Methods and Protocols
in Food Science

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Dhanasekaran Dharumadurai *Editor*

# Postbiotics

Humana Press

# Methods and Protocols in Food Science

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# Postbiotics

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ISSN 2662-950X ISSN 2662-9518 (electronic)
Methods and Protocols in Food Science
ISBN 978-1-0716-3420-2 ISBN 978-1-0716-3421-9 (eBook)
https://doi.org/10.1007/978-1-0716-3421-9



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The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

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# Foreword

The current consumer demands healthy and natural foods that allow them, through food, to prevent the development of diseases. In this sense, postbiotic compounds appear as an area of emerging research interest due to their beneficial effects on the digestive tract and the immune system. Postbiotics can be defined as natural soluble products or metabolic by-products generated by probiotics (released by secretion or after cell lysate) or even called non-viable microorganisms with biological activity and physiological benefits to the host. The main interest of postbiotics is their great capacity to exert beneficial effects on health, which, unlike probiotics, have advantages such as being easy to produce, store, and transport. In addition, postbiotics are soluble compounds with the ability to interact directly with the mucous layer and intestinal epithelial cells, where the postbiotics act by exerting their functional activity.

*Postbiotics* edited by Dr. D. Dhanasekaran and published by Springer Nature is a descriptive manual that details the techniques involved in the preparations of postbiotics, analysis of postbiotic types, evaluation of the functional activity of postbiotics, and different applications. It comprises more than 50 chapters, which deal with the world of postbiotics, how to produce them using probiotics, their analysis and identification, their beneficial effects, and applications in veterinary and aquaculture. This book contains meticulously detailed protocols to be able to advance in the development of ingredients and functional foods of the future, which allow us to offer products that are more adapted to the needs of today's consumers concerned about their health. In addition, it will enable companies to have established and standardized protocols to use in developing future foods personalized to the demands of the current consumer.

I would like to congratulate the editor and all the chapter contributors for their voluminous efforts in the meticulous drafting of each protocol and for sharing their knowledge with the wider scientific community and food industries. I am sure that with this book, the readers are getting updated information regarding the postbiotics, their health benefits, and protocols adapted to different fields of application.

*Vitoria, Spain* — *María Chávarri Hueda*

# Preface to the Series

*Methods and Protocols in Food Science* series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated, and used by food and related scientists as well as a theoretical basis is provided for each protocol. Aspects related to improvements in protocols, adaptations, and further developments in the protocols may also be approached.

*Methods and Protocols in Food Science* series aims to bring the most recent developments in research protocols in the field as well as very well-established methods. As such, the series targets undergraduates, graduates, and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such a way that the readers will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by comprehensive and step-by-step procedures on how to perform that experiment. The next section brings the dos and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions addressed.

The *Methods and Protocols in Food Science* series will fill an important gap, addressing a common complaint of food scientists, regarding difficulties in repeating experiments detailed in scientific papers. With this, the series has a potential to become a reference material in food science laboratories of research centers and universities throughout the world.

***Campinas, Brazil*** ***Anderson S. Sant'Ana***

# Preface

The book entitled *Postbiotics* is a manual that illustrates the techniques involved in the preparations of postbiotics from probiotic microorganisms like *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Saccharomyces*, and other groups of microbial flora in fermented food. This manual covers a wide range of postbiotics types and biological properties; isolation and identification of probiotics microorganisms; propagation of lactic acid bacteria (LAB); harvesting of postbiotics; next-generation sequence analysis of postbiotics in fermented food; postbiotics used in livestock feeding; antimicrobial, anticancer, antioxidant, and anti-inflamatory effects of postbiotics; biopreservation of dairy products using postbiotics; postbiotics in food packing; and other applications in functional food and neutraceuticals preparations.

The most relevant target audiences belong to the degree in BSc, MSc, PhD of Microbiology, Food Technology, Food Biotechnology, Food Science and Technology, Botany and Zoology, Microbial Biotechnology, Pharmacology, and Agriculture. The secondary audiences are the targeted readers of this manual. They include faculty members, researchers, and food industrial people who can use the protocol for standardizing and producing postbiotics on commercial basis.

