathogens in their tissues. While attention to shellfish-borne disease has mainly focused on bacterial and viral pathogens, protozoan pathogens including *Cryptosporidium* spp., *Giardia* spp., *Toxoplasma gondii*, and *Cyclospora cayetanensis* are recognized as pathogens that are likely underestimated as causes of illness through shellfish consumption [3]. Previous studies have detected *T. gondii* and *C. cayetanensis* in bivalves including oysters, mussels, and clams worldwide (summarized in [4, 5]).

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T. gondii is a zoonotic parasite, and felids are the only definitive hosts excreting *T. gondii* oocysts that can survive for prolonged periods in the environment and contaminate soil and water [4]. Any warm-blooded animals including humans can become infected through environmental transmission of *T. gondii* oocysts and upon ingestion of undercooked meat from infected animals. Symptoms of toxoplasmosis can vary depending on the route of infection, immune status of host, as well as the parasite genotype [6]. *T. gondii* infection can be asymptomatic or cause mild flu-like symptoms in immunocompetent individuals following acute infection; however, serious sequelae such as severe visual impairments can also occur. Congenital transmission can cause miscarriage, a stillborn child, and severe birth defects when a woman is first exposed to *T. gondii* during or just before pregnancy. Unlike *T. gondii*, *C. cayetanensis* is a human-specific parasite and is transmitted via the fecal–oral route through exposure to sporulated oocysts in contaminated food, water, or soil [5]. Cyclosporiasis can be asymptomatic or cause diarrheal illness depending on the age and immune status of infected individuals.

Detection of *T. gondii* and *C. cayetanensis* in environmentally contaminated bivalves are commonly based on molecular methods including polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) (summarized in [4, 5]). Gills, digestive tract, and hemolymph have been used as targeted tissues for protozoan pathogen detection in naturally contaminated marine bivalves because they may be most relevant organs in which protozoa concentrate [7]. To date, studies on comparison of different shellfish tissues for protozoan detection are inconclusive, and whole-tissue homogenate can serve as a useful approach to capture all pathogens in different tissues from shellfish. Enzyme digestion can facilitate downstream analysis by breaking down the complex whole-tissue matrix [8]. In this chapter, a sensitive nested PCR method is described for simultaneous detection of *T. gondii* and *C. cayetanensis* in hemolymph and whole-tissue homogenate using oysters as a model shellfish commodity.

2 Materials

2.1 Aspiration of Hemolymph

1. Nylon scrub brush (*see* **Note 1**).
2. Polystyrene weighing boats (diameter ~ 8 cm or larger).
3. Slim triangular taper file (*see* **Note 2**).
4. Hypodermic needles, regular bevel, 23-gauge, 1 in.
5. 3-mL syringes.
6. 2-mL microcentrifuge tubes.
7. Distilled water.

2.2 Pepsin-HCl Digestion of Whole Tissue

1. Nylon scrub brush.
2. Polystyrene weighing boats (diameter ~ 8 cm or larger).
3. Shucking knife and tear resistant gloves.
4. Isopropyl alcohol (*see* **Note 3**).
5. Distilled water.
6. 50-mL conical tubes.
7. Rotor stator homogenizer (*see* **Note 4**).
8. Motorized serological pipette controller and pipette tips.
9. Stir plates and magnetic stirrer.
10. Pepsin-HCl digestion solution: Mix 20 mL 1 N HCl and 80 mL of distilled water. Dissolve 1 g pepsin (*see* **Note 5**) into the solution and mix thoroughly using a magnetic stirrer for at least 10 min before use (*see* **Note 6**). Volume may increase depending on the number of samples (*see* **Note 7**).
11. PBS eluting solution: Dissolve 0.5 g sodium dodecyl sulfate (SDS), 0.5 mL Tween® 80, 50 µL Antifoam A into 500 mL phosphate-buffered saline (PBS) 1× solution. Adjust pH to 7.4 with 1 N NaOH or HCl. Store the solution at 4 °C.
12. Vortex mixer.
13. Incubator.
14. Pipettes and filtered pipette tips (*see* **Note 8**).
15. 2-mL microcentrifuge tubes.
16. Benchtop centrifuge.

2.3 Nucleic Acid Extraction

Extraction of protozoan DNA from hemolymph and whole-tissue homogenate is performed using the QIAGEN DNeasy Blood & Tissue Kit.

