Preface

Two of the recent books in the *Methods in Molecular Biology* series, *Yeast Protocols* and *Pichia Protocols*, have been narrowly focused on yeasts and, in the latter case, particular species of yeasts. *Food Microbiology Protocols*, of necessity, covers a very wide range of microorganisms. Our book treats four categories of microorganisms affecting foods: (1) Spoilage organisms; (2) pathogens; (3) microorganisms in fermented foods; and (4) microorganisms producing metabolites that affect the flavor or nutritive value of foods. Detailed information is given on each of these categories.

There are several chapters devoted to the microorganisms associated with fermented foods: these are of increasing importance in food microbiology, and include one bacteriophage that kills the lactic acid bacteria involved in the manufacture of different foods—cottage cheese, yogurt, sauerkraut, and many others. The other nine chapters give procedures for the maintenance of lactic acid bacteria, the isolation of plasmid and genomic DNA from species of *Lactobacillus*, determination of the proteolytic activity of lactic acid bacteria, determination of bacteriocins, and other important topics.

A substantial number of the chapters deal with yeasts, microorganisms which, after all, have also been associated with human foods and beverages for many thousands of years. The emphasis in *Food Microbiology Protocols* is on techniques for the improvement of methods for yeast hybridization and isolation, and for improvement of strains of industrially important yeasts, to be used in food and beverage production. For instance, the chapters by Katsuragi describe techniques for isolation of hybrids obtained by protoplast fusion and conventional mating, by the use of fluorescent staining, and by separation using flow cytometry. Other chapters discuss the identification of strains by analysis of mitochondrial DNA and other techniques. There are chapters on the isolation of strains of starches used in the production of human foods, and an important chapter on obtaining and isolating thermotolerant strains for the high temperature production of beverage and industrial alcohol. Finally, there are methods for the production of polyhydroxy alcohols for low-calorie sweeteners. The material on yeasts overlaps only slightly with that in the excellent book, *Yeast Protocols*, edited by Ivor H. Evans, so investigators interested in industrial yeasts should avail themselves of both volumes.

The chapters on spoilage organisms and pathogens include valuable information on the isolation and identification of most important species in these areas. Several of these are concerned with bacteria, yeasts, and molds, causing spoilage of poultry products, as well as causing disease in humans. Methods for identification by molecular biology techniques and by conventional plate counts are given. There are two reviews on topics of immediate interest.

Finally, the editors and the publishers would like to thank all those authors who gave so freely of their time and energy in preparing these chapters.

The editors wish especially to thank Dr. Faustino Siñeriz, Director of PROIMI, for allowing us to use the facilities at PROIMI in the preparation of this book, and for his kind encouragement in the work at all times. We also thank Dr. María E. Lucca for her able assistance in correcting the final version.

John F. T. Spencer
Alicia L. Ragout de Spencer

2

Biochemical Identification of Most Frequently Encountered Bacteria That Cause Food Spoilage

Maria Luisa Genta and Humberto Heluane

1. Introduction

When the microbial flora invades food, two major problems arise. First is the pathogenicity of several microbes, and second are the changes on the food characteristics, such as contents of nutrients (hydrocarbons, vitamins, aminoacids, metals, etc.), bad smell, color and flavor, texture modification, etc.

This chapter presents the identification techniques to the most usually encountered bacteria that could be present on food.

Salmonella may be present on raw or non-heat-treated food. Eggs, milk, mayonnaise, chicken, hamburger, and creams are the most frequent vehicles for this bacteria. *Salmonella* is sensitive to high temperatures and can survive at very low temperatures. It is a Gram negative, mobile, nonsporulated, and facultative anerobic bacteria. Only total absence of this bacteria on food is normally accepted by legal regulations.

As a consequence of the nature of the contamination, salmonellas are usually present together with large numbers of other enterobacteria. Therefore the use of enrichment and selective media is required.

Bacillus cereus is a gram positive, aerobic, and catalase-positive bacteria. The optimal growth for this microorganism is obtained at temperatures oscillating between 25 and 75°C and pHs between 6 and 7, even though this microorganism survives temperatures as low as –5°C. The spores are very resistant to heat. At pHs lower than 4 the growth of *B. cereus* is inhibited. This species is highly lipolytic, saccharolytic and proteolytic and it is pathogenic for humans. It may be present on flour, milk, dairy products, rice, chicken, spices, and herbs. Only total absence of this species is normally accepted by legal regulations.