The book consists of five broad sections under which different titles are prepared based on the total content of 56 protocols. It includes the main division of preparations; separations; identification; analysis of postbiotics types and mechanism; and uses of postbiotics in health, pharma, aquacultures, and food industry. This edited protocol is contributed by authorities from countries such as Canada, India, Spain, and Turkey. This manual help all researchers interested to work on preparation, chemical characterization of postbiotics, harvesting of postbiotics, postbiotics metabolites are antimicrobials, antibiofilm, anti-inflammatory, antiallergies, antiobesity, meurotransmitter activity, determining postbiotics concentration, dietary supplementation and immunomodulatory activity of postbiotics in fish, oyster, crab, shrimp, chicks, goat, and pigs Postbiotics as biopreservatives of meat, fish, dairy products, vegetables and fruits, as well as food packing material, biodegradation of chemical contaminants including pesticides and mycotoxins, preservation of postbiotics.

I am extremely thankful to all the authors who contributes chapters and for their prompt and timely responses. I extend my earnest appreciation to Ms. Monica Suchy, Springer Nature and their team for their constant encouragement and help in bringing out the volume in the present form. I am also indebted to Springer Nature and the authorities of Bharathidasan University, Tiruchirappalli, Tamil Nadu, India; and sincerely thank the INSA Visiting Scientist Fellowship, Indian National Science Academy, New Delhi (INSA/SP/VSP-24/2022–23/ 12th May 2022), for their support in the task of publishing book.

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# Part I

## Preparation of Postbiotics

# Chapter 1

# Isolation and Identification of Probiotics Microorganisms

**Gopikrishnan Venugopal, Radhakrishnan Manikkam, Kaari Manigundan, S. Usha Nandhini, Tamil Selvam Saravanan, Bavanilatha Muthiah, and Jestin Sheeba**

## Abstract

Probiotics are defined as nonpathogenic live microorganisms that, when administered in adequate amounts, confer health benefits on the host. Association of probiotics with human beings has a lot of history. Well known as "health-friendly bacteria," they are widely used commercially as a functional food. The popularity of probiotics has gone exponentially high due to an increasing number of clinical trials, supporting their beneficial effects. Several in vivo and in vitro experimental evidence supports strain-specific and disease-specific probiotic efficacy to prevent and ameliorate antibiotic-associated diarrhea, traveler's diarrhea, ulcerative colitis, and many more. Besides, numerous recent studies have reported that probiotics could have a significant effect in alleviating various metabolic-, lifestyle-, and diet-related disorders like obesity, type 2 diabetes, metabolic syndrome, irritable bowel syndrome. Strains of *Bifidobacterium, Lactobacillus, and Saccharomyces boulardii* are the most commonly used as probiotics. Safety, efficacy, pathogenicity, infectivity, intrinsic property, virulence factors are to be addressed during probiotic selection. The underlying mechanisms of probiotics effects are still not fully elucidated and have been under intensive research. Numerous diverse, strain-specific probiotic mechanisms have been proposed, which include early colonization of perturbed microbes, competitive exclusion of pathogens, short-chain volatile fatty acid production, alteration of gut pH, immunomodulation, and many more. Considering the remarkable influence on human health, probiotics seem to be alluring attractive agents to promote human health conditions and to improve the quality of life against several diseases. This chapter gives a protocol to isolate, identify, and screen the probiotics microbes.

**Key words** Microbes, Health, *Lactobacillus*, Microorganism, Probiotics, *Saccharomyces*

## 1 Introduction

Probiotic microorganisms are beneficial bacteria and yeast that live in the human gut and contribute to overall health and well-being. In order to use these microorganisms as dietary supplements or in food products, it is necessary to first isolate and identify them. Isolation of probiotic microorganisms can be done using various methods, such as culture-dependent techniques or culture-

Dhanasekaran Dharumadurai (ed.), *Postbiotics*, Methods and Protocols in Food Science,
https://doi.org/10.1007/978-1-0716-3421-9_1,

independent methods. Once the microorganisms are isolated, they can be identified using various methods such as microscopy, physiological and biochemical tests, and molecular biology techniques. For example, 16S rRNA gene sequencing can be used to identify bacteria at the genus and species level. In summary, probiotic microorganisms can be isolated and identified using a variety of techniques, but it is crucial to thoroughly evaluate the health benefits before incorporating them into supplements or food products.

