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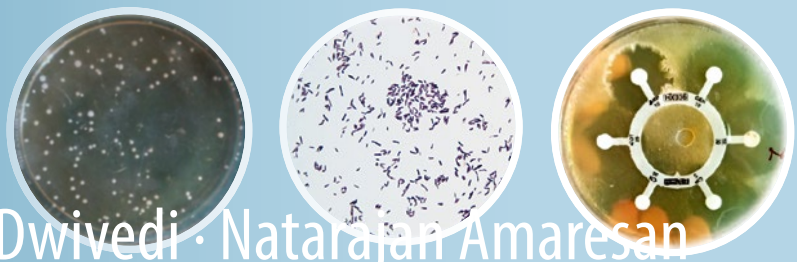

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A. Sankarnarayanan
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Biosafety Assessment of Probiotic Potential

Humana Press

Methods and Protocols in Food Science

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Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

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Biosafety Assessment of Probiotic Potential

Edited by

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ISSN 2662-950X ISSN 2662-9518 (electronic)
Methods and Protocols in Food Science
ISBN 978-1-0716-2508-8 ISBN 978-1-0716-2509-5 (eBook)
https://doi.org/10.1007/978-1-0716-2509-5

This Humana imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

Foreword

Probiotics have recently become an attractive term in modern science. *Lactobacillus* spp. are the most widely used probiotic bacteria. They colonize and prevent the colonization of pathogenic bacteria and provide health benefits to the host. Microbes derived from probiotics have been extensively studied for their various health benefits and potential applications in the control of various diseases. The beneficial effects of probiotics have been well documented.

However, research reports are also concerned about the use of probiotics among patients with immunosuppression, organ failure, and dysfunctional gut barrier, which can cause severe infections and other negative impacts on the host. In addition, recent research has insisted on the need for various *in vitro* and *in vivo* approaches before administering the probiotics to any host. The gut-microbial complex association, the triggering of various chronic diseases challenging the medical field, is also taken into account before the use of probiotics in a host for their beneficial activities.

In the light of the above, this book on *Biosafety Assessment of Probiotic Potential* edited by Dr. Mitesh Kumar Dwivedi, Dr. N. Amaresan, Dr. A. Sankaranarayanan, and Prof. Rasheedunnisa Begum is a timely needed protocol book. I would like to congratulate the editors and contributors for their strenuous efforts to bring the much-needed book for the wider benefit of the scientific community working in the said area. I hope the reader will get maximum benefit from this book.

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Preface to the Series

The *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 theoretical bases are provided for each protocol. Aspects related to improvements, adaptations, and further developments in the protocols may also be approached.

The *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 reader 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 the 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

There is an increasing trend in the use of probiotics as nutritional supplements and food ingredients that promote health and fight against various diseases worldwide. Researchers are pouring innovative ideas on probiotic-based research and reporting their benefits to humanity. Probiotics are sprawling their wings by their diverse applications as immune modulators, antioxidant agents, and anticarcinogenic agents, increasing the nutrient absorbing ability of host and as an ailment for various diseases. Although probiotic organisms have a wide history of safe usage, however, recent reviews and research reports have raised alarming concerns regarding the safety issues of probiotic usage. In summary, the adverse effects/negative lineages of probiotic usage include the production of harmful metabolites, excess immune stimulation, dysfunctional gut barrier, transfer of multidrug-resistant genes, and risk in immunocompromised hosts which have been seriously considered at this juncture.

In this context, our book entitled *Biosafety Assessment of Probiotic Potential* provides elaborate procedures for assessing the biosafety aspects of probiotics. This book comprises 39 protocols under two major sections dealing with *in vitro* biosafety assessment and *in vivo* biosafety assessment of probiotics. The *in vitro* biosafety assessment of probiotics includes various tests, including determination of biogenic amine production, D-lactic acid production, toxin production, production of various enzymes (e.g., gelatinases, glycosidases, enolase, β-glucuronidase, nitroreductase, and azoreductase), determination of toxicity, mutagenicity, presence of virulence genes, capsule formation, hemolytic activity, DNAse activity, bile salt deconjugation, antibiotic resistance and antibiotic resistance gene transfer, mucin degradation, platelet aggregation, and fibrinogen and fibronectin binding activity. The second section deals with the *in vivo* biosafety assessment of probiotics, which includes different tests and assays to monitor the *in vivo* toxicity of probiotics, assessment of infectivity in animal models, determination of reproductive and developmental toxicity in animal models, as well as evaluation of immunological parameters in animal models. These protocols are contributed by reputed senior scientists, researchers, and academicians working in these areas.

We hope that the protocols presented in this book will be informative to the reader. The detailed procedures mentioned in this book are similar to a pristine stream and will be helpful to scientists, researchers, Ph.D. scholars, and postgraduate students working in this area. We express our sincere gratitude to all the authors for their excellent contributions. We are also indebted to the publishers for their efforts to publish the book in a timely manner. Criticisms and concrete suggestions are always welcome for the improvement of this book.

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Surat, Gujarat, India — ***Natarajan Amaresan***
Kalaburagi, Karnataka, India — ***A. Sankaranarayanan***
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February 2022

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Part I

In Vitro Biosafety Assessment of Probiotics

Chapter 1

Determination of Biogenic Amine Production

Shilpika Pandey, Archana Chaudhari, and Mitesh Kumar Dwivedi

Abstract

Probiotics are live microorganisms that provide a variety of health advantages but also have the potential to be toxic. Biogenic amines are one of the harmful metabolites produced by probiotics, hence determining the safety of probiotics prior to intake is critical. We focused on qualitative and quantitative in vitro approaches for assessing biogenic amines generation by possible probiotics, as well as potential probiotic species identification utilizing 16S rRNA sequencing and whole genome sequencing.

Key words Biogenic amines (BA), Toxicological effects, Decarboxylase media (DCM), HPLC, 16S rRNA gene, whole genome sequencing

1 Introduction

A probiotic is defined as "a mono- or mixed culture of live microorganisms which, applied to man or animal (e.g., as dried cells or as a fermented product), affects beneficially the host by improving the properties of the indigenous microflora" [1]. Biogenic amines (BAs) are low molecular weight compounds released by probiotic microorganisms that are implicated in various biological activities. The food containing higher amount of BA causes human ailments leading to vomiting, hypertension, palpitations, and headache [2]. BAs can be found naturally in foods such milk, fruits, vegetables, meat, and fish, as well as synthesized by decarboxylase or deiminase activity of some probiotics [3, 4].

BAs are produced by variety of microorganisms such as Lactic acid bacteria (LAB), *Enterobacteriaceae* spp., *Enterococci* spp., *Pseudomonas* spp. [5–8]. The most harmful BAs are histamine and tyramine, which have been linked to "scombroid fish poisoning" and "cheese reaction," respectively [9, 10]. Consumption of fish such as mackerel, sardines, tuna, and others cause "scombroid fish

Shilpika Pandey and Archana Chaudhari contributed equally to this work.

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_1,

poisoning," which leads to flushing of the face, neck, upper arm, hives, headache, difficulty swallowing, heart palpitations, and other symptoms [11]. Cheese consumption is linked to "cheese reaction," which causes dietary-induced migraine, nausea, vomiting, elevated blood glucose, pulmonary, and cardiac problems [10, 12].BAs are also recognized to be carcinogenic nitrosamine precursors [13]. Apart from its toxicological effects, the amino acids catabolism by probiotics may affect safety and quality of foods [8, 14]. As a result, probiotics should not produce large amount of BA [2]. Study by Kurkutia et al., (2019) [15] suggested that the probiotic isolates derived from human breast milk (SP_1& B_2Enr) can be regarded as safe because they did not produce BA when subjected to different amino acids precursors.

The various techniques used to assess BA released by microorganisms are discussed in this chapter, including the qualitative decarboxylase media (DCM) plate and broth method, quantitative high-performance liquid chromatography (HPLC) method, molecular identification of microbial gene-producing BA, along with 16S rRNA gene sequencing and whole genome analysis.

2 Materials

Use distilled water and analytical grade chemicals to make all solutions and reagents. Keep all the prepared buffers/reagents at room temperature (unless indicated otherwise). Follow all trash disposal regulations when disposing of waste materials.

2.1 Media Composition

1. DeMan, Rogosa, and Sharpe (MRS)-broth composition (Sigma-Aldrich):10 g peptone, 8 g meat extract, 4 g yeast extract, 20 g D(+)-glucose, 2 g triammonium citrate, 5 g sodium acetate trihydrate, 2 g dipotassium hydrogen phosphate, 0.2 g magnesium sulfate heptahydrate, 0.05 g manganous sulfate tetrahydrate, 1 mL Tween 80 in 1 L of distilled water(in case using MRS-broth powder, add 51 g of the powder and 1 mL Tween 80 in 1 L of water). Adjust pH of the MRS-broth as 6.2 ± 0.2 at 25 °C.
2. MRS-agar composition(Sigma-Aldrich): 10 g peptone, 5 g meat extract, 5 g yeast extract, 20 g D(+)-glucose, 2 g diammonium hydrogen citrate, 5 g sodium acetate, 2 g dipotassium hydrogen phosphate, 0.1 g magnesium sulfate, 0.05 g manganous sulfate, 12 g agar and 1 mL Tween 80 in 1 L water. If using MRS-agar powder, add 61.15 g of the powder and 1 mL of Tween 80 in 1 L of water [To completely dissolve the powder components into water, heat MRS-broth/agar, stirring often. Autoclave at 121 °C for 15 min and store the media at 2–8 °C].

2.2 Decarboxylase Media(DCM) Plate and Broth Method of Screening Biogenic Amines

1. MRS-broth(modified) [6, 15]: MRS-broth (Sigma-Aldrich), 1 mL Tween 80, 0.2% final concentration of amino acids such as histidine, tryptophan, arginine, phenylalanine, and lysine. Autoclave the media at 121 °C for 15 min and store the media at 2–8 °C until use.
2. Decarboxylase media (DCM, modified) [6] based on Joosten and Northold (1989) media [16]: 0.5% tryptone, 0.5% yeast extract, 0.5% meat extract, 0.25% sodium chloride, 0.05% glucose, 0.1% Tween 80, 0.02% $MgSO_4$, 0.005% $MnSO_4$, 0.004% $FeSO_4$, 0.2% ammonium citrate, 0.001% thiamine, 0.2% K_2PO_4, 0.01% $CaCO_3$, 0.005% pyridoxal-5-phosphate, 1.0% amino acids, 0.006% bromocresol purple, 2% agar, pH 5.3. Autoclave the media at 121 °C for 10 min to avoid excessive hydrolysis of agar at low pH.
3. Sterile petri dish (90 mm).
4. Bacterial strain (s).
5. Disposable loop.
6. Incubator.

2.3 High-Performance Liquid Chromatography (HPLC)Method of Screening Biogenic Amines

1. Stock solution of BA: Prepare 7 mg/mL of cadaverine dihydrochloride (≥98%), putrescine dihydrochloride (≥98%), spermidine trihydrochloride (≥97%), spermine tetrahydrochloride (≥97%), tyramine (≥99%) in 0.1 M HCl. Store in glass bottle at 4 °C away from light. Use freshly prepared stock and working solutions for the experiment.
2. 0.6 M Perchloric acid ($HClO_4$): Add 65.2 mL of $HClO_4$ (60%) in 1000 mL of volumetric flask and fill with water.
3. 2 M NaOH: Dissolve 8 g of NaOH pellets in 100 mL distilled water. Store at room temperature.
4. Chemicals: NaCl, N-butanol, benzoyl chloride (≥98%), diethyl ether, acetonitrile (HPLC grade).
5. Liquid nitrogen.
6. C_{18} column.
7. RP-HPLC-DAD system.
8. Vortex mixer.
9. Benchtop centrifuge.
10. Whatman filter paper grade 1.
11. Sterile 1.5/2.0 mL microfuge tubes.
12. Micro-pipettors (20 μL, 200 μL, and 1 mL).
13. Incubator.
14. Millipore Milli-Q water system.
15. Ultrasonic bath for degassing acetonitrile and ultra-pure water.

2.4 Isolation of Genomic DNA

1. Bacterial sample.
2. MRS-broth.
3. Nuclease-free 1.5 mL microcentrifuge tubes.
4. Lysozyme (10 mg/mL): In a sterile microfuge tube, place 10 mg of lyophilized lysozyme powder and 1 mL of 10 mM Tris–HCl (pH 8.0). Alternatively, add 10 mg lysozyme powder in 1 mL of NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0 [used in this study]). For efficient mixing, pipette it several times. Dispense into aliquots and keep at −20 °C.
5. RNaseA (10 mg/mL): Dissolves 100 mg RNAseA/10 mL of 10 mM Tris–HCl (pH 7.5)/15 mM NaCl. Heat at 100 °C for 15 min, cool slowly at room temperature. Aliquot 10 mg/mL RNaseA and store at −20 °C.
6. 10% SDS: Dissolves 10 g sodium dodecyl sulfate (SDS) in 80 mL distilled water. Dissolve well and make up the volume up to 100 mL. Store the solution at room temperature (stable for 6 months).
7. 20 mg/mL Proteinase K: Dissolve lyophilized powder in 50 mM Tris (pH 8.0), 1.5 mM calcium acetate at concentration of 20 mg/mL. Aliquot the stock solution and store at −20 °C.
8. Sodium acetate (0.3 M, pH 5.2): Add 24.6 g of sodium acetate to 70 mL of MilliQ water in Duran bottle. Keep the bottle on a magnetic stirrer with magnetic flea into the bottle. Slowly add glacial acetic acid to adjust the pH of 5.2. Top up the solution to 100 mL. Filter the solution with 0.22 μM filter membrane (optional) and store at room temperature.
9. Tris-EDTA (TE; 1×) buffer: 10 mL of 1 M Tris–HCl (pH 8.0), 2.0 mL of 0.5 M EDTA (pH 8.0) in 988 mL distilled water. Autoclave the solution at 121 °C for 15 min and store at room temperature.
   (a) 1 M Tris–HCl (pH 8.0): 121.14 g of Tris base [tris (hydroxymethyl)aminomethane] in 800 mL distilled water. Adjust pH to 8.0 using HCl and finally make up the volume to 1 L by adding distilled water. Autoclave the solution (121 °C, 15 min) and store at room temperature.
   (b) 0.5 M EDTA (pH 8.0): 186.1 g EDTA in 800 mL distilled water. Using NaOH, adjust the pH to 8.0. Using a magnetic stirrer, vigorously mix the ingredients. Add distilled water to get the final volume 1 L. Sterilize by autoclave and store at room temperature.
10. Tris-Acetate-EDTA (TAE; 10×) buffer: In 800 mL distilled water, add 48.5 g Tris base [tris(hydroxymethyl)-aminomethane], 11.4 mL glacial acetic acid (17.4 M), 20 mL

of 0.5 M EDTA (pH 8.0). Set the buffer capacity to 1 L. Store the buffer at room temperature (no need to autoclave the buffer). To make 100 mL of 0.5× TAE buffer, dilute 5 mL of 10× TAE in 95 mL distilled water.

11. NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0).
12. Tris-saturated phenol (pH 8.0) (Thermo Fisher Scientific).
13. Phenol: Chloroform (1:1).
14. Isopropanol, 70% ethanol.
15. 0.8% agarose gel: 0.8 g agarose powder in 100 mL of 0.5× TAE buffer.
16. Water bath (capable of 55 °C).
17. Benchtop centrifuge.
18. Nanodrop spectrophotometer.
19. Gel imaging system.

2.5 Polymerase Chain Reaction (PCR) of Biogenic Amine Specific Genes

1. Bacterial DNA.
2. Primer pairs: HDC3/HDC4, tdcf/tdcr, odcf/odcr, AGDIf/AGDIR for histidine decarboxylase, tyrosine decarboxylase, ornithine decarboxylase, and agmatine dihydrolase (deiminase), respectively.
3. Thermal cycler.
4. Gel electrophoresis apparatus.
5. Ethidium Bromide solution (EtBr; 10 mg/mL): In 800 mL of distilled water, dissolve 10 g EtBr. Increase the volume of the EtBr solution to 1 L. Using a magnetic stirrer, stir the solution until the dye dissolves. At room temperature, store the EtBr solution in a dark bottle. To prepare 1.5% agarose gel with a final concentration of 0.5 μg/mL EtBr, mix 5 μL of 10 mg/mL EtBr solution in 100 mL of 0.5× TAE buffer.
6. 1.5% agarose gel: 1.5 g agarose dissolved in 100 mL of 0.5× TAE buffer (containing 0.5 μg/mL EtBr).
7. Tris-Acetate-EDTA (TAE; 10×) buffer: In 800 mL of distilled water, combine 48.4 g of Tris base [tris(hydroxymethyl)-aminomethane], 11.4 mL of glacial acetic acid (17.4 M), and 20 mL of 0.5 M EDTA (pH 8.0). Adjust the buffer volume to 1 L. The buffer should be kept at room temperature (autoclave not needed). To make 100 mL of 0.5× TAE buffer, dilute 5 mL of 10× TAE in 95 mL of distilled water.
8. Gel imaging system.

2.6 16S rRNA Gene Sequencing

1. Bacterial sample.
2. Genomic DNA isolation kit.
3. Universal primers for 16S rRNA: 16S_27f (5′-AGAGTTTGATC (A/C)TGGCTCA-3′) and 16S_1525r (5′-AGGAGGTGAT CCAGCC-3′).
4. Thermal cycler.
5. Tris-Acetate-EDTA (TAE; 10×) buffer: Add 48.4 g of Tris base [tris(hydroxymethyl)aminomethane], 11.4 mL of glacial acetic acid (17.4 M), 20 mL of 0.5 M EDTA (pH 8.0) in 800 mL distilled water. Make up the final volume to 1 L. Store the buffer at room temperature, no need for autoclave. To prepare 100 mL of 0.5× TAE buffer, dilute 5 mL of 10× TAE to 95 mL distilled water.
6. Ethidium bromide (EtBr; 10 mg/mL): Dissolve 10 g EtBr in 800 mL distilled water and adjust volume to 1 L. Dissolve the dye with frequent stirring the solution on a magnetic stirrer. Store the EtBr solution in amber bottle at room temperature. To prepare 1% agarose gel (containing 0.5 μg/mL EtBr), add 5 μL of 10 mg/mL EtBr solution in 100 mL 0.5× TAE buffer.
7. 1.0% agarose gel.
8. Gel electrophoresis apparatus.
9. QIAquick PCR Purification Kit.
10. Sanger sequencing device.
11. Basic Local Alignment Search Tool (BLAST).

2.7 Genome Sequencing and Analysis

1. Genomic DNA.
2. Covaris g-TUBE.
3. AMPure XP beads.
4. DNA polymerase and sequencing primers.
5. Sequence single-molecule real-time (SMRT) cell.
6. DNA/Polymerase Binding Kit P6.
7. DNA Sequencing Reagent 2.0 Kit.
8. Nanodrop spectrophotometer.
9. PacBio RS II instrument.
10. Software/databases such as SMRT Analysis v2.3.0, Hierarchical Genome Assembly Process (HGAP) software (v. 3.0), Pilon (v. 1.21), virulence factor database, PHAge Search Tool Enhanced Release (PHASTER) web-program, Genomic Island Prediction Software (GIPSy), antibiotic resistance genes database, ResFinder program and database.

Table 1
Biogenic amines (BA) production by probiotic isolates

Amino acids	Isolate 1	Isolate 2	Isolate 3	*Lactobacillus plantarum*
Histidine	+ve	+ve	+ve	−ve
Arginine	+ve	+ve	−ve	−ve
Phenylalanine	+ve	−ve	+ve	−ve
Tryptophan	+ve	+ve	−ve	−ve
Lysine	+ve	+ve	+ve	−ve

[+ve: BA production; −ve: No BA production]

3 Methods

3.1 Culture of Probiotic Isolates

1. Serially dilute the probiotic culture or the bacterial isolates with peptone water and spread them out on MRS-agar.
2. Incubate the MRS-agar plates in static incubator at 37 °C for 48 h.
3. Process the potential probiotics for further biosafety assessment (in vitro).

3.2 Qualitative Screening of Biogenic Amine Production [6, 15]

1. Subculture LAB strains 5–10 times in MRS-broth (modified) to enhance the enzyme induction for BA determination.
2. Use disposable loop to streak bacterial strain from MRS-broth on to DCM plates with and without amino acids (as control).
3. Incubate the plates at 37 °C for 4 days under aerobic and anaerobic incubation conditions.
4. Appearance for purple color around the colonies show strains positive for BA production.
5. Alternatively, add 0.2 mL of the bacterial suspension in 2 mL of MRS-broth (modified).
6. Incubate the culture for 3 days under aerobic and anaerobic conditions.
7. The observation of color change from purple to yellow and then to purple indicates strains producing BA (Table 1).

3.3 Quantitative Detection of Biogenic Amines Using Reversed Phase-High-Performance Liquid Chromatography-Diode Array Detector (RP-HPLC-DAD) [17]

1. Extract BA from the sample by treating it with 0.6 M perchloric acid for 30 min. Vortex for 1 min after every 10 min to ensure that the sample is thoroughly mixed.
2. Filter the supernatant using Whatman filter paperno.1 after centrifuging the sample mixture at 5500 × *g* for 30 min at 4 °C.
3. Alkalize 2 mL of the filtrate with 2.5 mL of 2 M NaOH, then vortex for 30 s.

4. Remove 1 mL of the supernatant after centrifuging the sample at 5500 × *g* for 15 min at 4 °C.
5. Saturate the supernatant with 4 g NaCl, then add 10 mL n-butanol, vortex for 1 min, and shake for 30 min to purify it.
6. Remove upper organic phase by centrifuging at 5500 × *g* for 5 min at 4 °C.
7. Using a steam of nitrogen, evaporate 1 mL of recovered phase to dryness.
8. Before derivatization, dissolve the residue in 1 mL of 0.6 M perchloric acid.
9. To derivatize the recovered supernatant, add 50 μL of benzoyl chloride and vortex for 1 min. At 30 °C, incubate the mixture for 40 min. Vortex for 1 min after 20 min of incubation interval.
10. Using 2.0 mL diethyl ether, extract BA derivatives twice. Allow the mixture to stand for 10 min after vortexing for 30 s prior to each extraction.
11. Aspirate the ether layer and use nitrogen gas to dry it out.
12. Dissolve the residue in 1 mL acetonitrile.
13. For BA detection, inject 50 μL of prepared sample onto an HPLC system with a C_{18} column (at 40 °C).
14. The mobile phase is made up of two components: (A) water and (B) acetonitrile. Chromatographic conditions include 0–6 min 35–55% B, 6–16 min 55–60% B, 16–24 min 60–90% B, 24–30 min 90–90% B (isocratic step), and 30–40 min 100% B with flow rate of 0.6 mL min^{-1}.
15. Use standard curves to detect and quantify BA at 254 nm based on retention time and peak area, respectively.
16. Using RP-HPLC-DAD, BA (mg. L^{-1}) in yoghurt sample is quantified as tyramine(7–23), cadaverine (2.5–13), spermine (1.5–6.0), putrescine (ND-4), spermidine (ND-5) (where ND is not detected) [17].

    Currently the only BA for which maximum limits have been set in food is histamine because of its toxicological effects. US FDA has set histamine limit to 50 mg/kg for all food [18], above this limit food will be considered spoiled.
17. Calculation for biogenic amines (BA):

    BA is produced by microorganisms and thus can be indicator of hygienic state of food. Biogenic amine index (BAI) first introduced in 1977 [19] to assess quality of fish meat uses formula:

$$\text{BAI} = (\text{histamine} + \text{putrescine} + \text{cadaverine})/(1 + \text{spermidine} + \text{spermine}).$$

Later, understanding of most important BAs (histamine, tyramine, cadaverine, and putrescine) in pork/chicken/tuna, meat, and/or meat product quality leads to new calculation for BAI [20–22].

$$\text{BAI (mg/kg)} = (\text{histamine} + \text{putrescine} + \text{cadaverine} + \text{tyramine})$$

3.4 Genomic DNA Isolation [23]

1. Harvest bacterial cells by centrifuging 2 mL of culture at 2800 × *g*, 4 °C for 5 min.
2. Decant the media and wash the pellet (3 times) with 1 mL of NaCl-EDTA solution.
3. Bacterial pellet is resuspended in 100 μL NaCl-EDTA and 100 μL lysozyme (stock 10 mg/mL in NaCl-EDTA).
4. Incubate the mixture at 37 °C for 30–60 min with intermittent shaking.
5. To remove RNA, add 4 μL of 10 mg/mL RNaseA solution (final concentration 100 μg/mL) to the above mixture before incubation.
6. After the incubation ends, make up the volume of mixture up to 500 μL with NaCl-EDTA and further add 50 μL of 10% SDS and 10 μL proteinase K solution (20 mg/mL).
7. Incubate this mixture at 55 °C for 1 h and after that add equal volume of Tris-saturated phenol (pH 8.0), mix thoroughly.
8. Centrifuge the mixture at 11,200 × *g*, 22 °C for 10 min and separate upper aqueous phase (take care not to disturb interphase containing proteins and cell debris).
9. Repeat this step once with Phenol-Chloroform mixture (1:1) and collect the supernatant in a sterile 1.5 mL microfuge tube.
10. Add 0.8 volumes of Isopropanol and 0.3 M Sodium Acetate (pH 5.2) to the supernatant to precipitate DNA.
11. Centrifuge the mixture at 11,200 × *g*, 4 °C for 5 min.
12. Discard the supernatant and wash the DNA pellet with 70% ethanol and air-dry it.
13. Resuspend the final pellet in 50 μL Tris-EDTA (10:1; pH 8.0) and store at −20 °C until use.
14. Measure the DNA concentration at 260 nm (A_{260}) using a Nanodrop spectrophotometer (Bio-Tek instruments, inc.).
15. Measure the purity of DNA using A_{260}/A_{280} ratio (1.8–2.0) and A_{260}/A_{230} (2.0–2.2).
16. To check the quality of DNA, load 2 μL of DNA onto 0.8% agarose gel and run the gel in 0.5 × Tris-Acetate-EDTA (TAE), pH 8.0.
17. Visualize the DNA band pattern in gel imaging system to indicate quality of isolated DNA.

3.5 Detection of Biogenic Amine Producing Strains

To detect the bacterial strain producing BA, carry out polymerase chain reaction (PCR) for histidine decarboxylase, tyrosine decarboxylase, ornithine decarboxylase, and agmatine dihydrolase (deiminase) genes on thermocycler (Biometra, T1) with the isolated bacterial genomic DNA as the template source [24].

1. Design primers for known BA-producing genes: histidine decarboxylase, HDC3/HDC4 (5′- GATGGTATTGTTTCKT ATGA-3′/ 5′-CCAAACACCAGCATCTTC- 3′), tyrosine decarboxylase,tdcf/tdcr (5′- CAAATGGAAGAAGAAGTT GG-3′/ 5′- GAACCATCAGCA ACAATGTG- 3′), ornithine-decarboxylase,odcf/odcr (5′- TGCA CTTCCATATCCT CCAG-3′/ 5′- GAATTTCTGGAGCAAATC CA-3′), agmatine dihydrolase (deiminase) AGDIf/AGDIR (5′- GAAC-GACTAGCAGCTAGTTAT-3′/ 5′- CCAATAGCCGATACT ACCTTG-3′).
2. Set up the PCR reaction mixture (25 μL) with 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 200 μM dNTP, 1 mM each primer, 1 U DNA polymerase, 2.5 mM $MgCl_2$and 12.5 ng of template DNA.
3. Set the PCR conditions (Initial denaturation (i.d), denaturation (d), annealing (a), extension (e), cycles (c)) for different genes: HDC3/HDC4 (i.d = 95 °C, 30 s, d = 95 °C, 30 s, a = 48 °C, 45 s, e = 72 °C, 2 min, c = 35), tdcf/tdcr (i.d = 95 °C, 30 s, d = 95 °C, 30 s, a = 52 °C, 30 s, e = 72 °C, 2 min, c = 30), odcf/odcr (i.d = 95 °C, 5 min, d = 95 °C, 30 s, a = 55 °C, 30 s, e = 72 °C, 30 s, c = 40) and AGD1f/AGDIR (i.d = 95 °C, 5 min, d = 95 °C, 30 s, a = 55 °C, 30 s, e = 72 °C, 2 min, c = 35).
4. Load 10 μL of the final PCR product on 1.5% agarose gel containing EtBr and run the gel in 0.5 × Tris-Acetate-EDTA (TAE) buffer, pH 8.0.
5. Visualize the amplicon size for histidine decarboxylase, tyrosine decarboxylase, ornithine decarboxylase, agmatine dihydrolase (deiminase) at the expected size of 435 bp, 213 bp, 127 bp, 90 bp, respectively.

3.6 Molecular Identification of Microbial Strain Using 16S rRNA Gene Sequencing [25]

1. Isolate the genomic DNA as described above in the protocol.
2. Quantify DNA using Nanodrop spectrophotometer.
3. Set up PCR reaction for amplification of 16S rDNA gene segment using universal primers 16S_27f (5′-AGAGTTTGATC (A/C)TGGCTCA-3′) and 16S_1525r (5′-AGGAGGTGATC CAGCC-3′).
4. To prepare the PCR reaction mixture (50 μL), add 100 ng bacterial DNA, 10 μL of HF (High Fidelity) buffer, 0.5 μL (1.5 U) Phusion high fidelity DNA polymerase, 2 μL of 2.5 mM dNTP mix and 2 μL of each primer (10 pm/μL) and make up the volume till 50 μL using sterile water.

5. Set up the PCR reaction in a thermocycler (BioRad, USA) with the following conditions: initial denaturation 98 °C for 1 min, denaturation 98 °C for 10 s, annealing 60 °C for 20 s, extension 72 °C for 45 s, final extension 72 °C for 5 min, cycles 30.
6. The amplicon of approximately 1.5 kb is separated on 1% w/v agarose gel.
7. Purify the PCR product using QIAquick PCR Purification Kit and proceed for DNA sequencing.
8. Use BLASTN (Basis Local Alignment Search Tool for Nucleotide) analysis from NCBI (https://www.ncbi.nlm.nih.gov/) to analyze 16S rRNA sequences.

3.7 Whole Genome Sequencing

Sequence the entire genome of bacteria utilizing a PacBio RSII machine and an Illumina platform to improve understanding of the bacterial genome (Macrogen, Seoul, Korea) [26, 27].

1. Briefly harvest bacterial culture and extract genomic DNA using DNA extraction kit (Omega Bio-Tek, USA) following manufacturer's instructions. High quality of DNA ([OD_{260}/OD_{280} 1.8–2.0] and [OD_{260}/OD_{230}2.0–2.2]) is used for whole genome sequencing.
2. Using the Covaris g-TUBE, shear the genomic DNA as per manufacturer's protocol.
3. Create SMRTbell libraries with C4 chemistry with PacBio RS II System (Pacific Biosciences, Menlo Park, CA).
4. Use 0.45× AMPure XP beads for purification of >1.5 kb amplicons and deleting short reads.
5. Quantify DNA using a Qubit Fluorometer (Thermo Fisher Scientific, Wilmington, DE).
6. Add DNA polymerase and sequencing primers (as per manufacturer's instructions) to initiate DNA replication.
7. Load enzyme/template complex and libraries onto zero-mode waveguides with the help of DNA/Polymerase Binding Kit P6 (Pacific Biosciences).
8. Using Pacific Biosciences' DNA Sequencing Reagent 2.0 Kit, sequence single-molecule real-time (SMRT) cell. Use a 120-min sequence capture method and stage start to increase the subread lengths.
9. Filter raw DNA sequence data and exclude SMRTbell adapters, subreads of <1000 bp, short reads <100 bp, reads with <80% accuracy using SMRT Analysis v2.3.0 software.
10. Preassemble the seed reads, produce consensus sequence, correct, and filter the reads to create contigs using Hierarchical Genome Assembly Process (HGAP) software (v. 3.0).

11. To fix errors in constructed contigs use Pilon (v. 1.21).
12. Predict genomic safety using virulence factor database [28], genomic stability using PHAgeSearchTool Enhanced Release (PHASTER) web-based program [29], Genomic Island Prediction Software (GIPSy) program [30], antibiotic resistance genes database [31], ResFinder program and database [32].

4 Inference

In bacterial strains, whole genome sequencing aids in the identification of critical BA genes that encode histidine decarboxylase, tyrosine decarboxylase, ornithine decarboxylase, N-carbamoylputrescine amidase, agmatinase, phenylalanine decarboxylase, and lysine decarboxylase. A strain that lacks the genes that produce BA is considered safe to use as a probiotic. If BA genes are present, calculating the specific BA protein accumulation level can aid in determining the strain's suitability as a probiotic candidate.

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Chapter 2

Determination of Gelatinases, Glycosidases, and Enolase Production

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Abstract

Probiotics confer several health benefits to the host but there are still safety concerns because theoretically they can cause systemic infections, excessive immune stimulation in susceptible individuals. Several in vitro tests are recommended to be performed on candidate microorganisms before they can be confirmed and accepted for use as probiotics. The methods for detection of virulent genes such as enolase, glycosidase, and gelatinases are described here. We also provide methods for determining gelatinases, glycosidases, and enolase production by these candidate probiotics using in vitro studies.

Key words Probiotics, Virulence genes, DNA sequence analysis, Gelatinase, Glycosidase, Enolase

1 Introduction

Consumers are increasingly becoming aware of the impact of diet on health. The International Scientific Association for Probiotics and Prebiotics (ISAPP) defined probiotics as "live microorganisms, that when administered in adequate amounts, confer a health benefit on the host" [1]. Despite health benefits caused by probiotic organisms there are still safety concern because theoretically they can cause systemic infections, excessive immune stimulation in susceptible individuals [2, 3]. Several tests have been recommended that needs to be performed on candidate microorganisms before they can be confirmed and accepted for use as probiotics [4]. A few of them in the list include antibiotic resistance patterns, Glycosidases and Enolase production gelatinase activity, DNase activity, lecithinase activity, and mucin degradation. The importance of these tests generally lies in ensuring efficacy and safety for the consumer [5].

Archana Chaudhari and Shilpika Pandey contributed equally to this work.

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_2,

Gelatinases are extracellular metalloproteinases containing zinc produced by pathogenic bacteria which are capable of degrading extracellular matrix and other membrane bound components [6]. The *gelE* gene responsible for gelatinase production is found on chromosomal DNA [7]. It is a proteolytic enzyme that acts on substrates such as gelatin, collagen, hemoglobin, and endothelial tissue in connective tissue. It provides bacterial migration and spreads by damaging the host tissue [8]. Also, the mucoid lining of the GIT constitutes the target across which several substances are exchanged in the body and gelatinase activity would disrupt it. This would interfere with the normal functioning of the lining and facilitate infections [9]. It is therefore important that candidate probiotic strains do not express gelatinase activity.

Glycosidases are known as a class of enzymes which are capable to efficiently catalyze the hydrolysis of glycosidic linkages [10]. Glycosidases are involved in a variety of important biological processes and applications because of their exquisite catalytic hydrolysis of glycans and the complex and multiple roles of glycans in biology [11]. More importantly, glycosidase enzymes are found to play a role in the pathology of several disease states, and thus considered as important biomarkers for some diseases including cancers [12]. Glycosidase activities are considered detrimental for a probiotic strain, as they might enable the breakdown of human glycoproteins and the synthesis and lysis of human fibrin clots [13]. Some strains of *Lactobacillus* produce these enzymes, suggesting that they may have an infective property in causing endocarditis [14].

Several pathogens including *Aeromonas hydrophila, Bacillus anthracis, Neisseria meningitidis, Staphylococcus aureus, Streptococcus pneumoniae, and Streptococcus pyogenes* [15–19] as well as the fungal pathogen *Paracoccidioides brasiliensis*, the protist *Trichomonas vaginalis*, and the filarial parasite *Onchocerca volvulus*, use an enolase to interact with the host [20–22]. Many of these surface-associated enolases bind plasminogen, facilitating cleavage by plasminogen activators to the proteolytic form, plasmin. Once active, plasmin degrades extracellular matrix proteins, allowing the pathogens to invade deeper into the host tissues [23–25]. Enolase (EnoA1) gene that codes for the glycolytic enzyme enolase is found on chromosomal DNA [26].

Therefore, the determination of gelatinases, glycosidases, and enolase production may be inevitable before considering the use of probiotic bacteria for human health.

2 Materials

To make all solutions and reagents, use analytical grade chemicals and distilled water. Unless otherwise stated, keep reagents at room temperature. When disposing of waste materials, follow the waste disposal guidelines.

2.1 Chemical Composition of Culture Media

1. DeMan, Rogosa, and Sharpe (MRS)-broth composition (BD Difco, Franklin Lakes, NJ, USA:10 g proteose peptone No. 3, 5 g yeast extract, 10 g beef extract, 20 g dextrose, 2 g dipotassium phosphate, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 1 g polysorbate 80 (also known as Tween 80) in 1 L of distilled water (If using MRS-broth powder, combine 55 g of the powder, 1 g polysorbate 80 in 1 L of water). Adjust pH of the MRS-broth as 6.5 ± 0.2.
2. MRS-agar composition: 15 g agar in 1 L MRS-broth (in case using MRS-agar powder, add 70 g of the powder in 1 L of water). [Heat MRS-broth /agar with frequent agitation for 1 min to completely dissolve the powder components into water. Autoclave at 121 °C for 15 min and store the media at 2–8 °C].

2.2 Bacterial Genomic DNA Isolation

1. Bacterial culture.
2. MRS-broth.
3. DNA extraction kit (Omega Bio-Tek, USA) includes HiBind® DNA mini columns, enzymes (RNase A, Proteinase K, Lysozyme), supplied buffers, glass beads S.
4. Nuclease-free 1.5 mL microcentrifuge tubes.
5. Benchtop centrifuge.
6. Vortex mixer.
7. Water bath (capable of 37 °C, 55 °C, 65 °C).
8. 100% ethanol and isopropanol
9. Tris-EDTA (TE; 1×) buffer: Add 1 mL of 1 M Tris–HCl (pH 8.0), 0.2 mL of 0.5 M EDTA (pH 8.0) in 98.8 mL distilled water. Mix thoroughly, autoclave for 15 min at 121 °C, and store at room temperature.
   (a) 1 M Tris–HCl (pH 8.0): 121.14 g Tris base [tris (hydroxymethyl) aminomethane] in 800 mL distilled water. Using HCl, adjust the pH to 8.0. Using distilled water, bring the final volume to 1 L. Autoclave and store the buffer at room temperature.

(b) 0.5 M EDTA (pH 8.0): Add 186.1 g EDTA in 800 mL distilled water. Using magnetic stirrer, mix NaOH to the solution and adjust the pH to 8.0. Bring the final volume of EDTA solution to 1 L by adding distilled water (if needed). Sterilize by autoclave and store at room temperature.

10. Tris-Acetate-EDTA (TAE; 10×) buffer: In 800 mL distilled water, add 48.4 gTris base [tris(hydroxymethyl)-aminomethane], 11.4 mL of glacial acetic acid (17.4 M), 3.7 g of EDTA, disodium salt and bring the volume of buffer to 1 L (no need to autoclave). Store the buffer at room temperature. To make 100 mL of 0.5× TAE buffer, dilute 5 mL of 10× TAE to 95 mL distilled water.
11. Phosphate buffer saline (PBS; 10×): 2 g KCl, 80 g NaCl, 2.4 g KH_2PO_4, 14.4 g Na_2HPO_4, in 800 mL distilled water. Modify pH to 7.4 with HCl. By adding water, increase the PBS volume to 1 L. Use an autoclave to sterilize. Allow to cool to room temperature before using. To make 100 mL of 1× PBS, dilute 10 mL of 10× PBS with 90 mL of distilled water.
12. 1% agarose gel: Dissolve 1 g of agarose in 100 mL of TAE buffer (0.5×).
13. Nanodrop spectrophotometer.
14. Gel imaging system.