From: *Methods in Biotechnology, Vol. 14: Food Microbiology Protocols*
Edited by: J. F. T. Spencer and A. L. Ragout de Spencer

Coliform is the name given to those microorganisms that have the following characteristics: rod shaped, gram negative, mobile or nonmobile, aerobic or facultative anerobic nonsporulating. They ferment lactose, producing acid and gas in presence of bile salts, at 30–38°C. Coliforms are usually found in the intestine of humans and animals.

The *Escherichia coli* genus belongs to the enterobacteracea family and it is usually a sign of fecal contamination. *E. coli* is a bacillus with the following characteristics: Gram negative, mobile or nonmobile, oxidase negative, catalase positive, and glucose and lactose fermentation positive. *E. coli* is found in the human intestine, synthesizes vitamin K, and is involved in the production of vitamin B. Humans eliminate *E. coli* through fecal residues. Some strains are enterohemorrhagic pathogens.

Staphylococci are Gram positive, nonmobile, nonsporulated, noncapsulated, catalase positive, oxidase negative and most of the strains grow in media containing 10% NaCl. They are anaerobically facultative, but they grow better in aerobiosis. Staphylococci are capable of growing at temperatures oscillating between 8 and 45°C, although the optimum growth temperature is 37°C and pHs oscillating between 4 and 9.3, but the optimum growth pHs are 7.0–7.5.

Staphylococci produce heat-resistant toxins that act at the digestive level. There are seven kinds of enterotoxins, A, B, C_1, C_2, C_3, D, and E. Toxins A and D are more frequently present in food intoxication. *Staphylococcus aureus* may cause skin infection (acne). Staphylococci could be present in, e.g., dairy, meat, sausages, fish, and eggs ***(1–6)***.

2. Materials

2.1. Method 1: Salmonellas

1. 500 mL and 250 mL sterile Erlenmeyer flasks.
2. Sterile Petri dishes.
3. 10 mL sterile pipets.
4. Loop.
5. Sterile blender (“Minipimer” type).
6. Buffered peptone water: Ingredients per liter: peptone 10 g, sodium chloride 5 g, sodium phosphate dibasic 9 g, potassium phosphate monobasic 1.5 g, pH 7.0.
7. Tetrathionate broth: Ingredients per liter: proteose peptone 5 g, bacto bile salts 1 g, sodium thiosulfate 30 g, calcium carbonate 10 g.

2.1.1. Method of Preparation

a. Prepare 100 mL of tetrathionate broth with distilled or deionized water and heat to boiling. Cool below 60°C.

b. Add 2 mL iodine solution (prepared by dissolving 6 g iodine crystals and 5 g potassium iodide in 20 mL distilled or deionized water) to medium. Do not heat after adding iodine.
c. Dispense 10–12 mL quantities into sterile test tubes. Use medium the same day it is prepared.

8. Selenite broth: Ingredients per liter: bacto tryptone 5 g, bacto lactose 4 g, sodium selenite 4 g, sodium phosphate 10 g, pH 7.0.

   Dissolve the ingredients 1 L distilled or deionized water and heat to boiling to pasteurize. Avoid excessive heating. Do not sterilize in the autoclave.

9. Wilson–Blair medium: Medium A: Ingredients per liter: bacto beef extract 5 g, proteose peptone 10 g, bacto dextrose 10 g, sodium chloride 5 g, bacto agar 30 g, pH 7.3.

   Solution 1: 40 g sodium sulfite anhydrous in 100 mL distilled water.

   Solution 2: 21 g sodium phosphate dibasic anhydrous in 100 mL distilled water.

   Solution 3: 12.5 g bismuth ammonium citrate granular in 100 mL distilled water.

   Solution 4: 0.96 g ferrous sulfate dried in 20 mL distilled water with two drops of hydrochloric acid.

2.1.1.1. Selective Reagent

The selective reagent consists of a combination of solutions 1–4. Each solution is made up separately, dissolved, then combined. Heat combined solution to boiling until a slate-gray color develops. Allow to cool and store at room temperature in a closed rubber-stoppered container. It is stable for up to 1 mo.

2.1.1.2. Basal Medium

a. Prepare 1 L of medium A with distilled or deionized water and heat to boiling to dissolve completely.
b. Sterilize in autoclave for 15 minutes at 121°C.
c. To prepare the complete medium, aseptically add 70 mL of selective reagent and 4 mL of a 1% titrate solution of Brillant Green. Mix thoroughly.
d. Dispense as desired.