1. DNeasy Blood & Tissue Kit (QIAGEN, cat. no. 69504).
2. 1.5-mL conical bottom screw cap microcentrifuge tubes that can withstand temperatures from −196 °C to +100 °C (*see* **Note 9**).
3. Microcentrifuge (capable of attaining 20,000 × *g*).
4. Pipette and filtered pipette tips.
5. Vortex mixer.
6. Liquid nitrogen and a benchtop dewar (*see* **Note 10**).
7. Hot plate and boiling water (>2 L) in a 4-L beaker (*see* **Note 11**).
8. Floating microtube rack and 12-in. forceps (*see* **Note 12**).
9. Additional proteinase K (*see* **Note 13**).
10. Dry heating block (*see* **Note 14**).
11. Ethanol (96–100%), molecular grade.
12. Nuclease-free water.

Table 1
Primer sets used in nested PCR for detection of *Toxoplasma gondii* and *Cyclospora cayetanensis*

Protozoa	Target gene	Primer	Direction	Nucleotide sequence (5′ – 3′)	Amplicon size (bp)	Reference
External reaction						
T. Gondii *C. Cayetanensis*	18S[a]	m18SeF	Forward	CGGGTAACGGGGAATTAGGG	751–779	[9]
		m18SeR	Reverse	TCAGCCTTGCGACCATACTC		
Internal reaction						
T. Gondii	18S	m18StoxF	Forward	GGTGTGCACTTGGTGAATTCTA	405	[9]
		m18StoxR	Reverse	TGCAGGAGAAGTCAAGCATGA		
C. Cayetanensis	18S	m18ScycF	Forward	TCGTGGTCATCCGGCCTT	359	[9]
		m18ScycR	Reverse	TCGTCTTCAAACCCCCTACTG		

[a]18S small subunit (ssu) ribosomal RNA (rRNA) gene

2.4 Polymerase Chain Reaction

1. Thermal cycler.
2. Mini centrifuge.
3. Pipette and filtered pipette tips.
4. PCR reagents including AmpliTaq Gold™ 360 Master Mix (*see* **Note 15**).
5. Bovine serum albumin (BSA), molecular biology grade aqueous solution (*see* **Note 16**).
6. Nuclease-free water.
7. Forward and reverse primers (Table 1).
8. 1.5-mL microcentrifuge tubes.
9. 0.2-mL PCR tubes and a 96-well PCR tube rack.

3 Methods

3.1 Aspiration of Hemolymph

1. Clean oyster shells with a scrub brush and distilled water.
2. Record the width and length of oysters.
3. Place an oyster on a weighing boat. Orient the oyster flat side facing up and cupped side down.
4. File a 2–3 mm notch approximately 2/3 to the left from the hinge (Fig. 1a; *see* **Note 17**), adjacent to the adductor muscle

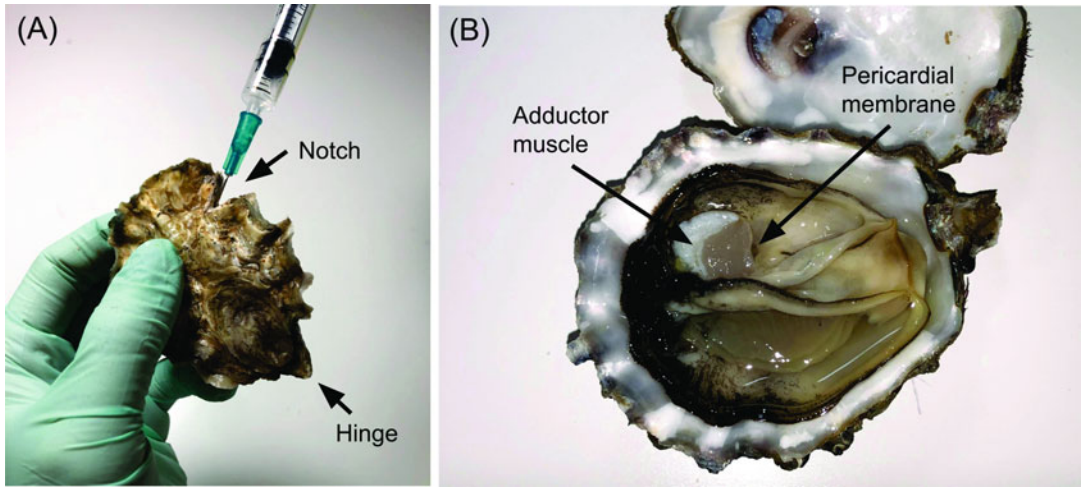

Fig. 1 (**a**) Illustration demonstrating the collection of hemolymph from the adductor muscle of an oyster through the notch created by a triangular taper file; (**b**) Anatomy of the oyster showing two hemolymph collection locations

(Fig. 1b; *see* **Note 18**). Alternatively, an electric tool can be used to make a notch (*see* **Note 2**).