2.3 Screening of Virulence Genes

1. Genomic DNA.
2. Primers for *gel* E gene sequence are (5′ ACCCCGTATCATT GGTT3′) and (5′ ACCCATTGCTTTTCCATC 3′) and *Eno*A gene sequence are EnoA1 FOR (5′CGGGATCCATGTTCTATTATTACAGATATTTATGC3′) EnoA1 REV (5′AACATGGTCGACTTACTTGCTAGTAATGGTG TTCCG3′) [26].
3. Thermal cycler.
4. Gel electrophoresis apparatus.
5. Polymerase chain reaction (PCR) purification kit.
6. Tris-Acetate-EDTA (TAE; 10×) buffer: In 800 mL distilled water, combine 48.4 g of Tris base [tris(hydroxymethyl)-aminomethane], 11.4 mL of glacial acetic acid (17.4 M) and 3.7 g of EDTA, disodium salt. Make the final volume of buffer to 1 L with distilled water. Do not autoclave buffer. Storage is at room temperature. To make 100 mL of 0.5× TAE buffer, mix 5 mL of 10× TAE with 95 mL distilled water.
7. Eva Green (0.05 mg/mL; fluorescence gel dye): Dissolve 0.5 g Eva Green in 80 mL distilled water. Top up the volume of the Eva Green solution to 100 mL. Using magnetic stirrer, dissolve the dye completely in the solution. Use amber bottle to store

solution at room temperature. In 100 mL 0.5× TAE buffer, add 5 μL of 0.05 mg/mL Eva Green solution for preparing 1.5% agarose gel (0.05 mg/mL Eva Green final).

8. Agarose gel (1.5%): Dissolve 1.5 g agarose in 100 mL TAE buffer (0.5×) with 0.05 mg/mL of Eva Green.
9. Gel imaging system.

2.4 DNA Sequence Analysis

1. Sanger sequencing device.
2. Basic Local Alignment Search Tool (BLAST).

2.5 Preparation for Gelatinase Assay

1. 1.MRS agar (HiMedia, India).
2. 3% (w/v)gelatin (HiMedia, India).
3. Ammonium sulphate solution.
4. Bacterial culture.
5. 5% (w/v) gelatin (BDH Chemicals, Ltd., Poole, UK).
6. Frazier reagent ($HgCl_2$ 12 g, HCl 20 mL, distilled water 20 mL) [23].

2.6 Preparation for Glycosidase Assay

1. MRS.
2. 145 mM NaCl in distilled water.
3. 0.2 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO4, pH 5.0).
4. Bacterial strain prepared in 145 mM NaCl.
5. p-nitrophenyl (p-NP) derivatives, p-NB-β-D-glucopyranoside (p-NP-β-D-Glu), p-NP-α-D-glucopyranoside (pNP-α-D-Glu), p-NP-β-D-galactopyranoside (p-NP-β-D-Gal), p-NP-β-D-fucopyranoside (pNP-β-D-Fuc), p-NP-β-D-xylopyranoside (p-NP-β-D-Xyl), and p-NP-α-Drhamnopyranoside (p-NP-α-D-Rham).
6. 0.5 M Na_2CO_3.
7. A cuvette (1 mL).
8. A spectrophotometer (Quant, Bio-Tek 128 Instruments Inc., Winooski, VT, USA).

2.7 Preparation for Enolase Assay

1. Bacterial culture.
2. MRS.
3. 100 mM HEPES buffer, pH 7.0, containing 10 mM MgSO4, 7.7 mM KCl, and 3 mM 2-phosphoglycerate.
4. PEP.
5. 2PGA.

3 Methods

3.1 Culture of Probiotic Isolates [27]

1. Grow the strains in de Man-Rogosa-Sharpe (MRS) broth (BD Difco).
2. Streak the isolates on MRS-agar and incubate the plates at 37 °C for 24 h.
3. Process the potential probiotics for further in vitro biosafety aspects.
4. Store the stock cultures at −40 °C in MRS broth with 40% glycerol.

3.2 Extraction of Genomic DNA

1. Select a single colony from bacterial plate and inoculate it in MRS-broth, allow it to grow overnight in incubator.
2. Harvest the bacterial cells at 4032 × *g*, 5 min, wash twice with phosphate buffer saline (PBS).
3. Isolate genomic DNA using a DNA extraction kit (GeneJet Genomic DNA Isolation Kit (Jena Bioscience)) following manufacturer's instructions [28].
4. Determine purity and concentration of DNA using Nanodrop spectrophotometer (BioPhotometer, Eppendorf, USA).
5. To check integrity of DNA, run on a 1% agarose gel in Tris-Acetate-EDTA (TAE, 0.5×) buffer pH 8.0.
6. Use gel imaging system to visualize the DNA.
7. For future use, keep the genomic DNA at −20 °C.

3.3 Screening of Virulence Genes

1. Analyze the genomic DNA of strains for the presence of *gel* E gene sequence using the primer sequences ACCCCGTAT-CATTGGTT and ACCCATTGCTTTTCCATC with Tm 51.2 °C [29, 30]. The genomic DNA of strains is analyzed for the presence of *Eno*A gene sequence using the primer sequences EnoA1 FOR CGGGATCCATGTTCTATTATTAC AGATATTTATGC EnoA1 REV AACATGGTCGACTTAC TTGCTAGTAATGGTGTTCCG [26].
2. 2.The PCR reaction mixture (45 μL) consists of 2.5 μL DNA (20 ng), 1 μL each primers (100 pmol/μL), 1 μL dNTP (10 mmol/L), 0.25 μL DNA Taq Polymerase (5 U/μL), 5 μL Taq Buffer (10×), 3 μL $MgCl_2$ (25 mmol/L), and 31.25 μL sterile dH_20.
3. The PCR conditions for all the genes are initial denaturation at 94 °C for 5 min, followed by 1 cycle of denaturation at 94 °C for 2 min, annealing at Tm °C for 2 min, extension at 72 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at Tm °C for 15 s, extension at 72 °C for 15 s, and a final extension at 72 °C for 10 min.

4. The amplification products are analyzed by gel electrophoresis on 1.5% (w/v) agarose gel 80 V for 1.5 h in 1× Tris-Acetate-EDTA buffer including 0.05 mg/mL EvaGreen (fluorescence gel dye).
5. The gel is photographed under ultraviolet light. The gel images are compared with the molecular weight standard (100–1000 bp DNA Ladder; Jena Bioscience) and examined in the Imaging System (Uvitec, Cambridge) [29, 31].

3.4 DNA Sequence Analysis

1. For sequencing studies, use high purity DNA with an OD_{260}/OD_{280} ratio of 1.8–2.0 and an OD_{260}/OD_{230} ratio of 2.0–2.2.
2. Perform sequence analysis on the PCR products detecting *gel*E.
3. Perform the alignment with GenBank sequences in the NCBI database using the BLAST algorithm with above gene. Compare the findings to the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). This confirms the presence of the genes in the respective isolates.

3.5 Gelatinase Activity

3.5.1 Protocol 1

1. Determine gelatinase production by the isolates as described by [32] Wang et al. (Wang et al. 2020). Briefly, Stab inoculate the culture into gelatin agar (Hi-Media) butts (1%, piercing 5 mm below the surface of medium). Incubate at 37 °C for 48 h.
2. Upon culture growth, place the tubes at 4 °C for 1 h to observe for liquefaction of gelatin.
3. The presence of liquid in the tube indicates gelatin has been hydrolyzed by expressed gelatinase.

3.5.2 Protocol 2

Determine Gelatinase enzyme production as described in [33].

1. Briefly, inoculate 20 μL of 12 h old cultures on MRS agar (Himedia, India) supplemented with 3% (w/v)gelatin (HiMedia, India). Incubate at 37 °C for 24 h.
2. The agar surface is inoculated by streaking the test strain grown in its usual MRS culture broth.
3. Flood the plates with 10 mL ammonium sulphate solution.
4. Assay the strains for gelatinase activity and presence of clear zone indicate presence of gelatinase activity (Fig. 1).

3.5.3 Protocol 3

1. Determine gelatinase activity using petri plates containing nutrient medium agar supplemented with 5% (w/v) gelatin (BDH Chemicals, Ltd., Poole, UK).
2. The agar surface is inoculated by streaking the tested strain grown in its usual culture broth.

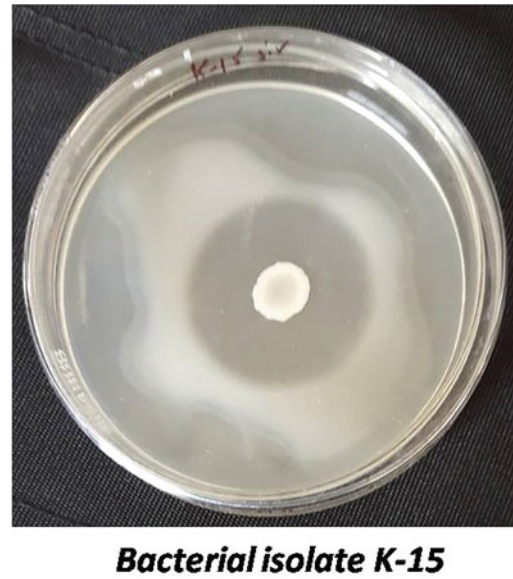

Fig. 1 The bacterial isolate K-15 shows gelatinase production as exhibited by zone of clearance surrounding the colonies

3. After incubation for 2–5 days at 37 °C, add Frazier reagent ($HgCl_2$ 12 g, HCl 20 mL, distilled water 20 mL) to show the hydrolysis.
4. Formation of a halo around a colony indicate gelatinase activity [28].
5. Inference: The isolate that produces gelatinase cannot be regarded safe for use as a probiotic.

3.6 Glycosidase Activity

Degree of glycosidase activity is performed as described in [34] with some modifications.

1. Grow strains in MRS overnight at 30 ° C.
2. Harvest bacteria by centrifugation (20,000 × *g*, 5 min).
3. Wash the pellet twice with 145 mM NaCl in distilled water.
4. To start with assay (400 µL), add 200 µL of 0.2 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO_4, pH 5.0), 100 µL of bacterial suspension prepared in 145 mM NaCl (OD_{600} 0.5 final).
5. Add 100 µL of 10 mM substrate solutions to the assay mixture. Different *p*-nitrophenyl (*p*-NP) derivatives are used as substrates, *p*-NB-β-D-glucopyranoside (*p*-NP-β-D-Glu), *p*-NP-α-D-glucopyranoside (*p*NP-α-D-Glu), *p*-NP-β-D-galactopyranoside (*p*-NP-β-D-Gal), *p*-NP-β-D-fucopyranoside (*p*NP-β-D-Fuc), *p*-NP-β-D-xylopyranoside (*p*-NP-β-D-Xyl), and *p*-NP-α-Drhamnopyranoside (*p*-NP-α-D-Rham).
6. Incubate the assay mixture at 37 °C for 1 h.

7. Stop the assay by the addition of 800 μL of 0.5 M Na_2CO_3 and clarify by centrifugation (10,000 × *g*, 3 min).
8. Transfer the supernatants (1000 μL) to a cuvette (1 mL) and determine the absorbance at 400 nm using a spectrophotometer (Quant, Bio-Tek 128 Instruments Inc., Winooski, VT, USA) set to automatic path-length correction.
9. Prepare blanks without bacterial cells but otherwise treated in the same manner. All reactions are performed in triplicate.
10. Calculate the Glycosidase activity as per the below mentioned formula. [Note: One unit of glycosidase activity is defined as the amount of enzyme that release 1 μmol of *p*-nitrophenol from substrate per minute under the conditions specified].

$$\text{Glycosidase activity (U/mL)} = (A_{400}/\text{reaction time in min}) \times (\text{total reaction volume in mL/enzyme volume in mL}) \times (1/\text{extinction coefficient of pNP}) \times \text{enzyme dilution factor}$$

3.7 Enolase Activity Assay

Enolase activity assays are carried out as described [15].

1. For whole cell assays, grow bacterial strains overnight in MRSat 37 °C.
2. Harvest various amounts of bacteria by centrifugation, wash once with 100 mM HEPES buffer, pH 7.
3. Resuspend bacteria in 100 mM HEPES, pH 7, 10 mM MgCl2, 7.7 mM KCl with or without substrate [3 mM 2-phosphoglycerate(2PGA)].
4. Incubate the bacterial suspensions for 5 min at 37 °C.
5. Remove bacteria by centrifugation.
6. Determine phosphoenolpyruvate (PEP) production spectrophotometrically ($\lambda = 240$ nm) using the extinction coefficient of PEP of $2.6 \times 10^3\ M^{-1}\ cm^{-1}$.
7. Measure change in absorbance of 2-PGA to PEP at 240 nm. The rate of PEP release is recorded as absorbance per minute.
8. Use GraphPad Prism software to determine Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) by fitting data into Michaelis–Menten equation with nonlinear regression analysis. Alternatively, we can use below mentioned formula:

$$\text{Enolase activity (U/mL)} = (A_{240}/\text{reaction time in min}) \times (\text{total reaction volume in mL/enzyme volume in mL}) \times (1/\text{extinction coefficient of PEP}) \times \text{enzyme dilution factor}$$

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Chapter 3

Determination of β-Glucuronidase Production

Dixita Panchal, Vrutika Lad, Meonis Pithawala, and Natarajan Amaresan

Abstract

Microbial encoded β-glucuronidase (EC 3.2.1.31) enzyme plays an important role in human health by metabolizing drugs in gastrointestinal tract. The screening of β-glucuronidase is achieved on methylumbelliferyl glucuronide plate (MUG) which contain, 4-methylumbelliferyl glucuronide as substrate. The fluorescent 4-methylumbelliferyl glucuronide is catalyzed in the presence of β-glucuronidase, which is detected by using UV light. The β-glucuronidase activity is also measured by the production of *p*-nitrophenol using *p*-nitrophenyl-β-D-glucuronide or phenolphthalein glucuronic acid as a substrate. Both the assays for the determination of β-glucuronidase are sensitive and require standard conditions.

Key words β-glucuronidase, Methylumbelliferyl glucuronide, *p*-nitrophenol

1 Introduction

The intestinal tract has a large surface area and constantly exposed to external influences which include microbial flora and their products [1]. The enzymes such as β-glucuronidase produced by intestinal bacteria may have histolytic capacity, that may lead to the decomposition of intracellular substances [2]. Microbes possessing β-glucuronidase enzyme activity play an important role in human health by metabolizing drugs in the gastrointestinal tract (GIT) [3]. The β-glucuronidases are a member of glycosidase family that catalyze the β-D-glucopyranosiduronic acid, glucuronides, and β-D-galacturonides. The β-glucuronidase is a structural protein of the endoplasmic reticulum (ER) and its occurrence in the lysosomes may be due to changes in the ER [4]. The β-glucuronidase activity is measured by methylumbelliferyl glucuronide as substrate and when it is hydrolyzed, strong fluorescent methylumbelliferone is liberated [2]. A quantitative measurement of enzyme is carried out by using glucuronides of phenolphthalein, and *p*-nitrophenol as substrate. The amount of aglycone liberated per unit time under standard conditions (e.g., substrate concentration, pH, and temperature) is a measure for the β-glucuronidase activity [4]. The

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_3,

chapter delivers both the qualitative and quantitative methods for detection of bacterial β-glucuronidase production.

2 Materials

2.1 Qualitative Method

- Bacterial culture.
- Methylumbelliferyl glucuronide plate (MUG Plate): 20 g tryptose, 10 g NaCl,15 g Noble agar, 1 L distilled water.
- 25 or 30 mL test tube.
- Sterile Petri plate.
- 0.45 μm pore size membrane filter.
- Stock Solution: 3 mg 4-methylumbelliferyl- β-D-glucuronide/mL. Filter the solution by using 0.45 μm pore size membrane filter.
- 50 mM glycine-NaOH buffer, pH 10.6.
- Fluorescence lamp.

2.2 Quantitative Method

1. Bacterial culture.
2. Reaction mixture solution: 0.1 mM EDTA and 1 mM *p*-nitrophenyl-β-D-glucuronide or phenolphthalein glucuronic acid in 20 mM potassium-phosphate buffer(pH 6.8).
3. 0.23 M glycine-NaOH buffer (pH 10.4).
4. Centrifuge.
5. Spectrophotometer.

3 Methods

3.1 Qualitative Method [2]

1. Prepare a methylumbelliferyl glucuronide medium (MUG) by dissolving the ingredients through gentle heating; then add 0.1% cysteine hydrochloride, and adjust pH to 7.2.
2. The medium is distributed in large tubes (approx. 15 mL in each tube), and sterilize at 121 °C for 20 min.
3. After sterilization, place the tubes in water bath at 46°Cand add 1 mL stock solution of methylumbelliferyl glucuronide in each tube.
4. Mix the contents well in the tubes and pour into sterile Petri plates.
5. After solidification, inoculate the bacterial culture as a dots or streak on the surface of MUG-plate.
6. Inoculate the plates at 37 °C for 24 h for aerobic bacteria and 3–5 days for anaerobic bacteria.

7. Observe the fluorescent zone around the bacterial growth, under the fluorescence lamp for the production of β-glucuronidase.
8. The fluorescent is most pronounced visible at alkaline pH; flood the plate with glycine-NaOH buffer, pH 10.6.
9. Observe the result immediately because the liberated methylumbelliferone may get diffuse into agar gel to form dispersed zones, which is difficult to interpret.

3.2 Quantitative Method [1, 3]

1. Add 0.1 mL of bacterial culture in 0.9 mL of reaction mixture solution.
2. Mix it well and incubate the tube for 30 min.
3. Add 2.5 mL of glycine NaOH buffer to reaction mixture.
4. Centrifuge the reaction mixture at 1000 × *g* for 30 min and collect the supernatant.
5. Determine the absorbance at 400 nm for *p*-nitrophenol or 540 nm for phenolphthalein.
6. The amount of *p*-nitrophenol and phenolphthalein released is determined by comparison with standard curve of *p*-nitrophenol and phenolphthalein, respectively (*see* **Note 1**).

4 Notes

1. The standard curve should be generated from the absorbance data of standard compound and a graph is plotted; the y-intercept is provided when the computer fits a line to the standard curve data. The absorbance is what you measure from your unknown.

   Calculation: $y = mx + b$.

   [Where y = Absorbance of unknown sample; m = Slop; x = concentration, and b = Intercept].

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Chapter 4

Determination of Nitroreductase Production

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Abstract

Most of the nitroaromatic compounds are toxic and mutagenic for living organisms, but some microorganisms have developed oxidative or reductive pathways to degrade or transform these compounds. One such enzymatic activity performed by human gut microbes is the production of nitroreductase. Some potential probiotics have the capacity to produce such enzymes, which prevent colon cancer and other health disorders. There are two types of nitroreductase enzyme; oxygen insensitive and oxygen-sensitive. Nitroreductase has raised a great interest due to their potential in biocatalysis and biomedicine, especially in prodrug activation for chemotherapeutic cancer treatments. Here, the activity of nitroreductase is estimated by the amount of m-aminobenzoic acid production.

Key words Colon cancer, Nitroreductase, Probiotics, m-aminobenzoic acid

1 Introduction

Heterocyclic and aromatic nitro compounds are extensively used in industry and medicine. They are important intermediates in the manufacture of thousands of consumer products and clinically are used as antibiotic, anti-parasitic, and radiosensitizing drugs [1]. The reduction of nitro groups by the intestinal microorganisms can be another source for the production of aromatic amines. The reduction of aromatic nitro groups is a complex reaction involving the addition of six electrons. The intermediates in this reaction include a nitro free radical, a nitroso group, and an N-hydroxy group; all three of these functional groups have been associated with potentially deleterious genotoxic events [2]. Nitro compounds such as chloramphenicol, p-nitrobenzoate, and nitrobenzene are reduced to primary amines by a liver enzyme system that can use either NADH or NADPH as its electron donor. Nitroreductase is active under anaerobic conditions but is virtually inactive in the air [3].

Bacterial nitroreductase reduces nitro ($-NO_2$) functional groups to the corresponding amines. Two types of bacterial

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_4,

nitroreductases have been described: Type 1 nitroreductases are oxygen in sensitive and catalyze the sequential reduction of nitro groups by adding electron pairs from NAD(P)H to produce the nitroso, hydroxylamino, and amino derivatives [4]. Type 2 nitroreductases are oxygen-sensitive that catalyze the oxygen-sensitive nitroreductases (type II), reducing the nitro group of nitroaromatic compounds by adding one electron. In the presence of oxygen, it forms a nitro anion radical; thereby generating the superoxide anion in a futile cycle that regenerates the nitro group [5]. The chapter delivers the qualitative and quantitative methods for detection of bacterial nitroreductase production.

2 Materials

2.1 Qualitative Methods

2.1.1 Method 1

- Bacterial culture.
- Brain heart infusion (BHI) agar medium.
- 1-nitropyrene.
- BHI broth (9 ml).
- Sterile pipettes (1 ml, 0.1 ml).
- Incubator.

2.1.2 Method 2

- Bacterial culture.
- Brain heart infusion (BHI) medium.
- 60% (W/V) ammonium sulfate.
- Nitroblue tetrazolium (NBT).
- Nondenaturing anaerobic gel.
- 4-Nitrobenzoic acid.
- M10 medium.
- FAD and NADH.
- 0.21% trichloroacetic acid.
- 0.007% sodium nitrite.
- 0.004% ammonium sulfamate.
- 0.027% NEDD(N-(1 naphthyl) ethylenediamine dihydrochloride).
- Ice box.
- Ziploc bag.
- Centrifuge.
- Incubator.

2.2 Quantitative Methods

2.2.1 Method 1

- Bacterial culture.
- Nitrobenzoic acid (final concentration 30 μg/ml).
- 0.007% Sodium nitrite.
- 0.35% NEDD (N-(1 naphthyl)ethylenediamine dihydrochloride).
- Incubator.
- Spectrophotometer.

2.2.2 Method 2

- Bacterial culture.
- 4-Nitrobenzoic acid.
- Centrifuge.
- 0.2 μm filter.
- Incubator.
- Spectrophotometer.

3 Methods

3.1 Qualitative Methods

3.1.1 Method 1 [6]

1. Prepare a BHI agar plates containing 16 μg of 1-nitropyrene per ml, which may produce a yellow background in the plates.
2. Make serial dilutions of bacterial culture in BHI broth.
3. Spread 0.1 ml of each dilution on each of the plates.
4. Incubate the plates at 37 °C under both aerobic and anaerobic conditions.
5. Observe the plates after 2 days for clearance of 1-nitropyrene around bacterial colonies. The zone of clearance suggests the production of nitroreductase by the bacterial isolate.
6. Use positive and negative strains for the confirmation.

3.1.2 Method 2 [6, 7]

1. Prepare an overnight culture of anaerobic bacteria.
2. Centrifuge the culture at 3000 × *g* for 15 min.
3. Collect supernatant and add 60% (W/V) ammonium sulfate. Incubate the mixer in an ice box for 1 h.
4. Centrifuge the mixer at 5000 × *g* for 15 min.
5. After centrifugation decant the supernatant and dissolve the pellet in BHI and subject to sonication for 6 min.
6. Load 50 μg of the sample in 10% polyacrylamide gel, and run the gel electrophoresis at 6 milliAmp.
7. Stain the gel with Nitroblue tetrazolium.

8. Incubate the gel anaerobically with occasional shaking for 1.5 h at 37 °C in a ziplock bag containing 30 ml of degassed M10 medium supplemented with 0.24 mg of 4-nitrobenzoic acid per ml with FAD and NADH (final concentration of each, 50 μg/ml).
9. Decant the solution, and incubate the gel for another hour.
10. Incubate the gel for another 20 min at room temperature in a solution containing 0.21% trichloroacetic acid and 0.007% sodium nitrite.
11. Add ammonium sulfamate (final concentration, 0.004%) to the gel.
12. After 3 min, add NEDD (final concentration, 0.027%) to the bag for the development of nitroreductase bands.

3.2 Quantitative Methods

1. Nitroreductase activity in bacterial cultures is determined by the conversion of 4-nitrobenzoic acid to 4-aminobenzoic acid.

3.2.1 Method 1 [6, 8]

2. Add nitrobenzoic acid (final concentration 30 μg/ml) into the cultures and incubate at 37 °C for overnight.
3. The amount of 4-aminobenzoic acid produced is detected in the cultures by diazotization of the amine in the presence of sodium nitrite(final concentration 0.007%) under acidic conditions at 4 °C and addition of NEDD (N-(1 naphthyl)ethylenediaminedi hydrochloride) (final concentration, 0.35%).
4. Measure the production of dye at 540 nm in spectrophotometer and compare it with the standard *p*-aminobenzoic curve (*see* **Note 1**). Use phosphate buffer as a blank.
5. One unit of enzyme is defined as the amount of enzyme necessary to produce 1 μg of 4-aminobenzoic acid at 37°Cunder anaerobic conditions in 1 h. The specific activity of the enzyme is measured by calculating the units of enzyme activity per milligram of total soluble protein in supernatant and cell extracts.

3.2.2 Method 2 [6, 8]

1. Nitroreductase activity of cultures is also evaluated by growth of the cultures aerobic condition.
2. To assay nitroreductase enzyme activity in extracellular fractions, centrifuge the cells at 10,000 × *g* and filter the supernatants through a 0.2 μm filter.
3. Add 4-nitrobenzoic acid to the sterilized supernatants and incubate at 37 °C overnight.
4. Measure the cell density at 600 nm and compare it with the standard *p*-aminobenzoic curve (*see* **Note 1**).

4 Notes

1. The standard curve should be generated from the absorbance data of standard compound and a graph is plotted; the y-intercept is provided when the computer fits a line to the standard curve data. The absorbance is what you measure from your unknown.

   Calculation: $y = mx + b$.

   [where, y = Absorbance of unknown sample; m = Slop; x = concentration, and b = Intercept].

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Chapter 5

Determination of Azoreductase Production

Vrutika Lad, Dixita Panchal, Meonis Pithawala, and Natarajan Amaresan

Abstract

The human body is inhabited by trillions of microbes that play essential roles in human health. One such enzymatic activity performed by human gut microbes in the production of azoreductases, enzymes that degrade azo dyes responsible for colon cancer in humans. Therefore, the detection of bacterial ability to produce azoreductase enzyme is mandatory before being used as a probiotic. The activity of azoreductase is determined by the amount of amaranth (azo dye) reduction. For this, the amount of amaranth reduced is determined by comparison with a standard amaranth curve.

Key words Amines, Azoreductase, Colon cancer, Direct Blue 15, Red azo dye, *N*-nitroso compounds

1 Introduction

Azoreductase is identified from various sources either monomeric or homo-dimeric. Fecal enzymes, such as azoreductase, have been reported to be involved in the etiology of colon cancer [1]. The enzymes are produced by members of the normal intestinal microflora. Predominant bacterial genera with azoreductase activity found in the human intestinal tract include *Clostridium, Pseudomonas, Bacillus, Geobacillus, Lysinibacillus, Enterococcus, Eubacterium,* and *Escherichia* [2]. Azoreductases catalyze the reductive cleavage of azo bonds (–N=N–) to give colorless aromatic amines [3], and these compounds induce the anti-apoptotic pathways, thereby facilitating the development of colorectal cancer (CRC). Some probiotic strains can metabolize carcinogenic compounds, especially amines and *N*-nitroso compounds [4], and alter metabolic activity (reduced the endogenous production of carcinogenic compounds) by intestinal microbiota. The binding and degradation of carcinogens are some of the mechanisms by which probiotic supplementation reduced the risk of CRC development.

It has been reported that probiotics decrease fecal concentrations of enzymes such as glycosidase, β-glucuronidase,

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_5,

azoreductase, and nitroreductase and secondary bile salts and reduce the absorption of harmful mutagens that may contribute to colon carcinogenesis. Therefore, it is necessary to test that probiotics for their ability to produce any azoreductase enzyme. A probiotics strain can influence carcinogenesis by producing enzymes such as glycosidase, β-glucuronidase, azoreductase, and nitroreductase that transform precarcinogens into active carcinogens. The chapter delivers the qualitative and quantitative methods for detection of bacterial azoreductase production.

2 Materials

2.1 Qualitative Method [5]

- Bacterial culture.
- Brain Heart Infusion (BHI) broth dilution tubes.
- Brain Heart Infusion (BHI) agar plates.
- Direct Blue 15 or Nitro Red (final concentration, 80 mg/ml).
- Spreader.
- Alcohol (for sterilization).
- Incubator.

2.2 Quantitative Method [5]

- Bacterial culture.
- Brain Heart Infusion (BHI) broth.
- Brain Heart Infusion (BHI) agar plate.
- Direct Blue 15 dye.
- Tetracycline.
- Incubator.
- Centrifuge.
- UV-Visible spectrophotometer.

3 Methods

3.1 Qualitative Method [5]

1. Transfer the bacterial isolate (probiotic strain) into BHI broth for their growth.
2. Make tenfold serial dilutions of the bacterial culture in BHI broth.
3. Spread 0.1 ml dilutions of 10^{-5} to 10^{-9} on BHI agar plates amended with Direct Blue 15 or Nitro Red (final concentration, 80 mg/ml).
4. Incubate the plates at 37 °C for overnight.

5. Observe for clearance of the dye surrounding the colonies. The zone of clearance suggests the production of azoreductase by the bacterial isolate.

3.2 Quantitative Method [5]

1. Grow the bacteria to be tested for azoreductase enzyme production for overnightin BHI broth.
2. Centrifuge the broth at 15,000 × *g* for 15 min.
3. Collect the supernatant for the assay of enzymatic activity.
4. Add Direct Blue 15 and tetracycline to the supernatants to final concentrations of 50 to 100 μM and 15 μg/ml, respectively.
5. Incubate the tubes at 37 °C for 24 h.
6. For control, use BHI broth with Direct Blue 15 alone.
7. Observe the cultures and monitor for a decrease in the A_{615} until the dye is completely cleared and reduced, at which point the absorbance reached a constant level.
8. Note the time required for the total reduction of the dye.
9. Determine the amount of reduced dye by the following formula:

$$\text{Decolorization } (\%) = \frac{\text{Initial absorbance} - \text{after decolorization absorbance}}{\text{Initial absorbance}} \times 100$$

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Chapter 6

Determination of Hemolytic Activity

Vrutika Lad, Dixita Panchal, Meonis Pithawala, Mitesh Kumar Dwivedi, and Natarajan Amaresan

Abstract

A substance that causes hemolysis (breakdown of red blood cells) is known as hemolysin. The presence of safety-related virulence factors like hemolysin production is determined by hemolytic activity. The hemolysin produces a clear β-hemolytic zone on a blood agar plate. Blood agar contains general nutrients, and 5% defibrinated mammalian blood (human, sheep, or horse). Therefore, it is useful for the cultivation of pathogenic microorganisms and determining the hemolytic capabilities of an organism. Hemolytic reactions are evaluated by observing both the partial hydrolysis of the red blood cells and the production of a green zone (α-hemolysis), as well as the total hydrolysis of red blood cells producing a clear zone around the bacterial colonies (β-hemolysis) or no reaction (γ-hemolysis).

Key words Blood agar, Defibrinated blood, Hemolysin, Hemolysis, Probiotics, α-hemolysis, β-hemolysis, γ-hemolysis

1 Introduction

The safety aspects of microorganisms, even belonging to a group of bacteria that is generally recognized as safe (GRAS) [1] such as lactic acid bacteria (LAB), involve determining its virulence potential. Among virulence determinants associated with pathogenicity, antibiotic resistance and the production of extracellular proteins such as hemolysin and gelatinase and surface proteins and aggregation substances are highlighted [2, 3].

Blood agar medium is an enriched and differential medium. It is a rich, complex medium that contains 5% of defibrinated mammalian blood (human, sheep, or horse). The appearance of the medium is red and opaque. The final pH of the medium at 25 °C is 7.3 ± 0.2. Blood agar is mostly used to cultivate pathogenic organisms capable of producing extracellular enzymes that cause hemolysis of the blood. The degree of hemolysis by these hemolysins helps differentiate the genera *Staphylococcus*, *Streptococcus*, and

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_6,

Enterococcus members. In addition, the blood added to the base provides more nutrition to the medium by giving additional growth factors required for these fastidious organisms. The blood also aids in visualizing hemolytic reactions of different bacteria. The sheep blood has been preferred source in the blood agar because sheep RBCs are most sensitive to the hemolytic toxins released by bacterial cells, thus causing hemolytic zones around the colonies over the period of time. Horse blood allows detection of hemolytic reactions and supplies both the X factor (heme) and the V factor (nicotinamide adenine dinucleotide), necessary for the growth of many bacterial species, including *Hemophilus influenzae*, which requires both the X and V factors.

Blood agar containing animal blood is not feasible in many developing countries due to technical and personnel issues and the high cost and adverse climate conditions for raising these animals [4, 5]. Therefore, a common practice in many developing countries is to prepare blood agar using human blood, mainly taken from expired blood transfusion bags or from volunteers, often the laboratory technicians themselves [5, 6]. The common tenet is that human blood in nutrient media results into poor bacterial isolation rates and hardly visible hemolysis or no hemolysis at all [7, 8]. The use of human blood is associated with safety risks to laboratory personnel, mainly due to transmission of blood-borne viral infections such as hepatitis B, C, and HIV and is therefore considered unsuitable for use in clinical diagnostic laboratories [4, 5].

Four types of hemolysis are observed on blood agar, which can be identified by a zone of hemolysis present around the growing colonies.

1.1 Alpha Hemolysis

- Alpha hemolysis is defined by a greenish-grey or brownish discoloration around the colony due to the partial lysis of the red blood cells.
- During α-hemolysis, H_2O_2 produced by the bacteria causes hemoglobin present in the RBC of the medium is converted into methemoglobin.
- Some of the α-hemolytic species are a part of the normal human flora, but some species like *Streptococcus pneumoniae* cause pneumonia and other severe infections.

1.2 Beta Hemolysis

- Beta hemolysis is defined by a clear zone of hemolysis around the colonies when grown on blood agar.
- The clear zone appears due to the complete lysis of the red blood cells present in the medium, causing hemoglobin denaturation to form colorless products.

- β-hemolytic bacteria include group A *Streptococci* such as *S. pyogenes* and group B *Streptococci* such as *S. agalactiae*, both of them are associated with severe infections in humans.

1.3 Gamma Hemolysis

- Gamma hemolysis is also called non-hemolysis, as no lysis of red blood cells occurs.
- As a result, no change of coloration or no zone of hemolysis is observed around the colonies.
- Species like *Neisseria meningitides* are non-hemolytic or gamma-hemolytic.

1.4 Alpha Prime or Wide Zone Alpha Hemolysis

- Alpha prime hemolysis is defined by a small zone of intact erythrocytes adjacent to the bacterial colony. A zone of complete lysis of RBCs surrounds the zone of intact erythrocytes.
- This might be confused with β-hemolysis due to the appearance of a clear zone around the colonies.

The growth and types of hemolysis depend on organisms' metabolic requirements; it is possible that some strains do not grow and/or can demonstrate hemolytic models other than expected. *Hemophilus influenzae*, which requires both factor X and factor V, will not grow on this medium [8]. *Neisseria, Mycobacterium, Bordetella,* and other microorganisms with highly specific nutritional requirements do not grow adequately; and hence specific culture media should be used for the detection of these microorganisms.

2 Materials

1. Blood agar medium or Nutrient agar medium.
2. 5% defibrinated sheep blood/Human blood/Horse blood.
3. Bacterial culture.
4. Incubator.
5. Nichrome wire loop.

3 Methods

1. Add about 5% of defibrinated mammalian blood (human, sheep, or horse) into the autoclaved basal medium (nutrient agar) to prepare blood agar.
2. Streak or inoculate the bacteria to be tested on to the blood agar supplemented with 5% defibrinated sheep/human/horse blood.

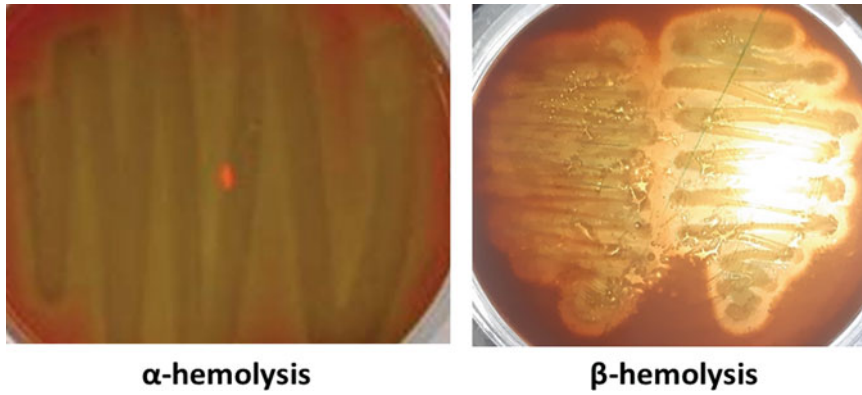

Fig. 1 Bacterial isolates showing α and β-hemolysis

3. Incubate the plates at 30 °C for 48–72 h.
4. After incubation, the hemolytic reaction is evaluated by observing the presence of hemolytic haloes (α-hemolysis/β-hemolysis/γ-hemolysis) (Fig. 1).
5. Confirm the different hemolytic activities by using a positive and negative control [9].

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Chapter 7

Determination of DNAse Activity

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Abstract

Probiotics which are beneficial microorganisms for humans provide several health benefits including immunomodulation of the intestines and gut microbiota. In vitro safety assessment of the probiotic isolates that are used for human consumption needs to be performed which includes screening for hemolytic, gelatinase, DNAse activities, etc. We provide the method for determining DNAse activity by these candidate probiotics using in vitro studies.

Key words Probiotics, In vitro safety assessment, DNase, DNase activity

1 Introduction

Probiotics are introduced as beneficial microorganisms for human health, and also favorable for the immunomodulation of the intestines and gut microbiota [1]. Probiotics can be obtained through consumption of naturally fermented foods, functional foods that are fortified in such bacteria, or in supplement form [2]. It has been reported that the probiotic microorganisms, which were basically isolated from local niches, must be safe as well as non-pathogenic, thus suitable for human consumption [3]. Several tests have to be carried out on candidate microorganisms before they can be confirmed and accepted for use as probiotics. Probiotic studies generally involve three major components: determining their survival in the gastrointestinal tract (GIT), safety for human or animal use, and establishing probiotic activity/benefit to the consumer. In vitro safety assessment of the probiotic includes screening for hemolytic, gelatinase, and DNAse activities.

Deoxyribonucleases (DNases) are extracellular endonucleases that cleave the phosphodiester bond in the backbone of DNA releasing free nucleotides and phosphate and thus, disrupting the cell functionality [4]. The DNase test is mainly used for identification of *Staphylococcus aureus* [5] but it can also be carried out for

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_7,

other microorganisms. *Serratia*, *Staphylococcus*, *Campylobacter* and *Moraxella* [6] are DNase producing genera. DNase evades the innate immune response in the host by degrading neutrophil extracellular traps and hydrolyzes human DNA interrupting protein synthesis [7]. The DNase helps in the growth of pathogen by amplifying the pool of available nucleotides through DNA hydrolysis process and aids in dispersal of the pathogens. DNase suppresses both macrophage bactericidal activity and TLR9-mediated innate immune response. This behavior is one of the bacterial innate immune avoidance mechanisms based on auto-degradation of CpG-rich islands by a bacterial DNase [8]. Therefore, the bacterial isolates producing DNase enzyme cannot be used as a probiotic in food and feed industry.