According to recent research, probiotics play an essential role in the treatment of diseases like diarrhea, allergic reaction, type 2 diabetes, high blood pressure, cancer, genetic conditions, as well as enhance immunity. It is crucial for the probiotic microorganism to be acid-resistant, bile-tolerant, noncarcinogenic, and nonpathogenic. It should also be able to adhere to the host epithelial tissue, enrich the intestinal microflora, reduce pathogenic adherence, and produce secondary metabolites that are antagonistic to pathogen microorganisms. The beneficial microorganism interacts with the raw food (substrate) and produces enzymes and vitamins, a valuable fermentation product with medical benefits. Additionally, probiotic microbes have a protective effect on the gut environment when consumed. It should be noted that probiotic effects vary by strain, so not all strains are effective for efficient fermentation or disease treatment.

Irradiation, immunosuppressive treatment, and excessive antibiotic use may alter the composition of the gut and harm the gut microbiota. As a result, introducing probiotic bacteria into the gastrointestinal tract has recently emerged as a useful method for establishing a healthy microbial equilibrium. The most common probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria, the genus *Bacillus*, or the genera *Pseudomonas* and *Burkholderia* [1–3]. The US Food and Drug Administration is researching a method for administering live microorganisms in animal and human feeds, which is referred to as "direct-fed microbial." Despite the fact that microbial natural products secrete a number of extracellular enzymes that break down organic matter and are excellent producers of antimicrobial secondary metabolites, these microorganisms have been overlooked as protective agents in aquaculture farming. Because they produce compounds with potential bioactivity against fish and shellfish pathogens, microbial strains could be promising probiotics for aquaculture [4–6].

## 2 Materials Required

1. Cotton swabs.
2. Sterile tips.
3. Sterile flask.
4. Sterile sea water.
5. Pipettes.
6. Well puncher.
7. Sterile disc.
8. Starch casein agar.
9. TCBS agar plates.
10. MHA plates.
11. NA plates.
12. ISP2.
13. Phosphate buffer saline.
14. Xylene.
15. Congo red.
16. Blood plate agar.
17. AMM plates containing filter sterilized cycloheximide and nalidixic acid.
18. Tween 20 agar media.
19. TBS.
20. NA + tryptone soy supplements with skimmed milk $HgCl_2$.
21. Marine agar plates (2% starch).
22. Humic acid vitamin agar.
23. Lugol solution.
24. Test organism.
25. *Vibrio harveyi.*
26. *Vibrio parahaemolyticus.*
27. *Vibrio alginolytians.*
28. *Vibrio vulnificus.*
29. *S. aureus* ATCC 25923.
30. *Salmonella enterica* ATCC 14028.
31. *P. aeruginosa* ATCC 27853.
32. *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 33591.
33. *Escherichia coli* (*E. coli*) ATCC 25922.
34. *S. mutans* ATCC 35668.

35. *Listeria monocytogenes* ATCC 13932.
36. *Bacillus cereus* ATCC 11778.
37. *Enterococcus faecalis* ATCC 29212.
38. *Candida albicans* ATCC 10231.
39. *Vibrio alginolyticus* (CAIM 57).
40. *Vibrio harveyi* (CAIM 1793).
41. *Vibrio vulnificus* (CAIM 157).
42. *Vibrio parahaemolyticus* (ATCC 17802).

## 3 Procedure

### 3.1 Sample Collection and Isolation of Bacteria

1. Milk samples are collected in sterile containers.
2. The samples are immediately transported to the laboratory and are stored at 4 °C and processed within 72 h post-collection.
3. 1 mL of each sample is suspended in 9 mL of sterile seawater by vortexing and is incubated for 6 min in a water bath at 55 °C and it is serially diluted tenfold (to 10–5).
4. Aliquots (100 μL) are spread onto (MRS) agar plates, containing filter-sterilized cycloheximide (100 μg/mL) and nalidixic acid (30 μg/mL).
5. The inoculated plates are incubated at 28 °C for 28 days [3].
6. The resulting colonies show different morphologies with a slimy, tough, or powdery texture and a dry or folded appearance.
7. The colonies adhere to the agar surface and have branching filaments with or without aerial mycelia.
8. These isolated colonies (pure cultures) are picked and maintained at 4 °C on NA slants and at −20 °C in 20% glycerol for further studies.

### 3.2 Biochemical and Morphological Characterization

1. Morphological characterization is carried out using the Gram staining technique.
2. Biochemical characterization is performed using the catalase test and analysis of carbohydrate fermentation profiles.
3. All catalase-negative and Gram-positive bacilli or cocci, the morphology of which is similar to LAB bacteria are classified as potential probiotic strains (Bergey's Manual, 1994).