5. Once the notch is made, gently insert a 23-gauge hypodermic needle attached to a 3-mL syringe through and locate the adductor muscle by gently moving the needle left and right parallel to the top shell (*see* **Note 19**).
6. Aspirate hemolymph using gentle intermittent suction (*see* **Note 20**). Typically, live oysters can yield 0.5–1.5 mL fluid.
7. Dispense the hemolymph from the syringe into a 2-mL microcentrifuge tube (*see* **Note 21**).
8. Record the volume of hemolymph collected.
9. (Optional) An alternative approach for obtaining a cleaner hemolymph sample can be done by shucking the oyster and collecting fluid from the pericardial membrane in which the oyster heart is located. Insert the needle and remove hemolymph from the pericardial cavity (Fig. 1b; *see* **Note 22**).

3.2 Pepsin-HCl Digestion of Whole Tissue

Here we described the procedure of a mechanical homogenization and pepsin-HCl digestion of whole oyster tissue [8, 10] with slight modifications. Other enzyme digestion methods including a trypsin digestion have been reported to detect protozoan pathogens in marine bivalves [11, 12].

1. Clean oyster shells with a scrub brush and distilled water.
2. Record the width and length of oysters.
3. Place an oyster on a weighing boat. Orient the oyster flat side facing up and cupped side down.

4. Hold the oyster with a non-dominant hand. Insert the tip of shucking knife at the hinge. Twist the blade until the knife pops the hinge open. Slide the blade along the interior flat side (top shell) of the oyster to slice through the adductor muscle.
5. Remove the top shell and transfer the oyster liquor (fluid within the oyster shell) to a 50-mL conical tube.
6. Scrape the knife underneath the oyster to detach the adductor muscle from the bottom shell. Pour the oyster whole tissue in the same conical tube containing oyster liquor (*see* **Note 23**). Clean the blade between different oysters using alcohol and distilled water (*see* **Note 24**).
7. Homogenize the oyster sample using the Omni tissue homogenizer with a hard tissue probe for 2 min, or until the whole tissue is completely blended (*see* **Note 25**). Use a sterile tissue probe for each oyster. Alternatively, clean the probe between oysters using alcohol and distilled water (*see* **Note 24**).
8. Measure the weight and volume of oyster samples (*see* **Note 26**).
9. Prepare fresh pepsin-HCl digestion solution (*see* **Note 27**).
10. Add 20 mL of pepsin-HCl solution to the homogenized samples, shake the tube for thorough mixing and then mix via vortexing at the maximum speed for 5 s.
11. Incubate the mixture at 35 °C for 75 min. Remove it from the incubator every 25 min and vortex for 10 s.
12. Centrifuge the resulting suspension at 900 × *g* for 6 min.
13. Pour off supernatant into a waste container (*see* **Note 28**).
14. Wash 1 (water): Bring volume up to 10 mL in the conical tube using distilled water and vortex to resuspend pellet into solution (*see* **Note 29**). For samples aliquoted into multiple tubes due to large sample volume (*see* **Note 26**), pool the aliquots by transferring the resuspended pellets until all aliquots are combined in one tube.
15. Centrifuge at 900 × *g* for 6 min. Pour off supernatant into a waste container.
16. Wash 2 (PBS): Add 10 mL of PBS eluting solution and vortex to wash the pellet.
17. Centrifuge at 900 × *g* for 6 min. Pour off supernatant into a waste container.
18. Wash 3 (water): Bring volume up to 10 mL in the conical tube using distilled water and vortex to resuspend pellet into solution.
19. Centrifuge at 900 × *g* for 6 min. Pour off supernatant into a waste container.