As one of the virulence factors that should be considered for the safety assessment of probiotic products is the production of DNase; we are focusing on protocols for determination of DNase activity of probiotic isolates.

2 Materials

All solutions and reagents should be made using distilled water and analytical grade chemicals. The prepared reagents are stored at room temperature (unless indicated otherwise). When disposing waste products, adhere to all the waste disposal requirements.

2.1 Chemical Composition of Culture Media

1. DeMan, Rogosa, and Sharpe (MRS)-broth composition (BD Difco, Franklin Lakes, NJ, USA:10 g proteose peptone No. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80 (also known as Tween 80), 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium phosphate in 1 L of distilled water(in case using MRS-broth powder, add 55 g of the powder in 1 L of water). Adjust pH of the MRS-broth as 6.5 ± 0.2.
2. MRS-agar composition: 15 g agar in 1 L MRS-broth (in case using MRS-agar powder, add 70 g of the powder in 1 L of water). [Heat MRS-broth /agar with frequent agitation for 1 min to completely dissolve the powder components into water. Autoclave at 121 °C for 15 min and store the media at room temperature].

3. *DNase agar pates* (HiMedia Laboratories, Pvt. Ltd., Mumbai, India).

Ingredients	g/L
Tryptone	15.00
Soya peptone	5.00
Deoxyribonucleic acid (DNA)	2.00
Sodium chloride	5.00
Agar	15.00

Final pH (at 25 °C) 7.3 ± 0.2

Directions: Suspend 42 g in 1000 mL purified/distilled water. Heat with frequent agitation to dissolve the medium completely. Sterilize by autoclaving at 12–15 lbs. pressure (118–121 °C) for 15 min. Cool to 45 °C and pour into sterile petri plates.

4. 10 mL 1 N HCl
5. DNAse methyl green agar (HiMedia Laboratories, Pvt. Ltd., Mumbai, India).

Ingredients	g/L
Tryptose	20.00
Deoxyribonucleic acid (DNA)	2.00
Sodium chloride	5.00
Methyl green	0.05
Agar	15.00

Final pH (at 25 °C) 7.3 ± 0.2

Directions: Suspend 42.05 g in 1000 mL purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121 °C) for 15 min. Cool to 45–50 °C. Mix well and pour into sterile Petri plates.

3 Methods

3.1 Culture of Probiotic Isolates

1. Test strains are propagated in Man, Rogosa, and Sharpe (MRS) broth and incubated at 37 °C for 48 h.
2. Then, 0.1 mL of the culture are spread on MRS agar and incubated for 48 h at 37 °C.

3. The colonies are randomly selected from MRS agar to be further evaluated for purity.
4. The isolates are also evaluated microscopically before being stored in MRS medium with 20% glycerol broth and stored at −80 °C [9].

3.2 Determination of DNase Activity

3.2.1 Protocol 1 (HCl-DNA Precipitation Method)

DNase Test Agar is used for detecting deoxyribonuclease activity of bacteria and fungi and particularly for identification of pathogenic Staphylococci. Jeffries et al demonstrated DNase activity by the agar plate method employing a semi-synthetic medium [10]. The DNase activity is tested by HCI-DNA precipitation method. The enzymatic activity is then assayed by flooding the DNase agar plates with 1 N Hydrochloric acid. Positive DNase activity was visualized as clear zones (around colonies) when the plates were flooded with 1 N Hydrochloric acid. The acid reacts with the nucleate salts in the medium, yielding free nucleic acid, and consequently a cloudy precipitate. A positive reaction is indicated by a clear zone around the colony. The width of this clear zone is related to the amount of the exocellular enzyme produced. This method involves the following steps:

1. The DNase activity is tested by HCI-DNA precipitation method.
2. The overnight cultures of isolates are spot inoculated on DNase agarplates (HiMedia) and incubated at 37 °C for 48 h (Fig. 1).
3. The cultures are flooded with 10 mL 1 N HCl. The absence of clear zones around the colonies confirm negative response in the production of DNase [11].

3.2.2 Protocol 2 (DNAse Methyl Green Agar Method)

This medium is based on modification of the procedure for detecting DNase-producing bacteria as per Smith, Hanoch, and Rhoden [12] and Jefferies, Holtman and Guse [10]. The medium supports growth of both gram-positive and gram-negative bacteria. Tryptose

Fig. 1 The bacterial isolate showed a negative response for the production of DNase

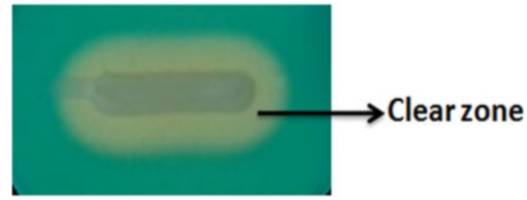

Fig. 2 The bacterial isolate showed a positive response for the production of DNase

serves as nitrogenous source for the organisms. DNase produced by microorganisms depolymerizes the DNA substrate in the medium. Methyl green fades into a colorless compound producing distinct clear zones surrounding colonies (or band/spot inocula) in an otherwise green colored medium. Methyl green requires a highly polymerized DNA substrate [13] and it combines with polymerized DNA to form a stable, green colored complex at pH 7.5 [14–16]. As hydrolysis progresses, methyl green is released and when not combined at this pH it fades and becomes a colorless compound. Therefore, clear zones are observed [14, 15].

1. DNAse activity is identified by spotting 1-μL aliquots of the cultures on the surface of DNAse methyl green agar (BD, Franklin Lakes, NJ, USA).
2. After incubation at 25 °C and 37 °C for 48 h, the formation of clear halos around the colonies is identified as a positive result (Fig. 2). [17].

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Chapter 8

Determination of Bile Salts Deconjugation

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Abstract

Probiotics are living microorganisms providing multiple health benefits to host, if present in adequate amount. Probiotic therapy is useful in multiple diseases such as inflammation, obesity, diabetes, and drug induced diarrhea to name a few. In order to enter in the consumer market, a probiotic need to cross several regulatory biosafety parameters. Bile salts conjugate produced by liver have important lipid emulsification and solubilization activity. Many microbial species reported for the presence of bile salt hydroxylase (BSH) enzyme. BSH catalyzes breakdown (deconjugation) of bile salt. If bile salt deconjugating enzymes are present in the intestine, the useful bile conjugates might be deconjugated, resulting in marked alterations in host physiochemical functions. Microbial deconjugation, particularly in the upper small intestine, may disrupt the lipid digestion and uptake of fat soluble vitamins. This makes analysis of probiotics BSH activity as an important biosafety criterion. Such indirect approach can save money and time for intestinal performance evaluation of probiotics in host animal. BSH activity can be analyzed by microbial screening in selective media, measuring enzyme activity, and high-performance liquid chromatography methods.

Key words Probiotic screening, Biosafety, Bile salt hydrolase, Deconjugation, Bile tolerance, Lactic acid bacteria, Cholesterol

1 Introduction

Liver secretes around one liter bile daily in gastrointestinal tract and it play crucial role in lipid metabolism. Bile salts are water soluble end products of cholesterol metabolism in liver. It contains organic (cholesterol, amino acids, phospholipid, bile acid, and biliverdin pigments) and inorganic compounds [1, 2]. Bile acid and salts are absorbed actively in the terminal ileum and are subsequently re-secreted, thereby forming an enterohepatic cycle [3].

Liver is a major site for conjugation processes. Conjugation is a mechanism by which the human regulates the metabolism, function, excretion, and re-circulation of many endogenous and exogenous compounds, including many drugs [4]. The four molecules most often used for conjugation are: glycine, taurine, glucuronic

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_8,

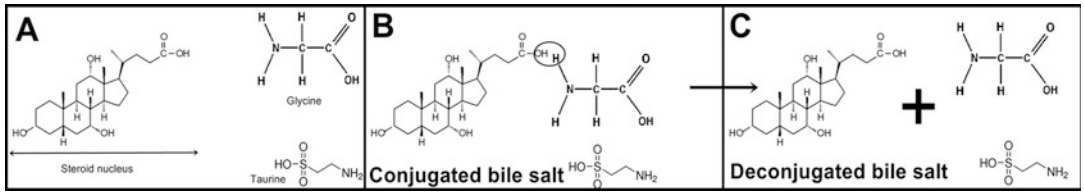

Fig. 1 (**a**) Precursors of bile salt. (**b**) Bile salt is formed in liver from after conjugation of cholesterol with amino acid glycine and taurine by amide bond (conjugated bile salt). (**c**) BSH breaks this peptide bond leading to removal of amino acids from steroid nucleus (deconjugated bile salt)

acid, and sulphate. Before secretion, conjugation of bile as N-acyl amidate takes place with taurine (tauroconjugation) or glycine (glycoconjugation) as shown in Fig. 1. Taurine and glycine conjugates are often called as bile salt [4].

During bile acid metabolism in microbes, the first step is deconjugation of dietary lipids. Deconjugation, particularly in the upper small intestine, may disrupt the lipid digestion and subsequent uptake of fat soluble vitamins [1, 3]. Microbial deconjugation takes part in the small intestine. If bile salt deconjugating enzymes are present in the intestine, the bile conjugates might be deconjugated, resulting in marked alterations in physiochemical properties. Intestinal deconjugation of glycine and taurine conjugates is always microbial; the same holds true for more than 99% of the deconjugation of glucuronic acid conjugates and most probably, for a substantial part of sulphate conjugates [5].

Bile salt hydrolase (BSH) (Cholylglycine hydrolase, E. C.3.5.1.24) present in bacterial community catalyzes deconjugation of bile acid leading to hydrolysis of amide bond and detachment of taurine/glycine from steroid backbone and formation of un(de)conjugated bile salt [6] (Fig. 1). BSH was investigated for its role in nutrition, bile detoxification, cholesterol lowering activity, modification in membrane characteristics, and gastrointestinal persistence with respect to host and bacteria [7–9]. BSH activity and its effects on the physiology of the host and the microbiota is topic of research in scientific community [8, 9].

Hydrolysis of bile salt is carried out by multiple dairy, non-dairy, and intestinal probiotics belonging to *Lactobacillus, Bifidobacterium* and less studies strains of *Leuconostoc, Brevibacillus, Enterococcus, Sporolactobacillus* [7, 9, 10]. Gram-positive commensals profoundly exhibit BSH activity as compares with Gram-negative strains [10].

2 Materials

1. Probiotic culture from animal intestine or fermented food.
2. Strains of the following genera: *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* species.
3. MRS agar plate containing 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA) and 0.037% calcium chloride.
4. Bile salt—MRS agar—Prepare 1 mM solutions of TDCA, taurocholic acid sodium salt hydrate(TCA), sodium taurolithocholate (TLCA), sodium glycocholate hydrate (GCA), and sodium taurochenodeoxycholate. Mix them in MRS agar and prepare plates for inoculation.
5. Bovine serum albumin (200 μg/mL), Bradford reagent, Ninhydrin (0.2% in acetone).
6. UV-Vis spectrophotometer or ELISA reader.
7. High performance liquid chromatography.
8. Sonicator.
9. Reverse phase HPLC column.
10. Pure standards of conjugated bile salts, sodium taurocholate, sodium glycocholate, sodium cholate.
11. 2-mercaptoethanol, trichloroacetic acid, methanol, acetic acid, sodium acetate, membrane filter.

3 Methods

3.1 Qualitative Determination of BSH Activity [11–13]

1. Take pure culture of probiotic bacteria and inoculate 10 μL culture on MRS agar plates (prepared MRS plate by any one method given in material section).
2. Incubate the plates separately under anaerobic and microaerophilic condition for 72 h at 37 °C (*see* **Note 1**).
3. After incubation appearance of zone of precipitation around colonies indicates positive BSH activity.
4. Measure diameter of the precipitate halos around colonies.
5. High diameter indicates high BSH activity.
6. Use non probiotic (non-BSH producing) strains as negative control.

3.2 Quantitative Determination of BSH Activity

3.2.1 Spectrophotometric Estimation BSH Activity [14]

1. Grow probiotic culture in MRS- bile salt broth for 24–48 h.
2. Centrifuge the broth at 12,298 × *g* and collect the cell pellet.
3. Wash the pellet with 0.1 M sodium-phosphate buffer and re-suspend in the same buffer containing 10 mM 2-mercaptoethanol.

Table 1
Amino acid estimation by ninhydrin method

Sr. No.	Standard Amino acid (glycine or taurine) (150 μg/mL) (mL)	D.W. (mL)	Conc. (μg/mL)	Ninhydrin (mL)			
Blank	0	4	0	1	Keep on boiling water bath for 15 min	Cool the test tube and add 1 mL ethanol and mix well	Measure the absorbance at 595 nm
1	0.66	3.34	25				
2	1.33	2.67	50				
3	2.00	2.00	75				
4	2.66	1.34	100				
5	3.33	0.67	125				
Test sample	0.1	3.9	–				

4. Disrupt the cells by sonicating for 3 min under cooling followed by centrifugation at 44,720 × *g* for 10 min at 4 °C.
5. Use the supernatant as enzyme source (*see* **Note 2**).
6. In an Eppendorf tube, add 190 μL of sodium-phosphate buffer (0.1 M, pH -6) containing 10 mM of 2-mercaptoethanol and conjugated bile salt.
7. Add 10 μL enzyme source in above mixture.
8. Incubate the mixture for 30 min at 37 °C.
9. Terminate the reaction by adding 200 μL of 15% trichloroacetic acid.
10. Centrifuge the mixture at around 12,298 × *g* to remove precipitate.
11. The supernatant contained released amino acid (glycine and taurine).
12. Determine released amino acids concentration by using standard graph of glycine and taurine (Optical density570 × concentration) by ninhydrin method (*see* Table 1) [15].
13. Use Bradford method to estimate protein concentration taking bovine serum albumin (BSA) as standard (*see* Table 2) [16].
14. One unit of BSH activity is enzyme amount that liberate 1 μM amino acid from substrate per minute.
15. Calculate the BSH activity by the given formula:

$$\text{BSH activity (μM amino acid from substrate per minute)} = \frac{\frac{\Delta A}{\text{min}} \times 1000 \times 0.190}{\text{Extinction coefficient of glyciine/taurine} \times 0.01}$$

Table 2
Protein estimation by Bradford method

Sr. No.	BSA (200 μg/mL) (mL)	D.W. (mL)	Conc. (μg/mL)	Bradford reagent (mL)	
Blank	0	1.0	0	5	Measure the absorbance at 595 nm
1	0.2	0.8	40		
2	0.4	0.6	80		
3	0.6	0.4	120		
4	0.8	0.2	160		
5	1	0.0	200		
Test sample	0.1	0.9	–		

3.2.2 HPLC Method [17]

1. HPLC parameters (*see* **Note 3**).
   (a) Mobile phase- 700 mL methanol and 300 mL 0.02 M acetic acid. Set the pH 5.6 by adding 5 M NaOH dropwise.
   (b) Flow rate- 1 mL/min for conjugated bile salt and 2 mL/min for free bile salt.
   (c) Sample injection volume- 20 μL.
   (d) Use UV detector at wavelength 205 nm.
2. Sample preparation.
   (a) Grow culture on MRS broth containing 1 mM sodium glycocholate and 1 mM sodium taurocholate for 24 h.
   (b) Centrifuge 200 mL broth culture for 5 min at 12,298 × *g* under cooling (5 °C).
   (c) Discard the pellet and use supernatant as for further processing.
   (d) Mix supernatant with methanol (2:1) and incubate for 1 h.
   (e) Centrifuge again 12,298 × *g* under cooling (5 °C) for 10 min to obtain precipitate.
   (f) Suspend the precipitate having enzyme in 20 mL of 50 mM sodium acetate in 1 mM EDTA buffer (pH 5.4). Use it as test sample for HPLC.
3. Assay protocol (*see* **Note 4**).
   (a) Take 25 μL of 0.01 M sodium taurocholate and 0.01 M sodium glycocholate in small test tube and add 100 μL of test sample.
   (b) Incubate reaction mixture for 30 min at 37 °C.

(c) Add 100 μL of HPLC mobile phase to stop enzymatic reaction. Filter the mixture through membrane filter and use to inject in HPLC system.

(d) Deconjugation of nanomoles of either sodium taurocholate or sodium glycocholate per minute is based on disappearance of each from the assay mixture and is used to calculate one BSH enzymatic unit.

4 Inference

Bile salt is vital for many physiological processes. Excessive deconjugation of bile salt due to BSH has pathological effects on host. Hence an ideal probiotic should have high bile salt tolerance and absence (or very low) of BSH activity.

5 Notes

1. Use McIntosh fildes jar, candle jar and anaerobic jar to maintain anaerobic condition.
2. BSH have very short half-life at storage conditions (50C) which can be somehow increase by adding EDTA during storage.
3. For HPLC troubleshooting, it is advised to wash the column with mobile phase up to 30 min before injection and sonicate the mobile phase before use.
4. Retention time of around 6.2 and 8 min for sodium taurocholate or sodiumglycocholate under above HPLC conditions.

Acknowledgments

Authors are thankful to Uka Tarsadia University and Kavayitri Bahinabai Chaudhari North Maharashtra University for providing necessary facilities for the chapter preparation.

Declaration of Competing Interest All authors of present manuscript declare no conflicts of interest.

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Chapter 9

Determination of D-Lactic Acid Production

Shilpika Pandey, Archana Chaudhari, and Mitesh Kumar Dwivedi

Abstract

Probiotics provide several health benefits and can aid in disease prevention. A safety assessment and logical design of probiotic formulations are essential for safe human intake of these probiotics. The methods for identifying potential probiotic species using 16S rRNA gene sequencing and whole genome sequencing (WGS) are described here. We provide methods for determining D-/L-lactate generation by these candidate probiotics using in silico and in vitro studies.

Key words Probiotics, D-lactic acidosis, Enzymatic spectrophotometry, 16S rRNA gene sequencing, Whole genome analysis, HMMER package

1 Introduction

Probiotics as defined by World Health Organization (WHO) are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [1]. Some common probiotics include *bacteria (Bacillus spp., Bifidobacterium spp., Propionibacterium spp., Enterococcus spp., Lactobacillus spp., Streptococcus spp.,* and some specific strains of *Escherichia coli*) and yeast (*Saccharomyces spp.)* [2].

There is no doubt that probiotics have several health benefits, yet there have been instances where probiotic use has resulted in negative health impacts [3–15]. Adverse effects have primarily been recorded in people with underlying medical disorders, with a few outliers. As a result, determining the safety of probiotics in the food business is critical [16]. It is also crucial to assess the safety of newly found strains of previously known species because these are eventually consumed by humans [16, 17]. L (+), D (−), or the combination D-L-lactate are produced by some lactic acid bacteria (LAB) genera [18, 19]. D-lactate is normally not hazardous to human

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Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_9,

health; however, at concentrations greater than 3.0 mmol/L, which can occur due to clinical conditions such as short bowel syndrome [20] or inefficient D-lactate metabolism [21, 22], it can cause health concerns [23].

This chapter covers how to assess a probiotic candidate's biosafety, with a special focus on estimating the candidate's ability to produce D-lactate using in silico and in vitro tests, as well as probiotic candidate identification using 16S rRNA gene sequencing and whole genome analysis.

2 Materials

All solutions and reagents should be made using distilled water and analytical grade chemicals. The prepared reagents are stored at room temperature (unless indicated otherwise). When disposing waste products, adhere to all the waste disposal requirements.

2.1 Chemical Composition of Culture Media

1. *DeMan, Rogosa, and Sharpe (MRS)-broth composition* (BD Difco, Franklin Lakes, NJ, USA: Add 10 g proteose peptone No. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80 (also known as Tween 80), 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium phosphate in 1 L of distilled water(in case using MRS-broth powder, add 55 g of the powder in 1 L of water). Adjust pH of the MRS-broth as 6.5 ± 0.2.
2. *MRS-agar composition*: Add 15 g agar in 1 L MRS-broth (in case using MRS-agar powder, add 70 g of the powder in 1 L of water). [Heat MRS-broth /agar with frequent agitation for 1 min to completely dissolve the powder components into water. Autoclave at 121 °C for 15 min and store the media at 2–8 °C].

2.2 Preparation for D-Lactate Assay

1. The D-lactate assay kit (Megazyme, Bray, Ireland) includes buffer (pH 10.0), NAD^+, D-Glutamate-Pyruvate Transaminase (D-GPT) suspension, D-Lactate Dehydrogenase (D-LDH) suspension along with lactic acid standard solution (0.15 mg/mL). [The majorities of the kit's reagents are used as supplied and remain stable for over 2 years at 4 °C. NAD^+, on the other hand, must be dissolved in 5.5 mL distilled water before use and is stable for over a year at 4 °C or over 2 years at −10 °C (avoid freeze/thaw cycles).]
2. Micro-pipettors (20 μL, 200 μL, and 1000 μL).
3. Bacterial culture.
4. Benchtop centrifuge.

5. Membrane filters (0.22 μm pore size).
6. Vortex mixer.
7. Stop clock.
8. Analytical balance.
9. Disposable plastic cuvettes (10 mm light path, 3.0 mL).
10. Spectrophotometer set at 340 nm.

2.3 Bacterial Genomic DNA Isolation

1. Bacterial culture.
2. MRS-broth.
3. Benchtop centrifuge.
4. DNA extraction kit (Omega Bio-Tek, USA) includes HiBind® DNA mini columns, supplied buffers, glass beads S, enzymes (Proteinase K, RNase A, Lysozyme).
5. Water bath (capable of 37 °C, 55 °C, 65 °C).
6. Vortex mixer.
7. Nuclease-free 1.5 mL microcentrifuge tubes.
8. 100% ethanol.
9. 100% isopropanol.
10. Phosphate buffer saline (PBS; 10×): 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 mL distilled water. Adjust pH to 7.4 with HCl. Adjust the PBS volume to 1 L by adding water. Sterilize by autoclave. Store at room temperature. Dilute 10 mL of 10× PBS to 90 mL of distilled water to make 100 mL of 1× PBS.
11. Tris-EDTA (TE; 1×) buffer: 1 mL of 1 M Tris–HCl (pH 8.0), 0.2 mL of 0.5 M EDTA (pH 8.0) in 98.8 mL distilled water. Mix well, autoclave the solution at 121 °C for 15 min, and store at room temperature.
    (a) 1 M Tris–HCl (pH 8.0): 121.14 g of Tris base [tris (hydroxymethyl)aminomethane] in 800 mL distilled water. Bring pH to 8.0 using HCl. Adjust final volume to 1 L by adding distilled water. Sterilize by autoclave and store at room temperature.
    (b) 0.5 M EDTA(pH 8.0): 186.1 g EDTA in 800 mL distilled water. Bring pH to 8.0 using NaOH. Mix vigorously with a magnetic stirrer. Adjust the final volume to 1 L by adding distilled water (if needed). Sterilize by autoclave and store at room temperature.
12. Tris-Acetate-EDTA (TAE; 10×) buffer: 48.4 g of Tris base [tris(hydroxymethyl)aminomethane], 11.4 mL of glacial acetic acid (17.4 M), 3.7 g of EDTA, disodium salt in 800 mL distilled water. Adjust the volume of buffer to 1 L. Do not sterilize the buffer. Store at room temperature. Dilute 5 mL of

10× TAE to 95 mL distilled water to make 100 mL of 0.5× TAE buffer.

13. 1% agarose gel: Dissolve 1 g of agarose in 100 mL of 0.5× TAE buffer.
14. Nanodrop spectrophotometer.
15. Gel imaging system.

2.4 16S rDNA Gene Sequencing

1. Genomic DNA.
2. Universal primers for 16S rDNA: fD1 (5′ AGAGTTT GATCCTGGCTCAG 3′) and rD1 (5′ AAGGAGGTGATC-CAGCC 3′).
3. Thermal cycler.
4. Gel electrophoresis apparatus.
5. Tris-Acetate-EDTA (TAE; 10×) buffer: 48.4 g of Tris base [tris (hydroxymethyl)aminomethane], 11.4 mL of glacial acetic acid (17.4 M), 3.7 g of EDTA, disodium salt in 800 mL distilled water. Adjust the volume of buffer to 1 L. Do not sterilize the buffer. Store the buffer at room temperature. Dilute 5 mL of 10× TAE to 95 mL distilled water to make 100 mL of 0.5× TAE buffer.
6. Ethidium bromide (EtBr; 10 mg/mL): 10 g EtBr in 800 mL distilled water. Adjust volume of the EtBr solution to 1 L. Stir the solution on a magnetic stirrer until the dye dissolves. Store the EtBr solution in dark bottle at room temperature. Add 5 μL of 10 mg/mL EtBr solution in 100 mL 0.5× TAE buffer for the preparation of 1% agarose gel with 0.5 mg/mL EtBr final concentration.
7. 1% agarose gel: 1 g agarose dissolved in 100 mL 0.5× TAE buffer containing 0.5 mg/mL EtBr.
8. Gel imaging system.
9. Polymerase chain reaction (PCR) purification kit.
10. Sanger sequencing device.
11. Basic Local Alignment Search Tool (BLAST).

2.5 Genome Sequencing and Analysis

1. Genomic DNA.
2. Nanodrop spectrophotometer.
3. PacBio RS II instrument.
4. Covaris g-TUBE.
5. AMPure XP beads.
6. Sequencing primers and DNA polymerase.
7. DNA/Polymerase Binding Kit P6.
8. DNA Sequencing Reagent 2.0 Kit.

9. Sequence single-molecule real-time (SMRT) cell.
10. Databases/software like SMRT Analysis v2.3.0, Hierarchical Genome Assembly Process (HGAP) software (v. 3.0), Pilon (v. 1.21), virulence factor database, antibiotic resistance genes database, ResFinder program and database, PHAge Search Tool Enhanced Release (PHASTER) web-programme, Genomic Island Prediction Software (GIPSy).

2.6 In-Silico Tools for D-Lactate Gene Identification

1. HMMER package, BLASTP tool.
2. UniProtKB, Pfam databases.

3 Methods

3.1 Culture of Probiotic Isolates [24]

1. Serially dilute the probiotic culture or the bacterial isolates with peptone water and spread it on MRS-agar containing 0.5% (w/v) $CaCO_3$.
2. Incubate the plates in a static incubator at 37 °C for 48 h.
3. Process the potential probiotics for further in vitro biosafety aspects.

3.2 Enzymatic Measurement of D-Lactate

Use a D-lactate assay kit (Megazyme, Bray, Ireland) to determine the amount of D−/L-lactic acid generated by *L. reuteri* IDCC 3701. The test is carried out in accordance with the manufacturer's instructions.

1. Inoculate *L. reuteri* IDCC 3701in MRS-broth and let the culture grow at 37 °C overnight.
2. Centrifuge the bacterial culture at 2300 × *g*, 4 °C for 30 min collecting the supernatant (50 μL) and filtering with a 0.22 μm-pore size membrane.
3. Combine 50 μL supernatant with 750 μL sterile distilled water and the kit's solutions, which include 250 μL buffer, 50 μL NAD^+, and 10 μL D-GPT.
4. After 3 min of incubation at 37 °C, measure the optical density (OD_1) at 340 nm.
5. Add 10 μL D-LDH and continue to incubate for another 5 min. Measure OD_2.
6. Measure OD_3 after adding 10 μL L-LDH for another 5 min.
7. Using equation, compute the total concentration of lactic acid (c) (Table 1) (*see* **Note 1**).

$$c = 0.3204 \times \Delta OD_D + 0.3232 \times \Delta OD_L$$

Table 1
Estimation of lactic acid production by probiotic bacteria [25, 26]

Bacteria	L-lactic acid	D-lactic acid	Total lactic acid (L + D)
Lactobacillus reuteri	19	5.0	24.0
Lactobacillus bulgaricus	0.3	8	8.3
Lactobacillus acidophilus	5.0	4.2	9.2
Bifidobacterium breve	0.4	0.1	0.5
Streptococcus thermophilus	5	0.1	5.1

$$\text{Concentration of D} - \text{lactic acid (mg/mL)} = 0.3204 \times \Delta OD_D \ (\text{Where}, \Delta OD_D = OD_2 - OD_1)$$

$$\text{Concentration of L} - \text{lactic acid (mg/mL)} = 0.3232 \times \Delta OD_L \ (\text{Where}, \Delta OD_L = OD_3 - OD_2)$$

Probiotic bacteria species are grown in MRS-broth and estimation of lactic acid is performed using D-lactate assay kit (Megazyme, Bray, Ireland). Values in the table represent concentration of lactic acid in mg/mL units.

3.3 Extraction of Genomic DNA

1. From the well grown bacterial colonies, pick up single colony and inoculate it in MRS-broth overnight.
2. Pellet the cells for 5 min at 4032 × *g* then wash with phosphate buffer saline (PBS) to remove interference from media components.
3. Isolate DNA using a DNA extraction kit (Omega Bio-Tek, USA) according to the manufacturer's instructions [27].
4. Using a Nanodrop spectrophotometer(BioPhotometer, Eppendorf, USA), determine the purity and concentration of isolated DNA.
5. For sequencing studies, use high purity DNA with an OD_{260}/OD_{280} ratio of 1.8–2.0 and an OD_{260}/OD_{230} ratio of 2.0–2.2.
6. To test the integrity of isolated DNA, run it on a 1% agarose gel in 0.5× Tris-Acetate-EDTA (TAE) buffer pH 8.0 and look at it with a UV light.
7. For future use, keep the genomic DNA at −20 °C.

3.4 16S rRNA Based Identification of the Isolate

1. To identify the bacterial isolate, use extracted DNA to perform polymerase chain reaction (PCR) amplification of the 16S rRNA gene region on a Touchgene Gradient Thermal Cycler (Techne, UK) [27].

2. Use the universal primers: fD1 (5′ AGAGTTTGATCCTGGCTCAG 3′) and rD1 (5′ AAGGAGGTGATCCAGCC 3’) for the amplification the 16S rDNA gene sequence.
3. Set up the PCR amplification program as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min.
4. Confirm the PCR product (i.e., amplicon) using gel-electrophoresis on a 1% agarose gel, run for 30 min at 100 V in 0.5× Tris-Acetate-EDTA (TAE) buffer pH 8.0 and observed under UV light.
5. Sequence the gel purified 16S rDNA amplicons and compare the findings to the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

3.5 Whole Genome Sequencing and Analysis of the Isolate

To enhance the understanding of bacterial genome, sequence the whole genome of bacteria using a PacBio RSII equipment and an Illumina platform (Macrogen, Seoul, Korea) [28, 29].

1. Centrifuge bacterial cells and extract genomic DNA according to the manufacturer’s instructions using a DNA extraction kit (Omega Bio-Tek, USA). The OD_{260}/OD_{280} ratio (1.8–2.0) and the OD_{260}/OD_{230}(2.0–2.2) ratio are used to assess the quality of genomic DNA.
2. Shear the genomic DNA using the Covaris g-TUBE, as directed by the manufacturer’s protocol.
3. Using the PacBio RS II System (Pacific Biosciences, Menlo Park, CA), create SMRTbell libraries with C4 chemistry.
4. Using 0.45× AMPure XP beads, purify the libraries by deleting <1.5 kb short reads.
5. Using a Qubit Fluorometer(Thermo Fisher Scientific, Wilmington, DE), quantify the sheared DNA.
6. For DNA replication to begin, add sequencing primers and DNA polymerase according to the manufacturer’s instructions.
7. Using the DNA/Polymerase Binding Kit P6(Pacific Biosciences), load the libraries and enzyme/template complexes onto zero-mode waveguides.
8. Sequence single-molecule real-time (SMRT) cell using Pacific Biosciences’ DNA Sequencing Reagent 2.0 Kit and a 120-min sequence capture methodology and stage start to maximize the subread lengths.

9. Using SMRT Analysis v2.3.0, filter raw sequence data to exclude SMRTbell adapters, short polymerase reads of <100 bp, subreads of <1000 bp, and low-quality reads with an accuracy of <80%.
10. Create contigs using Hierarchical Genome Assembly Process (HGAP) software (v. 3.0), which preassembles the seed reads, generates a consensus sequence of the mapped reads, and corrects and filters the reads.
11. Use Pilon(v. 1.21) to correct mistakes in built contigs.
12. Using whole genome sequencing data, predict genomic safety (using virulence factor database [30], antibiotic resistance genes database [31], ResFinder program and database [32], genomic stability (using PHAgeSearchTool Enhanced Release (PHASTER) web-based program [33], Genomic Island Prediction Software (GIPSy) program [34].

3.6 Identification of D-Lactate Related Genes

Use the HMMER software package to uncover homologs of lactate racemase, hydroxyacyl glutathione hydrolase, D-lactate dehydratase, D-lactate dehydrogenase, and 2-hydroxyglutarate-pyruvate transhydrogenase to forecast a bacterium's ability to make D-lactate [35–38].

1. Using the hmmbuild software, construct profile hidden Markov models (HMMs) using sequences from the UniProtKB database of genes implicated in D-lactate synthesis (included in HMMER package). In these enzymes, profile-HMMs reveal conserved amino acid sequence patterns.
2. Compare the created profile-HMMs with the protein sequence database using the hmmsearch tool (from the HMMER package) [39]. The results demonstrate comparable genes involved in D-lactate production in *L. reuteri* IDCC 3701.
3. To confirm the results, check the homology against SWISS-PROT and PFAM databases using BLASTP (protein-protein BLAST) method in NCBI's BLAST^{+}and hmmscan tool in HMMER package.

4 Notes

1. Multiply the concentration of lactic acid by the dilution factor, F, if the sample is diluted during preparation.

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Chapter 10

Determination of Antibiotic Resistance

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Abstract

The global emergence and spread of antimicrobial resistance posture a substantial risk for public health. Probiotics have become a very important component to common health food products. Lactic acid bacteria (LAB) colonizing gastrointestinal and urogenital tracts of humans and animals are frequently used as starter culture in the manufacturing of fermented products and as probiotics. LAB have potential to resist vancomycin, aminoglycosides, and many nucleic acid inhibitors. They may have ability to transfer antibiotic resistance genes to pathogenic bacteria. Determination of antibiotic resistance pattern of probiotic bacteria is essential to approve their safety status. Hence, assessment of antibiotic resistance profile of probiotic bacteria has to be determined by both phenotypic methods using antimicrobial susceptibility test by disc diffusion and molecular method by employing multiplex PCR to determine genetic determinants of antibiotic resistance.

Key words Probiotics, Antibiotic resistance, Lactic acid bacteria (LAB), Fermented food, Disc diffusion, Multiplex PCR

1 Introduction

The devastating use of antibiotics has betrayed a substantial role in the out stretch of antibiotic resistance bacteria. Probiotics are live microorganisms that endue a beneficial health on the host when inflicted in sufficient quantities. Many microbial species have probiotic properties, but those most commonly used are lactic acid bacteria (LAB). Several strains of *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, *Pediococcus*, and *Propionibacteria* present in foods and in dietary supplements are generally employed as probiotics [1]. Majority of probiotics are usual members of the human intestinal tract, and they are ingested in large amounts in functional foods [2].

The transmission of antibiotic resistant bacteria among human and animals may occur mainly through food [3]. Fermented dairy products and fermented meats are consumed without sufficient heat treatment may act as a vehicle for antibiotic resistant bacteria

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_10,

between the animal indigenous microflora and the human gut microbiome. Lactic acid bacteria widely used as probiotics or starter cultures in fermented food have the potential to serve as a host of antibiotic resistance genes with the risk of transferring the genes in many lactic acid bacteria and other pathogenic bacteria [4]. *Enterococci* are resistant to cephalosporins and low levels of amino glycoside and clindamycin [5]. *Lactobacilli*, *Pediococci,* and *Leuconostoc* spp. have been reported to have a high natural resistance to vancomycin. Many *Lactobacillus* spp. are resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin [6]. Therefore, bacteria used as probiotics for humans or animals should not carry any transferable antimicrobial resistance genes and methods for assessment of antibiotic resistance of probiotics is indispensable for safety evaluation.

Generally, methods used for antibiotic susceptibility testing are based on phenotypic detection of antibiotic resistance by disc diffusion and molecular identification of antibiotic resistance gene by multiplex polymerase chain reaction. Disc diffusion method is simple, reliable, and official method used for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) [7]. In this method disc impregnated with specific concentration of antibiotic are kept on the surface of agar medium previously inoculated with standardized inoculum of the test organism. The antibiotic diffuses into the agar and inhibits germination and growth of the test microbes and then the diameters of inhibition growth zones around the disc are measured. This method has ability to test many microorganisms and antibiotics and also easy to interpret result [8]. Multiplex PCR is used for the amplification of many target sequences in a single reaction mixture using multiple pairs of primers. In this method more than one target antibiotic resistance gene sequence can be amplified by saving time and make it simple. Furthermore, the PCR amplicon is characterized by agarose gel electrophoresis.

Lactobacillus strains isolated from commercial products cheese and yoghurt were resistant to vancomycin and ciprofloxacin in addition to gentamicin and streptomycin [9]. Different strains of *Lactobacilli* from Italian traditional fermented food exhibiting phenotypic antibiotic resistance to tetracyline and erythromycin were found to contain the tetM and ermB genes [10]. Many probiotics such as *Lactobacilli*, *Lactococcus*, *Leuconostoc*, *Enterococcus* were resistant to vancomycin, gentamicin, streptomycin, and ciprofloxacin [11, 12]. Isolates from traditional cheese *Leuconostoc mesenteroides* subsp. *dextranicum* and *Leuconostoc mesenteroides* subsp. *mesenteroides* were resistant to vancomycin, trimethoprim, kanamycin, chloramphenicol, erythromycin, streptomycin, and

tetracycline. Their determination for antibiotic resistance gene by molecular PCR method evidenced the presence of *erm(B)* and *tet (S)* gene [13]. Florez and Mayo [14] isolated 41 strains of *S. thermophilus* from raw milk, which exhibited phenotypic antibiotic resistance against tetracycline, erythromycin, clindamycin, streptomycin, and neomycin. PCR amplification confirmed *tet(S)* gene in the tetracycline-resistant strains, and ermB gene in erythromycin/clindamycin-resistant strains. Phenotypic and genotypic antibiotic resistance patterns of probiotics reported by several authors are listed in Table 1.

2 Materials

2.1 Antimicrobial Susceptibility Test of Probiotic Bacteria (Disc Diffusion Method)

- 70% v/v ethanol.
- Distilled water.
- Erlenmeyer flask.
- Measuring cylinder.
- Inoculation loop.
- Digital balance.
- Hot air oven.
- Water bath.
- Autoclave.
- Laminar airflow cabinet.
- Incubator.
- Non-absorbent cotton.
- 0.5 McFarland standard (Himedia).
- Antibiotic zone scale (Himedia).
- Petri plate (100 x 15 mm).
- Sterile cotton swab (Himedia).
- *Microbial culture*: Pure culture of probiotic bacteria.
- *Muller Hinton Agar mediumPlate (MHAMP)*: Accurately weigh 3.8 g of Muller Hinton agar medium (Himedia, M173) as per manufacturer recommendation and add 100 mL of distilled water. Completely dissolve the medium in a hot plate. Sterilize the medium in an autoclave for 15 min at 121 °C. Dispense the medium aseptically onto sterile petriplates and mix well before dispensing. Allow the medium to solidify under laminar air flow cabinet.
- *Antibiotic disc (Himedia)*: Chloramphenicol-30 μg, Ciprofloxacin-30 μg, Erythromycin-15 μg, Gentamicin – 50 μg, Norfloxacin-5 μg, Streptomycin-25 μg, Vancomycin-10 μg, Tetracycline-30 μg.