### 3.3 Analysis of Antimicrobial Activity

1. The pathogenic strains *S. aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, *P. aeruginosa* ATCC 27853, *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 33591, *Escherichia coli* (*E. coli*) ATCC 25922, *S. mutans* ATCC

35668, *Listeria monocytogenes* ATCC 13932, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231,*Vibrio alginolyticus* (CAIM 57), *Vibrio harveyi* (CAIM 1793), *Vibrio vulnificus* (CAIM 157), and *Vibrio parahaemolyticus* (ATCC 17802), obtained from the concern labs, are selected for antagonism assay using the well diffusion method [3].

2. Briefly, bacterial isolates cultured in nutrient broth at 37 °C for 24–48 h are centrifuged for 10 min at 10,000 rpm, and the resulting supernatants are then separated and used.
3. The test strains are inoculated on nutrient broth for 24 h.
4. Test strain's suspensions are prepared in optical density (OD) at 625 nm is adjusted to 0.08–0.1.
5. Cotton swabs from the test strain suspensions are spread on the surface of Mueller Hinton agar (MHA) plates.
6. Wells are made using a sterile cork borer. The wells are loaded with aliquots of 60 μL of the supernatant and incubated at 37 °C for 24 h [4].
7. The antimicrobial activity is monitored by measuring the diameter of the zone of inhibition (mm) around the wells after 24 h incubation at 37 °C [3, 4].

### 3.4 Analysis of Hemolytic Activity

1. The selected cultures are streaked on blood agar plates containing 5% human blood and 2.5% sodium chloride (NaCl).
2. The plates are incubated for 7 days at 30 °C.
3. Three types of hemolytic activity are examined: α (partial), ß (total), or γ (no hemolysis), using the ß-hemolytic strain *V. parahaemolyticus* as control.
4. All the selected strains showing γ hemolysis patterns are used for further studies [5].

### 3.5 Hydrophobicity Analysis

1. Hydrophobicity is examined using the Congo red method and the bacterial adherence to hydrocarbons (BATH) test.
2. Selected strains are streaked on TSA plates containing 1% sodium chloride and 0.03% Congo red, and the plates are incubated at 30 °C for 7 days.
3. Strains with a reddish color are considered positive for the test, whereas strains with a translucent to white color are considered negative [6].
4. The BATH test is performed by measuring the cellular affinity for organic solvents.
5. Briefly, the strains are grown in tryptic soy broth (TSB) at 30 °C for 7 days under shaking conditions.

6. The cells are harvested by centrifugation and then washed three times with phosphate-buffered saline (PBS).
7. The OD of the cells at 540 nm is adjusted to 0.8 in PBS.
8. Subsequently, 3 mL of each cell suspension is mixed with 1 mL of xylene and vortexed for 30 s at room temperature.
9. After 30 min, the OD of the aqueous phase at 540 nm is measured.
10. The percent hydrophobicity is calculated with the formula:

$$\text{Rate of hydrophobicity } (\%) = \text{OD initial–OD final/OD initial} \times 100$$

### 3.6 Sodium Chloride and Acidic pH Tolerance

1. Starch casein agar plates containing different sodium chloride concentrations (0%, 0.4%, 0.6%, 2%, 3% and 10%) are used to analyze the sodium chloride tolerance of the selected strains.
2. Tolerance to acidic pH is examined by growing the strains in TSB at a pH of 1, 2, 3, 4 and 7.2.
3. The isolates are seeded on agar and broth medium, incubated at 37 °C for 7–15 days, and the presence or absence of growth is recorded on the seventh day onwards [7].