20. Visually assess the pellet. If the resulting pellet is >0.5 mL, repeat **steps 16–19** (washes 2 and 3). If the pellet is <0.5 mL, proceed to **step 21**.
21. Add 0.5 mL nuclease-free water and vortex to resuspend the final pellet. Transfer the suspension in a 2-mL microcentrifuge tube.
22. Add 0.5 mL nuclease-free water to the emptied 50-mL conical tube and vortex to rinse the tube. Transfer the rinse suspension in the same microcentrifuge tube to pool the pellet and rinse suspensions.
23. Record the final volume of each sample as there is variation in pellet size between samples.

3.3 Nucleic Acid Extraction

1. Preheat a dry heating block to 56 °C. Prepare a liquid nitrogen dewar and a large beaker of boiling water (>2 L).
2. Transfer the hemolymph or tissue homogenate sample in microcentrifuge tubes to a 1.5-mL conical bottom screw cap tube for nucleic acid extraction. Centrifuge at 16,000 × *g* (or maximum speed of microcentrifuge) for 5 min and gently aspirate supernatant until a 100 μL final pellet volume is retained (*see* **Note 30**).
3. Add 180 μL Buffer ATL provided in the QIAGEN DNeasy Blood & Tissue Kit to the sample. Mix thoroughly by vortex mixing and/or pipetting. Include one extraction negative control consisting of only the 180 μL ATL Buffer (without added oyster sample).
4. Put the sample tubes in a floating microtube rack and place in liquid nitrogen for 4 min and immediately transfer the rack to boiling water for 4 min (*see* **Note 31**). Remove the rack and let cool for 2 min. Briefly spin it down to move liquid on the microcentrifuge wall to the bottom.
5. Add 40 μL Proteinase K. Mix thoroughly by vortex mixing at maximum speed for 10 s.
6. Place sample tubes in a dry heating block and incubate overnight at 56 °C (*see* **Note 32**).
7. Remove samples from the dry heating block and increase the dry heating block temperature to 70 °C.
8. Add 200 μL Buffer AL to samples and vortex mix for 10 s. Incubate samples in the dry block incubator at 70 °C for 10 min.
9. Remove samples from the dry heating block and add 200 μL ethanol (96–100%) to samples. Mix thoroughly by vortex mixing for 10 s. Turn on the dry heating block temperature to 95 °C.

10. Transfer the sample mixture to a DNeasy Mini spin column with a 2-mL collection tube. Centrifuge for 2 min at 9000 × *g* (≥6000 × *g*). Discard the flow-through and the collection tube bottom. Place the spin column in a new 2-mL collection tube.
11. Add 500 μL Buffer AW1 and centrifuge for 2 min at 9000 × *g* (≥6000 × *g*). Discard the flow-through and the collection tube bottom. Place the spin column in a new 2-mL collection tube (*see* **Note 33**).
12. Add 500 μL Buffer AW2 and centrifuge for 4 min at 14,000 × *g* (≥20,000 × *g*). In the meantime, make 1:10 AE buffer (for example, mix 130 μL Buffer AE with 1300 μL nuclease-free water in a 2-mL microcentrifuge tube). Heat the buffer in the dry heating block at 95 °C.
13. Following centrifugation, discard the flow-through and the collection tube. Place the spin column in a new 2-mL collection tube.
14. Centrifuge for additional 1 min at 14,000 × *g* (≥20,000 × *g*). Discard the flow-through and the collection tube bottom (*see* **Note 34**). Place a spin column to a new 1.5-mL microcentrifuge tube.
15. Add 50 μL 1:10 AE buffer preheated to 95 °C directly onto the center of the spin column filter. Close the cap of the spin column and incubate for 5 min at room temperature.
16. Centrifuge for 2 min at 9000 × *g* (≥6000 × *g*). Check to ensure the buffer filtered through to the 1.5-mL microcentrifuge tube.
17. Store eluted DNA at 4 °C for up to 48 h or freeze at −20 °C

3.4 Polymerase Chain Reaction (PCR)

A nested PCR assay which can coamplify *T. gondii* and *C. cayetanensis* in one external reaction followed by parasite differentiation in two internal (nested) reactions using parasite-specific primer sets [9] is described (*see* **Note 35**).