Table 1
Phenotypic and genotypic antibiotic resistance patterns of probiotics

Probiotic organisms	Source	Phenotypic antimicrobial resistance	Genotypic antimicrobial resistance	Reference
L. salivarius KKP 1835, *E. faecium* TR2	Fermented vegetables and fruits, probiotic drinks	Tetracycline	tet(M)	Stefanska et al. [15]
S. thermophilus M17PTZA496	Fontina cheese (cow)	Tetracycline	tet(S)	Tarrah et al. [16]
E. gallinarum DM29,DM26,*E. durans*DM4, DM20, DM34, *E. faecium* DM19	Dromedary raw milk	Streptomycin and kanamycin	entA and entB	Houicher et al. [17]
Enterococcus spp.	Rabbit feces	Ampicillin, vancomycin, and linezolid	entA, entB, and entP	Lengliz et al. [18]
Lactobacillus sp.	Yoghurt	Penicillin, streptomycin	–	Poonam and Srivastav [19]
L. rhamnosus, *L. plantarum*, *L. casei*, *L. paracasei*, *L. fermentum*	Cheese, yoghurt	Streptomycin and gentamicin vancomycin, and ciprofloxacin	–	Seifabadi and Baserisalehi [9]
L. casei, *L. brevis, L. delbrueckii*	Curd	Ciprofloxacin, streptomycin, and tetracycline,	–	Shirisha et al. [20]
Enterococcus, Pediococcus, *Weissella*	Animal feed	Ciprofloxacin, norfloxacin, teicoplanin, and vancomycin	vanC1, tetM, ermB	Akpınar &Tuncer [21]

Enterococcus spp. *E. faecium*, *E. faecalis*, *E. flavescens*	Raw and processed meat	Ciprofloxacin, erythromycin, gentamicin, norfloxacin, streptomycin, tetracycline, vancomycin	vanB, tetL	Maia et al. [22]
L. casei, *L. acidophilus*, *L. harbinensis*, *L. fermentum*, *L. plantarum*, *P. pentosaceus*, *W. paramesenteroides*	Dairy products	Oxacillin, sulfa/trimethoprim. Gentamicin, clindamycin, vancomycin, rifampicin, erythromycin, tetracycline, ampicillin, chloramphenicol	vanC1, VanC2, tet(S), tet(K), ermA, ermB, ant(4′)-Ia, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia	Colombo et al. [23]
L. lactis, *L. garvieae*, *L. plantarum*, *E. faceium*	Cheese	Rifampicin, tetracycline, vancomycin,	–	Kanak and Yilmaz [24]
L. plantarum *L. salivarius* *L. rhamnosus* *L. acidophilus* *L. paracasei*	Traditional fermented dairy products	Ciprofloxacin, vancomycin	–	Ali, et al. [25]
L. bulgaricus spp.	Traditional fermented milk	Kanamycin, streptomycin ciprofloxacin, trimethoprim	rpoB, erm(B), aadA, bla, cat, vanX.	Guo et al. [26]
L. bulgaricus, *S. thermophilus*	Yogurt, cheese	Vancomycin, gentamycin, neomycin, and streptomycin	van, aph, aadA2	Wang et al. [27]
Lactobacillus spp. *S. thermophilus*	Fermented food	Streptomycin, gentamycin	ant(6), aac(6′)-aph(2″)	Yang and Yu [28]
Lactobacillus spp.	Infant feces	Cephalosporins, oxacillin, aztreonam, meropenem, and polymyxin	blaCTX-M	Khan et al. [29]
L. reuteri BCS134, *P. acidilactici* R76, *P. acidilactici* X1, *P. pentosaceus, E. faecium* ISMMS VRE 2	Poultry	Erythromycin, vancomycin, ciprofloxacin, streptomycin, and tetracycline	–	Reuben, et al. [30]

(continued)

Table 1
(continued)

Probiotic organisms	Source	Phenotypic antimicrobial resistance	Genotypic antimicrobial resistance	Reference
L.paracasei	Serbian cheese	Tetracycline	tet(M) gene	Ledina et al. [31]
L. fermentum	Human feces and commercial dairy products	Erythromycin and tetracycline	tet(K) and tet(M), erm(B), erm(C)	Anisimova &Yarullina [32]
S. carnosus, *L. plantarum*, *L. brevis*, *L. sakei*, *W. confusa*, *W. cibaria*	Fermented smoked pork	Penicillin G, streptomycin, gentamycin, vancomycin, chloramphenicol, norfloxacin, ciprofloxacin, kanamycin, and neomycin	str(A), str(B), tet(O), tet(M), ere (A), catA	Wang et al. [33]

2.2 Determination of Antibiotic Resistance Gene in Probiotic Bacteria by Mutiplex PCR

- Measuring cylinder.
- Beaker.
- Distilled water.
- Thermalcycler (Himedia, LA949 Prima-96™).
- Electrophoresis system (Himedia, LA851).
- Vortex Mixer.
- Micropipettes and Tips.
- Adhesive tape.
- Hotplate.
- Molecular Biology Grade Water (Himedia, ML024).
- Agarose (Himedia, MB002).
- 50× TAE (Himedia, ML016).
- 6× Gel Loading Buffer (Himedia, ML015).
- Ethidium bromide Solution (EtBr;10 mg/mL) (Himedia, MB074).
- 0.2 mL (PCR Tubes) (Himedia, CG282).
- 100 bp DNA Ladder (Himedia, MBT049).
- UV transilluminator (Himedia,LA1067).
- *Microorganisms*: Pure culture of probiotic bacteria (Sample).
- *Positive controls*: Pure culture of *tet(A) - Escherichia coli* DSM 3876; *tet(K) - Staphylococcus aureus* subsp. aureus DSM 4911; *tet (L) - Enterococcus mundtii* IM 613.
- *Negative control*: Pure culture of *E. faecalis* JH2–2.
- *Nutrient broth*: Accurately weigh 1.3 g of nutrient broth (Himedia, M002) medium as per manufacturer recommendation and add 100 mL of distilled water. Completely dissolve the medium in a hot plate. Sterilize the medium in an autoclave for 15 min at 121 °C. Dispense 10 mL of aliquot in a sterile container.
- *PCR Mutiplex Master Mix (Himedia, MT118)*: It contains optimized composition of polymerase, dNTPs, $MgCl_2$ and reaction buffers for efficient amplification of DNA templates and suitable for multiple target gene amplification in a single tube. Thaw multiplex PCR master mix and vortex it. Centrifuge in microcentrifuge at the time of use as per instruction of the manufacturer.
- *Primer Mix*:

  Primer for suspected antibiotic resistance genes: *tet(A), tet (K), and tet(L)* [31].

Tetracycline resistance gene	Primer sequence (5′ to 3′)	T_{an} (°C)	Amplicon size (bp)
Tet(A)	F: GTAATTCTGAGCACTGT R: CCTGGACAACATTGCTT	55	670
Tet(K)	F: TTATGGTGGTTGTAGCTAGAAA R: AAAGGGTTAGAAACTCTTGAAA	55	348
Tet(L)	F: GTMGTTGCGCGCTATATTCC R: GTGAAMGRWAGCCCACCTAA	55	696

Antibiotic resistance genes tet(A), tet(K), and tet (L) specific primer 10 μM (Invitrogen): Add equal volume of each forward and reverse primer in an Eppendorf tube and mix well before use.

- *Template DNA*: Inoculate a single pure colony of probiotic bacteria, positive controls - (*Escherichia coli* DSM 3876, *Staphylococcus aureus* subsp. aureus DSM 4911, and *Enterococcus mundtii* IM 613) and negative control (*E. faecalis* JH2–2) bacteria into 10 mL of sterile nutrient broth tube. Incubate the culture tube at 37 °C for 24 h. Extract the DNA (genomic/plasmid) depend upon the presence of antibiotic resistance gene using bacterial genomic DNA Purification Kit (Himedia, MB505)/plasmid DNA purification kit (Himedia, MB518)as per manufacturer instruction.
- *Preparation of 1× TAE*: Add 10 mL of 50× TAE Buffer to 490 mL of sterile distilled water. Mix well before use.
- *Preparation of agarose gel* (2%):
  - Add 1 g agarose to 50 mL of 1× TAE buffer in a glass beaker.
  - Heat the mixture on a hot plate until agarose dissolves.
  - Allow it to cool down to about 55–60 °C.
  - Add 0.5 μL ethidium bromide solution (EtBr; 10 mg/mL), mix well, and pour the gel solution into the gel tray.
  - Allow the gel to solidify for about 30 min at room temperature.

3 Methods

3.1 Antimicrobial Susceptibility Test of Probiotic Bacteria by Disc Diffusion Method

3.1.1 Preparation of 0.5 McFarland Standard Probiotic Inoculum

1. Transfer four or five colonies of pure culture of probiotic bacteria to 2 mL of sterile saline aseptically.
2. Make smooth suspension by vortexing it.
3. Add more colonies or dilute with sterile suspension to make up the turbidity of bacterial suspension to 0.5McFarland standard [34].
4. Use this inoculum suspension (1–2 × 10^8 CFU/mL) within 15 min of preparation.

3.1.2 Inoculation of MHAM Plate

1. Immerse the sterile cotton swab into the probiotic bacterial inoculum.
2. Turn round the swab on top of suspension against the inner side wall of the tube to clear away the surplus inoculum [35].
3. Streak the swab backwards and forwards intently above the whole surface of the agar thrice.
4. Turn round the plate almost 60° at all times to ensure even distribution of inoculum to produce lawn of growth.
5. Close the petri plate with lid and let the agar exterior to dry for 5–15 min.

3.1.3 Application of Antibiotic Discs

1. Dispense 6–8 antibiotic discs onto the inoculated MHAM plate by using antibiotic disc dispenser (HiMedia, LA971).
2. Gently press each disc with sterile stick, if needed to ensure attachment of disc to the agar.
3. Incubate the MHAMP at 37 ± 2 °C for16–24 h in an incubator.
4. Measure the diameter (in mm) of zone of complete growth inhibition for each antibiotic using antibiotic zone scale or ruler (Fig. 1).
5. Interpret the probiotic bacteria as sensitive, intermediate or resistance according to CLSI standard [36].

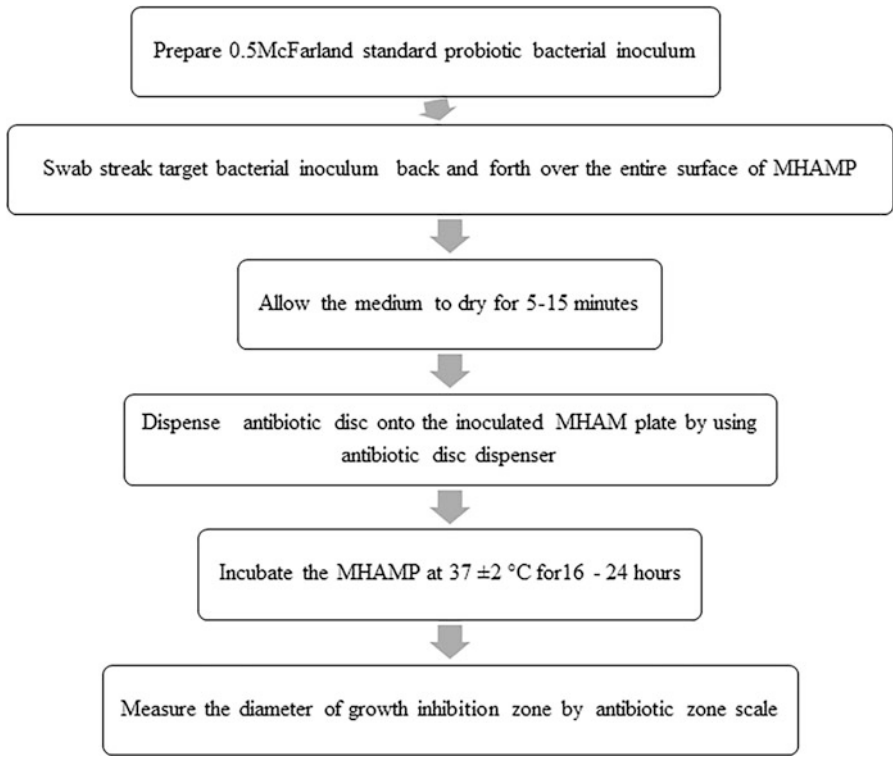

Fig. 1 Steps involved in the determination of antimicrobial susceptibility of probiotic bacteria by disc diffusion method

3.2 Detection of Antibiotic Resistant Gene in Probiotic Bacteria by Mutiplex PCR

3.2.1 Preparation of PCR Reaction Mix

1. Prepare the 25 µL of PCR reaction mix by adding 12.5 µL 2× Multiplex PCR Master mix, 2 µL of stock primer mix, 2 µL of template DNA (Probiotic) /negative control/Positive control [*tet (A) / tet (K)/tet (L)*] in a PCR tube.
2. Make the volume to 25 µL with Molecular Biology Grade Water.
3. Mix the contents of the tube by vortex.
4. Keep the tube in a thermocycler and set the program.

3.2.2 PCR Amplification Cycle

1. Perform Amplification in a thermocycler for 35 Cycles with the Following Reaction Conditions
   Initial denaturation at 94 °C for 10 min; Denaturation at 94 °C for 60 s; Annealing at 55 °C for 60 s; Extension at 72 °C for 60 s; Final Extension at 72 °C for 10 min, and Cooling at 4 °C.
2. Observe and confirm the presence of antibiotic resistance gene in PCR amplicon by 2% agarose gel electrophoresis.

3.2.3 Agarose Gel Electrophoresis

Loading of the PCR Amplicon

1. Load 5 µL of ready to use DNA ladder into the first well of 2% agarose gel.
2. Add 2 µL of 6× Gel loading buffer to 10 µL of PCR amplicons in negative control, probiotic sample, and positive controls [PC1 *tet(A)*, PC2 *tet(K)*, PC3 *tet(L)*].
3. Load 10 µL of negative control, test probiotic amplicon, and positive controls in the second, third, fourth, fifth, and sixth well.

Electrophoresis

1. Connect the power cord to the electrophoretic power supply.
2. Electrophorese the gel at 100–120 volts and 90 mA for 45 min or until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

Visualization

1. Carefully remove the gel from electrophoresis unit.
2. Keep the gel in a UV transilluminator and close the lid.
3. Illuminate the gel under UV light (254–366 nm) and observe for the presence of specific PCR amplicon (fluorescent band) [37] (Fig. 2).

4 Observation

4.1 Antimicrobial Susceptibility of Probiotic Bacteria by Disc Diffusion Method

Measure the zone of inhibition diameter with the aid of antibiotic zone scale (Himedia) and interpret the result (Table 2). Compare the inhibition zone diameter with reference to zone size interpretative chart as per CLSI standard and interpret the probiotic organism as sensitive, intermediate or resistant to the respective antibiotic (Fig. 3).

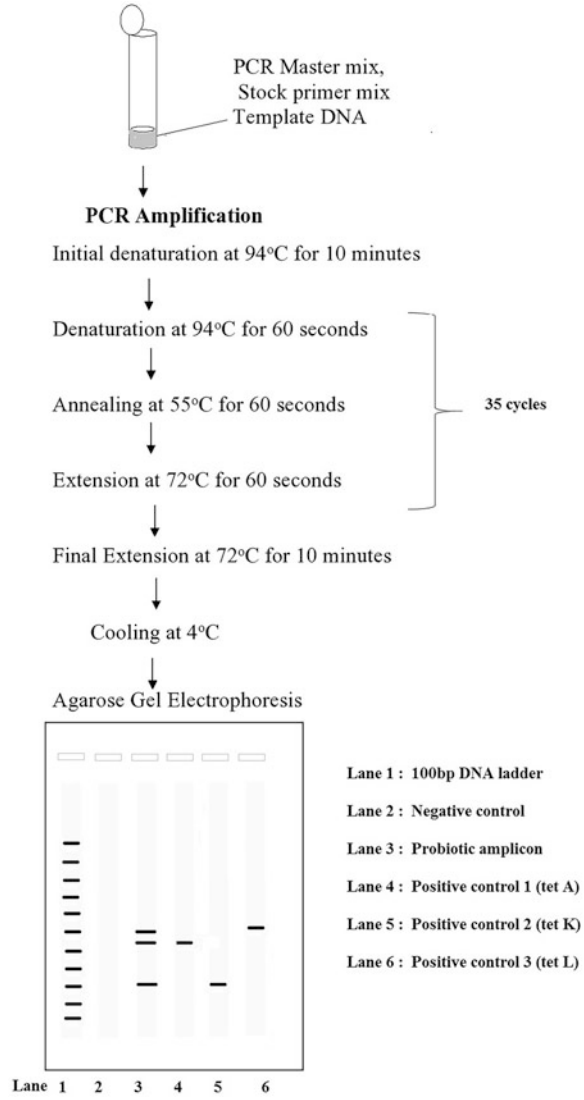

Fig. 2 Detection of antibiotic resistant gene in probiotic bacteria by Mutiplex PCR

4.2 Detection of Antibiotic Resistant Gene by Multiplex PCR

Presence of respective antibiotic resistance gene is interpreted as (+) and absence as (−) (Table 3). In the negative control no band appears indicating the absence of antibiotic resistance gene. Observation of one fluorescent band in each positive control amplicon confirms the presence of respective *tet(A)*, *tet(K)*, and *tet(L)* genes in PC1, PC2, and PC3. Presence of three distinct bands in the probiotic amplicon confirms three tetracycline resistance genes *tet (A)*, *tet(K)*, and *tet(L)* in the probiotic bacteria. No distinct fluorescent bands in the probiotic amplicon under UV indicates the absence of *tet(A)*, *tet(K)*, and *tet(L)* tetracycline resistance genes in the probiotic bacteria.

Table 2
Observation table for antimicrobial susceptibility test of probiotic bacteria by disc diffusion method

Probiotic bacteria	Antibiotic	Concentration (μg)	Zone of Inhibition diameter (mm)	Interpretation (sensitive/ intermediate/resistant)
PB1	Chloramphenicol	30		
PB2	Ciprofloxacin	30		
PB3	Erythromycin	15		
PB4	Gentamicin	50		
PB5	Norfloxacin	5		
PB6	Streptomycin	25		
PB7	Vancomycin	10		
PB8	Tetracycline	30		

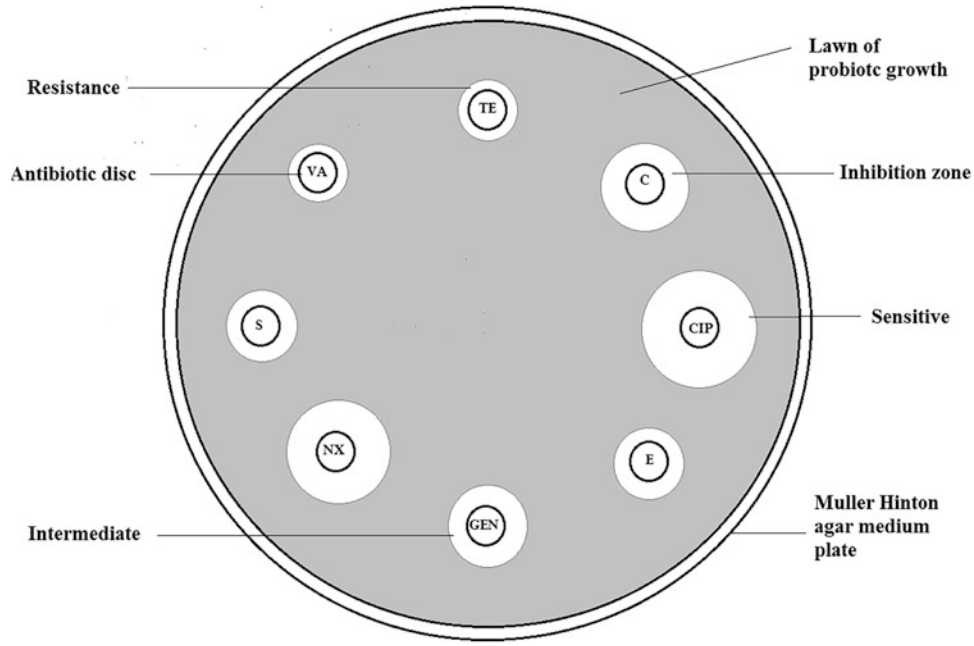

Fig. 3 Antimicrobial susceptibility test of probiotic bacteria by disc diffusion method

Table 3
Observation table for detection of antibiotic resistant gene in probiotic bacteria by multiplex PCR

	Antibiotic resistance gene		
Sample	***tet(A)***	***tet(K)***	***tet(L)***
Probiotic bacteria			
Negative control			
Positive control (PC1) - tet(A)			
Positive control (PC2) - tet(K)			
Positive control (PC3) - tet(L)			

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Chapter 11

Determination of Antibiotic Resistance Gene Transfer

K. R. Jeya, Ashraf Khalifa, M. Veerapagu, and A. Sankaranarayanan

Abstract

Probiotics are recognized as safe by the US Food and Drug Administration (USFDA) and the European Food Safety. But many probiotic bacteria serve as a reservoir of antibiotic resistance genes. Antibiotic resistance (ABR) in bacteria is recognized worldwide as an important threat to humans and animal health, and it is imperatively essential to know the mechanisms of antibiotic resistance together with its emergence and dissemination. An important way of transmission of antibiotic-resistant bacteria in humans and animals occurs through food especially consumption of fermented food containing live probiotic bacteria which serve as a vehicle for antibiotic resistance, with a direct link between the animal indigenous microflora and the human gastrointestinal tract (GIT). Therefore, the safety evaluation of probiotics for antibiotic resistance gene transfer has been assessed by conjugation filter mating assay.

Key words Probiotics, Fermented food, Antibiotic resistance, Safety evaluation, Conjugation filter mating assay

1 Introduction

Almost from a millennium of years fermented food consisting of probiotic live bacteria are consumed since most of them are known to produce lactic acid in the natural fermentation. They inhibit pathogenic microbes, reduce food spoilage, and extend the shelf life of food. Many lactic acid bacteria are a significant part of gut microbiota in a wholesome community and play a vital role in manifold metabolic processes. Numerous LAB are considered as GRAS (Generally Regarded As Safe by the US Food and Drug Administration (USFDA) [1]. However, many intestinal bacteria have antibiotic resistance genes having the potential to transfer to pathogens [2]. *Bifidobacteria* isolated from human, animal, and probiotic products have tetracycline resistance activity, and the *tetW* gene is dependable for drug resistance in entire strains [3].

Probiotic bacteria intercommunicate with native microbes in the gut and gene shift may occur. They may bestow antibiotic-resistant genes to another commensal or pathogen in the

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_11,

gastrointestinal tract. Hence, there is a potential risk of acquiring antibiotic resistance by pathogen and the consequent case of unsuccessful [4]. Horizontal gene transfer (HGT] or lateral gene transfer mechanism is associated with the dissemination of bacterial antibiotic resistance. HGT can operate as a significant mechanism in the prokaryotic gene transfer [5]. Well-organized mobile elements, namely plasmid, transposon, and integron containing antibiotic-resistant genes play a vital role in horizontal gene transfer and are amenable for the transfer of genetic material from one bacterium to another [6].

Conjugation seems to be the predominant mechanism in the transfer of one or more genes conferring resistance to a different group of antibiotics through conjugative transposon present in the chromosome or plasmid [7]. Human and animal intestinal microbes comprising of several thousands of bacteria which include opportunistic pathogens also acquire virulence genes [8]. The prime danger concomitant with antibiotic resistance in non-harmful bacteria is accordingly the risk of horizontal gene transfer of resistance to pathogens of humans and animals and impairs antibiotic treatment.

Antibiotic resistance is principal safety distress. The presence of antibiotic resistance genes in probiotics is not of primary concern as far as they are not transferable/mobilizable to other bacteria. Lactic acid bacteria are also subject to interexchange genetic determinants as a means to survive in an adverse antibiotic environment [9]. The prime interest, however, with the presence of antibiotic resistance in probiotic bacteria is their ability to transfer resistance to pathogenic organisms in vivo also. Lactic acid bacteria have been well characterized for the presence of several antibiotic resistance genes on the plasmid or transposons [10].

Probiotics can also disperse antibiotic resistance factors, hence the risks associated need to be considered before its commercial exploitation. In this view, the safety of probiotic bacteria has been evaluated for the transfer of antibiotic resistance genes by conjugation mating assay. In conjugation mating experiment antibiotic-resistant donor and antibiotic susceptible recipient bacteria are cultivated independently, later combined in the liquid medium, solid agar medium, filters, and or in animals [11] and permitted to conjugate for a limited time. Afterwards, conjugation frequency is measured by the ratio of the average number of transconjugants to the initial average number of donors or recipients by selective plating [12]. Traditional in vitro conjugation mating experiments have shown that LABs are capable of transferring their resistance determinants to other bacterial species [13]. Safety assessment of probiotics for antibiotic resistance gene transfer by employing conjugation mating assay reported in several literatures are listed in Table 1.

Table 1
Evaluation of antibiotic resistance gene transfer in probiotic bacteria by conjugation mating assay

Probiotic donor	Antibiotic Resistance gene	Recipient	Transconjugant frequency	References
L. mesenteroides subsp. *dextranicum* LbE15	Erythromycin erm(B)	*E. faecalis*	3.2×10^{-8}	Florez et al. [14]
L. helveticus and *L. casei*	Ciprofloxacingyr(A) Tetracycline tet(W)	*L. lactis* MG1614, *E. faecalis*	No transconjugants	Guo et al. [15]
E. faecium M3G	Erythromycinerm(B), Tetracyclinetet(M), tet(L), vancomycin	*E. faecalis* JH2–2	6.72×10^{-7},	Preethi et al. [16]
L. plantarum S11T			2.16×10^{-7}	
L. fermentum	Erythromycinerm(B), and Tetracyclinetet(K) and tet(M)	*S. aureus*, *S. epidermidis*, *L. monocytogenes*, *A. baumannii*, *C. freundii*, and *E. coli*	No transconjugants	Anisimova et al. [17]
E. faecium 37BA, *E. faecalis* 13BA, *E. faecalis* 27TO, *and E. avium* 35PA	Erythromycinerm(B) and erm(A)	*E. faecalis* JH2–2	$5.7 \times 10^{-9}$to 1.7×10^{-6}	Milanovic et al. [18]
L. delbrueckii subsp. bulgaricus R6	Tetracycline tet(M), tet(S)	*L. monocytogenes*	7.3×10^{-7}	Yang & Yu [19]
L. plantarum R41			2.9×10^{-6}	
Lactobacillus salivarius(CHS-1E *and* CH7-1E)	Erythromycin erm(B) and Tetracyclinetet(M), tet(L), and tet(W)	*E. faecalis* JH2–2	$1 \times 10^{-4}$and 3.8×10^{-3}	Thumu et al. [20]
Lactobacillus reuteri(CH2–2)			2×10^{-3}	
Enterococcus faecium	Vancomycin van A	*E. faecalis*	Transconjugants appeared	Terra et al. [21]
Lactobacillus bulgaricus spp.	Kanamycinaph(3″)-III, streptomycinaad(A), ciprofloxacingyr(A), trimethoprimdfrA	*Lactobacillus bulgaricus* spp.	No transconjugants	Guo et al. [22]
Enterococcus strains	Tetracyclines(tetM, tetL, tetK, tetO, and tetW) Aminoglycosidesaac(6′)-Ie-aph(2″)-Ia, aph(2″)-Ib, aph(2″)-Ic,aph(2″)-Id, ant(4′)-Ia, ant(6′)-Ia Macrolides(ermA, ermB, ermC, msrC, mefAB)	*E. faecalis* JH2–2	1.3×10^{-6} to 8.7×10^{-7} 1.7×10^{-6} to 3.1×10^{-8} $3.2 \times$ 10–6 to $2.4 \times$ 10–8	Chajęcka-Wierzchowska et al. [23]
Bifidobacterium animalis subsp. lactis AD011	Tetracycline	*L. fermentum*	No transconjugant	Ku et al. [24]

(continued)

Table 1
(continued)

Probiotic donor	Antibiotic Resistance gene	Recipient	Transconjugant frequency	References
E. faecalis VA02-2, *S. anginosus* VA01-10AN	Tetracycline tet(L) tet(M)	*L. lactis* subsp. *Cremoris* MG1614, *L. lactis* subsp. *lactis* biovar. *diacetylactis* Bu2-60, *E.faecalis*OG1RF, *L. plantarum* NC8	No transconjugants	Sirichoatet al. [25]
S. thermophilus M17PTZA496	Tetracycline tet(S)	*L.rhamnosus*CG, *L. delbrueckii* DSM20,355	No transconjugants	Tarrah, et al. [26]
Weissellacibaria CMU	Vancomycin Kanamycin	*E. faecalis* *L. rhamnosus*	No transconjugants No transconjugants	Kang et al. [27]

2 Materials

2.1 Equipment and Accessories

1. Conical flask.
2. Micropipette.
3. Water bath.
4. Membrane filters (0.45 μm).
5. Bunsen burner.
6. Inoculation loop.
7. Digital balance.
8. Automatic colony counter.
9. Autoclave.
10. Incubator.
11. Refrigerator.
12. Vortex mixer.
13. L-Spreader.
14. Petri plate.

2.2 Media, Reagents and Recipes

2.2.1 Brain Heart Infusion Broth (BHIB)

Dissolve 3.7 g of Brain Heart Infusion broth (Himedia) as per manufacturer recommendation and add 100 mL of distilled water. Completely dissolve the medium on a hot plate. Dispense 10 mL aliquots in a tube and sterilize the medium in an autoclave for 15 min at 121 °C.

2.2.2 MRS Broth (for Lactic Acid Bacteria -LAB)

Dissolve 5.515 g of MRS broth (Himedia) as per manufacturer recommendation and add 100 mL of distilled water. Completely dissolve the medium on a hot plate. Dispense 10 mL aliquots in a tube and sterilize the medium in an autoclave for 15 min at 121 °C.

2.2.3 Peptone Physiological Saline (PPS)

Dissolve the ingredients in 100 mL of distilled water. Dispense 10 mL aliquots in a tube and sterilize in an autoclave for 15 min at 121 °C.

2.2.4 Tetracycline (Tet)

Dissolve 60 mg of tetracycline in 2 mL of 70% ethanol and mix well to give a final concentration of 30 mg/mL. Store it in a refrigerator until use and can be used within a month.

2.2.5 Rifampicin (Rif)

Dissolve 0.3 g of rifampicin in 10 mL of 100% methanol completely to get a concentration of 30 mg/mL and store it in a refrigerator.

2.2.6 Physiological Saline

Dissolve 0.85 g of sodium chloride in 100 mL of distilled water. Autoclave it at 121 °C for 15 min. Cool to room temperature.

2.2.7 Brain Heart Infusion Agar (BHIAM)

Dissolve 53 g of Brain Heart Infusion agar (Himedia) as per manufacturer recommendation and add 1000 mL of distilled water. Completely dissolve the medium on a hot plate. Dispense 200 mL aliquots in a conical flask and sterilize the medium in an autoclave for 15 min at 121 °C.

Add the 0.2 mL of tetracycline solution, rifampicin solution and both tetracycline and rifampicin solution separately to the sterile BHIA medium, when it is around 65 °C to get the final concentration of (30 μg/mL) and aseptically pour approximately 25 mL of each medium to sterile petriplate. Allow the medium to solidify under a laminar airflow cabinet for about 20–30 min. Mark the plate as BHIAM+(Tet) plate, BHIAM+(Rif) plate, BHIAM +(Tet + Rif) plate.

2.3 Microbial Culture

1. **Donor probiotic bacteria**: (Tet^R,Rif^S), i.e., resistant to antibiotic Tetracycline and sensitive to rifampicin (R) (*see* **Note 1**).
2. **Recipient bacteria**: *Enterococcus faecalis* JH2–2plasmid-free recipient(Tet^S,Rif^R), i.e., sensitive to antibiotic tetracycline and resistant to rifampicin (Rif)(*see* **Notes 1** and **2**).

3 Method

The diagrammatic representation of determination of antibiotic resistance gene transfer in probiotics by conjugation filter mating method has been shown in Fig. 1. The method involves the following steps:

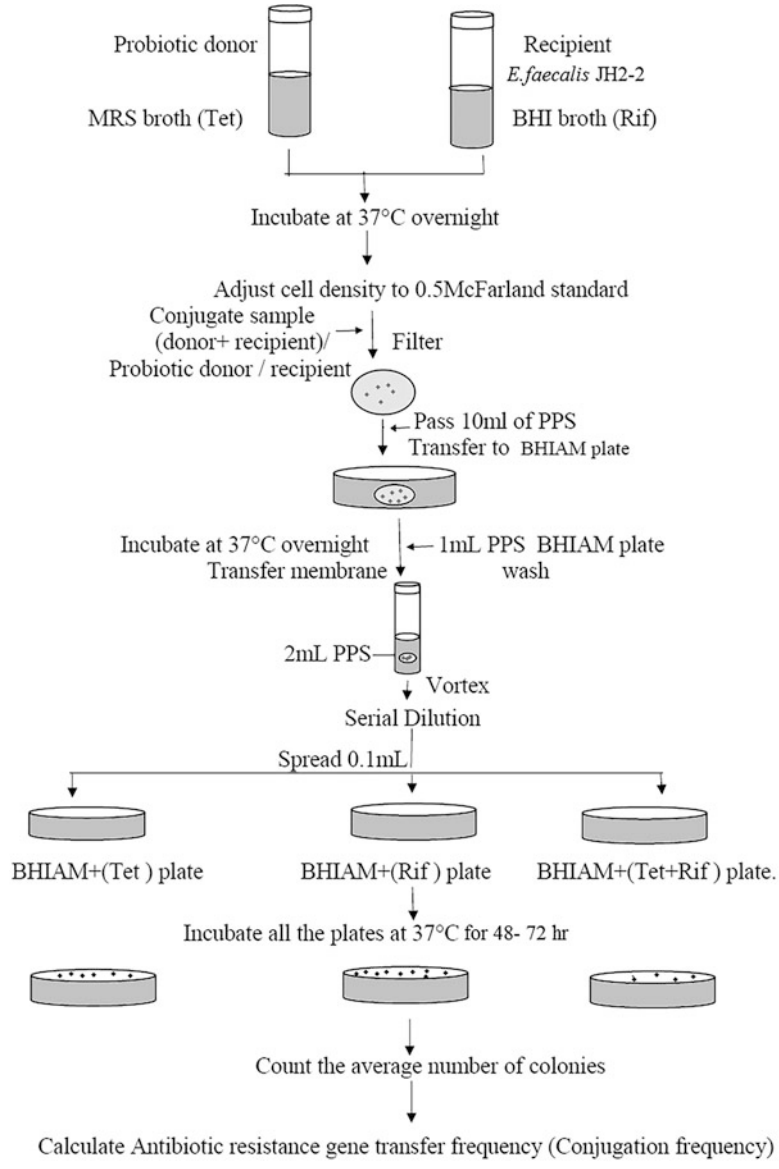

Fig. 1 Determination of antibiotic resistance gene transfer in probiotics by conjugation filter mating method

1. Inoculate a pure colony of donor probiotic bacteria (LAB) into 10 mL of sterile MRSbroth with tetracycline (30 μg/mL) and incubate at 37 °C for overnight.
2. Inoculate a pure colony of recipient bacteria into 10 mL of sterile BHI broth with rifampicin (30 μg/mL) and incubate at 37 °C for overnight.
3. Adjust the cell density of recipient and donor to 0.5 Mc Farland standard using sterile physiological saline (0.85%w/v) [28].

4. Transfer 0.5 mL of donor probiotic culture and 0.5 mL of recipient culture to a sterile tube.
5. Filter the culture mix through a sterile nitrocellulose membrane filter 0.45 μm pore size.
6. Pass 10 mL of sterile peptone physiological saline solutions through the membrane to entrap cells firmly onto the membrane.
7. Incubate the membrane with the cell side facing upward on the BHIAM plates at 37 °C overnight [15].
8. Aseptically transfer membrane to 2 mL of sterile peptone physiological saline solution in a sterile tube.
9. Vigorously mix in a vortex mixer to separate all cells from the membrane.
10. Wash the plate with 1 mL of sterile peptone physiological saline solution and add it to the above tube [27].
11. Serially dilute it appropriately and label the tube as a transconjugant sample.
12. Perform the same procedure for donor culture and recipient culture separately and label the tube as donor and recipient properly.
13. Aseptically transfer 100 μL of culture from each tube into three plates, i.e., BHIAM+(Tet) plate, BHIAM+(Rif) plate, BHIAM +(Tet + Rif) plate. Spread the culture and incubate the plates at 37 °C for 48–72 h.
14. Perform the assay in triplicates.
15. Observe BHIAM plates for the growth of bacterial colonies.
16. Count the average number of colonies by using an automatic colony counter (Table 2).

Table 2
Observation table for assessment of antibiotic-resistant gene transfer in probiotics by conjugation filter mating assay

	The average number of colonies (CFU/mL)		
Sample	**BHIAM + (Tet)**	**BHIAM + (Rif)**	**BHIAM + (Tet + Rif)**
Transconjugant			
Probiotic donor			
Recipient			

17. Calculate antibiotic resistance gene transfer frequency (conjugation frequency) which is expressed as the ratio of an average number of transconjugant colonies on BHIAM+(Tet + Rif) plate by an average number of recipient colonies on BHIAM + (Rif) plate by the given formula [29].

$$\text{Antibiotic resistance gene transfer frequency(Conjugation frequency)} = \frac{\text{Avg. no. of Transconjugant colonies (CFU/mL)}}{\text{Avg. no. of Recipient colonies (CFU/mL)}}$$

4 Interpretation

1. Probiotic donor bacteria resistant to tetracycline antibiotic growth occurs on BHIAM + (Tet) plate and no growth on BHIAM + (Rif) plate and BHIAM +(Tet + Rif) plate.
2. Recipient bacterial strain resistant to antibiotic rifampicin and sensitive to antibiotic tetracycline growth occurs only on BHIAM + (Rif) plate and no growth on BHIAM + (Tet) plate and BHIAM +(Tet + Rif) plate.
3. Transconjugants formed by the transfer of a tetracycline-resistant gene from donor probiotic bacteria to the recipient growth occurs on all the BHIAM plates. The presence of colony growth on BHIAM + (Tet + Rif) plate with both antibiotic tetracycline and rifampicin confirms the ability of probiotic bacteria to transfer antibiotic resistance genes to the recipient bacteria by conjugation (*see* **Note 3**).

5 Notes

1. Perform the antibiotic resistance pattern and MIC of probiotic donor strain and recipient bacterial strain by the standard method as recommended by CLSI to select the antibiotic and concentration of antibiotic.
2. Select the plasmid-free/ plasmid cured recipient bacterial strain to prevent the transfer of an antibiotic-resistant gene from the recipient to donor bacterium.
3. Growth of donor and recipient bacterium on BHIAM +(Tet + Rif) plate may occur by spontaneous mutation. Differentiate and confirm the transconjugant colonies from mutant by detecting the presence of antibiotic resistance gene using PCR or other molecular methods.