10. *Salmonella–Shigella* agar (SS agar): Ingredients per liter: bacto beef extract 5 g, proteose peptone 5 g, bacto lactose 10 g, bacto bile salts 8.5 g, sodium citrate 8.5 g, sodium thiosulfate 8.5 g, ferric citrate 1 g, bacto agar 13.5 g, bacto Brillant Green 0.33 mg, bacto Neutral Green 0.025 g, pH 7.0.
    a. Prepare 1 L of SS agar in distilled or deionized water and heat to boiling.
    b. Boil for 2–3 min with frequent and careful swirling to dissolve completely. Avoid overheating. Do not autoclave. Cool to 55–60°C.
    c. Dispense into sterile Petri dishes. Allow the surface of the medium to become quite dry by partially removing the covers while the medium solidifies (about 2 h) ***(3,7,8)***.

2.2. Method 2: Salmonellas

1. 500 mL and 250 mL sterile Erlenmeyer flasks.
2. Sterile Petri dishes.
3. 10 mL sterile pipets.
4. Loop.
5. Sterile blender ("Minipimer" type).
6. Test tubes.
7. Buffered peptone water: Ingredients per liter: peptone 10 g, sodium chloride 5 g, sodium phosphate dibasic 9 g, potassium phosphate monobasic 1.5 g, pH 7.
8. Chromogenic *Salmonella* esterease agar (CSE)
   a. Basal medium: Ingredients per liter: peptone 4 g, Lab-Lemco powder (Oxoid Ltd.) 3 g, tryptone 4 g, lactose 14.65 g, L-cisteine 0.128 g, trisodium citrate dihydrate 0.5 g, Tris base 0.06 g, Tween-20.3 g, Roko agar (Industrias Roko S.A., La Coruna, Spain) 12 g, pH basal medium 7.
   b. Chromogenic substrate: SLPA-octanoate [bromide form], 0.3223 g per liter in the final medium formulation (*see* **Note 1**).
   c. UV-absorbing compound: ethyl 4-dimethylamilobenzoate (0.035%, wt/vol) dissolved in 8 mL of methanol per liter of medium.
   d. Novobiocin: 70 mg per liter (Sigma-Aldrich Co., Ltd.) (*see* **Note 2**).

2.2.1. Preparation

   a. Prepare required volume of basal medium.
   b. Sterilize in autoclave for 15 minutes at 121°C.
   c. Cool down to about 55°C.
   d. Add other compounds (e.g., chromogenic substrate, UV-absorbing compound, and Novobiocin).
   e. Pour complete agar medium into Petri dishes.
   f. After setting, let plates to be surface dried. Use immediately or store in dark at room temperature for up to 2 wk.
9. Saline solution: Sterile solution: 0.85% NaCl in distilled water ***(8,9)***.

2.3. Bacillus cereus

1. 1 mL sterile pipets.
2. Drigalski spatula.
3. Loop.
4. 500 mL and 250 mL Erlenmeyer flasks.
5. Sterile Petri dishes.
6. Mossel agar: Ingredients per liter: bacto beef extract 1 g, peptone 10 g, D-mannitol 10 g, sodium chloride 10 g, phenol red 0.025 g, bacto agar 12 g, pH 7.0
   a. Make up 90 mL of the medium and dispense into a flask. Autoclave 15 min at 121°C. Cool to 50–55°C.
   b. Add 10 µL of a suspension 1:1 of egg yolk in physiological solution. This suspension must be maintained at 50°C.

   c. Add 100 µg of polymixin B sulfate per milliliter of medium.
   d. Use immediately.
7. Gelatinase reaction medium: Ingredients per liter: bacto beef extract 3 g, peptone 5 g, gelatin 120 g, pH 7.0. Make up the desired volume of medium and autoclave 15 min at 121°C.
8. Clark–Lubs medium (peptone broth): Ingredients per liter: peptone 7 g, glucose 5 g, dibasic potassium 5 g, pH 7.5.
   a. Dissolve ingredients in distilled or deionized water.
   b. Adjust pH to 7.5.
   c. Sterilize in the autoclave for 15 min at 121° Gay Lussac.
9. Methyl red: Make up a 0.5% solution in 60°C ethanol.
10. Diluted acetic acid.
11. Malachite green.
12. Sudan black: Make up a 0.3% solution in 70% ethanol.
13. Xylol.
14. Safranin ***(3,7,8,10)***.