1. Disinfect PCR workstations (or equivalent clean counter space for DNA-free work) using 10% bleach (*see* **Note 36**).
2. Remove PCR reagents and primers from the freezer and place in a rack on ice to thaw them while keeping them chilled.
3. Inside a PCR workstation, prepare an external PCR master mix containing all PCR reagents except for the DNA template in a 1.5-mL microcentrifuge tube according to Tables 1 and 2. The master mix can be scaled up appropriately depending on the number of samples. Include PCR negative/positive controls and an estimated 10% extra volume for pipetting loss (*see* **Note 37**). Mix reagents thoroughly by pipetting.

Table 2
PCR mixture

Component External reaction	50-μL reaction	Final concentration	Component Internal reaction	50-μL reaction	Final concentration
Nuclease-free water	18.6 μL		Nuclease-free water	21.6 μL	
m18S forward primer (20 μM)	0.5 μL	0.2 μM	Pathogen specific forward primer (50 μM)	0.5 μL	0.2 μM
m18S reverse primer (20 μM)	0.5 μL	0.2 μM	Pathogen specific reverse primer (50 μM)	0.5 μL	0.2 μM
BSA (10%)	0.4 μL	0.8 μg/μL	BSA (10%)	0.4 μL	0.8 μg/μL
2× AmpliTaq gold 360 Mastermix	25.0 μL	1×	2× AmpliTaq gold 360 Mastermix	25.0 μL	1×
Subtotal	45.0 μL		Subtotal	48.0 μL	
Template DNA Template DNA, added at **step 5**	5.0 μL		**Template DNA** Template DNA, added at **step 11**	2.0 μL	
Total	50.0 μL		Total	50.0 μL	

Table 3
PCR thermal cycle condition

			3-step cycling (35 cycles)				
Reaction	Step	Initial denaturation	Denature	Anneal	Extend	Final extension	Hold
External	Temp.	95 °C	95 °C	58 °C	72 °C	72 °C	4 °C
	Time	10 min	40 s	40 s	90 s	7 min	∞
Internal	Temp.	95 °C	95 °C	59 °C	72 °C	72 °C	4 °C
	Time	10 min	40 s	40 s	90 s	7 min	∞

4. Dispense 45 μL external master mix into each 0.2-mL PCR tube.
5. Ideally, use separate workspaces for preparation of master mix and addition of DNA to avoid cross-contamination of PCR reagents. In such a setting, transfer PCR tubes with the master mix to another clean workspace. Add 5 μL template DNA to the PCR tubes. Mix thoroughly by pipetting several times.
6. Briefly spin down PCR tubes using a mini centrifuge to move all liquid on the PCR tube wall to the bottom.
7. Place PCR tubes in a thermal cycler and start the cycling as detailed in Table 3 (*see* **Note 38**).

8. Once the external PCR is completed, remove PCR tubes from the thermal cycler and place on a rack on ice for internal PCR. The external product can also be stored at 4 °C (for up to 48 h) until used in internal reactions.
9. For nested reactions, prepare internal PCR master mix in two separate microcentrifuge tubes in a DNA-free workspace (one for *T. gondii* and one for *C. cayetanensis*) according to Tables 1 and 2 (*see* **Note 39**).
10. Dispense 48 μL internal master mix into each PCR tube.
11. Add 2 μL of the external reaction amplicon to the PCR tube as template (*see* **Note 40**).
12. Place the PCR tubes in the thermal cycler and start the cycling as detailed in Table 3 (*see* **Note 41**).
13. After the internal reaction is complete, analyze the presence of the target DNA amplification by running the internal amplicons in a 2% agarose gel containing DNA staining 1× (e.g., 1 g of agarose powder dissolved in 50 mL of 1× TBE buffer and 2.5 μL 20,000× RedSafe) until DNA products move 70–80% downwards through the gel (*see* **Note 42**).
14. Visualize the PCR product using an ultraviolet transilluminator. Use a DNA ladder to infer the size of amplicons and compare them with the target amplicon size in Table 1.
15. It is recommended that sequence confirmation of suspect positives be always performed for conclusive molecular identification (*see* **Note 43**). Purify internal PCR amplicons from the excised target gel using a gel purification kit (*see* **Note 44**) for sequence analysis and identity confirmation. Store remaining PCR products at −20 °C.