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Chapter 12

Determination of Toxin Production

Urjita V. Sheth

Abstract

Nowadays, strains of the *Bacillus* species are widely used as probiotics for human beings, animal feed as well as plant promoting bacteria. Probiotics based on *Bacillus* strains are gaining increasing attention as prophylactic and therapeutic agents for several gastro-intestinal diseases. Probiotic organisms are consumed as live organisms and *Bacillus cereus* and other *Bacillus* species are found to cause two types of food poisoning, emetic [by a small cyclic polypeptide (cereulide)] and diarrheal (by three different enterotoxins). Therefore, safety evaluations of the probiotic strains are necessary. Efforts are made to assess the safety of *Bacillus* strains for their supposed virulence factors. Various methods for the detection of bacterial toxins are animal model based in vivo assays, tissue culture assays or biochemical techniques but these methods are expensive, time-consuming, and labor intensive. Commercially available kits can be used for easy and rapid detection of bacterial toxins. Presently, three commercial kits for *Bacillus cereus* enterotoxins Nhe and/or Hbl detection are available, namely, the Bacillus diarrheal enterotoxin visual immunoassay (BDE-VIA) kit (3M Tecra), *B. cereus* enterotoxin reversed passive latex agglutination (BCET-RPLA) kit (Oxoid), and the Duopath® Cereus Enterotoxins (Merck). These kits can be used to monitor food quality.

Key words Enterotoxins, TECRA-VIA, BCET-RPLA, Duopath®, ELISA, Immunochromatography

1 Introduction

Currently, variety of microorganisms including lactic acid bacteria (LAB; such as species of *Lactobacillus*, *Streptococcus,* and *Enterococcus*), *Bifidobacterium, Propionibacterium, Bacillus,* and *Escherichia coli* with variety of health benefits are used as probiotics. These probiotics are live when administered unlike other food or drug ingredients and can possess the potential for infectivity or in situ toxin production. Thus, the safety of probiotics must be evaluated. Since numerous types of microbes are used as probiotics, safety is also intricately tied to the nature of the specific microbe being used [1]. One of the safety aspects include evaluation of toxigenicity which must be assessed according to the genus and species of the probiotics. For instance, few of the widely used *Bacillus* species such as *Bacillus cereus*, *B. thuringiensis*, *B. mycoides, B. anthracis,*

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_12,

B. weihenstephanensis, B. pseudomycoides have the capacity to produce toxins [2–4]. These Bacilli enterotoxins can cause food poisoning with main symptoms being diarrhea and vomiting [5, 6] and emetic syndrome. The main causative agents for the diarrheal syndrome are the two enterotoxin complexes, the hemolysin BL (Hbl; [7]) and the non-hemolytic enterotoxin (Nhe; [8]), as well as a single protein, cytotoxin K (cytK1; [9, 10]) and for emetic syndrome the causative agent is a cyclic peptide, cereulide [11]. The Hbl consists of lytic proteins L2 and L1 as well as binding protein B, encoded by hblC, hblD, and hblA, respectively [12, 13]. Protein components NheA, NheB, and NheC of the non-hemolytic enterotoxin complex are transcribed from genes *nheA, nheB,* and *nheC* [14]. The non-ribosomal production of cereulide is encoded by peptide synthetase genes (*ces* genes; [15, 16]).

Multiple detection methods for enterotoxins exist such as biological assays, mass spectrometry analysis, etc. Conventional methods rely upon in vivo testing procedures such as animal feeding, guinea pig's skin reaction, mouse lethality, rabbit ileal loop, and vascular permeability reaction (rabbit skin). These biological assays are functional assays which can determine the overall toxicity, i.e. toxicity by all biologically active toxins with high sensitivity. However, the downside of biological assays is the uncertain specificity, resulting in false-positive results for samples which contain other toxins. Besides cytotoxicity assays utilizing tissue culture cells (HeLa, CHO, Vero, and HEL), have also been used for the detection of the diarrheal toxin [17]; however, positive cytotoxicity assay needs further analysis to determine the specific enterotoxin produced by the test organism. For instance, enterotoxins Nhe, Hbl, and CytK all are toxic for Vero cells [18]. The further identification of *B. cereus* cereulide [19] and other *B. cereus* enterotoxins [20] can be done by liquid chromatography mass spectrometry (LC-MS) assay with high specificity and sensitivity, but its disadvantages include extensive, labor-intensive sample preparation and high investment, running, and maintenance costs. Due to these drawbacks fast and reliable enterotoxins detection methods are needed. Molecular biological methods and immunoassays which can provide consistent results can be implemented for the same. The relatively fast, easy, and cheap immunological detection of enterotoxins makes it suitable for research and routine analysis. Their sensitivity and specificity for a particular toxin are high, and depends upon the antibody quality [21].

Currently, three commercial kits for *Bacillus cereus* enterotoxins Nhe and/or Hbl detection are available. The TECRA *Bacillus* Diarrheal Enterotoxin Visual Immunoassay (BDE-VIA) is manufactured by Bioenterprises Pty Ltd. (Roseville, Australia) and

Table 1
Comparison of commercial test kits available for enterotoxin detection

	TECRA-VIA	BCET-RPLA	Duopath®
Target toxin component	Nhe-AB	Hb-1-L2	Nhe-B and Hbl-L2
Principle	Immunoassay	RPLA	Immunosorbent assay
Duration of the test (h)	4–5	20–24	0.5
Sensitivity/ detection limit (ng/ml)	<1 ng/ml of prepared sample	2 ng/ml of prepared sample	>1 *B. cereus*/g or ml of food sample
Minimal sample volume (μ)	200	50	150
Advantages	Simple and easy to use Automation is possible for large scale		Detects both Nhe and HBl Easy to use Control whether the test is conducted correctly in included Rapid and sensitive screening No false positive result due to cross-reactivity with non-toxic exoproteins No apparatus necessary for reading the result
Disadvantages	Only detection of the Nhe proteins (Nhe A component)	Only detection of HBl protein (L2 component)	
References	[21, 27, 28]	[21, 25]	[26, 29]

supplied by TECRA diagnostics (Batley, UK). It detects mainly NheA (45-kDa) protein. The kit is a microtiter plate-based immunoassay, which can be read visually or with an automated platereader. OXOID (Basingstoke, UK) markets the *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination (BCET-RPLA) kit, which is manufactured by Denka, Japan, and it is specific to the HblC (L2) component, and the Duopath Cereus Enterotoxins (Merck) [22, 23]. Overview of common commercial kits available for the detection *of B. cereus* diarrheal enterotoxins is given in Table 1 [22]. In this chapter, methods for three immunological kits for detection of *B. cereus* enterotoxins components Nhe-A, Nhe-B, and Hbl-L2 are mentioned.

1.1 Bacillus Diarrheal Enterotoxin Visual Immunoassay (BDE VIA) Kit (3M Tecra)

The TECRA *Bacillus* Diarrheal Enterotoxin (BDE) is a visual immunological ELISA assay (BDE-visual immunoassay (VIA)), developed for rapid in vitro detection of *B. cereus* diarrheal enterotoxin in food and food-related samples. It makes use of a sandwich ELISA [21].

1.1.1 Principle [24]

This kit is comprised of a microtiter plate coated with antibodies which react with the enterotoxin. When the test sample is added, it binds to the antibodies on the plate if having the desired enterotoxin. Excess of unbound material is thoroughly washed off. Then a second antibody with specificity to other epitope of the enterotoxin is added. This second antibody has an enzyme attached to it. The unbound second antibody is thoroughly washed off. Furthermore, if the second antibody binds to the antigen on the plate, upon addition of enzyme substrate, it produces a color that gives a measure of the amount of antigen present. The result may be read by eye (a simple presence or absence) or the optical density (O.D.) may be read in a plate reader. A standard curve of toxin will make the assay more quantitative. This standard curve can be produced in the laboratory from a series of dilutions of a culture supernatant of a known toxigenic strain.

1.2 BCET-RPLA Toxin Detection Kit

BCET-RPLA toxin detection kit was developed with the purpose of detecting the diarrheal enterotoxin by reversed passive latex agglutination (RPLA), which enables the soluble antigen such as bacterial toxins to be detected in an agglutination assay. In contrast to the standard agglutination assay where the soluble antibody reacts with particulate antigen such as bacterial cells, in reversed agglutination assay, the antibodies attached to particles, react with the soluble antigen. The particles (in this case, latex) are passive as they do not take part in the reaction and the cross-linking of the latex particles by the specific antigen/antibody reaction results in the visible latex agglutination reaction. The BCET-RPLA test may be used to detect *B. cereus* enterotoxin in a variety of foods and to give a semi-quantitative result. The test may also be used to demonstrate enterotoxin production by isolates of *B. cereus* grown in culture.

1.2.1 Principle [25]

Polystyrene latex particles are sensitized with purified antiserum taken from rabbit immunized with purified *B. cereus* diarrheal enterotoxin. If *B. cereus* enterotoxins are present, the antisera sensitized latex particles will agglutinate. The latex particles sensitized with non-immune rabbit globulins are provided as control reagent. The test is performed in V-well microtiter plates. Dilutions of the culture filtrate are made in two rows of wells and a volume of the appropriate latex suspension is added to each well and the contents are mixed. A diffuse layer on the base of the well will be formed upon settling, if *B. cereus* enterotoxin is present and

agglutination occurs due to the formation of lattice structure. A tight button will be observed, if *B. cereus* enterotoxin is absent or at a concentration below the assay detection level, as no such lattice structure can be formed.

1.3 Duopath® Cereus Enterotoxins (Merck)

Duopath® Cereus Enterotoxins is an immunological screening and confirmation test for the detection of *B. cereus* enterotoxins. It is based on the immune flow principle. It is less time consuming and less labor intensive.

1.3.1 Principle [26]

Duopath® Cereus Enterotoxins is an immunochromatographic rapid test. It is based on gold-labeled antibodies. The test device has a circular sample port, and an oval shaped test (NHE, HBL) and control (C) window. When the sample to be tested is applied to the circular sample port, the sample will be absorbed through the chromatographic pad and move to reaction zone containing colloidal, gold-labeled antibodies specific to NHE and HBL enterotoxins of *B. cereus*. If the enterotoxin is present, the antigen and gold-labeled antibody will migrate through the port until it encounters the binding zone in the test area. In binding zone, another antibody for Nhe and another antibody for HBL are located which immobilize any enterotoxin-antibody complex present. Because of the gold-labeling a red line appears when enterotoxins are present. The rest of the sample continues to migrate to another binding reagent zone within the control (C) zone, and also forms a further distinct red line (positive control). Regardless of whether any enterotoxin is present or not, this distinct red line is always formed in the control (C) zone, thus ensuring the test is working correctly.

2 Materials

2.1 Enterotoxin Detection Kits [25–27]

1. The TECRA *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (BDE-VIA) manufactured by Bioenterprises Pty Ltd. (Roseville, Australia) and supplied by TECRA diagnostics (Batley, UK).
2. *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination (BCET-RPLA) kit manufactured by Denka, Japan and marketed by OXOID (Basingstoke, UK).
3. Duopath Cereus Enterotoxins (Merck).

2.2 Media for Bacterial Culture Growth

1. Brain Heart Infusion (BHI) broth with 1% glucose (BHIG).

2.3 Bacillus Diarrheal Enterotoxin Visual Immunoassay (BDE VIA) Kit (3M Tecra) [27]

2.3.1 Kit Components

1. Target (BDE) specific antibody captured 96-Well plate.
2. Positive control.
3. Diluent.
4. Conjugate, i.e. enzyme linked secondary antibodies specific to BDE.
5. Substate specific to enzymes for color development.
6. Wash solution.

2.3.2 Materials Required but Not Provided with the Kit

1. Moisture box.
2. Micropipettes with capacity to dispensing 200 µl of the content.
3. Sterile Distilled/deionized water.
4. ELISA plate reader.

2.4 B. Cereus Enterotoxin Reversed Passive Latex Agglutination (BCET-RPLA) Kit (Oxoid) [25]

2.4.1 Kit Components

1. Sensitized Latex: Latex sensitized with specific *B. cereus* anti-enterotoxin (rabbit lgG).
2. Latex control: Latex suspension sensitized with non-immune rabbit globulins.
3. Enterotoxin control (lyophilized): Lyophilized *B. cereus* enterotoxin.
4. Diluent: Phosphate buffered saline containing bovine serum albumin.

2.4.2 Materials Required but Not Provided with the Kit

1. Microtiter plates; V-well microtiter plates (Greiner).
2. Fixed or variable pipette and tips (25 µl).
3. Centrifuge capable of generating 900 × *g* (typically 3000 rpm in a small bench top centrifuge) or membrane filtration unit using low protein-binding disposable filters with a porosity of 0.2 µm to 0.45 µm.
4. Moisture box.

2.5 Duopath Cereus Enterotoxins [26]

2.5.1 Materials Required but Not Provided with the Kit

1. Incubator.
2. Distilled or deionized water.
3. Autoclave.
4. Micropipettes with capacity to dispensing 150 µl of the content.

3 Methods

3.1 Sample Preparation

1. Inoculate a loopful of biomass of test organism from a 24 h grown culture in BHIG broth.
2. Incubate at 32–37 °C for 6–18 h, preferably with shaking (250 cycles/min) (*see* **Note 1**).
3. After growth, either centrifuge at 900 × *g* for 20 min at 4 °C or membrane filters using a 0.2 μm–0.45 μm low protein-binding filter.
4. Retain the filtrate for assay of toxin content.

3.2 Bacillus Diarrheal Enterotoxin Visual Immunoassay (BDE VIA) Kit (3M Tecra) [27]

3.2.1 Reagent Preparation

1. Prepare Kit reagent: positive control, conjugate, and substrate, in accordance with the instructions of the manufacturer, supplied with the kit.
2. The positive control, diarrheal enterotoxin (BDE) antigen which the TECRA kit reacts against, comes in a dried format; therefore, it must be resuspended using the supplied diluent.
3. The diluent which is used to resuspend the positive control acts as the negative control.
4. The conjugate is an enzyme labeled antibody, specific for BDE. The conjugate binds to associated BDE, and its enzyme reacts with the substrate. This enzyme labeled antibody is specific for the antigen, i.e., the diarrheal enterotoxin.
5. The substrate is initially colorless, but the conjugate enzyme can cleave the substrate producing a green coloration.

3.2.2 Assay Procedure

1. Fill the test wells with wash solution supplied with the kit. Allow it to stand for 10 min at room temperature (20–25 °C). This is to presoak the antibody coated test wells.
2. Empty the wells and add samples (200 μl) and positive and negative controls (200 μl) in their respective wells. Cover the wells to avoid evaporation and incubate for 2 h at 37 °C.
3. Empty wells and wash four times using the wash solution provided in the lit.
4. Add 200 μl of conjugate to empty wells. Cover wells and incubate for 1 h at room temperature (20–25 °C) to allow the conjugate to bind.
5. Empty the wells and wash it for five times.
6. Add 200 μl of substrate to each well. The substrate is clear in color when it is added into the test wells. Incubate at room temperature (20–25 °C) for minimum of 30 min.
7. Observe for color change.

3.2.3 Observation of Result

1. If test sample has BDE, the conjugate binds to the toxin in the wells and substrate will react with the enzyme linked to the conjugate which changes its color from clear to green.
2. If after the incubation of 30 min, positive control well obtains the color indicated in color card or show absorbance of 1.0 at 405 nm, then add 20 μl of stop solution supplied with the kit to each well to prevent further color development.
3. Gently mix the content. Read the results against the provided color card and on a plate reader at 405 nm.
4. If after the incubation of 30 min, color of positive control well fails to reach to the color of color card supplied with the kit, extend the incubation for further 15 min. If color reached to desired, add 20 μl of stop solution. And then also, if it fails to get color in positive control well, the test is invalid.
5. For the test to be valid, also check for the negative well color. It should have the color fall within the specified range of negative control in color card or show <0.2 absorbance at 405 nm.
6. The sample can be considered as negative, if its color falls within the negative range on the color chart or if its absorbance reading is less than 0.2 at 405 nm. The sample can be considered as positive, if color of the well fall within the positive range on the color chart or if its absorbance reading is equal to or greater than 0.2 at 405 nm.

3.3 B. cereus *Enterotoxin Reversed Passive Latex Agglutination (BCET-RPLA) Kit (Oxoid) [25]*

3.3.1 Reagent Preparation

1. The latex reagents; Sensitized Latex, Latex control, and Diluent are ready to use. The latex reagents should be thoroughly shaken before use to ensure a homogenous suspension.
2. To reconstitute the enterotoxin control, add 0.5 ml of diluent to each vial. Shake gently until the contents are dissolved.

3.3.2 Assay Procedure

1. Two rows of 8 wells on a 96 V-well microtiter plate are required to test a cell free culture supernatant for toxicity using the OXOID kit. Arrange the plate, so that each row consists of 8 wells.
2. Add 25 μl of diluent in each well of the 2 rows except the first well in each row.
3. Add 25 μl of test sample to the first and second well of both rows.
4. Make serial dilutions of test sample using a pipette or diluter and starting at the second well of each row by withdrawing 25 μl with a pipette and adding the content in third well. Again from third well withdraw 25 μl and add to fourth well. Repeat the same till the seventh well of a row. From the seventh well discard 25 μl test solution after proper mixing.

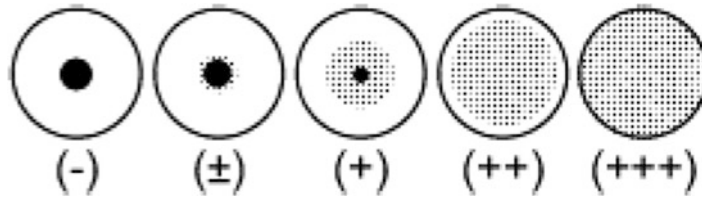

Fig. 1 Interpretation for *B. cereus* enterotoxin reversed passive latex agglutination (BCET-RPLA) kit (Oxoid)

5. Leave the 8th (last) well of raw containing diluent only. This is used as a negative control.
6. To each well in the first row, add 25 µl of sensitized latex.
7. To each well in the second row, add 25 µl of latex control.
8. To mix the contents of each well, rotate the plate by micro-mixer or agitate by hand. Take care that no spillage occurs from the wells. To avoid evaporation, cover the plate with a lid. Placing the plate in a moisture box is an acceptable alternative.
9. Leave the plate undisturbed on a vibration-free surface at room temperature for 20 to 24 h. It will help the subsequent reading of the test, if the plate is placed on black paper for the duration of this incubation.
10. Examine each well in each row for agglutination against a black background.
11. Discard all the used items properly (*see* **Note 2**).

3.3.3 Observation of Result

1. The agglutination pattern should be judged by comparison as shown in the Fig. 1.
2. Results classified as (+), (++) are considered as positive.
3. Results in the row of wells containing latex control should be negative. In some cases, non-specific agglutination may be observed. In such cases the results should be interpreted as positive, provided that the reaction with sensitized latex is positive to a higher dilution of test sample that seen with the latex control.
4. The last well in all rows should be negative. If positive patterns are observed in some of these wells, the reaction should be regarded as invalid.

3.4 Duopath® Cereus Enterotoxins (Merck) [26]

3.4.1 Assay Procedure

1. Take required number of Duopath® Cereus Enterotoxins strips and remove the foil pouches.
2. Place the test device on a flat surface and label with appropriate sample identification.
3. Add 150 µl of test sample in the circular sample port with the help of micropipette and disposable pipette tip.
4. Observe the test result 30 min after applying the sample to the device.

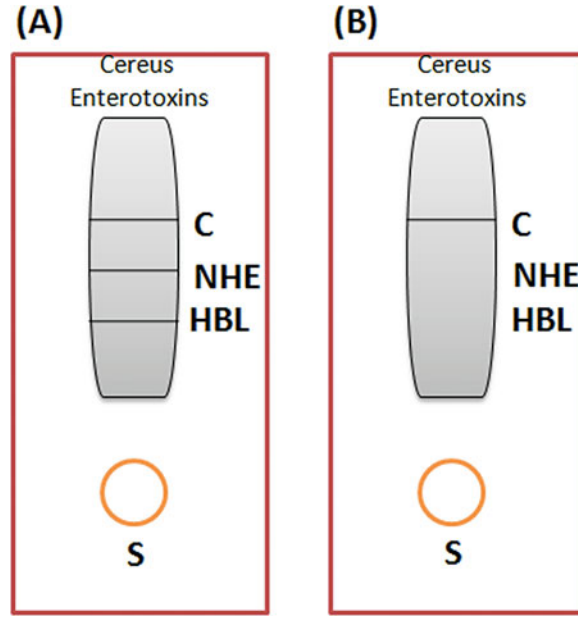

Fig. 2 (**a**) Positive result in Duopath® Cereus Enterotoxins strip test which is indicated by all three red lines, and (**b**) Negative result in Duopath® Cereus Enterotoxins strip test indicated by only one red line in C region

3.4.2 Interpretation of Results

1. If a distinct red line appears in the control zone (C) within 30 min, the test can be regarded as working correctly.
2. A sample can be considered POSITIVE if at or prior to 30 min, red lines appear on both test (NHE and/or HBL) and control (C) zones.
3. A sample can be considered NEGATIVE if no red line appears in the test (NHE and HBL) zone but does appear distinctly in the control (C) zone 30 min after application of sample to the device.
4. Figure 2a shows three red lines which indicate positive result and Fig. 2b shows only one line in "C" which shows negative result.

4 Notes

1. It is advisable to check the particular cultural method of use with a standard enterotoxin-producing strain such as *B. cereus* NCTC 11145.
2. Centrifuge tubes, membrane filters, microtiter plates, lids, and pipette tips should be sterilized by autoclaving at 121 °C for 15 min or disinfected, before disposal. Dispose of the culture extracts, food extracts, samples and enterotoxin controls in hypochlorite solution (>1.3% w/w).

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Chapter 13

Detection of Toxin Genes by PCR Based Methods

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Abstract

Recently, *Bacillus* species have gained wide acceptance to be used as probiotics for human consumption. *Bacilli* are Gram-positive, spore forming bacteria, commonly found in the soil, plants, and various food sources. Some *Bacillus* strains are reported as human pathogens. The food poisoning by the *Bacillus* species can be diarrheal or emetic type. Therefore, along with the characterization of probiotic characteristics, safety of the organism for their putative virulence factor is an important consideration. The diarrheal type food poisoning is caused by five different types of enterotoxins and emetic poisoning is caused by circular dodecadepsipeptide commonly known as "cereulide." All these toxins are coded by various genes and sequences of these genes are known. Polymerase chain reaction (PCR) is a rapid method used for the detection of the presence of toxins genes. This protocol chapter describes assessment of safety of the probiotic strains by detecting the genes encoding bacterial toxins by PCR method. These genes include five different enterotoxin genes (*nheA,B,C, hblCDA, entFM, cytK,* and *bceT*) and one emetic toxin, cereulide synthetase gene (*ces*). If the isolated bacterial strain with probiotic properties is found to have any of these genes, the bacterial strain cannot be considered as a safe for human consumption.

Key words PCR, *Bacillus* spp., Probiotic, Enterotoxin, Emetic toxin, Toxigenic pattern, Safety assessment, Toxin genes, *ces*, *nheA*, *nheB*, *nheC*, *hblCDA*, *entFM*, *cytK*, *bceT*

1 Introduction

The *Bacillus* genus is the heterogenic assemblage of Gram positive, endospore forming, rod shaped, and facultative anaerobic bacteria with widespread occurrence in the nature. Because of its endospore forming ability, they can endure adverse/unfavorable conditions and proliferate under variety of environmental conditions and found in the food production environments. These endospores are resistant to heat, desiccation, and disinfectants and therefore can contaminate many kinds of foods during various production stages and storage [1, 2]. Both the endospores and vegetative forms of *Bacillus* spp. are being marketed as probiotics now-a-days with advent of their ability to survive in extremes conditions of heat, gastric acid and moisture. *B. cereus*, *B. clausii*, *B. coagulans*,

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_13,

B. licheniformis, *B. polyfermenticus*, *B. pumilus*, and *B. subtilis* with antimicrobial, anticancer, antioxidant, and vitamin production properties are commercial *Bacillus* probiotic strains used currently. However, *Bacillus* probiotics can also produce toxins and biogenic amines and transfer antibiotic resistance genes; therefore, their safety is a concern [3]. Therefore, the major safety concern with the *Bacillus* probiotics is the risk of occurrence of food poisoning after the ingestion [4]. *Bacillus cereus* is the most important cause of food poisoning amongst the *Bacillus* species, as it produces enterotoxins and emetic toxin. Three different heat-labile enterotoxins, produced by *B. cereus* in the small intestine after ingestion are responsible for diarrheal type food-borne illness and emetic toxin (cereulide), produced during growth in food under variety of conditions are responsible for emetic symptoms [1]. Other *Bacillus* species such as *B. subtilis*, *B. pumilus*, and *B. licheniformis* are also recognized to produce both enterotoxins and emetic toxins [1, 5–7]. However, it is believed that the enterotoxins produced by *Bacillus* species other than *B. cereus* are proteins transcribed by genes similar to those of *B. cereus* enterotoxins [1]. There are five distinctive diarrheal type of food poisoning causing enterotoxins; enterotoxin T (encoded by gene bceT), cytotoxin K (encoded bygene CytK), enterotoxin FM (encoded by gene EntFM), non-hemolytic enterotoxin complex (composed of the protein constituents NheA, NheB, and NheC encoded by genes *nheA*, *nheB,* and *nheC,* respectively) [8], hemolysin BL (Hbl) complex (consists of the protein components B, L1, and L2 [9, 10], encoded by genes *hblA*(B), *hblC*(L2), and *hblD*(L1), respectively [9])(HblA, HblC, HblD) and one emetic toxin, a circular dodecadepsipeptide, known as "cereulide" (encoded by gene ces), causing the emetic type of food poisoning have been identified [11]. The diarrheal type of food poisoning causes abdominal pain and watery diarrhea after 8–16 h of the latent period [7]. The emetic type of food poisoning caused by *B. cereus* is characterized by vomiting and nausea [2].The appearances of both types of food poisoning are comparatively mild; occasionally it can be more severe which can results in death [12]. These toxins can cause disease due to their ability to induce necrosis of human tissues and/or gastrointestinal infections [13, 14]. Conventionally, cultural and biochemical methods were used to detect and identify production of emetic and enterotoxins which are time-consuming and labor intensive. For the detection of enterotoxins, and cytotoxicity commercially available immunoassay kits, such as BCET-RPLA (specific to HBL; Oxoid, Ogdensburg, N.Y.), can also be used [15–21].Currently, a rapid PCR method targeted for each toxin gene has been developed [14, 17–19, 22–25]. For detection of emetic toxin also, cytotoxicity assay was used initially [26], but now PCR method has been developed for rapid detection [27]. Even for detection of five different enterotoxins and one emetic toxin of *Bacillus cereus*, a multiplex PCR with 12 primers pairs has also been established [28].

2 Materials

2.1 Positive and Negative Enterotoxigenic Reference Strains [22]

Bacillus cereus DSM-31 (ATCC-14579 T) can be used as a positive enterotoxigenic reference strain and *Bacillus subtilis* subsp. *Spizizenii* DSM-347 (ATCC-6633) can be used as a negative enterotoxigenic reference strain to demonstrate the sensitivity of the assay.

2.2 Bacterial Culture Media for Maintaining Reference Strains

1. **Brain heart infusion (BHI)** (*see* **Note 1**): Readymade media is available from different manufacturer. The composition of BHI medium is as follows [29]:

Ingredients	Gms/Liter
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5
Final pH (at 25 °C)	7.4 ± 0.2

2. **Preparation of BHI medium**: Dissolve all the components in 600 mL of distilled water. Heat if necessary to dissolve medium completely and make up final volume up to 1 L. Dispense into bottles or tubes as desired. Sterilize by autoclaving at 15 lbs. (121 °C) for 15 min. Store the culture medium at 2–8 °C sealed in plastic bags to reduce the chances of contamination under dark.

2.2.1 DNA Isolation

Instruments and Equipment

1. Incubator shaker.
2. Centrifuge.
3. Refrigerator.
4. Micropipettes 1–100 µL and 100–1000 µL.

Reagents [30]

1. **TE buffer:** 10 mM Tris–HCI (pH 8.0), 1 mM Na_2EDTA, in sterile, deionized H_2O (sterilize by autoclaving).
2. **Sodium dodecyl sulfate (SDS; 10%)**: Dissolve 10 g SDS in 100 mL of sterile deionized water.
3. **Proteinase K (20 mg/mL)**: Weigh 0.02 g Proteinase K and dissolve in 1 mL of sterile deionized water. Store vialsin small single-use aliquots at −20 °C.
4. **NaCl (5 M)**: Prepare in sterile, deionized H_2O (sterilize by autoclaving).

5. **CTAB/NaCl solution**: 10% (w/v) hexadecyltrimethyl ammonium bromide in sterile 0.7 M NaCI solution. (Heat solution to 65 °C before bringing to final volume), 0.7 M NaCI in sterile, deionized H_2O (sterilize by autoclaving).
6. **24:1 Chloroform/isoamyl alcohol:** Add 24 volumes chloroform to 1 volume isoamyl alcohol.
7. **25:24:1 phenol/chloroform/isoamyl alcohol (PCI):**
   (a) **Saturate phenol using following method:**
   Phenol: 250 mL redistilled, Tris-equilibrated, phenol in TE Buffer (pH 8.0) [250 mL redistilled phenol (melted at 65 °C) and 0.25 g 8-hydroxyquinoline is equilibrated twice with 250 mL 50 mM Tris–HCI (pH 9.0); a final equilibration is made with 50 mM Tris–HCI (pH 8.0) -the pH of the phenol should be approximately 8.0; add 125 mL TE buffer for storage (covered with aluminum foil) at 4 °C].
   (b) **Preparation of 25:24:1 PCI:**
   - Isopropanol (2-propanol): Molecular Biology Reagent grade.
   - Ethanol (EtOH): 70% (v/v) in sterile, deionized H_2O.

2.2.2 PCR Amplification

1. Thermal cycler.
2. PCR buffer, 10×, supplied by the manufacturer of the DNA polymerase.
3. dNTPs.
4. $MgCl_2$.
5. Forward and reverse primers.
6. DNA template.
7. Taq DNA polymerase.
8. Q.S. sterile distilled water.
9. PCR tubes and caps.
10. PCR tube rack.
11. An ethanol-resistant marker.

2.2.3 Agarose Gel Electrophoresis

1. Electrophoretic assembly.
2. Microwave.
3. Agarose.
4. 10× TBE buffer: 108 g Tris + 55.65 g boric acid + 40 mL 0.5 M EDTA (pH 8.0) stored at room temperature.

5. 1× TBE buffer: take 100 mL of 10× TBE buffer and dilute it to 1000 mL by adding 900 mL of distilled water.
6. EtBr.
7. Gel loading dye (6× concentration): 0.25% bromophenol blue + 0.25% xylene cyanol + 30% glycerol in water Store at 4 °C.
8. Marker DNA: molecular size marker (100–1000 bp).

Preparation of 1% Agarose Gel

Weigh an appropriate amount of agarose to the appropriate volume of 1× electrophoresis buffer in a conical flask. For Making a 1% agarose Gel, weigh 0.5 g agarose and dissolve it in 50 mL of 1× TAE Buffer (*see* **Note 2**). Heat in a microwave or using hot plate to boiling consistency, rotate the flask occasionally, until the agarose is dissolved, marked with a clear solution. (If the volume reduces during heat due to evaporation, makeup the original volume with distilled water. This will ensure that the agarose concentration is maintained).

Sample Preparation

1. 8 μL of DNA sample (0.1 μg to 1 μg) and 2 μL of 5× gel loading dye.
2. Add 1 volume of sample buffer to 5 volumes of DNA sample and mix.

3 Methods

3.1 Bacterial DNA Isolation

Many methods have been published for the isolation of whole genomic DNA from bacteria and many kits are also available for the same. Here, Miniprep of Bacterial Genomic DNA is explained. The method use the following steps including the lysis of bacterial cells, removal of protein and other cell wall debris, polysaccharides, and extraction and purification of DNA [30, 31].

1. Take 1.5 mL of overnight grown bacterial culture in microfuge tube.
2. Spin in a microcentrifuge for 2 min, or until a compact pellet forms.
3. Discard the supernatant and resuspend pellet in 567 μL TE buffer by repeated pipetting.
4. Add 30 μL of 10% SDS and 3 μL of 20 mg/mL proteinase K to give a final concentration of 100 μg/mL proteinase K in 0.5% SDS.
5. Mix thoroughly and incubate 1 h at 37 °C till the solution become viscous.
6. Add 100 μL of 5 M NaCl and mix thoroughly (*see* **Note 3**).
7. Add 80 μL of CTAB/NaCl solution.
8. Mix thoroughly and incubate 10 min at 65 °C.

9. Add an approximately equal volume (0.7–0.8 mL) of chloroform/isoamyl alcohol, mix thoroughly, and spin 4 to 5 min in a microcentrifuge. A white interface (CTAB–protein/polysaccharide complexes) should be visible after centrifugation.
10. Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind.
11. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge for 5 min.
12. Transfer the supernatant to a fresh tube.
13. Add 0.6 mL isopropanol to precipitate the nucleic acids. Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. At this point it is possible to transfer the pellet to a fresh tube containing 70% ethanol by hooking it onto the end of a micropipette that has been heat-sealed and bent in a Bunsen flame. Alternatively, the precipitate can be pelleted by spinning briefly at room temperature.
14. Wash the DNA with 70% ethanol to remove residual CTAB and respin 5 min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet.
15. Redissolve the pellet in 100 μL TE buffer.

3.2 Detection of Enterotoxin Genes by PCR Amplification [32]

3.2.1 PCR Amplification

DNA sequences that coded enterotoxin can be obtained based on an alignment of the gene sequences publicly available for *B. toyonensis* BCT-7112T and *B. cereus* DSM-31 from National Center for Biotechnology (NCBI) GenBank (Bethesda, Massachusetts, USA) [22]. Conserved regions can be selected and using various software available, primer can be designed [28] (*see* **Note 4**). Reverse and Forward primers for the toxin genes, published in literature are listed in Table 1.

Primer Design

PCR Master-Mix

It is advantageous to prepare a mixture of reagents common to all reactions. This mixture is known as master mix. PCR Master-mix contains 1X Buffer, 200 μM dNTPs (50 μM of each of the four nucleotides), 15 mM $MgCl_2$(if it is not present in the buffer), 20–50 pmoles of each, forward and reverse primers, 1–1000 ng of DNA template, 0.5–2.5 units of DNA Polymerase per 50 μL of reaction and Q.S. sterile distilled water to obtain final decided volume. Calculated amount of ingredients to get final 50 μL of PCR Master-mix is shown in Table 2.

Pipette all the ingredients of PCR Master-mix in order as follows:

Sterile Water, 10× PCR buffer, dNTPs, $MgCl_2$, primers, and template DNA (*see* **Note 5**).

Table 1
Oligonucleotide Primer sequences for Diarrheal and emetic toxin genes

Gene	Primer Sequence (5′ → 3′)	Origin of DNA sequence	Annealing temperature (°C)	Product Size (bp)	References
bceT	F: CGT ATC GGT CGT TCA CTC GG R: TTT CTT TCC CGC TTG CCT TT	*Bacillus cereus*	55	924	[36]
	F: GAC TAC ATT CAC GAT TAC GCA GAA R: CTATGC TGA CGA GCT ACATCC ATA	*Bacillus cereus*	55	303	[2]
	F: CGTATCGGTCGTTCACTCGG R: GTTGATTTTCCGTAGCCTGGG	*Bacillus cereus*	55	661	[17]
CytK	F: ATC GGK CAA AAT GCA AAA ACA CAT R: ACC CAG TTW SCA GTT CCG AAT GT	*Bacillus cereus*		515	[28]
	F: ACAGATATCGG(G, T) CAAAATGC R: GAACTG(G, C)(A, T) AACTGGGTTGGA	*Bacillus cereus* *Bacillus thuringiensis*	54	809	[17]
	F: GTAACTTTCATTTGATGATC R: GAATACATAAATAATTGGT	*Bacillus cereus*		505	[1]
	F:CGACGTCACAAGTTGTAACA R: CGTGTGTAAATACCCCAGTT	*Bacillus cereus* *Bacillus thuringiensis*	58	565	[6]
EntFM	F: AAA GAA ATT AAT GGA CAA ACT CAA ACT CA R: GTATGTAGC TGG GCC TGT ACG T	*Bacillus cereus*		596	[37]
	F: GTTCGTTCAGGTGCTGGTAC R:AGCTGGGCCTGTACGTACTT	*Bacillus cereus* *Bacillus thuringiensis*	62	486	[6]
nheA	F: TAC GCT AAG GAG GGG CA R: GTT TTT ATT GCT TCA TCG GCT			499	[36]
	F: ATTACA GGG TTATTG GTTACA GCA GT R: AAT CTT GCT CCATACT CT CTT GGATGC T	*Bacillus cereus*		625	[28]
	F: GTTAGGATCACAATCACCGC R: ACGAATGTAATTTGAGTCGC	*Bacillus cereus*	56	755	[17]
	F: GTTAGGATCACAATCACCGC R: CCATATGCATTTGTAAAATCTG	*Bacillus cereus*		264	[1]

(continued)

Table 1
(continued)

Gene	Primer Sequence (5′ → 3′)	Origin of DNA sequence	Annealing temperature (°C)	Product Size (bp)	References
	F: TAAGGAGGGGCAAACAGAAG R: TGAATGCGAAGAGCTGCTTC	*Bacillus cereus* *Bacillus thuringiensis*		759	[6]
nheB	F: CTA TCA GCA CTT ATG GCA G R: ACT CCT AGC GGT GTT CC			769	[36]
	F: GTG CAG CAG CTG TAG GCG GT R: ATG TTT TTC CAG CTATCT TTC GCA AT	*Bacillus cereus*		328	[28]
	F: TTTAGTAGTGGATCTGTACGC R: TTAATGTTCGTTAATCCTGC	*Bacillus cereus*	54	743	[17]
	F:CGGTTCATCTGTTGCGACAGC R:GATCCCATTGTGTACCATTGG	*Bacillus cereus*		335	[1]
	F: CAAGCTCCAGTTCATGCGG R: ATCCCATTGTGTACCATTG	*Bacillus cereus* *Bacillus thuringiensis*		935	[6]
nheC	F: CGG TAG TGA TTG CTG GG R: CAG CAT TCG TAC TTG CCA A			581	[36]
	F: GCG GAT ATT GTA AAG AAT CAA AAT GAG GT R: TTT CCA GCT ATC TTT CGC TGT ATG TAA AT	*Bacillus cereus*		557	[28]
	F: TGGATTCCAAGATGTAACG R: ATTACGACTTCTGCTTGTGC	*Bacillus cereus*	54	683	[17]
	F:GCGATTGATCAAAAGGATAG R:CGACTTCTGCTTGTGCTCCTG	*Bacillus cereus*		411	[1]
	F: ACATCCTTTTGCAGCAGAAC R: CCACCAGCAATGACCATATC	*Bacillus cereus* *Bacillus thuringiensis*		618	[6]
hblA	F: AAG CAA TGG AAT ACA ATG GG R: AGA ATC TAA ATC ATG CCA CTG C		56	1154	[17, 36]
	F: GCA AAATCT ATG AAT GCC TA R: GCATCT GTT CGT AAT GTT TT	*Bacillus cereus*	54	884/ 1025	[6]
hblB	F: AAGCAATGGAATACAATGGG R: AATATGTCCCAGTACACCCG	*Bacillus cereus*	58	2684	[17]
hblC	F: GAT AC(T,C) AAT GTG GCA ACT GC	*Bacillus cereus*	58	740	[17, 36]

(continued)

Table 1
(continued)

Gene	Primer Sequence (5′ → 3′)	Origin of DNA sequence	Annealing temperature (°C)	Product Size (bp)	References
	R: TTG AGA CTG CTC G(T,C)T AGT TG				
	F: CCT ATC AAT ACT CTC GCA A R: TTT CCT TTG TTATAC GCT GC	*Bacillus cereus*		351	[6]
	F: AGAAACTCAACAAGAAAACA TGG R: TTGCGCAGTTGCCACATTAG	*Bacillus cereus*		444	[1]
	F:CCTATCAATACTCTCGCAA R: TTTCCTTTGTTATACGCTGC	*Bacillus cereus* *Bacillus thuringiensis*	54 56	695	[6]
hblD	F: ACC GGT AAC ACT ATT CAT GC R: GAG TCC ATA TGC TTA GAT GC	*Bacillus cereus*	58	829	[17, 36]
	F: GAA ACA GGG TCT CAT ATT CT R: CTG CAT CTT TAT GAATAT CA	*Bacillus cereus*		1018	[6]
Ces	F: TTG TTG GAATTG TCG CAG AG R: GTA AGC GAA CCT GTC TGTAAC AAC A	*Bacillus cereus*		405	[2]

[F, forward primer; R, reverse primer]

PCR Protocol

1. Use a 96 well plate to hold 0.2 mL thin-walled PCR tubes and place it on ice bucket to prevent nuclease activity and nonspecific priming.
2. Add 50 μL of PCR Master-mix.
3. Keep positive and negative control for PCR too.
   (a) PCR tube with all the reagents except template DNA can be used as negative control. Use sterile distilled water instead of template DNA.
   (b) Positive control contains all the reagents but the primer used for the reaction is previously known to amplify under the same conditions as the experimental PCR tubes.
4. Gently mix the contents well by pipetting up and down at least 20 times.
5. Cap PCR tubes and place them into the thermal cycler. Close the lid of thermal cycler and start program.