2.4. Coliforms

1. 1 mL Sterile pipets.
2. Loop.
3. Fermentation vials (Durham tubes).
4. Brilliant Green lactose bile broth media: Ingredients per liter: bacto peptone 10 g, bacto lactose 10 g, bile salts 20 g, Brillant Green 0.0133 g, pH 6.9.

2.4.1. Preparation

   a. Suspend ingredients in distilled or deionized water and warm slightly to dissolve completely.
   b. Dispense required amount in test tubes.
   c. Place an inverted fermentation vial (Durham tube) in each tube.
   d. Place caps on tubes and sterilize in the autoclave for 15 min at 121°C.
   e. Before opening the autoclave, allow the temperature to drop below 75°C to avoid entrapment of air bubbles in the inverted vials.
5. Tryptone water: Ingredients per liter: tryptone 10 g, sodium chloride 5 g.
6. Levine agar: Ingredients per liter: bacto peptone 10 g, bacto lactose 10 g, dipotassium phosphate 2 g, bacto agar 15 g, bacto eosin Y 0.4 g, bacto methylene blue 0.065 g, pH 6.8–7.0.
   a. Suspend the ingredients in distilled or deionized water and heat to boiling to dissolve completely.
   b. Sterilize in the autoclave for 15 min at 121°C.
7. Simmons citrate agar: Ingredients per liter: magnesium sulfate 0.2 g, ammonium dihydrogen phosphate 1 g, dipotassium phosphate 1 g, sodium citrate 2 g, sodium chloride 5 g, bacto agar 15 g, bacto brom thymol blue 0.08 g.

a. Suspend the ingredients in distilled or deionized water and heat to boiling to dissolve completely.
b. Sterilize in the autoclave for 15 min at 121°C.

8. Clark–Lubs medium (peptone broth): Ingredients per liter: peptone 7 g, glucose 5 g, phosphate bipotasic 5 g, pH 7.5.
   a. Dissolve ingredients in distilled or deionized water.
   b. Adjust pH to 7.5.
   c. Sterilize in the autoclave for 15 min at 121°C.
9. Kovacs reagent: Ingredients per liter: paradimethyl-aminobenzaldehyde 5 g, amyl alcohol 75 mL, hydrohydrochloric acid 25 mL.

   Dissolve the aldehyde in the amyl alcohol heating in a water bath at 50°C. Let it cool down. Add the hydrochloric acid very slowly (in drops). The yellow-golden solution obtained must be stored in a dark bottle with a ground glass stopper.
10. Methyl red solution: Prepare a 0.5% solution of methyl red in 60° Gay Lussac ethanol.
11. Creatinine.
12. Potassium hydroxide: 40% Solution in water ***(3,7,8,13)***.

2.5. Staphylococcus aureus

1. 1 mL sterile pipets.
2. Drigalsky spatula.
3. Sterile Petri dishes.
4. Test tubes.
5. Hemolysis tubes.
6. Loop.
7. Hydrochloric acid 1 *N*.
8. Rabbit citratade plasma.
9. Baird–Parker agar: Ingredients per liter: bacto tryptone 10 g, bacto beef extract 5 g, bacto yeast extract 1 g, glycine 12 g, sodium pyruvate 10 g, lithium chloride 5 g, bacto agar 20 g, pH 6.8–7.0.

2.5.1. Preparation

a. Suspend the ingredients in distilled or deionized water.
b. Heat to melt agar and adjust pH.
c. Autoclave for 15 min at 121°C.
d. Cool down to 45–50°C.
e. Add 50 mL of a suspension 1:1 of egg yolk in physiological solution. This suspension must be maintained at 45–50°C.
f. Add 3 mL of a 3.5% potassium tellurite solution sterilized by filtration. The solution must be warmed up to 45–50°C.

10. DNase test agar: Ingredients per liter: bacto tryptose 20 g, deoxyribonucleic acid (DNA) 2 g, sodium chloride 5 g, bacto agar 15 g, pH 7.3.
    a. Suspend compounds in distilled or deionized water. Heat to boiling to dissolve completely.
    b. Sterilize in the autoclave for 15 min at 121°C.

11. Brain–heart infusion**:** Ingredients per liter: Infusion from calf brains 200 g, infusion from beef heart 250 g, proteose peptone 10 g, bacto dextrose 2 g, sodium chloride 5 g, disodium phosphate 2.5 g, pH 7.4.
    a. Suspend the ingredients in distilled or deionized water.
    b. Dispense as desired.
    c. Sterilize in the autoclave for 15 min at 121°C ***(3,7,8,10)***.