4 Notes

1. Scrub brush with hard nylon bristles (Anchor Brush Company, cat. no. 750233, or equivalent).
2. The file (Nicholson, cat. no. 14290M, or equivalent) is used to create a notch into the shell to facilitate insertion of a needle for hemolymph aspiration. Alternatively, an electric rotary tool with a cutting wheel (Dremel, cat. no. 4300, or equivalent) can be used to speed the procedure. If using an electric tool, wear appropriate PPE for protection from dust.
3. Alcohol is used to rinse the blade of shucking knife and/or the tissue homogenizer probes in-between samples.
4. Mechanical homogenization using the Omni Tissue Homogenizer (Omni International, cat. no. TH115, or equivalent) with Hard Tissue Omni Tip™ plastic homogenizing probes (cat. no. 30750H) is described in this chapter.

5. Pepsin, Proteomic grade (VWR cat. no. 97063-862, or equivalent).
6. Pepsin-HCl digestion solution should be prepared immediately before use for each experiment.
7. For example, 100 mL is for 5 samples.
8. Use DNase-, RNase-free filtered barrier pipette tips ideal for molecular biology applications.
9. For the freeze–thaw cycle using liquid nitrogen and boiling water, use screw cap, conical bottom, microcentrifuge tubes that can withstand temperatures from −196 °C to 100 °C to prevent accidental pop-up of flip-top tubes in boiling water. Avoid using skirted bottom microcentrifuge tubes, as the seams can leak during rapid temperature change during the freeze–thaw cycle.
10. Use a benchtop cryogenic dewar that can withstand temperature of −196 °C for safe short-term transport and storage of liquid nitrogen. Wear safety goggles, a face shield, lab coat, insulated gloves when handling liquid nitrogen. Any unused liquid nitrogen remaining in a dewar should be allowed to evaporate. Do not dispose of liquid nitrogen into a sink.
11. To prevent rapid decrease of boiling water temperature when frozen samples are placed during the freeze–thaw procedure, use a large volume of boiling water (>2 L).
12. Use a floating microtube rack and 12-in. forceps or similar tool to grasp the floating microtube rack for gentle placement and removal from liquid nitrogen and boiling water. Do not touch liquid nitrogen with bare skin or disposable gloves, or place tools (e.g., forceps) in contact with liquid nitrogen.
13. Additional proteinase K may be needed to add 40 μL per sample as the proteinase K provided in the QIAGEN kit is intended for use at 20 μL per sample. Proteinase K can be purchased separately (QIAGEN, cat. no. 19133, or equivalent).
14. Dry heating block (also referred as to dry bath, block heater, or dry block incubator) that can hold 2-mL microcentrifuge tubes (Fisher Scientific, cat. no. 88-860-022, or equivalent).
15. AmpliTaq Gold 360 Mastermix (Applied Biosystems™, cat. no. 4398882, or equivalent)
16. Nuclease-free bovine serum albumin (BSA) 10% aqueous solution (Sigma-Aldrich, cat. no. 126615, or equivalent). BSA can be sub-aliquoted to 1.5-mL microcentrifuge tubes and stored at −20 °C.
17. Hinge is the v-shaped end of the oyster where two valves (shells) join (Fig. 1a).

18. Adductor muscle is a partially translucent and white organ than functions to hold the shells closed (Fig. 1b).
19. You will feel the needle touching the oyster adductor muscle, which is connected to the upper and lower shells.
20. It is normal to feel negative pressure in the syringe during aspiration.
21. Quick dispensing can minimize hemocytes loss from the inner wall of the syringe.
22. Hemolymph can be collected from an adductor muscle without opening the shells (i.e., without sacrificing oysters) and/or from a pericardial membrane containing the heart after the shells are opened. Once oysters are opened, very low amount of hemolymph is to be available. Pericardial membrane is located next to the adductor muscle (Fig. 1b). Refer Subheading 3.2 how to shuck oysters.
23. Avoid shell debris.
24. Label three 50-mL conical tubes: first water, alcohol, second water. Use the first water to rinse the blade (or homogenizer probe). After the tool is rinsed inside the conical tube by gently shaking, discard the water and replace with fresh water. Then rinse the tool with the alcohol and then with the second water. The alcohol and the second water can be reused for up to eight samples, or sooner if they look turbid.
25. Use maximum or the second highest speed of the Omni tissue homogenizer. Move the homogenizer up and down to thoroughly blend the whole tissue.
26. If the oyster homogenate is >15 mL, divide the homogenate into multiple tubes (maximum 15 mL per tube). Aliquoted samples will be pooled at **step 14** (Wash 1).
27. Refer **item 10** (pepsin-HCl digestion solution) in Subheading 2.2 pepsin-HCl digestion of whole tissue.
28. The oyster pellets will stick to the bottom of the conical tube.
29. A wash bottle with a dispensing spout helps to easily add in the desired amount.
30. The optimal volume that can be processed for DNA extraction in the spin column of QIAGEN DNeasy Blood & Tissue kit is 100 μL. Samples for DNA extraction can be stored at −20 °C at this step. If concentrated pellet volume cannot be reduced to 100 μL due to large pellet size, then up to 200 μL of partial sample can be placed in the spin column for DNA extraction.
31. This freeze–thaw cycle ruptures oocyst walls. The performance of one freeze–thaw cycle was previously tested on *Cryptosporidium parvum* and *T. gondii* oocysts [13].