### 3.7 Simulated Gastric Juice Tolerance Test

1. Simulated gastric juice is prepared using 3 g/L pepsin, 7 mM KCl, 45 mM NaHCO3, and 125 mM NaCl, adjusting at pH 3 (assay) and pH 7 (control) with 1 M HCl and 1 M NaOH, respectively [8].
2. Overnight grown bacterial broth culture is taken and centrifuged at 5000 rpm at 5 °C for 15 min. The bacterial pellet is resuspended in 10 mL PBS buffer, followed by incubation in simulated gastric juice (both assay and control).
3. The viable cell counts at 1, 2, and 3 h are recorded.
4. The survival percentage of the isolate is determined by the following formula:

$$\text{Bacterial survival rate } (\%) = \text{CFU assay CFU control} \times 100$$

### 3.8 Bile Tolerance Test

1. One hundred microliters of overnight grown bacterial culture is inoculated in freshly prepared MRS broth containing 0.3% bile salts (HiMedia Pvt. Ltd).
2. Test isolates are also inoculated in MRS broth without bile, which acts as a control.
3. Both the test tubes (with and without bile) containing test isolates are incubated at 37 °C for 4 h, and their growth at a different time interval and percentage resistance is noted by measuring the absorbance of MRS broth at 600 nm.

4. After the incubation period of 4 h, the viability of bacteria in 0.3% bile is also evaluated by spreading 100 μL of the bacterial sample onto the MRS agar plate [8].

### 3.9 Cellular Auto-Aggregation Assay

1. The overnight grown bacterial broth is centrifuged at 5000 rpm for 10 min in order to harvest the cell pellets.
2. Pellets are repeatedly washed with PBS (pH 7.2), resuspended in PBS buffer, and the initial absorbance is noted at 600 nm.
3. The bacterial suspension is incubated at 37 °C for 2 h, and the final absorbance of the supernatant is measured at 600 nm [9].
4. The percentage of cellular auto-aggregation is measured by the formula:

$$\text{Auto} - \text{aggregation rate } (\%) = \text{OD initial–OD final/OD initial} \times 100$$

### 3.10 Glucose Fermentation Test

1. Eighteen-hour-old bacterial cultures are centrifuged at 4000 rpm for 15 min, and the bacterial pellets are recovered.
2. The pellets are washed twice by PBS buffer and resuspended in the PBS buffer.
3. Thereafter, 500 μL of PBS buffer containing the bacterial cells are inoculated into MRS broth supplemented by 1% glucose and 0.5% phenol red (dye) and incubated at 37 °C for 24 h [10].

### 3.11 Pancreatin Tolerance Test

1. One hundred microliters of overnight grown bacterial culture is inoculated in 10 mL of MRS broth containing 0.5% (v/w) pancreatin and without pancreatin (control).
2. Inoculated test tubes are kept in a shaker incubator for 48 h at 37 °C.
3. Pancreatin tolerance is determined by measuring the OD (at 600 nm) at an interval of 0, 24, and 48 h.
4. The viable cell count of test and control cultures in MRS agar plates after 48 h of incubation is also determined [8].

### 3.12 Enzymatic Activity

1. Different substrates are used to determine the ability of the selected strains to hydrolyze macromolecular polymers such as proteins, lipids, and carbohydrates.
2. Salt is added to each culture medium at a concentration of 3% when testing enzymatic activities [11].

#### 3.12.1 Starch Hydrolysis

1. Amylolytic activity is determined by the radial diffusion method using marine agar plates supplemented with 2% starch.
2. Plates seeded with the test organisms are incubated at 30 °C for 4–5 days [11, 12].

#### 3.12.2 Tween 80 Hydrolysis

1. Lipolytic activity is examined by seeding the test organisms on tween-agar media [containing 1% Tween 80].
2. After incubation at 30 °C for 4–5 days, the tween 80-hydrolysing strains forms a precipitation halo around their colonies because of the combination of released fatty acids and Ca2+ ions [11, 12].

#### 3.12.3 Protein Hydrolysis

1. Tryptone soy and nutrient agar supplemented with skim milk (1%) and gelatin (0.4%) are used to determine the protein hydrolysis capacity of selected strains.
2. To measure casein hydrolysis, the diameter of clear halos around colonies on skim milk plates is measured after incubation at 30 °C for 4–5 days.
3. Qualitative gelatin hydrolysis is monitored by flooding plates with a $HgCl_2$ solution ($HgCl_2$ 15 g, HCl 20 mL and distilled $H_2O$ 100 mL) after incubation at 30 °C for 4–5 days [11, 12].

#### 3.12.4 Cellulose Hydrolysis

1. NA agar plates supplemented with 1% carboxy methyl cellulose (CMC) are used to measure the cellulolytic activity of the test organisms.
2. Diameters of clear halos around the colonies are measured after incubation at 30 °C for 4–5 days and addition of a lugol solution [13, 14].