3. Methods

3.1. Salmonellas: Method 1

1. The sample of the solid or liquid food must be representative and weight not less than 25 g.
2. Mix 225 mL of the buffered peptone water with 25 g of the food sample. Blend the mixture with a Minipimer-type blender during 2 min. Afterward, the pH of the mixture must be adjusted to 6–7 with a buffer solution.
3. Transfer aseptically the mixture obtained in **step 2** to a 500-mL sterile Erlenmeyer flask. Incubate at 37°C for 16–20 h.
4. After incubation, transfer 10 mL of the culture to a flask containing 100 mL of tetrathionate broth (culture A). Incubate at 42–43 °C for 48 h.
5. Transfer 10 mL of the culture obtained in **step 3** to a flask containing 100 mL of selenite broth (culture B). Incubate at 37°C for 48 h.
6. After 24 h incubation in both tetrathionate broth (culture A) and selenite broth (culture B), samples of each culture must be plated on to Wilson–Blair medium and SS agar to obtain isolated colonies. Petri dishes must be incubated at 37°C for 48 h (*see* **Note 3**).
7. The same plating procedure indicated in **step 6** must be followed after 48 h of cultivation in both cultures (tetrathionate broth and selenite broth), but this time Petri dishes are incubated for 24 h.
8. *Salmonella* colonies on Wilson–Blair agar will appear as brown or black with shiny colonies surrounded by a dark halo. Some strains develop green colonies and do not darken the medium.

Salmonella colonies on SS agar appear as colorless or pinkish colonies at 18 h of incubation. Afterward, they become bigger and opaque and they can develop a gray or black central spot ***(1,3,5,10,11)*** (*see* **Note 4**).

3.2. Salmonellas: Method 2

1. Follow **steps 1–3** of **Subheading 3.1.**
2. Transfer a sample of the culture into test tubes containing buffered peptone water.
3. Incubate for 4–6 h at 37°C.
4. In order to obtain plates showing well-isolated colonies, the cultures must be serially diluted in saline solution.
5. Spread 100 µL of appropriate dilutions onto chromogenic medium.
6. Incubate plates at 37 or 42°C.
7. Observe for colony coloration for up to 48 h.

8. *Salmonella* spp. could be differentiated from nonsalmonellae by the production of burgundy-colored colonies. Nonsalmonellae appeared as white or colorless colonies ***(3,9)***.

3.3. Bacillus cereus

1. Take 25 g of the sample and add 225 mL of peptone broth. Mix thoroughly for 1–2 min. The solution thus obtained is a 10^{-1} dilution of the original sample. The amount of peptone broth to add to the sample depends on the product to test. In case of flour it is convenient to predetermine the volume of medium to use.
2. Add 1 mL of the 10^{-1} dilution to 9 mL of sterile peptone broth to obtain 10^{-2} dilution. Make up a 10^{-3} and 10^{-4} dilutions following the same procedure.
3. Plate 0.1 mL of the dilutions on Mossel agar. Spread the inocula using a Drigalski spatula. The plates must be completely dry.
4. Incubate for 24–48 h at 30°C. Examine plates to find colonies that have grown (*see* **Note 5**).

3.3.1. Biochemical Confirmation

The colonies must be transferred to plates containing gelatinase reaction medium. Incubate for 2–3 d at 30°C. Cover the plates with diluted acetic acid. Colonies that are gelatinase positive will show a halo.

The doubtful colonies must also be transferred to test tubes containing 5 mL of Clark–Lubs medium (peptone broth). Incubate for 3 d at 30°C and then add to the cultures 4–5 drops of methyl red indicator, mix to homogenize, and check color. If the colonies are methyl red positives the culture color will turn to red, otherwise the culture color will remain yellow. *B. cereus* is methyl red (+), gelatinase (+), and lecitinase (++) (*see* **Note 6**).

The number of cells per gram of product is obtained multiplying the number of colonies developed on Mossel agar by the dilution factor used for the suspension plated on the Petri dishes ***(3,4,10–12)***.