32. The QIAGEN DNeasy Blood & Tissue Handbook indicates that samples can be incubated with proteinase K at 56 °C until they are completely lysed. Depending on sample types, the treatment time can vary (e.g. as short as 10 min for animal blood or cells to overnight for animal tissues). It shows that samples can be lysed overnight for convenience; this will not affect them adversely.
33. Add the appropriate amount of ethanol (96–100%) as indicated on the Buffer AW1 and Buffer AW2 bottles before the first use to make a working concentration.
34. As residual ethanol on the spin column may interfere with subsequent reactions, additional 1 min of centrifugation step is included to ensure complete removal of ethanol from the spin column. Often, no residual ethanol (or liquid) is visible in the collection tube after the last centrifugation.
35. The nested PCR assay was developed to simultaneously amplify a target region of the 18S ribosomal RNA (rRNA) gene of *T. gondii, C. cayetanensis,* and *Cryptosporidium* spp. in the external reaction [9]. The assay has a capability to be multiplexed with an additional primer set to coamplify four key target parasites (*T. gondii, C. cayetanensis, Cryptosporidium* spp. *and Giardia* spp.). Here we describe the procedure with slight modification for focusing on *T. gondii* and *C. cayetanensis* detection in shellfish. Spiking experiments using hemolymph and whole-tissue homogenate were performed for assay validation (ongoing experiments). Lowest limits of detection of *T. gondii* and *C. cayetanensis* using the nested PCR assay were 5 oocysts per extract in hemolymph and 5–10 oocysts per extract in whole-tissue homogenates.
36. PCR workstations and micropipettes should be thoroughly disinfected using 10% household bleach and ultraviolet (UV) before use. Because of the nested design of the PCR assay, DNA amplification is particularly sensitive, and cross contamination can occur if proper separation of DNA from reagents is not maintained, or if trace DNA material is present on surfaces or equipment. Thoroughly apply 10% bleach to all surfaces, ideally allow for 30 min of contact time, followed by ultrapure water (or 70% alcohol) to remove residual bleach. Preferably, preparation of PCR master mix and addition of DNA template into the master mix should be done in separate PCR workstations to reduce cross-contamination among samples.
37. For PCR negative control, nuclease-free water is used instead of DNA template. PCR-positive controls consist of target parasite DNA. Extracted DNA from 1000 oocyst stock solution can be used for PCR-positive control.

38. Initial denaturation temperature and time may vary depending on the PCR reagents.
39. To discriminate the parasites via internal nested reaction, two pathogen-specific internal primer sets are separately used in the nested reactions. The specificity of each set of internal primers was validated [9], and no cross reactivity between *T. gondii* and *C. cayetanensis* for their specific primer set will occur in the internal reactions [9].
40. While 5 μL template DNA (genomic DNA) is used for external reaction, 2 μL of external PCR amplicon is used as DNA template for internal reaction.
41. PCR thermal cycle conditions for the internal reactions are similar to those of the external reaction except for the annealing temperature, which is increased 1 °C.
42. Use safe alternatives to highly mutagenic ethidium bromide (EtBr) such as RedSafe (Bulldog Bio, cat. no. 21141, or equivalent) for detecting nucleic acid in agarose gel. RedSafe can be added in agarose solution before it is solidified. RedSafe can be also added to the running buffer to avoid depletion of RedSafe in the bottom portion of the gel during gel electrophoresis.
43. The identity of DNA amplicons can be confirmed via Sanger sequencing.
44. QIAquick gel extraction kit (QIAGEN cat. no. 28704, or equivalent).

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