## Acknowledgements

Authors thank the management of Sathyabama Institute of Science and Technology, Tamil Nadu, and Department of Biotechnology, New Delhi, for their support in the form of research grant (BT/PR41474/NDB/39/760/2020 dated 28.09.2021).

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# Chapter 2

# Isolation and Identification of Lactic Acid Bacteria

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## Abstract

Lactic acid bacteria (LAB) comprise a wide range of genera. LAB have been isolated from various sources such as raw and fermented foods, human and animal intestinal tracts, and mucus membranes. LAB also play an important role as a probiotic culture, usually belonging to *Lactobacillus*, *Enterococcus*, and *Bifidobacteria* genera. This chapter summarizes the isolation and identification steps widely used in LAB strains.

**Key words** Lactic acid bacteria, Isolation, Identification, Protocol

## 1 Introduction

Lactic acid bacteria (LAB) cover a large group of microbial groups. The first pure culture of LAB was obtained in 1873. However, the similarity between milk-souring bacteria and isolation of lactic acid-producing bacteria from other habitats was identified in the early 1900s. The basis of the systematic classification of LAB was also elaborated and published in 1919 by Orla-Jensen. LABS play a major role in agriculture, food, and clinical sectors [1]. LABs have been used in various alcoholic and food fermentations for 6000 years [2]. LABs are Gram-positive, non-spore-forming rods, cocci, and coccobacilli, non-respiring but aero-tolerant, fastidious, acid-tolerant and catalase-negative devoid of cytochromes. This group produces lactic acid as one of the key fermentation products by utilizing carbohydrates during fermentation. These bacteria also produce organic substances that contribute to flavor, texture, and aroma, thus aiding the unique organoleptic characteristics of the products [3].

In recent taxonomic classification, LAB comes under the phylum of *Firmicutes*, class *Bacilli*, and order *Lactobacillales* [2]. LAB has been classified into different genera/species based on their acid production properties by fermenting sugars and their growth at specific temperatures. Moreover, LAB can be classified as

Dhanasekaran Dharumadurai (ed.), *Postbiotics*, Methods and Protocols in Food Science,
https://doi.org/10.1007/978-1-0716-3421-9_2,

homofermentative or heterofermentative organisms based on their ability to ferment carbohydrates. The homofermentative LAB such as *Lactococcus* and *Streptococcus* yields two molecules of lactates from one glucose molecule, while heterofermentative LAB such as *Leuconostoc, Wiessella*, and some lactobacilli produce lactate, ethanol, and carbon dioxide from one molecule of glucose. The conventional approach of LAB classification has relied on physiological and biochemical characteristics, but molecular characterization has become an important tool for classifying and identifying LAB [1]. The major genera of LAB include *Lactobacillus, Lactococcus, Weissella, Pediococcus, Enterococcus, Streptococcus, Melissococcus, Lactosphaera, Leuconostoc, Carnobacterium, Oenococcus, Vagococcus,* and *Tetragenococcus*. Other genera include *Alloiococcus, Dolosigranulum, Globicatella, Aerococcus, Microbacterium, Propionibacterium*, and *Bifidobacterium*. However, this group's largest genus is Lactobacillus, which consists of more than 80 recognized species. LABs are typically isolated from decayed plants and animal matter, faecal substances, in numerous raw materials. These materials are used to produce fermented foods like milk, meat, and flour and even in the gut of herbivorous animals and humans as a symbiotic [2]. LAB is widespread in niches of dairy, meat and vegetable origin, the gastrointestinal and urogenital tracts of humans and animals, and soil and also water. The mammalian intestine is a repository of 100 trillion microorganisms and thus generally called microbiota [1].

## 2 Materials

All buffers, medium, and solutions must be prepared using sterilized, deionized, or ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. All reagents should be stored at room temperature (25 °C) and 4 °C. If you store them at 4 °C, you must wait for their warming up to 25 °C before use.

## 3 Methods

### 3.1 Isolation and Identification of LAB

Isolation and identification protocol of LAB are below [4–6]:

1. Take 10 mL (or 10 g) of samples aseptically and then homogenize them with 90 mL of sterile physiological NaCl solution (0.85%) or buffered peptone water.
   (a) Dissolve 8.5 g NaCl in 1000 mL water (*see* **step 2**). Autoclave 15 min at 121 °C. Cool to room temperature.