3.4. Coliforms

1. Add 225 mL peptone water to 25 g of the sample. Shake during 1–2 min. The final suspension is a 1:10 dilution of the sample.
2. Prepare 9 sterile test tubes containing 9 mL each one of BGBL medium and the inverted fermentation tubes (*see* BGBL culture medium, **Subheading 2.4., item 4**).
3. Add with sterile pipet 1 mL of the 1:10 dilution of the sample to each of three of the test tubes mentioned earlier. The suspension obtained will be a 1:100 dilution of the original sample. Follow the same procedure to obtain a 1:1000 and a 1:10000 dilutions of the original sample. Note that three test tubes containing each dilution will be obtained.
4. Incubate the test tubes for 48 h at 30°C.

5. After the incubation period, observe the test tubes. Tubes where 10% of the total volume of the inverted vial is occupied by gas are considered as coliform positive. The results of these test tubes are useful to determine the more probable number of microorganisms per gram or milliliter in the original sample. For each dilution (1:100, 1:1000, and, 1:10000) the tubes with positive gas production are counted. The number of positive tubes are used to obtain the more probable number from **Table 1** ***(1,2,4,10,12,13)***. The positive tubes will also allow the identification of *E. coli*.

3.5. E. Coli

3.5.1. General Procedure

E. coli must be investigated in all test tubes where gas is present when the coliform identification technique was followed (*see* **Subheading 3.4.**). *E. coli* has the following biochemical characteristics: gas production when incubated in BGBL broth for 48 h at 44°C and indole production when incubated in tryptone water during 48 h at 44°C.

1. Mix each test tube where gas is observed when the coliform identification technique was followed. Transfer a loopful of the suspension from each tube to a new sterile tube containing 10 mL of BGBL medium and an inverted vial.
2. Repeat the foregoing procedure with test tubes containing 10 mL of tryptone water.
3. Incubate for 48 h at 44°C.
4. After the incubation period check the gas production. Write down the number of positive tubes.
5. Investigate indole presence in the tubes containing tryptone water with the following technique:
   a. Add 1 mL of Kovacs reagent.
   b. An indole positive reaction will develop a red ring on the surface of the medium after 5 min.
6. Consider *E. coli* positive subcultures that, having produced gas during the 30°C incubation, give an indole-positive reaction in tryptone water and gas production in BGBL at 44°C.

3.5.2. Specific Media for Confirmation of E. coli

In order to reinforce the identification of *E. coli,* use the following procedures:

1. Take samples with a loop from the *E. coli* positive test tubes containing BGBL medium. Plate the samples onto Levine agar.
2. Incubate plates for 48 h at 37°C.
3. Observe colonies. Different characteristics between *E. coli* and *Enterobacter aerogenes* are given in **Table 2**. Colonies of *Salmonella* and *Shigella* are transparent, amber colored.
4. Confirm the presence of *E. coli* by the IMVIC (test using the following techniques: indole, methyl red, Voges-Proskauer, and sodium citrate).
   a. Indole: Proceed as indicated in **step 5** of protocol for *E. coli* identification.

Table 1
More Probable Number of Microorganisms per Gram or Milliliter (*see* Note 7)

Positive tubes			Number per gram milliliter
10^{-2}	10^{-3}	10^{-4}	
0	0	1	3
0	1	0	2
1	0	0	4
1	0	1	7
1	1	0	7
1	1	1	11
1	2	0	11
2	0	0	9
2	0	1	14
2	1	0	15
2	1	1	20
2	2	0	21
2	2	1	28
3	0	0	23
3	0	1	39
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	2	0	93
3	2	1	150
3	2	2	210
3	3	0	240
3	3	1	460
3	3	2	1,100

b. Methyl red:
(1) Prepare a pure culture from the doubtful colonies using peptone water as the culture medium. This culture must be incubated for 6–8 h. Use a loopful of the latter culture as the inoculum to a test tube containing 10 mL of Clark–Lubs medium.
(2) Incubate for 72 h at 30°C.
(3) Divide culture in two identical volumes, one of which will be used in the methyl red test and the other one in the Voges–Proskauer test.
(4) Add to one of them five drops of methyl red solution.
(5) Observe results: Red color: methyl red +; Yellow color: methyl red –.