   Setting up of Thermal Cycling Conditions:

   Thermal cycle heat and cool reaction mixture in order to have heat-induced denaturation of duplex template DNA and

Table 2
PCR reagents in order and in amount they should be added to get 50 μL final solution

Ingredients	Concentration of stock	Working concentration	Amount to be added
Sterile distilled water			Q.S. to 50 μL
PCR buffer	10×	1×	5 μL
dNTP's	10 mM	200 μM	1 μL
$MgCl_2$	25 mM	1.5 mM	3 μL
Forward primer	20 μM = 20 pmol/μL	20 pmol	1 μL
Reverse primer	20 μM = 20 pmol/μL	20 pmol	1 μL
Template DNA	Variable	~105 molecules	Variable
Taq DNA polymerase	5 Units/μL	2.5 Units	0.5 μL

[Q.S. = Quantum statis = Add as much of this ingredient as is needed to achieve the desired result, but not more]

primer, annealing of primers to strand of template DNA and elongation/amplification of targeted strand. Time of the cycles depends upon the size of the template and GC content of DNA.

Typically PCR reaction starts with a 1 min denaturation at 94 °C (optimum temperature for DNA Polymerase). Then 25–35 cycles of denaturation (94 °C for 10–60 s) (Excess of PCR cycle may lead to unwanted secondary products.), annealing (annealing temperature depends upon the primers used, *see* Table 1; for 30 s), and elongation (70–80 °C, time of which depend on amplicon and DNA Polymerase) followed by final extension at 70–80 °C for 5 min (allows synthesis of many uncompleted amplicons to complete) (*see* **Note 6**). Termination of the reaction is achieved by chilling the mixture to 4 °C and/or by the addition of EDTA to a final concentration of 10 mM.

For detection of *Bacillus* endotoxin genes, thermal cycles published in literature are mentioned in Table 3.

6. Once finished, remove PCR tubes from thermal cycler and store at 4 °C.
7. Detect the PCR products by agarose gel electrophoresis.

3.2.2 Agarose Gel Electrophoresis [33]

Gel Preparation

1. Take 1% boiled agarose gel and cool to 55–60 °C using running tap water.
2. Add 2 μL of EtBr solution mix it well by gentle swirling (*see* **Note 7**).
3. Insert the comb before pouring the gel on to the gel tray (*see* **Note 8**).

Table 3
PCR Thermal Cycling Conditions used in different products

Initial denaturation		Thermal cycle							Final extension		
			Denaturation		Annealing		Extension				
Temp [°C]	Time	Number	Temp [°C]	Time	Temp [°C]	Time	Temp [°C]	Time	Temp [°C]	Time	References
94	–	30	94	20 s	54	1 min	71	2 min	72	–	[4]
95	3 min	35	95	30 s	55	30 s	72	30 s	72	2 min	[35]
		35	95	30 s	60	30 s	72	1 min	72	5 min	[2]
94	3 min	30	94	1 min	50	1 min	72	1 min	72	10 min	[1]
95	5 min	30	94	45 s	54	1 min	72	2 min	72	5 min	[6]
95	5 min	30	95	30 s	60	30 s	72	45 s	72	7 min	[28]

4. Seal the open edges of the tray provided with the apparatus with the help of cellophane tape before pouring the agarose.
5. Pour the agarose solution on to the gel tray.
6. Leave the gel to solidify for 30–40 min (*see* **Note 9**).
7. Remove cellophane tape from the gel tray.
8. Fill the electrophoresis tank containing the gel with 1× electrophoresis buffer (*see* **Note 10**).
9. Now carefully remove the comb so as to eliminate presence of air bubble.

Loading of Samples

1. Apply prepared samples to the wells formed in the gel (*see* **Note 11**).

Gel Electrophoresis

1. Connect appropriate electrodes to the power pack and run it at 50–100 volts for 20 min (*see* **Note 12**).
2. Monitor the progress of the gel with reference to tracking dye (Bromophenol blue). Stop the run when the marker has run 3/4th of the gel.

Examining the Gel

1. Place the gel on the UV-transilluminator and check for orange-colored bands in the gel.
2. Visualize the gel under UV light (254–366 nm) (*see* **Note 13**).

Observation

While running agarose gel electrophoresis, first lane is loaded with molecular size markers to determine the size of the DNA bands in rest of the lanes. Also amplified DNA products of positive and negative control strains are used to determine the exact product

size of both enterotoxins and emetic toxins. Test organisms' amplified DNA products run in the same agarose gel are then compared with the molecular size marker to determine the size of the amplified product and with that of control bands to determine which toxin genes are present or absent. Lane 2 and 3 are the positive control strain DNA and negative control strain DNA respectively. The rest of the test organisms' DNA can be loaded in subsequent lanes. Thus, by comparing the bands with that of molecular size markers and the bands of positive and negative control organisms, one can determine the presence or absence of the toxin genes.

4 Inference

If any of the toxic genes is found to be present, the organism cannot be considered safe for the use as a probiotic.

5 Notes

1. *Bacillus* species organism, isolated from different sources and to be evaluated for probiotic activities should be cultivated and maintained using Brain heart infusion (BHI) medium at 30 °C and can be stored using glycerol-containing BHI medium at −80 °C [18, 34]. For the same, Tryptic soy broth [4], Luria-Bertani (LB) broth [35] can also be used. Bacterial strains should be grown at 30 °C on nutrient agar or in nutrient broth with shaking for preparation of DNA template for PCR [6].
2. The flask should not be more than half full and covered to minimize evaporation. Use 250 mL conical flask for preparing 50 mL solution to avoid overflow of gel solution while heating and to avoid its loss.
3. This step is required to retain nucleic acid as CTAB can precipitate nucleic acid if salt concentration drops below 0.5 M at room temperature and if NaCl is added, cell wall debris, denatured protein, and polysaccharides complexed to CTAB will be removed whereas the nucleic acids will be retained in solution.
4. Many programs/software are available for designing primers. NCBI Primer design tool: http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and Primer3: http://frodo.wi.mit.edu/primer3/ are recommended websites for this purpose [32].
5. While preparing PCR reaction mixture, keep all the reagents in a freshly filled ice bucket, and let them thaw completely before setting up a reaction. Keep the reagents on ice throughout the experiment.

6. This temperature and time regimen depends on the melting temperature of the primer. Annealing temperature should be less than melting temperature of primers. So determination of primer melting temperature is mandatory and this can be done by the help of software available [32]).
7. Ethidium bromide (EtBr) is commonly used dye which intercalates and fluoresces in nature, and can be either used before or after electrophoresis. Stock solutions of this stain (10 mg/mL) should be stored in dark bottles or wrapped in aluminum foil. EtBr at a concentration of 0.5 μg/mL is also normally incorporated in the gel before electrophoresis. The gel is soaked otherwise in electrophoresis buffer or water containing 0.5 μg/mL EtBr for 30–40 min. The gel is rinsed with buffer or water before analyzing it to remove excess EtBr) Wear gloves during the addition of EtBr and while handling the casted gel as EtBr is a potent carcinogen.
8. Ensure that there is enough space between the bottom of the comb and the gel tray, about 0.5–1.0 mm to allow proper formation of wells and avoid sample leakage.
9. Make sure that there are no air bubbles in the gel or in the well.
10. Add enough buffer to cover the gel with 1 mm liquid above the surface of the gel. If too much buffer is used the electric current will pass through the buffer instead of the gel.
11. Prior to sample loading remove any air bubbles from the wells and rinse with electrophoresis buffer; insert pipette tip into the well to load samples and expel the sample slowly; after sample loading do not move the electrophoresis tank or tray as this may cause samples to float out of the wells; include always a lane for appropriate molecular weight marker.
12. Appropriate current to be used is 1–10 V/cm of the gel. One should avoid the use of high voltages, which can cause trailing and smear of DNA particularly with high molecular weight DNA. Melting of agarose during electrophoresis indicates incorrect preparation of the gel or exhausted ions in the buffer during the run. Recycle the buffer using a pump for very long runs.
13. This increases fluorescence of EtBr and DNA complexes. Handling the gel should be careful as the gel may break due to improper handling. While performing the UV-trans illumination for visualizing the bands, avoid direct contact and exposure to eyes. UV light damages the DNA and if DNA fragments are to be extracted from the gel, use a lower intensity UV source and minimize the exposure of the DNA to UV light. UV light can damage the eyes and skin. Wear suitable eye and face protection when working with UV light.

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Chapter 14

Evaluation of Pathogenicity Potential by Phenotypic and Genotypic Methodologies

Teresa Semedo-Lemsaddek and Maria João Fraqueza

Abstract

Probiotic microorganisms can be included in various types of fermented food products or in supplements. Safety aspects are fundamental in the selection process of putative probiotics, namely origin, non-pathogenicity, and antibiotic resistance features. A careful evaluation of the virulence potential is fundamental during the selection of food-related microbes, being a prerequisite to assure the biosafety of putative probiotics. Screening for the production of known traits (e.g., hemolysin), as well as searching for virulence determinants, is mandatory. For a microorganism to be included in the GRAS—Generally Recognized as Safe—list (in the United States of America) or to harbor QPS—Qualified Presumption of Safety—status (in Europe) "the lack of pathogenic properties must be established and substantiated." This chapter will address methodologies available for the evaluation of the pathogenicity of lactic acid bacteria -LAB-, with main emphasis on the *Enterococcus* genus, the most controversial LAB.

Key words Lactic acid bacteria (LAB), *Enterococcus*, Probiotic, Virulence factor, Biosafety

1 Introduction

According to the World Health Organization (WHO), probiotic cultures are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. Probiotics are presented in food or as supplements; they are presumed to be safe, since they have been consumed globally by many people without any safety issues. The main microbial groups linked to probiotics in food are *Lactobacillus* genera, or others from the lactic acid bacteria group, with recognized health benefits, and *Bifidobacterium* genera; yeasts such as *Saccharomyces* spp. have also been used. Emerging research studies support the use of selected strains of bacteria, or yeasts, to treat specific medical conditions, so certain probiotics do have well-defined health benefits [2, 3]. However, theoretical and proven adverse events from probiotic consumption may exist, and safety should be cautiously assessed [4].

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_14,

The Food and Drug Administration (FDA) and/or external experts can assign *Generally Recognized as Safe* (GRAS) status to a microorganism based on the history of use, the body of knowledge, and the absence of adverse effects at the strain level. The *Qualified Presumption of Safety* (QPS) list is the fast track risk assessment tool used by the European Food Safety Authority (EFSA) panels, when evaluating microorganisms intentionally introduced into the food chain, particularly products with microorganisms requiring a pre-market authorization (e.g., feed additive cultures, cell factories producing enzymes/additives/vitamins, novel microorganisms, and plant protection). This approach is based on history of use, body of knowledge, and absence of adverse effects at the taxonomic unit level [5].

Another recent definition has outcome; the term synbiotic means "a mixture comprising live microorganisms and substrate (s) (prebiotic(s)) selectively utilized by host microorganisms that confers a health benefit on the host" [6]. The safety of the synbiotic for the intended use, and its consistent performance, needs to be always established. Live microbial component(s) of the synbiotic should have an openly available genome sequence and annotation, be assessed for any genes of safety concern (for example, toxin production or transferrable antibiotic resistance), named using current taxonomic nomenclature and carry a traceable strain designation.

Moreover, the safety assessment of the strains used as probiotics, synbotics, or other starter or protective cultures for fermented foods production, should discard the presence of antibiotic resistance genes in the selected strains, to avoid transmission to commensal or pathogenic bacteria [7].Another major concern is the virulence potential (presence of virulent genes) or other traits, such as biogenic amines production.

It is also acknowledged that some members of the lactic acid bacteria, such as enterococci, occurring in the human gastrointestinal tract, may confer health benefits; however, many of them possess virulence characteristics and harbor virulence determinants. Hemolysis is a common virulence factor among pathogens, facilitating iron availability to the microbe and causing anaemia and edema in the host, so its screening is of utmost importance. There is no consensus in terms of virulence related genes in lactobacilli and probiotic bacteria should be checked for any virulence related genes, as an additional safety measure. Numerous virulence determinants have been identified in enterococci (including probiotic strains) such as gelatinase, or the presence of structural *cyl* genes *(*Cytolysin-Hemolysin), *ace (*Collagen adhesion), *asal* (Aggregation substance), *esp (*Enterococcal surface protein), g*elE* (Gelatinase), and hylE_{fm} (Hyaluronidase) [8].

The presence of one or more virulence traits does not make a strain necessarily pathogenic, although the presence of mobile genetic elements drives the evolution of bacteria to specialization

and adaptation of specific genetic lineages, with the acquisition of genes that increase the cell's fitness. A complete picture of all the genes and biological processes that determine pathogenicity is still not elucidated, so a case-to-case study of selected *Enterococcus* should be performed [7]. In fact, due to a complete high effort, there are few authorized commercial probiotics, such as *E. faecium* SF68® (NCIMB 10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland) and *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany) [9].

Overall, it is of utmost importance that all novel microorganisms used as probiotic or synbiotic cultures in foods are evaluated to warrant safety, since these microorganisms will be present in supplements or foods with high concentrations. Hence, this chapter will address methodologies available for evaluating the pathogenicity of lactic acid bacteria -LAB-, with main emphasis on the *Enterococcus* genus, the most controversial LAB.

2 Materials

Prepare all solutions using previously autoclaved glassware, ultrapure water, and analytical grade reagents.

2.1 Phenotypic Approach

For phenotypic tests disposable materials (e.g., Petri dishes, inoculation loops, microtubes) will be needed, as well as autoclave for media sterilization and incubators at different temperatures.

1. Columbia agar supplemented with 5% blood (horse or sheep; *see* **Note 1**). Store at 4 °C until use.
2. Gelatin medium formulation per litter: peptone 5 g, yeast extract 3 g, gelatin 30 g, agar 15 g, pH 7.0. Sterilize by autoclave (121 °C, 15 min), distribute by Petri dishes and store at 4 °C until use.
3. Saturated solution of ammonium sulphate (4.1 M at 25 °C). Prepare as follows: For 800 mL of distilled water, add 761 g of ammonium sulfate, dissolve with stirring and gentle heating, cool to room temperature, and adjust the pH to 7.0. Store at room temperature.

2.2 Genotypic Approach

For genotypic tests use gloves and disposable DNAse-free material. Manipulation of organic reagents must occur in a laboratorial hood. Micropipettes and disposable tips are essential.

1. TE buffer (Tris 10 mM, EDTA 1 mM pH 8.0). Prepare as follows: For 800 mL of distilled water add 15.759 g of Tris-HCl and 2.92 g of EDTA, dissolve with stirring, adjust the pH to 8.0, add distilled water to a final volume of 1 L. Store at room temperature.

2. TE buffer with 0.1% Tween 20: add 100 μL of Tween 20 to 100 mL of TE buffer. Homogenize by gentle shaking. Store at room temperature.
3. Water or dry bath.
4. Centrifuge for microtubes.
5. Lysozyme.
6. Mutanolysin.
7. GES reagent (5 mol/L guanidium thiocyanate, 100 mmol/L EDTA and 0.5% v/v sarkosyl). Prepare as follows: guanidium thiocyanate (60 g), 0.5 mol/L EDTA at pH 8.0 and ultrapure deionized water (20 mL), heat at 65 °C with mixing until completely dissolved; after cooling, add 5 mL of 10% v/v sarkosyl, complete up to 100 mL with ultrapure deionized water, filter through a 0.45 μm pore size membrane and store at room temperature.
8. Ammonium acetate 7.5 mol/L: Dissolve 57.81 g of ammonium acetate in ultrapure water to a final volume of 100 mL and sterilize by filtration (0.2 μm filter). Store at 4 °C.
9. Chloroform: 2-pentanolmixture (24:1): to prepare 500 mL, add 240 mL of chloroform to 10 mL of 2-pentanol. Mix by inversion and store at room temperature.
10. TE with RNAse A: add 500 μg of RNAse A to 10 mL of TE, mix vigorously and store at −20 °C.
11. Conventional PCR apparatus.
12. Taq DNA polymerase, adequate buffer, dNTPs.
13. Primers, specific for the intended purpose (for details *see* Tables in the Methods section).
14. TBE 5× (Tris 450 mM, boric acid 450 mM, EDTA 10 mM): Prepare by mixing and dissolving 54 g of Tris base, 27.5 g of Boric acid, and 20 mL of 0.5 M EDTA in ultrapure water to a final volume of 800 mL. Adjust the pH to approximately 8.3, complete the volume to 1000 mL, sterilize by autoclaving and store at room temperature. To prepare TBE 0.5×: add 100 mL of TBE 5× to 900 mL ultrapure water, mix and store at room temperature.
15. Agarose for DNA electrophoresis.
16. Agarose 1.2% in TBE 0.5×: Mix 2.4 g of agarose with 200 mL of TBE 0.5×, stir and heat until complete dissolution (total transparency), stabilize at 50 °C for 30 min, pour into the electrophoresis mold and allow to solidify.
17. Loading buffer (bromophenol blue).
18. A DNA intercalating fluorochrome (e.g., gelstar, gelred, ethidium bromide).
19. Molecular size marker.

20. Electrophoresis apparatus and power source.
21. Ultraviolet transiluminator.
22. Image acquiring apparatus.
23. Real time PCR apparatus.
24. SYBR green I.

3 Methods

Both phenotypic and genotypic approaches must be applied for a fully reliable characterization of the microorganisms of interest.

3.1 Phenotypic Approach

Among lactic acid bacteria, the most relevant virulence factor is the production of hemolysins. If the bacteria belong to the *Enterococcus* genus, gelatinolytic activity must also be screened [8, 10, 11].

3.1.1 Hemolysis

1. Evaluation of the hemolytic ability requires the easy procedure of streaking the interest microbe on Columbia agar supplemented with 5% blood (*see* **Note 1**), followed by incubation at 37 °C for 48 h [8].
2. Results interpretation should be considered as follows: β-hemolysis (true hemolysis) corresponds to the development of a clear halo due to erythrocyte destruction; α-hemolysis (incomplete hemolysis) present a greenish-brown coloration around the streaking, while γ-hemolysis (non-hemolysis) correspond to growth without medium alteration. It is also possible to detect the absence of growth ability in Columbia agar supplemented with blood; this putative probiotic such be considered non-hemolytic.

3.1.2 Gelatinase Production

1. For the phenotypic assay, regarding gelatinase detection, isolates should be grown on agar plates containing 3% (w/v) gelatin, followed by incubation at 37 °C for 48 h [8, 12].
2. Results interpretation: After incubation, the plates must be flooded with a saturated solution of ammonium sulphate. Residual gelatin precipitates, and a transparent halo appears around and beneath gelatinase producers.

3.2 Genotypic Approach

3.2.1 Total DNA Isolation (Genomic and Plasmidic)

Despite the subsequent methodology, the use of genotypic methods always begins with DNA isolation. Two strategies may be applied: (1) recur to a rapid DNA extraction method, which is fast but may isolate impure DNA or (2) select a more complex protocol and obtain high-quality DNA. Methods described below extract both genomic and plasmidic DNA.

Rapid DNA Isolation

1. Overnight growth of the interest microorganism.
2. Resuspend one bacterial colony on TE Buffer with 0.1% Tween-20, using a microtube.
3. Submit to heat (100 °C, 10 min), on a water or dry bath.
4. Quick cool on ice (heat-shock).
5. Centrifuge for 5 min at high speed and recover the supernatant to a new tube.
6. Maintain at −20 °C until use.
7. Use 1 μL for subsequent PCR-based procedures.

High-Quality DNA Isolation (Guanidium Thiocyanate Methodology, Adapted from [13]

1. Overnight growth of the interest microorganism.
2. Resuspend a substantial loopful of bacterial culture on TE Buffer, using a microtube.
3. Centrifuge (~25,000 × *g*) for 5 min.
4. Discard the supernatant and resuspend the pellet in 200 μL TE with 10 mg/mL of lysozyme (Sigma-Aldrich) and 1 mg/mL of mutanolysin (Sigma-Aldrich).
5. Incubate at 37 °C (water bath) for 30 min to 1 h.
6. Add 500 μL of GES reagent.
7. Cool the lysates on ice for 10 min (*see* **Note 2**).
8. Add 250 μL of cold 7.5 mol/L ammonium acetate, mix by inversion and place on ice for 10 min.
9. Add 1 mL of chloroform: 2-pentanolmixture (24:1), and homogenize by inversion.
10. Centrifuge at maximum speed (~12,000 × *g*) for 10 min at 4 °C.
11. The aqueous (top) and organic (bottom) phases will separate. Carefully recover the upper phase to a new microtube.
12. Add 1 mL of 2-propanol (ice cold) and mix cautiously by inversion.
13. Centrifuge at maximum speed (~12,000 × *g*) for 10 min at 4 °C.
14. Discard the supernatant and add 1 mL of ethanol 70%, mix by inversion.
15. Centrifuge at maximum speed (~12,000 × *g*) for 10 min at 4 °C.
16. Discard the supernatant and let the pellet dry at room temperature (invert the microtubes to facilitate the process) or under vacuum.
17. Resuspend the pellet in TE with 50 μg/mL RNAse A (Sigma-Aldrich).

18. Incubate 30 min at 37 °C or overnight at room temperature.
19. Use the remaining DNA for PCR amplification (~1 μL per reaction tube).

Commercial Kits

Several options are available for high-quality bacterial DNA isolation, like innuPREP Bacteria DNA Kit (Analytik Jena GmbH), AllPrep Bacterial DNA/RNA/Protein Kit or DNeasy PowerLyzer Microbial Kit (Qiagen), MagMAX™-96 DNA Multi-Sample Kit or PureLink™ Pro 96 Genomic DNA Purification Kit (Thermo Fisher Scientific), DNA Isolation, Bacterial DNA Mini Kit, peqGOLD (Avantor), among many others.

3.2.2 Screening Procedures

Nowadays, there are many genotypic-related protocols available for the evaluation of the pathogenicity potential of putative probiotics, which mostly include conventional end-point PCR, real-time quantitative PCR or Whole Genome Sequencing (WGS) [14–21].

Conventional PCR

Over the years, a large number of manuscripts described primers and PCR amplification conditions, which may be applied for the molecular screening of virulence factors, associated with lactic acid bacteria, mainly *Enterococci* [8, 11, 14, 15, 22, 23].

The preferred approaches use several primer sets in the same reaction (multiplex PCR) to simultaneously recognize numerous determinants prone to be present in the samples under study, saving time and money. Due to the great number of previous studies, which applied this methodology, a careful selection of the primer sets and amplification conditions is fundamental to achieve the expected aims.

Tables 1 and 2 display primers and conditions for PCR amplification of the most common virulence factors associated with the putative pathogenic species *E. faecalis* and *E. faecium*. Moreover, for enterococcal genus confirmation and species allocation, using molecular procedures, the approach reported by [24] can be easily applied.

1. Multiplex-PCR reaction mixture:

   0.2 mL reaction tubes with mixtures (25 μL each) using PCR buffer (pH 8.4, 2.5 mM $MgCl_2$), 0.1 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM of each primer, 2 U of Taq DNA polymerase, and 1 μL of enterococcal DNA.

   (a) **Amplification conditions:**

   Initial cycle of 94 °C for 3 min, 35 cycles of 94 °C for 1 min, annealing temperature of 55 °C for 1 min, 72 °C for 1 min, a final extension step of 72 °C for 10 min, and thereafter cooled to 4 °C.

   (b) **Electrophoresis:**

   An 8 μL aliquot of the amplification mixture is combined with 2 μL of loading buffer (bromophenol blue)

Table 1
Virulence factors associated with *E. faecalis*

Target	Primers (5′–3′)	Amplicon size (bp)	References
Aggregation substance—*asa1*	For- CGGTACAGTTGGCAGTGTTTCG Rev-GGCTTGTGGGTCTTTGGCAGAG	518	[25]
Cytolysin activator—*cylA*	For-CGGGGATTGATAGGCTTCATCC Rev-TAACCATCTGGAAAGTCAGCAG	628	
Enterococcal surface protein—*esp*	For-TTGCTAATGCTAGTCCACGACC Rev-GCGTCAACACTTGCATTGCC GAA	933	[14]
Gelatinase—g*elE*	For- ACCCCGTATCATTGGTTT Rev- ACGCATTGCTTTTCCATC	419	

Table 2
Virulence factors associated with *E. faecium*

Target	Primers (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)	References
Enterococcal surface protein - Esp_{fm}	For- TTGCTAATGCTAGTCC ACGACC Rev-	945	63	[26]

GCGTCAACACTTGCATTGCCGAHyaluronidase $hylE_{fm}$For-ACAGAAGAGCTGCAGGA AATG27656[27]Rev-GACTGACGTCCAAGTTT CCAA

and 2 μL GelStar 6× (Lonza), the preparation is resolved by electrophoresis using 1.2% (w/v) agarose gel on TBE 0.5× at 90 V for 2 h, a molecular size marker must be included for comparison. DNA amplification is visualized under UV light.

2. PCR reaction mixture (individual reactions):
   0.2 mL reaction tubes with mixtures (25 μL each) using PCR buffer (pH 8.4, 2.5 mM MgCl2), 0.1 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM of each primer (forward and reverse for the selected gene target), 2 U of Taq DNA polymerase and 1 μL of *Enterococcal* DNA.
   (a) **Amplification conditions:**
   Initial cycle of 94 °C for 3 min, 35 cycles of 94 °C for 1 min, adequate annealing temperature(56 or 63 °C,

depending on the selected target) for 1 min, 72 °C for 1 min, a final extension step of 72 °C for 10 min and thereafter cooled to 4 °C.

(b) **Electrophoresis:**

An 8 μL aliquot of the amplification mixture is combined with 2 μL of loading buffer (bromophenol blue) and 2 μL GelStar 6× (Lonza), the preparation is resolved by electrophoresis using 1% (w/v) agarose gel on TBE 0.5X at 90 V for 2 h, a molecular size marker must be included for comparison. DNA amplification is visualized under UV light.

Real-Time PCR

Quantitative real time PCR (qRT-PCR) is one of the most widespread methodologies used for the detection and/or quantification of virulence traits present in diverse microorganisms present in a variety of settings [20, 21]. qRT-PCR differs from end-point (conventional) PCR because it allows the monitoring of amplification in real time, based on the increasing fluorescence as cycles of amplification are repeated [28]. The observed fluorescent signal is directly proportional to the number of amplicons generated, turning it quantitative. Signal detection may result from (1) non-specific detection based on the use of fluorochromes, such as SYBR green I, which intercalate double stranded DNA or (2) specific detection, based on the use of specific oligonucleotides (primers and/or probes) labeled with fluorescent reporters, which hybridize with complementary targets on the sequence of interest.

This versatile methodology can be applied to DNA, RNA or cDNA targets. Thus, it can be used for the detection/quantification of specific determinants, for the evaluation of gene expression or to determine viral load, among many others (*see* **Note 3**). Major disadvantages include the initial investment in the purchase of the equipment and personnel training. Furthermore, it is difficult to optimize reactions aiming the amplification of more than two targets (multiplex qRT-PCR). Table 3 displays the targets and primer sets suggested for real-time PCR for detecting virulence determinants.

1. Real-time PCR reaction mixture (individual reactions):

In a total volume of 20 μL: 1 μL DNA 1 μL, 8 μL deionized ultrapure water, primers forward and reverse 10 pmol each, 10 μL SYBR Green mix [29].

(a) **Amplification conditions:**

Initial cycle of 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s [29].

Table 3
Targets and primers sets for real-time amplification of enterococcal virulence factors [29]

Target	Primers (5′–3′)	Amplicon size (bp)
Aggregation substance—*asa1*	For- ATGACAAACCCAAAGCCAAT Rev- CGCAATTCGTTCTACACCAA	260
Cytolysin—*cylB*	For- GCTCTAATTGACTCGGGGATT Rev- CACTCTTGGAGCAATCGTGT	180
Enterococcal surface protein— *esp*	For- TCGCTCCAAATGAAAAAGATG Rev- CGGTTGAACCTTCTTCTGGT	200
Gelatinase – *gelE*	For- CGGATTGGTTACACCATTATCC Rev- TGCCACTCCTTATCCATTTTT	207

Whole Genome Sequencing (WGS)

Relevant advances in high-throughput-sequencing significantly reduced the costs associated with state-of-the-art methodologies, such as whole genome sequencing (WGS), turning them affordable for numerous laboratories.

As the name suggests, WGS correspond to the obtainment of the complete DNA sequence of the putative probiotic, including both chromosome and extra-chromosomal elements. Due to the large amount of data generated, this approach allows the identification of virulence, antibiotic resistance or probiotic/technological-related determinants [30–33], being fundamental for the assessment of safety and/or for the selection of microbes with probiotic potential. Nevertheless, the major challenge continues to rely in deciphering bacterial potential from genetic information, due to the large amount of complex data analysis and the low quality of the databases available for comparison. In the near future, the progress of multi-OMIC technologies and application of systems biology approaches [34] will may shed light on microbial safety assessment and help explore the health benefits associated with their uptake.

WGS workflow protocols depend on the sequencing platform selected, such as Roche 454, Illumina Hiseq series (GA, Hiseq, Miseq, X), SOLiD, Complete Genomics, or Ion Torrent [35–39], among others.

1. Usual WGS steps.
   A. DNA extraction.
   B. Target amplification (*see* **Note 4**).
   C. Library preparation.
   D. Target enrichment.
   E. Library quantification.
   F. Sequencing.
   G. Data analysis (*see* **Note 5**).

Overall, WGS may be applied to the interest microorganism and, if pathogenic properties are absent, biosafety can be inferred, allowing the selection of the microbe as putative probiotic. The comprehensive data obtained may also lead to the identification of health-benefit traits, further consolidating the decision.

4 Conclusions

As aforementioned, the selection of novel microbes to be used as probiotics must involve the detailed analysis of the microorganism (s) of interest, regarding reliable identification procedures and comprehensive safety assessment, involving the screening for virulence factors, both at phenotypic and genotype level, as here described. However, due to the possibility of horizontal gene transfer events, regular monitoring of the selected probiotic is advised.

5 Notes

1. The blood type used for medium supplementation must be carefully considered. Sheep blood is the most widely used, but the enterococcal hemolysin has difficulties lysing sheep erythrocytes. Hence, if testing enterococci, horse blood is better to avoid false negatives [8, 23].
2. If the lysate is not transparent incubate for 10 min at 50 °C and cool on ice for a further 10 min, before proceeding.
3. If the evaluation of gene expression is intended, RNA must be used instead of DNA. For that purpose, check information available on the literature [20, 21, 29].
4. The use of an amplification step, for target enrichment, may lead to the introduction of incorrect nucleotides thus, this error-prone phase should only be applied if the amount and/or quantity of target DNA are reduced.
5. Alignment against reference genomes available online or, if no reference genome is available, alignment and assembling "*de novo* sequencing."

Acknowledgments

Teresa Semedo-Lemsaddek is financially supported by national funds through FCT—Foundation for Science and Technology, I.P. under the Transitional Standard—DL57/2016/CP1438/CT0004. We thanks projects PTDC/CVT-CVT/29510/2017,

PTDC/OCE-ETA/1785/2020 and UIDB/00276/2020 (CIISA) supported by National Funds through FCT-Foundation for Science and Technology.

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Chapter 15

Determination of Toxicity Through Cytotoxicity Assays

Urjita V. Sheth

Abstract

The purpose of present protocol is to assess the toxigenic potential of selected/isolated probiotic organism using MTT assay. Various bacteria have been isolated from different sources and believed to be safe for using them as probiotics. However, in vitro toxicity must be assessed as some bacteria may be toxic to the host. *Bacillus* species organisms are found to produce diarrheal enterotoxins and emetic types of toxins. Earlier toxicity of probiotics has been detected using in vivo experiments on rats, rabbits, etc. Various in vitro methods have also been designed to detect the toxicity of probiotic strains. One of the in vitro methods is determination of cytotoxicity of probiotics on mammalian cells using MTT assay. It is well reported in the literature that the *Bacillus cereus* enterotoxin has a cytotoxic effect on cultured cells. This assay measures total cellular metabolic activity using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). As only viable cells are able to produce formazan crystal by reducing MTT using mitochondrial dehydrogenase enzyme, the assay is considered as a very sensitive assay to express cellular respiration, cell viability, and cytotoxicity. This protocol explains method for determination of cytotoxic effect of enterotoxin on Vero cell line using MTT assay and of emetic toxin on HEp-2 cells using cell vacuolation assay.

Key words Probiotics, Cytotoxicity, MTT assay, Enterotoxin, Emetic toxin, Vero cell line, HEp-2 cell line

1 Introduction

Probiotics are live microbes used as a food or drug which upon ingestion expected to confer various health benefits to the hosts [1]. Main concern with the use of probiotic organism is that the organism should be documented as generally recognized as safe (GRAS) for human consumption at the effective concentration of organisms [2]. Though traditionally used probiotic bacteria such as lactic acid bacteria or *Bifidobacteria* were considered as safe, evidences have raised much dispute over the safety of probiotics for recently isolated various probiotic bacteria from infection sources [3]. Thus, for a particular organism which proved to be an efficient probiotic for its clinical applications, the safety evaluation is also

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_15,

very important concern [4]. Certain microorganisms such as *Enterococci*, which may harbor transmissible antibiotic resistance and *Bacilli* may produces toxins. Especially *Bacillus cereus* group is known to produce enterotoxin and emetic toxin are problematic. *Bacillus* organisms are widely used as a probiotic organisms [5]. The current legislation in the European Union on probiotics recommends safety assessment for the target animal species, consumers, and workers [6]. For evaluation of safety of probiotics, various factors need to be considered such as pathogenicity, infectivity, and virulence factors comprising toxicity, metabolic activity, and intrinsic properties of the microbes [3].

Bacillus cereus is a potential problem to the food industry, as it causes food spoilage and it is also associated with two distinct types of food poisoning. The symptoms of *Bacillus cereus* food poisoning have been classified into two types; (1) emesis caused by an emetic toxin and (2) diarrhea caused by an enterotoxin [7]. The diarrheal syndrome is characterized by abdominal cramps and watery diarrhea which lasts for 12–24 h [8]. The emetic syndrome is commonly associated with farinaceous foods and is caused by a heat-stable dodecadepsipeptide [9]. The intoxication is characterized by nausea and vomiting, which is often accompanied by diarrhea, although this is not the major symptom [10, 11]. Two different three-component enterotoxins, hemolysin BL (Hbl) and non-hemolytic enterotoxin (Nhe) [12, 13] and a one-protein enterotoxins (-T, -K, and -FM (cytotoxin K- CytK)) [14] and an emetic toxin (cereulide) are reported to be produced by *Bacillus cereus* [15–17] and all of which are found to adversely affect the metabolic status of cultured mammalian cells and induce membrane damage, which can be measured by different endpoints for general cytotoxicity in vitro [13, 18–24].

Safety evaluation of probiotic strains such as *Bacillus* includes screening of enterotoxins. Earlier, the presence of enterotoxins was determined by laboratory experiments. Therefore, establishment of proper in vitro assays for determination of human risk assessment of *Bacillus cereus* putative enterotoxins is necessary [5]. In vitro cytotoxicity assays performed earlier on various *Bacillus* strains have raised question of continuing use of *Bacillus* species and have driven the Scientific Committee on Animal Nutrition (SCAN) (European Commission) to endorse necessities for testing industrial strains for production of *Bacillus cereus* toxins [25, 26]. Determination of production ability of enterotoxin in probiotic containing spores of *Bacillus* species by PCR, cytotoxicity, and ELISA is becoming necessary for the approval of probiotics [25, 27, 28]. In addition, in vivo assays on various laboratory animals are required such as intestinal loop tests in rabbits or feeding trials. However, all these assays have the following limitations. Since Nhe and Hbl are three component toxins, presence of any of the three genes by PCR may not determine the presence of functional protein and marketed ELISA based test kits determines presence of only one of the three protein components [28].

Cytotoxicity assays have been proven to be a sensitive and reliable method for the detection of toxins than other assays [20]. Numerous cell lines were utilized and found to have different responses to *Bacillus* toxins [31] and hence, none can be considered as standard. In vitro cytotoxicity to Vero cells, CHO-K1 cells, and Caco-2 cells have demonstrated to be a suitable assay extensively used to distinguish enteropathogenic and non-enteropathogenic strains of *Bacillus cereus*. With Vero cells and Caco-2 cells, all three components are required for maximum cytotoxicity [13, 29, 30]. However, Vero cells because of its availability and rapid growth are a common choice. A diagnostic method of detecting the enterotoxins is commercially available. However, the emetic toxin has not been fully examined. The emetic toxin lowers antigenicity, and its detection by immunochemical methods is difficult.

The only assay method currently available for emetic toxin has many disadvantages; since it involves oral challenge of primates. Whilst investigating the application of tissue culture for the detection of food poisoning toxins, it was noted that culture filtrates from an emetic syndrome *B. cereus* isolate produced vacuoles inHEp-2 cells [19]. Hughes et al. [19] have reported that vacuoles are produced in HEp-2 cells by the emetic toxin, and they explained the relationship between these vacuoles and the toxin. Additionally, diarrheal enterotoxins are heat-labile and are fully denatured by treatment like autoclaving the while treating cells with untreated cultures, cytotoxicity assays do not differentiate among the effects of emetic and enterotoxins. But the *B. cereus* emetic toxins are resistant to such treatments. Thus, this concept of treating Hep-2 cell with heat treated cell extract forms the basis of detection of emetic toxin by Hep-2 cell vacuolation assay. Szabo et al. [31] reported that the toxin affected the HEp-2 cells by changing the vacuoles, and by acid production, cell rounding, cell granule promoting, and cytostatic activity [31]. SCAN recommended the Hep-2 cell vacuolation assay for the emetic toxin [19]. However, this assay is laborious, subjective, and unreliable because mitochondrial swelling, the diagnostic marker for the presence of toxin, is transient and easily missed [24]. The determination of in vitro cytotoxicity of probiotic isolates (bacteria) on Vero cell line and of heat-treated bacterial culture extract on Hep-2 cell line using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is described here.