Table 2
Different Characteristics between Colonies of *E. coli* and *A. aerogenes*

	E. coli	*A. aerogenes*
Size	Well-isolated colonies are 2–3 mm in diameter	Well-isolated colonies are usually larger than *E. coli*
Elevation	Colonies are slightly raised; surface flat or slightly	Colonies considerably raised and markedly convex concave
Appearance by reflected light	Colonies dark, buttonlike, often concentrically ringed with a greenish metallic sheen	Much lighter than *E. coli*, centers are deep brown Metallic sheen is not observed

c. Voges–Proskauer test: This test is also known as of acethyl-methyl-carbinol production.
(1) Add to the other portion of the culture in the Clark–Lubs medium a small amount of creatinine and 5 mL of the solution 40% of KOH.
(2) Mix during 2 min.
(3) Observe results: Pink Color: Voges–Proskauer +; no color change: Voges–Proskauer –.

d. Sodium citrate test: Some microorganisms are able to grow with citrate as the sole carbon source. This characteristic is used to differentiate the Enterobacteriaceae.
(1) Inoculate the doubtful culture with a loop in the middle of the plate.
(2) Incubate for 48 h at 37°C.
(3) Observe results. Blue color: citrate +. No color change: citrate – ***(3,4,6,8,13–15)***.

The results of the IMVIC tests for different bacteria genus are given in **Table 3**.

3.6. Staphylococcus

1. Add 225 mL of sterile water to 25 g of the sample. Shake during 1–2 min. The final suspension is a 1:10 dilution of the sample.
2. Prepare 1:100, 1:1000, and 1:10000 dilutions of the sample.
3. Spread 0.1 mL of each dilution onto Petri dishes containing Baird–Parker agar. Use a Drigalski spatula to spread properly. Make duplicates of the plates.
4. Incubate for 24–48 h at 37°C.
5. Coagulase-positive colonies of staphylococci are black with a sheen surrounded by a clear zone due to the action of the enzymes on the egg yolk.
6. Investigate the presence of enzymes in the doubtful colonies using the following techniques.
   a. DNase test: *S. aureus* is DNase positive.
   (1) Plates containing DNase test agar are inoculated by streaking or spotting with the material or culture being tested. Make only three streaks or spots per plate.

Table 3
IMVIC Test Results for Different Bacteria

	Indole	Methyl red	Voges–Proskauer	Citrate
Shigella	±	+	–	–
Escherichia	±	+	–	–
Salmonella	–	+	–	+
Arizona	–	+	–	+
Citrobacter	–	+	–	+
Klebsiella	±	–	+	+
Enterobacter	–	–	+	+
Hafnia	–	–	+	+
Proteus	+	+	–	+
Providencia	+	+	–	+

(2) Incubate for 18–24 h at 37°C.
(3) Flood plates with 1 *N* hydrochloric acid and observe for clearing around the streak or spot indicating DNase activity.

b. Coagulase test: Most of the *S. aureus* strains are coagulase positive, although in very few cases it could be coagulase negative.
(1) Test tubes containing 5 mL of brain–heart infusion are inoculated with the material being tested.
(2) Incubate for 24 h at 37°C.
(3) Add 2–3 drops of the culture to a hemolysis tube containing 0.5 mL rabbit plasma.
(4) Incubate at 37°C, periodically observing coagulation. Coagulation usually takes place before 4 h of inoculation.

c. Thermonuclease test: *S. aureus* DNase is thermoresistant.
(1) Plates containing Baird–Parker agar are inoculated by streaking or spotting with the material or culture being tested.
(2) Incubate at 37°C for 24 h.
(3) The dishes that show colony development are then incubated at 65°C for 150 min. After this treatment the cells become inviable.
(4) Add to the plates DNase test agar containing toluidine blue. Incubate at 37°C for 4 h.
(5) DNase hydrolysis is shown by the presence of pinkish brilliant halos. The clearing is due to the action of *S. aureus* DNase that is thermoresistant.

Although *S. aureus* cells could have been destroyed in the sample by any reason, some toxins could remain active. Therefore, even though *S. aureus* cells are not detectable, it is important to investigate the presence of toxins. Specific kits are the more usual way to detect these toxins.

To isolate a toxin from the food sample, proceed as follows.

(1) Take 10 g of the food and add to it 10 mL of sterile 0.85% NaCl solution. Cool to 4°C.
(2) Centrifuge at 2000*g* for 30 min at 4°C.
(3) The supernatant is then filtered through a 0.2-μm filter.
(4) The filtrate is used to detect the presence of the toxin using adequate kits ***(2,4,5,11,13–15)***.