1.1 Principle of MTT Assay [32, 33]

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a simple colorimetric assay for determination of cell proliferation and survival. The method has been developed by Mosmann in 1983 [34] and adapted by Cole in 1986 [35] to measure chemosensitivity of human lung cancer cell lines [36]. The main purpose of the MTT assay is to measure/quantify viable cells in comparatively high throughput (96-well plates)

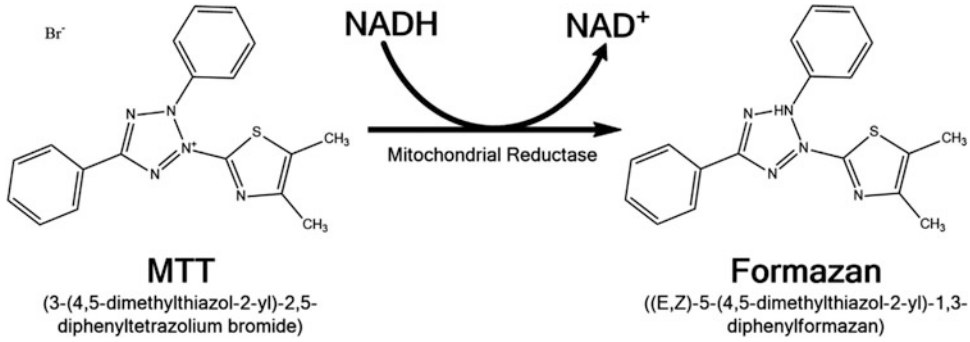

Fig. 1 Reduction of MTT to formazan crystals

without the need of cell counting after treating cells with cytotoxic elements [33]. This assay involves the principle of conversion of colorless tetrazolium salt, MTT into purple colored water-insoluble formazan crystals by mitochondrial dehydrogenase enzyme of living cells at 37 °C as shown in Fig. 1. MTT because of its lipophilic group and positive net charge, pass the cell membrane and is reduced in viable cells by mitochondrial or cell plasma enzymes like oxidoreductases, dehydrogenases, oxidases, and peroxidases using NADH, NADPH, succinate, or pyruvate as electron donor. Besides enzymatic reactions there are different non-enzymatic reactions with reducing molecules like ascorbic acid, glutathione, or coenzyme A that are able to interact with MTT forming the formazan product and produce a higher absorbance accordingly [33].

The formation of needle-like formazan crystals pierce the cell's membrane and destroys the cell's integrity and thus leads to cell death and the cell metabolism breaks down; therefore, further formation of formazan is interrupted very quickly [32, 33]. As the dead cells do not have the capacity to further reduce the MTT, the assay is considered to be the most sensitive assay for determination of cell respiration, viability, and cytotoxicity [4]. These kinds of cell death associated with terminations of reactions assays are known as endpoint determination. Formazan crystals are formed intracellularly; therefore, to quantify the amount of formazan produced, cells are lysed and formazan crystals are dissolved in suitable solvent [33]. Various solubilizing agents used are acidified isopropanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), sodium dodecyl sulfate (SDS), and combinations of detergent and organic solvent [37–39]. Mitochondrial activity is constant for most viable cells, so an increase or decrease in the number of viable cells can be directly correlated to mitochondrial activity which is reflected by the amount of formazan crystals produced [33]. The purple-colored formazan product has an absorbance maximum near 570 nm. Thus, the intensity of the colored product is directly proportional to the number of viable cells present in the culture [33].

2 Materials

2.1 Reference Bacterial Strains [8, 26]

To demonstrate the sensitivity of the cytotoxicity test system to both kinds of toxins, the toxigenic reference strains of *Bacillus cereus* NCTC 11145 (DSM4313) (diarrheagenic) and *Bacillus cereus* NCTC 11143 (DSM4312) (emetic) should be used as toxin producer strains and *Bacillus licheniformis* (DSM 13 = ATCC14580) should be used as non-toxigenic strain in the assay.

2.2 Bacterial Culture Media [8, 26]

LB agar should be used to maintain isolated bacterial cultures. Brain-heart infusion broth (BHI) added with 1% Glucose (BHIG) or skim milk (SMP) should be used to prepare cell culture extract/supernatant for cytotoxicity assay.

2.3 Cell Line and Cell Culture Media/Reagents [8, 26]

Vero cells are recommended to determine toxicity of enterotoxins using MTT assay and HEp-2 (human carcinoma of the larynx) cells are recommended to determine toxicity of emetic toxin using cell vacuolation assay.

2.4 Reagents for Cell Culture

To culture Vero cells under in vitro conditions, the growth medium used is DMEM supplemented with 5–10% fetal calf serum.

1. DMEM supplemented with 10% (v/v) (FBS) and 1% penicillin–streptomycin should be prepared as follows:
   (a) Inactivate the FBS by incubating for 1 h in a 56 °C water bath.
   (b) Take 1 L sterile/autoclaved glass bottle and add:
   - 1 pouch of DMEM powder.
   - 900 mL sterile/autoclaved Milli-Q water.
   - Mix it well to dissolve powder.
   - Then add 10 mL, 10,000 U Penicillin/mL, and 10,000 mg/mL Streptomycin solution to get concentration of 100 U Penicillin and 100 μg Streptomycin.

   (c) Make final volume 1000 mL using sterile Milli-Q water.
   (d) Sterilize the medium by filter and store it at 2–8 °C.
   (e) Add heat inactivated FBS at concentration of 10%, immediately before use after filter sterilization.
2. To cultivate HEp-2 cells in Basal Medium (Eagle) with Earle's Salts (EBME) containing 2 mM L-glutamine, 10 mM sodium bicarbonate, 1% non-essential amino acids, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 20 mM Hepes buffer (pH 7.8), and 10% (v/v) fetal calf serum should be used.

2.5 Reagents for MTT Assay [33]

1. Phosphate-buffered saline(PBS) solution:

Ingredients	Quantity
NaCl	8 g
Potassium Chloride (KCl)	0.2 g
Disodium phosphate ($Na_2HPO_4{\cdot}2H_2O$)	1.44 g
Monopotassium phosphate (KH_2PO_4)	0.2 g
Deionized distilled water	1000 mL

Dissolve all the ingredients in deionized distilled water
Adjust pH to 7.4 using sodium hydroxide (NaOH) or hydrogen chloride (HCl)

2. 5 mg/mL MTT solution/MTT-Medium Master-mix solution:
   (a) Dissolve 0.5 g MTT in100 mL 0.9% NaCl solution by stirring with a magnetic stirrer for approximately 1 h in dark.
   (b) Filtrate the solution through 0.22 μm filter to sterilize the solution and remove all solid particles like non-specifically formed formazan crystals (*see* **Note 1**).
   (c) Divide solutions in small aliquots (approx. 10 mL) and store in light protected container at −20 °C. Avoid refreezing of thawed aliquots to prevent accumulation of formazan by non-specific conversion of MTT.
   (d) For conducting assay, concentration of MTT used is 1 mg/mL. For that, prepare a 20% (v/v) MTT-Medium Master-mix solution for the desired amount of wells to be measured (e.g., 20 μL of MTT solution and 80 μL of fresh medium per well in a 96-well plate).
3. Solvent (to dissolve formazan crystals):
   (a) To dissolve the formazan crystals, different solvents can be used such as methanol, ethanol, isopropanol, and DMSO.
   (b) Dimethyl sulfoxide (DMSO): A purity of 99.5% is sufficient.
   (c) Acidified Isopropanol: Add 50 mL of 2 M HCl to 2.5 L isopropanol. Store the solution at least a month at room temperature before use. When the isopropanol is not acidified correctly, the suspension becomes cloudy [32].

2.6 Equipment/ Apparatus [32]

1. Hemocytometer.
2. Plate shaker.
3. Pipettes: 0.001–1 mL, single channel and 0.01–0.3 mL multi-channel.
4. Class 2B biosafety cabinet.
5. Bench-top centrifuge.
6. Microplate reader.

7. Incubator with 5% CO_2 at 37 °C.
8. Micro-well plates (96 wells plates).
9. For cells culture, Flat bottom Carrel flask (T-25/T-75 flasks).

3 Methods

The cytotoxicity of enterotoxins produced by probiotic strains is guided by European Commission and Opinion of the Scientific committee on Animal Nutrition [40] has approved the protocol as explained by Sandvig and Olsnes (1982) [41].

3.1 Preparation of Bacterial Toxin/ Supernatant for Cytotoxicity Assay [8, 26]

1. Inoculate single colony of bacterial isolate grown on LB agar plate into 10 mL BHI with 1% extra glucose (BHIG) and incubate it overnight.
2. Transfer 1 mL of this overnight grown culture into 50 mL of BHIG in 500 mL flask, and incubate it with shaking (100 rpm) for 6 h at 32 °C (*see* **Note 2**).
3. Harvest the culture by centrifugation at 5000 × *g*, at 4 °C for 30 min and use the supernatant for cell toxicity assay.
4. Concentrate thee supernatant ten-fold by protein precipitation with ammonium sulphate (*see* **Note 3**).
5. Sterilize the supernatant by filtration through 0.45 μm sterile filter.
6. Keep the supernatant frozen until further assessment of cytotoxicity.
7. Assays for the presence of emetic toxin were conducted on filter-sterilized culture supernatant fluids that had been heated for 10 min at 100 °C to inactivate the enterotoxin [8].

3.2 Plate Setup for Cytotoxicity Assay [32]

1. Grow Vero cells in MEM medium supplemented with Fetal calf serum and antibiotics.
2. Culture the cells at 37 °C in a humid atmosphere containing 5% CO_2.
3. Harvest the cells from the flask with a cell scraper upon arriving confluency.
4. Seed 1 mL of Vero cells suspension grown in MEM medium supplemented with 5% fetal calf serum into each wells of 24-well plate two-three days before the testing; except for the outer wells (*see* **Note 4**) and first three wells (*see* **Note 5**) are referred as blank wells.
5. Incubate the plate at 37 °C with 5% CO_2.
6. Before the start of the toxicity test, check that the growth of the Vero cells is confluent, i.e. the wells should be completely covered with Vero cells.

3.3 Treatment of Cells

1. Remove the growth medium from the wells and wash each well once with MEM (1 mL).
2. Add 1 mL of pre-heated (37 °C) MEM to each well.
3. Add 100 µL of bacterial supernatant/cell-free filtrate.
4. For positive control, add 100 µL supernatant of a positive *B. cereus* reference strain.
5. For negative control, add 100 µL of PBS instead of bacterial supernatant (*see* **Note 6**).
6. For calculation of the average toxicity, treatment should be given in triplicates.
7. Incubate the micro-well plate for further 8 and 24 h.

3.4 Cytotoxicity Assay

1. Measure the percentage of cells surviving using MTT assay.
2. Add 100 µL of MTT solution to each well.
3. Incubate the plate for 2–4 h.
4. After incubation, add 100 µL of dimethylsulfoxide (DMSO) to dissolve the blue crystals.
5. Measure the absorbance/optical density (O.D.) of each wells at 570 nm using a microplate reader.

3.5 Calculation and Inference

1. The toxic effect of the cell free bacterial culture supernatant fluids on the Vero cell line can be calculated from the following equation:

$$\left[1 - \frac{\mathrm{OD}_{\text{Negative control}}}{\mathrm{OD}_{\text{Control}}}\right] \times 100$$

2. Samples would be considered toxic, if the optical density of the test well is 20% less than that detected in the negative control wells. Above 20% inhibition is considered to indicate the cytotoxicity.

3.6 HEp-2 Cell Vacuolation Assay

3.6.1 Filtrate Preparation

1. Inoculate single colony of test strain to 10 mL of Brain Heart Infusion (BHI) broth and incubate it for 8–15 h at 37 °C.
2. Transfer 0.1 mL aliquot to 10 mL of BHI supplemented with 0.1% (w/v) glucose (BHIG).
3. Incubate it for 6 or 15 h at 32 °C with moderate agitation (250 rpm).
4. Centrifuge the cultures at (2000 × *g* for 10 min) and filter the supernatants through 0.8 µm Millex-AA filters (Millipore, S.A., France).
5. Give heat treatment to the filtrate at 100 °C for 10 min prior to assay.

3.6.2 Tissue Culture Assay

1. Cultivate Hep-2 cells up to confluency and trypsinize the cells with 0.25% trypsin-EDTA solution.
2. Take 25 μL of bacterial aliquots/supernatant prepared in Subheading 3.6.1 to first well of the 12-wells in 96-well plate and subsequently dilute it to two-fold in 0.15 M NaCl across 12 wells of a 96-well tissue culture plate.
3. Add 100 μL of freshly trypsinized HEp-2 cells in maintenance medium (EBME as mentioned above but with only 1% v/v fetal calf serum) to each well.
4. Seal the plates and incubate them overnight at 37 °C.
5. Perform all assays in duplicates.

3.6.3 Observation

1. Presence of more than 10 vacuoles/cell should be indicated by the expression "+ + +", 3–10 vacuoles/cell as "+ +" and 1–2 as "+".
2. Record the titer of filtrates with the vacuole response.

4 Notes

1. After preparing MTT solution, divide solution into small aliquots (approx. 10 mL) and store in light protected container at −20 °C. Avoid refreezing of thawed aliquots to prevent accumulation of formazan by unspecific conversion of MTT. MTT is toxic and harmful. MTT is light sensitive, hence, protect it from light.
2. Incubate at 32 °C (*Bacillus cereus*) or 37 °C (*Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus amyloliquefaciens*) for 6 h (BHIG) and 18 h (SMP), respectively, with shaking (100 rpm) in an orbital incubator.
3. The supernatant should be concentrated ten-fold by protein precipitation with ammonium sulphate to 80% saturation (561 g/L). After recovery of the protein by centrifugation (10,000 × *g* for 20 min) the pellet should be resuspended in 20 mM phosphate buffer at pH 6.8 in about 1/20 of the original volume. The remaining ammonium sulphate should then be removed by dialysis against the same buffer at 4 °C for at least 6 h and the volume then should be adjusted to one-tenth of the original volume. Cytotoxins usually are easily detected in supernatants before concentration.
4. The outer wells are not used for the experiment due to evaporation and are filled with phosphate buffered saline (PBS) to keep the evaporation of the plate to the minimum.

5. Additional control wells (without cells but with supernatant) should be kept to assess non-specific formazan conversion, i.e. to eliminate quantification of formazan crystals produced either by media components of bacterial supernatant.
6. Control wells (without bacterial supernatant) in triplicate should be assessed to determine the maximum number of viable cells.

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Chapter 16

In Vitro Evaluation of the Nitric Oxide Pathway

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Abstract

Regulatory organizations proposed that safety of each probiotic strain should be analyzed in sufficient details by all appropriate in vitro and in vivo methods. Nitric oxide (NO) exerts bactericidal action and it helps macrophages to kill pathogenic bacteria. It is generally observed that pathogenic bacteria show resistance to high level of NO as compared to probiotics. A probiotic having very low NO tolerance is considered to be a more health beneficial. NO sensitivity and resistance is an important safety criterion for probiotic strain. Moreover some reports suggested role of probiotics in clearing foreign matter by enhancing NO production by macrophages. Because few probiotic strains exhibited resistance to the host defense system such as macrophage and NO, it would act as inflammatory lesion and hence creates questions about generally regarded as safe label. Therefore analyzing sensitivity and resistance of probiotic strains to NO have its own safety importance. Due to the unstable nature of NO, its stable intermediates are used as an indirect indicator of NO amount. Griess reaction for NO estimation is based on nitrite estimation. Conversion of metmyoglobin to nitrosomyoglobin is another method for determination of NO produced by probiotics.

Key words Nitric oxide (NO), Probiotics, Nitric oxide resistance, Macrophage, Nitric oxide synthetase, Nitrite, Griess reaction, Nitrosomyoglobin

1 Introduction

In mammals, nitric oxide (NO) plays an important role in cell signaling, cell-host response, neuronal function, and immune system regulation [1]. In human during birth, NO concentration in intestine is very low. But soon after bacterial colonization in gut; intestinal NO in healthy infants upsurge rapidly [2, 3]. NO level in human need to be optimal as deficiency lead to atherosclerosis, coronary vasopasm while excess amount can cause hypotension in liver cirrhosis and failure, hemorrhagic, and anaphylactic shock [4].

It has been experimentally proved that NO and related reactive nitrogen intermediates such as nitrite (NO_2^-) and nitrate (NO_3^-) exert bacteriostatic and microbiocidal effects [5]. NO play vital role to help macrophages in killing (invade) of pathogenic bacteria

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_16,

[6]. Microbial resistance to NO mediated phagocytic pathway is thought to permit the bacteria to escape the phagocytic activity of innate immunity [4, 5]. Therefore, an ideal probiotic should not show resistant to NO.

Infective endocarditis (IE), also called bacterial endocarditis, is bacterial infection in bloodstream and population with some cardiac conditions have high risk of developing IE [7]. In rabbit IE model, it was observed that pathogenic strain of *L. monocytogenes* completely resist macrophage mediated intracellular killing as compared with probiotic *L. casei* strain Shirota. More than 50% IE clinical isolates were able to survive a high dose of 500 μg/mL *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrezino)-ethanamine (NOC12), a NO donor; whereas, the Shirota strain was sensitive to 125 μg/mL NOC12 [6]. Variability occurred among different probiotic for virulence and escaping host defense like macrophage mediated killing, hence safety of each strain should be analyzed by appropriate methods.

In diabetic human patients and rats, macrophages are deficient, affecting the production of NO, which affect immune and inflammatory response as well as liberation of cytokines [8]. It was observed that inflammation lead to increase in NO level. In healthy volunteers, rectal NO level was 3–25 parts per billion by volume (ppbv) whereas in inflammatory bowel disease (IBD) it increased significantly up to 71–8978 ppbv [9].

Furthermore, absence of NO resistance is considered as useful property regarding probiotic safety. As in the gastrointestinal tract (GIT); NO is involved in the protective mechanisms and may contribute to some of the beneficial effects of probiotics [5, 10].

NO is highly lipophilic diatomic free radical. It is extremely unstable (short half-life in vivo of a few seconds or less) and undergoes rapid oxidative degradation to nitrite (NO_2^-) and nitrate (NO_3^-) [11, 12]. Therefore, the levels of the more stable NO metabolites such as nitrite, metmyoglobin have been used in the indirect measurement of NO in biological samples (1) and (2). Variety of direct and indirect NO detection methods such as spectrophotometry, high performance liquid chromatography, fluorometric, electron spin resonance spectroscopy, and magnetic resonance imaging are reported in literature [13].

$$\text{Nitrite} + \text{sulfanilic acid} = \text{diazonium salt}$$

$$\text{Diazonium salt} + \text{N(1} - \text{naphthyl)ethylenediamine} = \text{Azo dye} \quad (1)$$

$$\text{Metmyoglobin} + \text{NO} = \text{nitrosomyoglobin (red)} \quad (2)$$

The chapter describes the protocols for assessing the NO sensitivity of probiotic isolates as well as the estimation of NO in vitro.

2 Materials

2.1 NO Sensitivity

1. Probiotic culture.
2. deMan Rogosa Sharpe (MRS) broth.
3. MRS agar (1.5%, w/v).
4. Hanks' Balanced Salt Solution (HBSS).
5. *N*-Ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrezino)-ethanamine (NOC12) (0–500 μg/mL).
6. Phosphate-buffered saline (PBS) (pH 7.3).
7. Incubator.
8. Anaerobic jar.
9. Centrifuge.
10. Glassware's and Plastic wares.

2.2 Estimation of NO

1. Probiotic culture.
2. MRS broth.
3. MRS agar (1.5%, w/v) (*see* **Note 1**).
4. Sodium nitrite standard (0–200 μM).
5. Naphthylethylenediamine dihydrochloride.
6. Sulphanilamide.
7. Argon gas.
8. Metmyoglobin.
9. Griess reagent—0.2% naphthylethylenediamine dihydrochloride, and 2% sulphanilamide in 5% phosphoric acid. Prepare both the compounds separately in an amber colored bottle label it and store it in 4 °C. Mix equal volume of both the solutions before estimation (*see* **Notes 2** and **3**).
10. Single/double beam spectrophotometer, ELISA reader.
11. Incubator, centrifuge, anaerobic jar, sterile syringe.
12. Glassware's and Plastic wares.

3 Methods

3.1 Estimation of NO Sensitivity of Probiotic Species [6]

1. Grow probiotic culture in MRS broth at 37 °C for 24 h in an anaerobic atmosphere of 7% H_2 and 5% CO_2.
2. Perform repetitive (2–3 times) centrifugation (12,298 × *g*) and washing of culture in phosphate-buffered saline(PBS) (pH 7.3).
3. Resuspend the culture in PBS to obtain optical density of 0.9 ± 0.1 Au at 600 nm and use it for further step.

4. Inoculate above culture suspension in Hanks' Balanced Salt Solution (pH −7.3) supplemented with 0–500 μg/mL NOC1−.
5. Incubate for 37 °C for 12 h.
6. Determine minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) to understand NO sensitivity and tolerance of probiotic (*see* **Notes 4** and **5**).
7. Various reports suggested sensitivity of probiotics strain to 125–250 μg/mL as opposed to pathogenic strains capable of tolerating more than 500 μg/mL NO [6].

3.2 Estimation of NO in Probiotic Using Griess Reaction [12]

1. Grow probiotic culture in MRS broth at 28–30 °C for 2–3 days.
2. After incubation, remove the cells by centrifugation at 12,298 × *g* for 20 min at 4 °C.
3. Collect supernatant and sterilize by passing through 0.45 μm filter.
4. Take 100 μL of culture filtered supernatant as test sample.
5. Take 100 μL of different concentrations of sodium nitrite (0–200 μM) as standard.
6. Add equal volume of Griess reagent separately in test and standards.
7. Prepare blank using distilled water and Griess reagent.
8. Maintain anaerobic conditions by purging reaction mixture with argon gas for 5 min.
9. Incubate the reaction mixture for 5 min at room temperature (30 °C).
10. Measure the absorbance at 550 nm immediately.
11. Prepare the standard curve of sodium nitrite and to estimate nitrite present in test sample. (*see* Table 1; **Note 6**).
12. Calculate the NO by using the given formula:

$$\text{NO}\ (\mu\text{M nitrite/mL}) = \frac{A\,\text{test}}{A\,\text{standard}} \times \text{concentration of standard nitrite}$$

wherein
A test = Absorbance of test sample.
A standard = Absorbance of standard nitrite.

3.3 Estimation of NO Using Metmyoglobin Method [12]

3.3.1 Qualitative Method Using MRS Agar

1. Add filtered sterilized metmyoglobin in sterile MRS agar at 50 °C to obtain concentration of 2–5 mg/mL (*see* **Note 7**).
2. Prepare plate aseptically and allow them solidification for 2 h then inoculate probiotic culture by stabbing in plate.
3. Incubate the plates at 28–30 °C for 2–3 days.

Table 1
NO estimation in probiotic using Griess reaction

Sr. no.	Standard nitrite conc. (μg/mL)	Standard nitrite (mL)	Griess reagent (mL)	D/W (mL)			
Blank	0	0	0.1	0.1	Purge argon gas for 5 min	Incubate the mixture for 5 min at room temperature	Measure the absorbance at 550 nm immediately
1	40	0.1	0.1	0.0			
2	80	0.1	0.1	0.0			
3	120	0.1	0.1	0.0			
4	160	0.1	0.1	0.0			
5	200	0.1	0.1	0.0			
Test	–	0.1 (Test spl)	0.1	0.0			

4. After incubation observe the colonies for red coloration around stabbing area.
5. Red colored colonies indicate conversion of metmyoglobin to nitrosomyoglobin due to NO.

3.3.2 Quantitative Method Using MRS Broth

1. Prepare a sterile 10 mL MRS broth having 0.2% glucose then add filtered sterile metmyoglobin (0.5–2 mg/mL) by sterile syringe or pipette.
2. Prepare suspension of the probiotic organism from previously screen (red color colonies) and inoculate 1% to the glucose-metmyoglobin MRS broth.
3. Incubate it for 18 h at 30 °C.
4. After incubation, remove the cells by centrifugation at 12,298 × *g* for 20 min at 4 °C.
5. Collect the supernatant and sterilize it by passing through 0.45 μm filter.
6. Measure the color change in filtrate between 450 and 650 nm against suitable control.
7. Take aliquots of standard nitrosomyoglobin (0.2–2 mg/mL) for preparing standard curve and for estimation of nitrosomyoglobin in supernatant.
8. Calculate the Nitrosomyoglobin concentration by using the below mentioned formula:

$$\text{Nitrosomyoglobin (mg/mL)} = \frac{A\,\text{test}}{A\,\text{standard}} \times \text{conc.of standard nitrosomyoglobin}$$

wherein
A test = Absorbance of test sample.
A standard = Absorbance of standard nitrosomyoglobin.

4 Notes

1. To prevent fungal growth add 0.01 mg/g cycloheximide in MRS agar.
2. The stability of Griess reagent is very less hence use fresh reagent during analysis. However, if stored in amber color bottle at 4 °C, it can be used up to a week.
3. Do not mix both the reagent (naphthylethylenediamine dihydrochloride, and sulphanilamide) used for preparing Griess reagent together before analysis because of auto oxidation and hence after mixing it has to be used within half an hour.
4. Minimum inhibitory concentration (MIC) is the smallest amount of an agent needed to inhibit growth of a microorganism. After addition of probiotic culture in different concentrations of NO (*see* Subheading 3.1) followed by incubation, observe the lowest NO containing test tube showing no visible growth. This is MIC value.
5. Minimum bactericidal concentration (MBC) is the smallest amount of an agent needed to kill the microorganism. Incolulate 0.1 mL probiotic culture (from the test tubes having probiotic culture exposed to different concentrations of NO, *see* Subheading 3.1) separately in MRS agar. Incubate for 48 h under anaerobic condition. The lowest NO concentration showing absence of microbial colonies on plate is MBC value.
6. Limit of nitrite detection up to 5 μM.
7. The myoglobin in an oxidized met-form, a physiologically inactive form that is not capable of binding oxygen.

Acknowledgments

Authors are thankful to the Uka Tarsadia University and Kavayitri Bahinabai Chaudhari North Maharashtra University for providing necessary facilities to write this chapter.

Declaration of Competing Interest All authors of this chapter declare no conflicts of interest.

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Chapter 17

Assessment of Capsule Formation

Rushabh Shah, Natarajan Amaresan, and Mitesh Kumar Dwivedi

Abstract

Microorganisms produce a diverse range of carbohydrates, including cytoplasmic storage polymers (glycogens) and structural polymers (glycans) that make up a portion of the microbial envelope. Glycan polymers include capsular polysaccharides (if polymer tightly bound to the cell surface) or exopolysaccharides (if loosely attached to the extracellular surface). Many Gram-positive and Gram-negative bacteria have been found to have capsular polysaccharides as virulence features. Therefore, the bacterial isolates which produce capsules cannot be considered probiotics. The capsules can be detected by various microscopy methods. Moreover, polymerase chain reaction (PCR) based method can detect specific capsule gene harbored by the bacterial strain.

Key words Capsular polysaccharides, Exopolysaccharides, Probiotics, Staining, Polymerase chain reaction (PCR)

1 Introduction

Bacterial capsular polysaccharides are a different group of high molecular weight polysaccharides that contribute to the virulence of many human pathogens in the gut, urinary tract, respiratory tract, and other host tissues by masking cell-surface components that would otherwise elicit an immune response from the host [1]. Pathogenic bacterial polysaccharide capsules have been shown to sterically shelter antigens from opsonizing antibodies, protect against antimicrobial peptides, and alter immunological responses. Capsules polysaccharide from probiotic bacterial species might have similar effects. The previous study indicated that biosynthesis of high-molecular-weight, galactose-rich surface polysaccharide molecules negatively impacts *Lactobacillus rhamnosus* GG ability to bind to intestinal epithelial cells, which may be due to adhesins on the bacterial cell surface is shielded [2]. Similarly, the purified capsular polysaccharide of *Lactobacillus casei shirota*, which was demonstrated to mediate the repression of pro-inflammatory responses in macrophages, has been postulated to play a direct role

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_17,

in host signaling [3]. Bacterial capsules have been detected by serological reactions, molecular genetic approaches, and electron microscopy but light microscopy is one of the most readily available and inexpensive techniques. After staining bacterial cells with nonspecific dyes, capsular polysaccharides can be seen using a light microscope. Standard dyes do not stain the capsules well, basic dyes are frequently combined with acidic dyes to stain the cells and background, respectively, while leaving the capsules transparent.

2 Materials

2.1 Microscopy Methods

1. Anthony's Method: 1% crystal violet and 20% copper sulfate.
2. Hiss's Method: 0.1% crystal violet and 20% copper sulfate.
3. Maneval's Method: Congo red and Maneval's stain {(distilled water (89.26 mL): CH_3COOH (4.68 mL): C_6H_6O (3.21 g): $FeCl_3 \cdot 6H_2O$ (2.80 g): acid fuchsin (0.05 g)}.
4. M'Fadyean's Method: 96% ethanol/methanol and 1% methylene blue or Loeffler's blue: {methylene blue (0.30 g): 95% ethanol (30 mL): 0.01% KOH (100 mL): K_2CO_3 (0.65 g)}.
5. Duguid's Method: India ink.
6. India ink staining Method: India ink and Crystal violet.
7. Negative–positive staining Method: Negative stains (India ink/Congo red/nigrosin) and Positive stains: (1% crystal violet/1% methylene blue).
8. Bacterial culture.
9. Microscope slide.
10. Covers slips.
11. Filter paper.
12. Nichrome wire loop (for inoculation).
13. Compound light microscope.

2.2 PCR Based Method

1. Ethidium bromide.
2. Agarose gel.
3. Tris–HCl.
4. KCl.
5. $MgCl_2$.
6. dNTPs.
7. Taq DNA polymerase.
8. Primers for Capsule genes.
9. Extracted bacterial DNA.

10. Molecular size marker (50 or 100 bp DNA ladder).
11. PCR tubes.
12. Thermal cycler.
13. Microtips and micropipettes.
14. UV transilluminator.

3 Methods

3.1 Microscopy Methods

3.1.1 Anthony's Method [4]

1. Place three loopful cultures of test bacterium (probiotic) from broth culture on a glass slide.
2. Obtain another clean microscope slide and place it at an angle to the end of the slide containing organisms. Spread out the drop into a thin film along with the first slide.
3. Allow the smear to air dry without heat fixation.
4. Apply 1% crystal violet to smear and allow it to react for 2 min.
5. Wash the slide with 20% copper sulfate solution.
6. Let the slide air dry and then examine under oil immersion.
7. Bacterial cells will appear purple while the capsules will appear light blue.

3.1.2 Hiss's Method [5]

1. Place a drop of 1% crystal violet stain on a clean microscope slide.
2. Using a sterile loop add a sufficient amount of bacteria from the culture tube or plate and mix it into the drop of crystal violet.
3. Prepare a very thin smear over the entire surface of the slide with the help of another slide.
4. Wait for 5–7 min to dry the slide, do not heat fix the slide.
5. Wash the slide thoroughly with 20% $CuSO_4$ solution.
6. Allow it to air dry and examine under oil immersion.
7. Bacterial cells will appear purple while the capsules will appear light blue.

3.1.3 Maneval's Method [6]

1. Place a drop of 1% congo red at one end of the microscope slide.
2. Using a sterile loop, collect bacteria from the culture tube or plate and mix it into the drop of congo red.
3. Then spread it gently on the slide to form a smear and allow it to air dry.
4. Flood the Maneval's solution to the smear and allow it to react for 5 min.

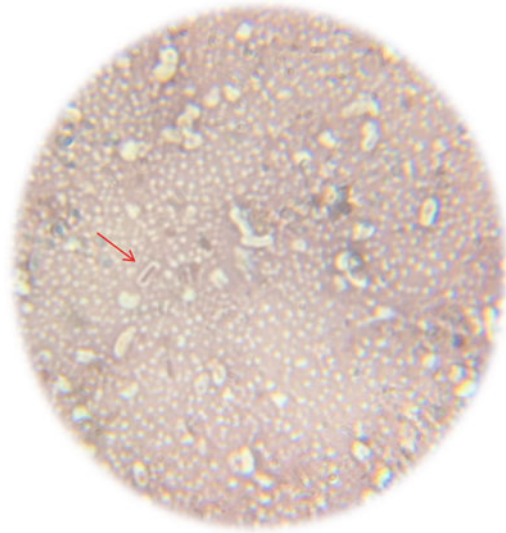

Fig. 1 Capsule staining by Maneval's method**.** The arrow indicates the colorless capsule surrounding the red colored bacterial cell against the red background

5. Afterward, gently wash the slide with distilled water, allow it to air dry and examine under oil immersion.
6. In the microscopic field, bacterial cells will appear red and capsules will be colorless against the red background (as shown in Fig. 1).

3.1.4 M'Fadyean's Method [7]

1. Make a thin smear by spreading a small drop of culture on a microscope slide using another slide.
2. Allow it to air dry and then add 96% ethanol or methanol for 30–60 s.
3. Allow the remaining solution to evaporate.
4. Add methylene blue or Loeffler's blue to smear for 30–60 s.
5. Wash the slide with distilled water and leave it to air dry.
6. Examine the slide under oil immersion.
7. The pink capsules will appear surrounding the bluish bacterial cells.

3.1.5 Duguid's Method [8]

1. Place a drop of India ink on a microscope slide.
2. Using a sterile loop, mix bacterial culture with an India ink and cover with a cover slip.
3. Blot off the excess sample and then examine under 400× magnifications.
4. The capsules will appear as brighter halos against dark bacterial cells and dark background of the slide.

3.1.6 India Ink Staining Method [9]

1. Place a drop of India ink on a microscope slide.
2. Using a sterile loop, mix bacterial culture with an India ink and spread as thin film with another slide.
3. Allow it to air dry, and then stain with crystal violet for 1 min.
4. Wash off the excess dye with distilled water.
5. Allow it to air dry and examine under oil immersion.
6. The bacterial cells will appear purple and the background black. The capsules will appear as clear zones around the cells.

3.1.7 The Negative–Positive Method [10]

1. Place a drop of a negative stain such as nigrosin, India ink, congo red, or eosin on the microscope slide.
2. Using a sterile loop, add a loopful of bacterial culture to slide and mix with stain.
3. Use another slide to spread the mixture into a thin film along with the first slide.
4. Allow it to air dry for 5–7 min. and do not heat fix it.
5. Then, apply the positive dye such as crystal violet for 1 min.
6. Remove the crystal violet by a positioned slide at 45° and then wash off with distilled water.
7. Allow it to air dry and examine under oil immersion.
8. If we use a combination of India ink as a negative dye and crystal violet or phenol fuchsin as a positive one then the cell will appear purple or pink against a blue background and capsules are revealed as a bright halo around bacterial cells.

3.2 PCR Based Method

The amplification of capsule gene can be carried out by using polymerase chain reaction. The steps for PCR based detection of capsule gene are given below.

1. Extract the DNA from the bacterial strain by following any standard protocol.
2. Analyze the extracted DNA on 1% agarose gel stained with ethidium bromide.
3. Use genomic DNA isolated from bacterial strain as DNA template and select pairs of primers for PCR amplification to screen the genes involved in capsular polysaccharide formation (e.g. cps2A-J and cps4A-J in *Lactobacillus plantarum* WCFS1 [11]). Refer Table 1 for the list of capsule biosynthesis related genes primers.
4. Make the final volume of 25 μL PCR mixture which include {10× (100 mM Tris–HCl pH 8.8 and 500 mM KCl), 1.7 mM $MgCl_2$, 0.2 mM dNTPs, and 1.5 U of Taq DNA polymerase}, 10 pmol/μL of each primer, and 100 ng of target DNA.

Table 1
List of capsule biosynthesis related genes′ primers

Primer name/Target	Sequence (5′ → 3′)	Capsulated organisms	Reference
CPS-F	CGACCTGGCCTGGCTTTCCGATCG	*K. pneumoniae*	[12]
CPS-R	CAAGCAACAGATCGGGGTTGTCGG	*K. pneumoniae*	
CPS-F2	GCCGGGTTAGTGGTAAA TGACAACG	*K. pneumoniae*	
CPS-R2	CGAGGGATTCAACAAACTCT	*K. pneumoniae*	
Flanking sequence of CPS region VP0219-0237	AATACTAGTGAGCTGTGTTCTTCA TTATTAATCCT	*Vibrio parahaemolyticus VP53*	[13]
	CGAAGTGATCTTCCGTCACAGG TGCAGTGAATGTCTGTTAACTCT	*V. parahaemolyticus VP53*	
	CGCGCCATTTAAATGAAGTTCC TCAGGCTCGTTACCAATGTGCT	*V. parahaemolyticus VP53*	
	GCCAATTATCCTAGACTCACCACT	*V. parahaemolyticus VP53*	
	cps2::cat (lp_1197–1206)		[11]
is60 lp1197F	TAATCCCAATTGAAATCCAGCC	*L. plantarum WCFS1*	
is63 lp1206R	ACAACTGCTTAATAGCCGGC	*L. plantarum WCFS1*	
is130 lp1197R	GCATACATTATACGAACGGTAGA TTTTTGATCCATCATTCACTC TCC	*L. plantarum WCFS1*	
is131 lp1206F	CGGTTACAGCCCGGGCA TGAGACAATATTGAAACTGTCG TAAAG	*L. plantarum WCFS1*	
	cps4::cat (lp_2108–2099)		
is 66 lp2108F	AGATCTATCATCAGGACGGC	*L. plantarum WCFS1*	
is132 lp2108R	GCATACATTATACGAACGGTAGA TTTGTGCGAAAACCAATC TAGCCG	*L. plantarum WCFS1*	
is133 lp2099F	CGGTTACAGCCCGGGCATGAGTA TTACTTGCTCCATTCCACACG	*L .plantarum WCFS1*	
is69 lp2099R	TGATGAAGCTGCATCGACCC	*L. plantarum WCFS1*	
cpsAup	ATCTTTAAGTACAATATCCT	*Streptococcus pneumoniae*	[14]
cpsAdown	TCAATAGCACATAGTAAT	*S. pneumoniae*	
cpsBup	CATATCGTTTTTGATGTAGA	*S. pneumoniae*	
cpsBdown	ATTGTGCATGTCACTTGC	*S. pneumoniae*	
CapA F	TGCCAAAATCGCAGTCAG	*Pasteurella multocida*	[15]
CapA R	TTGCCATCATTGTCAGTG	*P. multocida*	
CapD F	TTACAAAAGAAAGACTAGGAGCCC	*P. multocida*	
CapD R	CATCTACCCACTCAACCATATCAG	*P. multocida*	

5. Setup the PCR conditions in a thermal cycler for the capsule gene amplification as follows: 5 min denaturation at 94 °C followed by 35 cycles of amplification with 1 min denaturation at 94 °C, 45 s of annealing at 45–55 °C (depending on capsule gene primers) and 2 min extension at 72 °C followed by final extension step 72 °C for 10 min.
6. Analyze the PCR product using agarose gel (1%) electrophoresis and observe for specific bands by staining the gel with ethidium bromide and visualize under UV transilluminator.
7. Compare the size of the amplified product with the molecular size marker (50 or 100 bp DNA ladder) for identification of the capsule gene.