4. Notes

1. Chromogenic *Salmonella* esterase agar is based on the detection of C_8–esterase activity in salmonellae.
2. Alternatively, the chromogenic substrate and UV-absorbing compound could be added directly to the basal medium if this is heated only to the boiling point. UV-absorbing compound is added to protect the substrate against photochemical degradation. The use of novobiocin reduce the growth of nonsalmonella strains in the chromogenic medium.
3. *Salmonella*: following the procedure four plates will be obtained:
   a. Culture A on Wilson–Blair medium.
   b. Culture A on SS agar.
   c. Culture B on Wilson–Blair medium.
   d. Culture B on SS agar.
4. *Salmonella*: It is recommended to confirm the presence of *Salmonella* that either the colonies are typical or they are doubtful. The appearance of the colonies varies not only for the different species but also with different batches of culture media. An API-20E kit could be used as a method to confirm *Salmonella*.
5. *B. cereus:* the polymixin added to the Mossel agar allows the growth of *B. cereus* but inhibits the growth of the secondary flora. The lecithin of the egg yolk will precipitate by the action of the lecithinase of *B. cereus*. *B. cereus* does not produce an acid from mannitol, therefore its presence does not turn the phenol red to yellow. The colonies of *B. cereus* are pink surrounded by an opaque zone due to the action of the lecithinase on the egg yolk. The colonies are invasive, irregular, and rough.
6. *B. cereus:* the other test to confirm the presence of *B. cereus* consists of transferring the doubtful colonies onto any nutrient agar for bacteria and incubating them for 24 h. The cells are then fixed by heat on a slide. The spore staining is done by adding the malachite green solution to the slide and heating with direct flame until vapor emission. Keep heating for 5–10 min. Wash the sample with water, dry it, and add the Sudan Black solution for lipid staining. Keep the Sudan Black solution in contact with the preparation for 15 min, wash it with xylitol for 5 s, and let dry. Add safranin and keep in contact for 1 min. Afterward, wash with water, dry, and observe. Spores show green, lipid compounds black, and the cytoplasm will show red.
7. Coliforms: use **Table 1**, e.g.,
   a. Dilution 1:100 positive tubes 1.
   b. Dilution 1:1000 positive tubes 2.
   c. Dilution 1:10000 positive tubes 0.

   Then the more probable number of microorganisms is 11 per gram or milliliters of the original sample.

References

1. Banwart, G. J. (1981) *Microbiología Básica de los Alimentos,* Ediciones Bellatterra S.A. Anthropos, Barcelona, Spain.
2. Cliver, D. O. (1990) *Foodborne Diseases,* Academic Press, San Diego, CA.
3. International Commission on Microbiological Specifications for Foods (ICMSF). *Microorganismos de los Alimentos. Vol. 1. Técnicas de Análisis Microbiológicos,* Editorial Acribia S.A., Zaragoza, Spain.
4. Sharf, J. M. (1965) Recomended methods for the microbiological examination of foods. American Public Health Association Inc. Washington, DC.
5. Vila Aguilar, R. (1994) Instituto de Agroquímica y Tecnología de Alimentos (IATA), Valencia, Spain.
6. Martialay Valle, F. (1989) *Prontuario de Técnicas en Microbiología de Alimentos,* Ministerio de Defensa, Sec. Gral. Técnica, Madrid, Spain.
7. Marchal, N., Bourdon, J. L., and Richard, C. L. (1982) Les milieux de culture pour l'isolement et l'identification biochimique des bactéries. Doin éditeurs. París.
8. Difco Manual, Difco Laboratories, USA (1995).
9. Cooke, V. M., Miles, R. J., Price, R. G., and Richardson, A.C. (1999) A novel chromogenic ester agar medium for detection of Salmonellae. *Appl. Environ. Microbiol.* **65,** 2.
10. Pascual Anderson, M. R. (1982) *Técnicas para el Análisis Microbiológico de Alimentos y Bebidas,* Ministerio de Sanidad y Consumo, Madrid, Spain.
11. Larpent, J. P. and Larpent Gourgaud, M. (1975) *Memento. Techniques de microbiologie,* Technique et Documentation, París.
12. Fung, D. Y. C. and Matthews, R. F. (1991) *Instrumental Methods for Quality Assurance in Foods,* Marcel Dekker, ASQC Quality Press, New York.
13. Collins, C. H. and Lyne, P. M. (1989) *Métodos Microbiológicos*, Editorial Acribia S.A., Zaragoza, Spain.
14. Speck, M. L. (1976) *Compendium of Methods for the Microbial Examination of Foods*, American Public Health Association, Inc., Washington, DC.
15. Thatcher, F. S. and Clark, D. S. (1973) *Análisis Microbiológico de los Alimentos*, Editorial Acribia S.A., Zaragoza, Spain.