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Chapter 18

Assessment of Platelet Aggregation

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Abstract

Probiotics, a kind of beneficiary microbes, confers growth and development of other organisms. Notable probiotic groups incorporate bacterial genera viz. *Lactobacillus* and *Bifidobacterium*. Before utilizing a probiotic, performing various safety assessments is essential to evade avoidable health concerns. Of them, platelet aggregation, also called platelet adhesion, is an essential criterion to be acknowledged. The "gold standard" technique for platelet aggregation assessment is Light Transmission Aggregometry (LTA), which yield an index curve proportionally signifying level of platelet aggregation present in platelet-rich plasma (PRP) obtained from healthy donors. In this protocol, we described level of platelet aggregation by blending *Lactobacillus* strain with PRP blood samples along with Adenosine Di Phosphate and some inhibitors of platelet aggregation. From initial lag phase observed in aggregometry, we can conclude ability of different probiotic strains to cause platelet aggregation in blood.

Key words Probiotics, Aggregometry, Platelet rich plasma, *Lactobacillus*, Adenosine di phosphate (ADP), Aggregate inhibitor

1 Introduction

Probiotics are alive nonpathogenic microbes that enhances the host's intestinal microbial balance [1]. The term "probiotic" was coined by Lilly and Stillwell [2], indicating probiotics as substances produced by one microorganism that arouses the growth of another organism. Most usually used bacterial genera includes *Lactobacillus, Enterococcus, Escherichia*, *Bacillus*, *Bifidobacterium*, and *Streptococcus*, and are directed as dietary supplements and food [3]. Before using certain probiotics, it needs to satisfy some reliable safety evaluations like pathogenicity, toxicity, antibiotic resistance, and deleterious metabolic activities like platelet aggregation [4]. *Lactobacillus* acting as a beneficiary probiotic which had previously been reported in patients having septicemia and infective endocarditis (IE). IE had believed to be a progression of blood platelet aggregation, several experiments had been conducted regarding this disorder [5]. Platelet aggregation in simple terms

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_18,

can be defined as adhesion of platelets with each other at a vascular injury site [6]. The canonic model for the formation of platelet aggregation requires a platelet stimulus (ADP, thrombin, TXA2, or collagen), a soluble adhesive protein (Von Willebr and factor (VWF), fibrinogen, or fibronectin), and a membrane-bound platelet receptor (integrin αIIbβ3 or GPIIb-IIIa) [7]. Platelet aggregation assessment can be practiced in several methods, viz. skin Bleeding time (BT), Light Transmission Aggregometry (LTA), flow cytometry platelet analysis, Platelet Function Analyzer—PFA-100, IMPACT Cone and Plate(Let) Analyzer, and viscoelastic methods [8]. Platelet aggregometry had considered a "gold standard" technique for platelet aggregation assessment, which works on the principle of detection of light transmission difference with the help of photometer subsequently by adding platelet-rich plasma (PRP). Aggregator provides result in the form of an index curve, which describes transmitting light intensity via PRP samples. Extent of aggregation is generally manifested in parameters like percentage, lag phase, and slope of curve [9]. The requisite advantage of platelet aggregometry is, it measures the most inherent function of platelet is their aggregation with each other via a receptor-dependent manner [10]. Thus, in this chapter, we provided a brief protocol for platelet aggregation assessment with the assistance of Light Transmission Aggregometry.

2 Materials

1. MRS broth.
2. Phosphate-buffered Saline (PBS)—Add about 100 mL water to a 1 L graduated cylinder or a glass beaker. Weigh 8.0 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4 and transfer to cylinder. Add water to a volume of 900 mL. Mix and adjust pH to 7.3. Make up to 1 L with water and store at 4 °C.
3. Instruments like Spectrophotometer, Centrifuge, Incubator.
4. 0.1 M-trisodium citrate.
5. Dual-channel aggregometer.
6. Adenosine Di Phosphate.
7. Tris-NaCl-EDTA buffer—0.05 M Tris-hydrochloride, 0.1 M NaCl, 0.02 M EDTA.
8. Inhibitors of platelet inhibition like EDTA (40 mM), apyrase (10 mg/mL), imipramine (2 mM), quinacrine (1 mM), and acetylsalicylic acid (20 mM).
9. Dipyridamole (5 mM), 0.5% conc. HCl.
10. The peptide "arginine-glycine-aspartic acid-serine" (RGDS).
11. Pronase.
12. Bradford reagents.

13. 0.125 M Tris (pH 6–8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol.
14. Coomassie Blue R250.

3 Methods

3.1 Preparation of Lactobacillus *Culture*

1. Prepare and sterilize the MRS broth in autoclave at 121 °C with 15 lbs pressure for 15 min.
2. Inoculate *Lactobacillus* strains in 10 mL MRS broth under aseptic conditions.
3. Incubate inoculated *Lactobacillus* culture tubes at 37 °C for 18 h in an anaerobic jar with an atmosphere of 5% carbon dioxide in nitrogen.
4. Check purity of culture by plating on MRS agar by standardized plate counting methods.
5. Centrifuge cultures for platelet aggregation at 3075 × *g* at 4 °C for 15 min.
6. Collect the resulting cell pellet, and wash it three times in cold phosphate-buffered saline (PBS, pH 7.3).
7. Resuspend cells in PBS to an OD_{660} of 1.0 ± 0.02 (5×10^8 CFU/mL) and then concentrate it about fourfold, to give a suspension containing approximately 2×10^9 CFU/mL.

3.2 Preparation of Blood Samples

1. Collect blood samples from the healthy donors and mix fresh blood immediately with 0.1 M-trisodium citrate (9:1 vol/vol).
2. To produce Platelet-rich plasma (PRP), centrifuge the citrated blood sample at 150 × *g* for 10 min at 20 °C similarly to produce platelet-poor plasma (PPP) further centrifuge remaining blood at 2450 × *g* for 10 min at 20 °C.
3. Adjust the PRP with the PPP to an OD_{660} of 0.5 ± 0.01, giving a platelet concentration of 2.27×10^8 platelets/mL.

3.3 Platelet Aggregometry Assay

1. Carry out platelet aggregation in a recording aggregometer, with light transmission through PPP representing 100% aggregation and that through PRP representing 0% aggregation.
2. Lactobacilli can induce platelet aggregation by adding 25 μL bacterial suspension in 25 μL PRP and PPP which is pre-incubated at 37 °C for 5 min (final ratio of platelet to bacteria should be 1:1).
3. A lag phase of greater than 25 min should be assumed to be negative aggregation. Add Adenosine Di Phosphate (ADP) (20 μM) to negatively aggregating strains to confirm platelet function.

4. Perform all aggregations in triplicate, utilizing different batches of platelets, and get the mean and standard deviation of results.

3.4 Inhibition of Platelet Aggregation Assay

1. To test the effect of various inhibitors of platelet aggregation with representative strains, carry out all studies on the dual-channel recorder with one channel for a control aggregation without inhibitors.
2. Add 25 μL of PBS to PRP and PPP as a control to monitor the dilution effect of adding inhibitors and it does not cause changes in the length of the lag phase or final percentage aggregation.
3. Dissolve the potential inhibitors to the required concentration in PBS; EDTA (40 mM), apyrase (10 mg/mL), imipramine (2 mM), quinacrine (1 mM), and acetylsalicylic acid (20 mM).
4. At the same time, dissolve dipyridamole (5 mM) in PBS containing 0.5% (v/v) conc. HCl, a control aggregation of PBS with 0.5% (v/v) conc. HCl. Then dissolve the peptide "arginine-glycine-aspartic acid-serine" (RGDS), known to inhibit binding of fibrinogen to platelets in PBS (11.5 mM).
5. In all cases, add 25 μL of the inhibitor at the required concentration to PRP and PPP and incubate at room temperature for 10 min before the addition of bacteria. Platelet aggregation follows until aggregation is completed or until 25 min had passed.

3.5 Pronase Treatment

1. Incubate bacterial suspensions (10 mL, OD_{660} 1.0) with an equal volume pronase (5 mg/mL) at 37 °C for 20 min and then cool on ice before centrifugation and wash it three times with PBS.
2. Resuspend the cells to correct the optical density required for use in platelet aggregation.

3.6 Heat Treatment

1. Heat the bacterial suspensions (10 mL) for 1 h at 60 °C, then cool on ice and check the optical density before using in aggregation.

3.7 Extraction of Bacterial Surface Components

1. Harvest the cultures (400 mL) and wash them once in distilled water and resuspend the cells in 10 mL 0.1 M Tris–HCl (pH 8.5) and incubate it with shaking at 37 °C for 1 h.
2. Centrifuge the suspension and dialyze the extract immediately against distilled water for 18 h at 4 °C.
3. Determine the protein concentration of the extract by the Bradford assay method, lyophilize and redissolve the extract in PBS to give a protein concentration of 800 μg/mL.

4. Inhibition of aggregation can be tested by adding 25 μL extracted material to PRP and incubating along with stirring for 5 min before adding bacteria.

3.8 SDS-PAGE

1. Dissolve lyophilized protein extracts (50 μg) in 50 μL sample buffer containing 0.125 M Tris (pH 6–8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue, and 5% (v/v) 2- mercaptoethanol.
2. Then boil the dissolved extracts for 2 min and electrophorese through 12.5% (w/v) acrylamide gels, containing 0.1% SDS.
3. Stain the gels with Coomassie Blue R250 for 30 min and then de-stain and photograph it.
4. Molecular masses of proteins can be estimated concerning the following standards (kDa): α-lactalbumin, 14; trypsin inhibitor, 20; carbonic anhydrase, 30; ovalbumin, 43; albumin, 67; phosphorylase b, 94.

4 Observation

The bacterial strains *Lactobacillus rhamnosus*, *L. plantarum*, *L. acidophilus*, *L. fermentum*, and *L. salivarius* are tested for their aggregation activity, when mixed with ADP and other inhibitors such as EDTA, apyrase, imipramine, quinacrine, and acetylsalicylic acid (ASA), dipyridamole, RGDS and their aggregation percentage are recorded in Table 1.

Table 1
Observation table for recording the aggregation percentage

	L. rhamnosus		*L. plantarum*		*L. acidophilus*		*L. fermentum*		*L. salivarius*	
Agonist	**Lag (min)**	**Aggn (%)**	**Lag (min)**	**Aggn (%)**	**Lag (min)**	**Aggn (%)**	**Lag (min)**	**Aggn (%)**	**Lag (min)**	**Aggn (%)**
ADP										
EDTA										
Dipyridamole										
Apyrase										
ASA										
Quinacrine										
Imipramine										
RGDS										

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Chapter 19

Assessment of Fibrinogen and Fibronectin Binding Activity

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Abstract

Microbial adhesion is a high-affinity binding of a microbe to the host cell or its components, especially glycoproteins (fibrinogen, fibronectin, collagen, and mucin). An adhesion is a prerequisite for microbial attachment, colonization, biofilm formation, and tissue tropism (to support the growth of a particular microbe). Both pathogen and probiotics make adhesion to the host cells involving different host cell proteins. Probiotic bacteria attach to gut epithelium involving fibronectin of host cells. Pathogens penetrate gut epithelium, reach to blood, and make adhesion with fibrinogen. The binding affinity of both kinds of bacteria can be assessed by "in vitro adhesion assay" using a 96 well microplate coated with fibronectin or fibrinogen. Such an adhesion assay model can be used to explore the host cellular components involved in microbial adhesion and its efficiency.

Key words Microbial adhesion, Fibrinogen, Fibronectin, Probiotic, Pathogen

1 Introduction

Bacteria were predominantly considered as pathogen to human until the beneficial role of commensal microbes in health as established. Bacteria has the well-developed mechanism for the adhesion, invasion, or evasion in respective host cells [1]. Microbial adhesion to the human cells or cellular components is not always harmful. The best example of the beneficial bacterium in human is probiotic bacterium. Bacteria express several virulence factors collectively known as cell wall-anchored (CWA) proteins. The CWA proteins are crucial for its success as a commensal bacterium or pathogen. The structure and cognate interaction with host ligands of the CWA proteins may vary among different strains of bacterium. The key host cell protein that interacts with CWA is the glycoprotein (collagen, fibronectin, fibrinogen, and mucin). Fibronectin (FN) is extracellular, dimeric 454-kDa, glycoprotein that assembles into extracellular matrix (ECM) fibrils at cell surfaces. Fibronectin is secreted by fibroblast in gut epithelium also has important implications to maintain normal epithelial integrity. This protein is found

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_19,

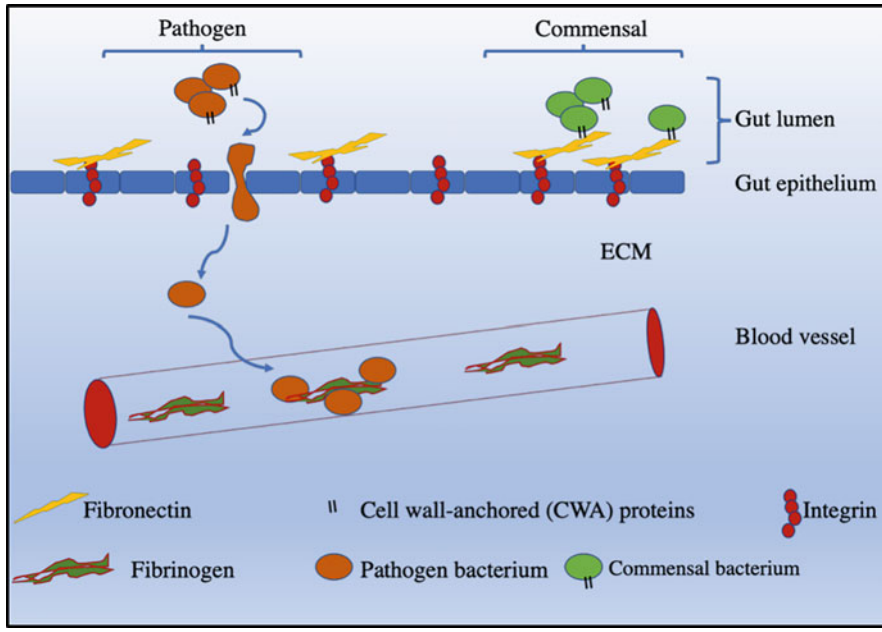


Fig. 1 Schematic representation of commensal and pathogenic bacterial interaction with fibronectin and fibrinogen, respectively

in blood plasma in soluble form or remains immobilized on host cells surfaces. Gut epithelium derived fibronectin potentiates the bacterial cell attachment and wound healing through epithelial-matrix interactions. Mucosal adhesion of the commensal microbes is also considered an essential attribute for their colonization [2]. Furthermore, it has been reported to regulate epithelial response during injury due to colitis [3].

Fibrinogen is a 340 kDa protein produced by the liver cells. It plays a critical role in blood clot formation during injury. Although fibrinogen has potential to limit bacterial pathogenesis, pathogenic bacteria also significantly interact with host fibrinogen to favor bacterial infection [4]. Thus, adhesion of the bacteria to the cellular components of the host is a critical event for the pathogenesis and symbiotic association. The above-mentioned procedure is simple, quick, and appropriate to assess the adhesion efficiency of a bacterial stain to its host cells. This procedure also can be used to explore the unknown cellular or bacterial components involved in the process of cell adhesion. It is well known that cancer cell encounter loss of cell adhesion for cancer progression and metastasis. Similar procedure also can be used to assess the adhesion property of cancer cell to the extracellular matrix protein [5] (Fig. 1).

1.1 Fibrinogen Binding Is an In Vitro Infectivity Measure

Fibrinogen forms a major formation of the ECM and the most abundant protein in blood. In blood it plays essential role in thrombosis, blood coagulation cascade, and immune response to pathogens [6]. Therefore, binding of bacteria to fibrinogen is crucial determinant of the bacterial pathogenesis. Hepatocytes mainly secrete fibrinogen in blood; however, endothelial cells also secrete a small amount of it. Many bacteria have developed mechanisms to bind fibrinogen to promote host cell adherence and disrupt blood coagulation to escape immune response [4, 7]. Several fibrinogen-binding proteins in pathogenic bacteria (*Staphylococcus aureus* and *Streptococcus* spp.) have been identified which play an important role in virulence [1, 8]. Hence, fibrinogen binding can help in measuring the infectivity potential of a bacteria.

1.2 Fibronectin Binding Is an In Vitro Probiotic Measure

A major component for probiotic adhesion in gut mucosa is fibronectin. Fibronectin is an ECM glycoprotein secreted by fibroblast in the gut. Probiotics are potential therapeutic options for a variety of diseases. Exploring the probiotic components involved in interaction with host fibronectin may help to decide the use of the antimicrobial drug with lesser harm to probiotics in the gut [9]. The cell surface proteins (e.g., FbpA) in probiotic strains mediate the microbial adhesion to respiratory tract epithelial cells [10]. The selection of probiotics for a given patient is very crucial. In vitro adhesion assay can be used to test pathogenicity, bile and acids tolerance, and adherence efficiency of probiotics before recommendation to the patient.

2 Materials

2.1 Adhesion of Probiotic Bacteria with Fibronectin Coated Microtiter Plate

- Bacterial strain (*Lactobacillus*).
- MRS Broth (De Man, Rogosa, and Sharpe agar); (#GM369; HiMedia).
- Centrifuge (4 °C);(Eppendorf).
- 50 mM Tris–HCl buffer at pH 7.5, (#71033, SRL).
- Dulbecco's modified Eagle's medium (DMEM); (#AT186; HiMedia).
- Fetal bovine serum (FBS); (#12103C; Sigma).
- 96-Well flat bottom microtiter plates, (#CLS3340; Corning®).
- Fibronectins, (#ECM001; Sigma).
- Human Collagen Solution (#C2249, Sigma).
- Mucin (#M2378, Sigma).
- HEPES-Hanks buffer (#37150, Stem Cell Technology).
- Bovine serum albumin (BSA), (#A3294; Sigma).
- Phosphate-buffered saline with 0.05% Tween™ 20 (PBST).

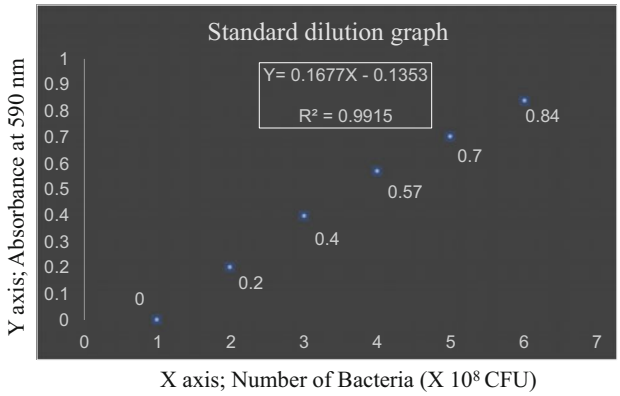
2.2 Adhesion of Probiotic Bacteria with Fibrinogen Coated Microtiter Plate

- Bacterial strain (*Staphylococcus aureus*).
- Human fibrinogen (#F3879, Sigma).
- 96-Well flat bottom culture plate, (#CLS3340; Corning®).
- 1× Phosphate-buffered saline (PBS)(#D8537, Sigma).
- 5% skimmed milk in PBS.
- 25% formaldehyde (#252549; Sigma).
- Crystal Violet (#C3886, Sigma).
- 5% Acetic acid (v/v) (#100063, Merck).
- Microplate Readers (Thermo fisher scientific).

3 Methods

3.1 Adhesion of Probiotic Bacteria with Fibronectin Coated Microtiter Plate

1. Grow *Lactobacillus* spp. in MRS broth for 12 h at 30 °C.
2. Coat the 96 well microtiter plates with fibronectin (7 μg/cm^2 in 1× PBS) at 4 °C overnight and subsequently block with 2% BSA for 1 h at 37 °C.
3. Harvest bacterial cells (3000 × *g*, 15 min at 4 °C) and resuspend the pellets in Tris–HCl buffer (50 mM, pH 7.5) and incubate at 30 °C for 1 h.
4. Again, centrifuge the cells (3000 × *g*, 15 min, and 4 °C) and resuspend in DMEM (with 2% FBS).
5. Wash the blocked plate three time with PBST and add 100 μL bacterial suspension containing 7 × 10^8 CFU and incubate for 2 h at 37 °C.
6. Wash the wells three times with 1× PBST and fix the adherent cells with 25% formaldehyde for 30 min at room temperature.
7. Stain the fixed cells with 0.5% (v/v) crystal violet for 3 min.
8. Wash the cells twice with 1× PBST and add 100 μL 5% acetic acid (v/v) to solubilize the stain taken by bacterial cells.
9. Analyze the intensity of color generation using microplate reader at 590 nm wavelength.
10. Use several dilutions of respective bacteria (1 × 10^8, 2 × 10^8, 3 × 10^8, 4 × 10^8, 5 × 10^8, 6 × 10^8 CFU/mL) as an internal standard.
11. Using the intensities of the internal standard, prepare a line chart (CFU of standard dilutions vs. intensity) in Microsoft excel and get the respective equation of the chart. Using the equation of the standard chart calculate the number of adherent bacterial cells. Figure 2 shows the representative illustration of graph of standard dilution and calculation of unknown experimental sample (absorbance intensity = 0.27). Similar way of calculation also has been demonstrated previously by Wilson et al. [11].



Concentration of bacteria in unknown sample (Absorbance-0.27)

$X = (0.27+0.1353)/0.1677$
$= 2.4 \times 10^8$ CFU/ml

Adhesion efficiency:
$=(2.4 \times 10^8/7 \times 10^8)$
$= 34.28\%$

Fig. 2 Representative Microsoft Excel graph and related formula of standard dilutions vs. crystal violet intensity (left). All the intensities are mean of triplicate sample. Calculation of concentration of unknown (experimental value) sample using graph formula (right). Value of R^2 evaluate the quality of the standard curve and expected to be more than 0.99 (note that all the intensities are mean of triplicate)

12. This protocol also can be used to measure the adhesion efficiency of probiotics to other two host cell glycoproteins (collagen and mucin). In this case, 96 well microtiter plates will be coated with 50 μL collagen (3 mg/mL) or 100 μL mucin (1 mg/mL in HEPES-Hanks buffer) solution at 4 °C overnight.

3.2 Adhesion of Probiotic Bacteria with Fibrinogen Coated Microtiter Plate

1. Grow the bacterial (*S. aureus*) culture in Tryptic soy broth (TSB).
2. Add 100 μL of human fibrinogen (10 μg/mL) solution in 50 mM sodium carbonate buffer (pH 9.6) to wells in a 96-well plate and incubate overnight at 4 °C.
3. Wash fibrinogen-coated wells three times with 1× PBS to remove the unbound fibrinogen.
4. Block the wells with 100 μL, 8% (w/v) skimmed milk-PBS for 2 h at 37 °C.
5. Harvest and wash the bacterial (*S. aureus*) culture once in PBS and resuspend it to 5×10^8 CFU/mL.
6. Add 100 μL of above cell suspension to fibrinogen coated and skimmed milked blocked wells and incubate for 2 h at 37 °C for proper bacterial cell adhesion.
7. Wash the wells three times with 1× PBS and fix the adherent cells with 25% formaldehyde for 30 min at room temperature.
8. Stain the fixed cells with 0.5% (v/v) crystal violet for 3 min.
9. Wash the cells with 1× PBS and add 100 μL, 5% acetic acid (v/v) to solubilize the stain taken by bacterial cells.

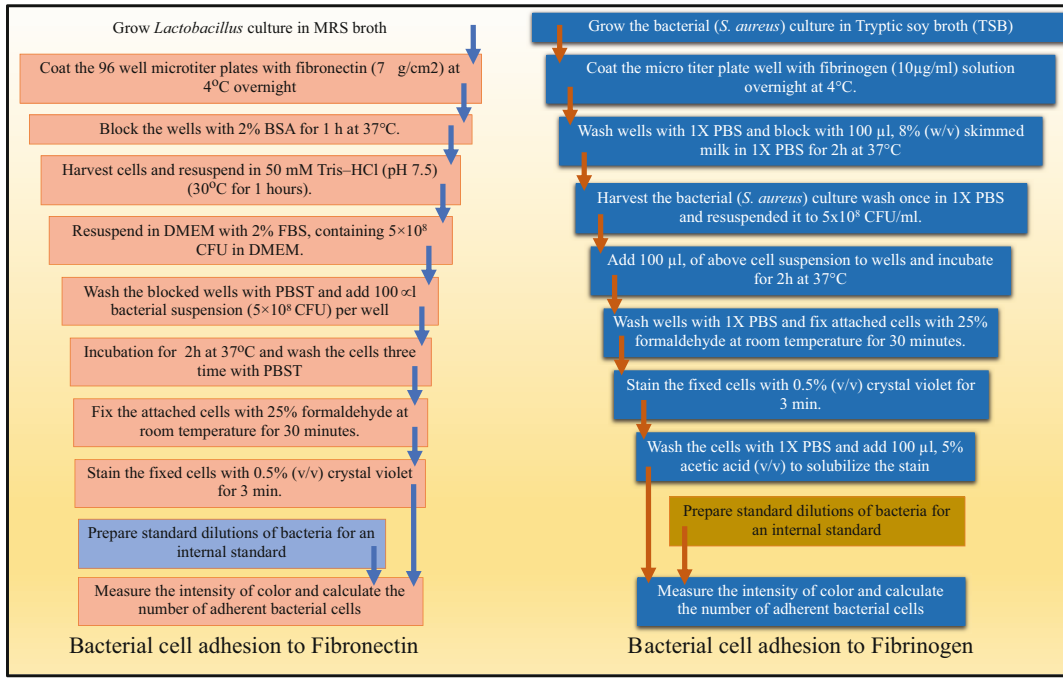


Fig. 3 Schematic representation of protocol for the bacterial adhesion to fibronectin (left panel) and fibrinogen (right panel)

10. Analyze the intensity of color generation using microplate reader at 590 nm wavelength.
11. Use the several dilutions of respective bacteria (1×10^8, 2×10^8, 3×10^8, 4×10^8, 5×10^8, 6×10^8 CFU/mL) as an internal standard.
12. Calculate the number of adherent bacteria similar to above mentioned (*see* Subheading 3.1) graph, formula, and calculation.
13. Figure 3 depicts the schematic protocol for the bacterial adhesion to both fibronectin and fibrinogen.

4 Inference

Adherence efficiency of probiotics to gut ECM proteins varies from 1% to 34% in healthy individuals depending on the strain under investigation. In general disease and gut infection condition decreases the adhesion efficiency of probiotics and consequently its beneficial functions. Combination of specific probiotic strains may enhance the adhesion efficiency to gut epithelium [12].

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Chapter 20

Assessment of Probiotics Adhesion to Mammalian Cells

Guhanraj Radhamanalan and Dhanasekaran Dharumadurai

Abstract

Probiotics are live microbial feed additives that help the host animal's microbial balance and gut function better. Probiotics are thought to colonize the gut for a short time via attaching to intestinal surfaces. As a result, the ability of bacteria to attach to intestinal cells is one of the selection criteria for probiotic strains. *Lactobacilli* resemble a large component of the human mucosal commensal flora. *Lactobacilli* that adhere to intestinal cell lines have been shown to offer health benefits, particularly in terms of pathogen adhesion inhibition. The bacterial strains can be screened by examining their adhesion capabilities with the HT-29 and Caco-2 cell lines. *Lactobacilli* closely resemble the commensal flora of the human mucosa. *Lactobacillus*, a probiotic adhesive, has been described as having beneficial health effects, especially in terms of avoiding disease attachment to intestinal cell lines. The chapter is focused on delivering the protocol to evaluate the in vitro adherence of bacterial strains (such as *Lactobacillus* isolates) to mammalian cells.

Key words Probiotics, *Lactobacillus*, Mammalian cell line, HT29 cell line, Caco-2 cell line

1 Introduction

Lactobacilli are Gram-positive rods that are non-sporulating and typically facultative in anaerobic circumstances [1]. *Lactobacilli* closely resemble the commensal flora of the human mucosa. There has been a growing attempt to isolate novel *Lactobacillus* strains with beneficial effects on human health [2]. In vitro adhesion experiments are considered to influence a probiotic's capacity strain to remain in the human stomach for a short period of time. The adhesion of lactic acid bacteria (LAB) in human primary organ culture models has gotten a lot of attention. Although only temporary, these cultures are identical to those seen in the field. Furthermore, a serious drawback is the shortage of human tissue. Human intestinal cell lines developed in vitro, such as HT-29 and Caco-2 cells, have been routinely used to select for probiotic strains in vitro in recent years. The differentiated human gut is typically modelled using HT-29 and Caco-2 cells. Despite the fact that both cell lines are generated from colon cancer, they have distinct

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_20,

absorptive enterocyte characteristics. This cell line has been used to evaluate a wide range of probiotic microbes for adhesion characteristics [3]. At initially, non-specific physical binding, such as hydrophobic interactions, may promote bacterial adhesion to gut surfaces. In the presence of surface proteins such as cell wall-anchored proteinases, certain lactic acid bacteria have been shown to have enhanced hydrophobicity and adhesion. Bacterial adherence to the gut is aided by adhesins found in the cell wall. Mucin adsorbed onto abiotic surfaces and human tumorigenic cell lines such as HT-29 were used to explore probiotic bacteria adherence in vitro [4, 5].

The initial stage in the adhesion process appears to be auto-aggregation, which allows bacteria to adhere to unwanted microorganisms. Another factor that influences total adhesion capability is cell surface hydrophobicity. Microbial adhesion to hydrocarbons (MATH) is a technique for determining the hydrophobicity of bacterial membranes on the cell surface [6]. The purpose of this chapter is to provide the protocol to evaluate the bacterial strains' adhesion to mammalian cells using in vitro method.

2 Materials

2.1 Assessment of Adhesion Ability

- Intestinal epithelial cell line HT29, Caco-2.
- McCoy's medium.
- Bovine fetal serum.
- Penicillin, Streptomycin, Gentamicin, Amphotericin B.
- EDTA.
- Dulbecco's Modified Eagle Medium.
- MRS broth.
- MRS agar.
- Trypsin.
- 96 or 24 well plates.
- Overnight *Lactobacillus* culture.
- Phosphate buffer saline.
- Centrifuge tube.
- Centrifuge.
- CO_2 incubator.

3 Methods

3.1 Assessment of Adhesion Ability

1. Grow the cell line in McCoy's medium supplements with 10% (v/v) heat inactivated bovine fetal serum with antimicrobial agents (Gentamicin, Amphotericin B).
2. Change the culture medium every 2 days.
3. Trypsinize the cell line with 0.25% trypsin-EDTA solution.
4. For the adhesion assay, seed the cells at a concentration of 10^5 cells/mL in 24-well plates and grow to confluence (11 ± 1 days) at 37 °C with a 5% CO_2 in an SL water jacketed CO_2 incubator.
5. Grow overnight culture in MRS broth at 37 °C and wash twice with PBS buffer to make the bacterial suspension.
6. Re-suspend the acquired bacteria in McCoy's medium without antimicrobials at a concentration of 10^8 CFU/mL.
7. Finally, wash the HT29/Caco-2 monolayers in Dulbecco's PBS twice.
8. Fill each well with 1 mL bacterial suspension and incubate for 1 h at 37 °C in a 5% CO_2 incubator.
9. Discard the supernatant and gently wash the wells twice with PBS Dulbecco's solution after incubation.
10. To determine the number of adherent bacteria, trypsinize the monolayers and count the bacterial cells in MRS agar (Table 1).

Table 1
Examination of *Lactobacillus* isolates for adherence to HT 29/Caco-2 cell line

Isolate code	Range of adhesion capacity	Value of adherence capacity	Number of isolates	Percentage (in relation to the total number of isolates)
	HT 29/Caco-2 cell with high adherence (Above 50 adherent bacteria)			
	Adhesion (10–50 attached bacteria/(HT 29/Caco-2 cell is good)			
	Moderate adhesion (1-ten attached bacteria per (HT 29/Caco-2 cell)			
	Weak adhesion (HT 29/Caco-2 cell with 1 attached bacteria)			
	No adherence			

Table 2
Recommendations for improved in vitro probiotic adhesion analysis

Use a variety of models	Pre-screening can be done with tissue culture cells or intestinal mucus, and the refined model can be a whole tissue or organ culture
The intestine's natural microbiota	To account for the average gut microbiota's influence, use complete tissue or organ culture models
Optimal circumstances for growth	Microbes are cultivated in the future application's growth medium, using characteristics (temperature, stirring, atmosphere, etc.) that will be used in the future application (milk, juice, etc.)
Incubation period for adhesion	Depending on the simulated location, standardize incubation periods and utilize physiologically relevant incubation times
Flux in the intestines	The substratum should be gently shaken to imitate intestinal activity. This will also prevent the germs from sediment after an extended period of incubation (see above)
The concentration of bacteria	Use physiologically adequate probiotic doses to avoid saturation of the substratum; Digestion will dilute the probiotics 10,100-fold and may destroy up to 90% of them
Buffers	Physiologically appropriate buffers should be used to mimic gastrointestinal tract conditions
The matrix of food	Food as a matrix before the adhesion research, expose the probiotic to the food matrix or other foods. It is also possible that exposure to partially digested meals is advantageous, however this might be difficult to reproduce
Digestion	Allow for in vitro digestion of the probiotics, including saliva

11. Calculate the adhesion percentage by comparing number of adhered cells to the number of bacteria added (% CFU bacteria adhered/CFU bacteria added) (Table 1).
12. For each probiotic isolate, perform the adhesion assay in triplicates.

Lactobacillus isolates have been discovered to have beneficial probiotic characteristics. The recommendations for better probiotic adhesion in vitro investigation are given in (Table 2). These isolates are promising candidates for future exploration in vitro and in vivo to investigate whether they have any health benefits.

4 Inference

Understanding the molecular processes of adhesion of a certain strain/species in vitro and subsequent modification by the host–microbe interaction is required. Different probiotic strains' in vitro adherence may be compared using Caco-2/HT 29 cells and intestinal mucus. Finally, human intestinal epithelial cell lines, such as

Caco-2/HT 29 cells can be used to perform comparative investigations on probiotic strain's adhesive abilities. In addition to human intestinal cell lines, intestinal mucus glycoproteins may be used to evaluate in vitro adhesion probiotic strains. It may be able to choose certain probiotics with targeted adhesion sites for in vivo experiments by using distinct in vitro models for gut surfaces. To produce a standard in vitro model of human origin for adherence investigations, more research comparing different sources of mucus and different ways for obtaining intact mucus glycoproteins is needed. Because in vivo adhesion cannot be replicated in vitro, the results of in vitro adhesion experiments are suspected. The hypothesized relationship between in vitro and in vivo adhesion should be investigated as soon as possible, employing adherent and low-adherent isogenic strains using in vitro and in vivo models from the same target host. Adherent and low-adherent isogenic strains may also be useful in assessing the importance of adhesion for probiotics in vivo.

Acknowledgements

The authors express their appreciation to the DST-FIST (Department of Science and Technology-Fund for Improvement of Science and Technology Infrastructure), New Delhi, for the instrumentation facility (DST Sanction Order No.: SR/FIST/LSI-013/2012/Dt.13.08.2012). We gratefully acknowledge the Department of Science and Technology's (DST) financial support for the research facility through the DST Promotion of University Research and Scientific Excellence (PURSE) scheme-Phase II, Rashtriya Uchchatar Shiksha Abhiyan (RUSA)-2.0- Biological Sciences (TN RUSA: 311/RUSA (2.0)/2018 dt. 02/12/2020).

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Chapter 21

Assessment of Mutagenicity

K. R. Jeya, Ashraf Khalifa, M. Veerapagu, and A. Sankaranarayanan

Abstract

Probiotics are live microorganisms that empower conducive to health on the host when dispensed in ample supply. Probiotics have been conventionally employed in fermented foods under being safe to use. They include *Lactobacillus*, *Bifidobacteria*, *Enterococcus*, *Streptococcus*, *Bacillus*, *Lactococcus*, *E. coli*, *Pediococcus*, *Tetragenococcus*, *Aerococcus*, and *Weissella* and some yeasts. They may be found in milk, cheese, yoghurt, fermented sausages fermented meat, fermented vegetables (olives, sauerkraut), sourdough bread. Probiotics have a multifarious and miscellaneous impact on the host namely improvement of mucosal defenses of the gastrointestinal tract which include antimicrobial activity, enhancement of mucosal barrier function and immunomodulation, etc. They have been used in food preservation and as food additives to improve flavors and texture. However, a multidisciplinary approach for the safety evaluation of probiotic bacteria are of considerable significance since as most probiotic bacteria are marketed in foodstuffs or feed supplements. Hence the mutagenicity potential of probiotic bacteria has been assessed by bacterial reverse-mutation assay (Ames test). The Ames test by plate incorporation method is the most commonly used adaptable technique to detect the mutagenic aptitude of probiotics.

Key words Probiotic, Safety evaluation, Bacterial reverse mutation assay, Ames test, Mutagenicity, Plate incorporation method

1 Introduction

Fermented foods have been renowned for their nourishing health effects over several centuries. Nevertheless, it was presumed that consumption of live microbes has a favorable impact on the intestinal microflora and thereby, ameliorates individual health and endurance [1]. Henceforth, multiple efforts have been adopted to regulate the intestinal microflora utilizing live microbes to advance the functional state of mankind or animal life. Such live advantageous microbes are presently profitable market as probiotics in capsule and sachet forms and also included in the formulation of food [2].

Probiotics are live microbes which when consumed have a favorable effect on the host by recuperating intestinal microbial

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_21,

equilibrium [3]. Probiotic approach for human wellness has been almost over several years [4]. They have been disclosed to exhibit a curative function in diversifying allergy, bowel movements eczema, reducing cholesterol, progressing lactose tolerance, and avert cancer and more diseases [5, 6]. *Lactobacillus* and *Bifidobacterium* are increasingly being employed as probiotics in food and feed supplement [7]. Some species of yeast *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, and several *Escherichia coli* and *Bacillus* spp. are also used as probiotics.

Probiotic bacteria are utilized in fresh milk, yogurt, cheese, and other fermented products such as fruit and vegetable based drinks, fermented cereal probiotic beverages as starter cultures to ameliorate digestion since ancient times [8]. Yoghurt is manufactured using *Streptococcus thermophilus* and *Lactobacillus delbrueckii* as starter cultures [9]. In nondairy products such as dry sausages, sauerkraut, and smoked salmon *Lactobacillus* and *Pediococcus* sp. are starters, *Lactobacillus plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Pediococcus pentoseceus*, and *Lactobacillus acidophilus* in fermented vegetable juices, pickles, and silages [10]. *Lactobacillus* spp. and *Enterococcus* spp. are utilized as starter cultures in cultured buttermilk, kefir, cheeses, ice cream, and other dairy products [11]. Probiotics are extensively exploited for improving the health of humans and animals. The undesirable effects of probiotics are only unusual. Nevertheless, the safety assessment of new fangled probiotic strain must be deployed before usage. Probiotics may be able to produce bacteremia, affect metabolic function and immunity [12]. There is a potential risk of viable probiotics in immunosuppressed hosts or individuals with a defective immune system. However, infective endocarditis caused by probiotics is rare [13]. Thus, there is a prerequisite to assess the safety of probiotics before commercialization for human usages.

Numerous in vitro and in vivo methods including human clinical trials are used for the safety assessment of probiotics which may include interaction between host and probiotics, mode of infections, pathogenesis, virulence, etc. [14]. Since there is no commonly accepted method for the evaluation of the safety of probiotics, Organization for Economic Cooperation and Development (OECD) guidelines [15] are used for safety assessment of chemicals in rodents are also now used as common standards [16]. OECD guidelines based Bacterial Reverse Mutation test have been applied to determine the genotoxic potential of probiotic strain [17]. One of the essential methods for the evaluation of the safety of probiotics is the ability of the probiotic to induce a mutagenic effect on the host [18]. The Bacterial Reverse mutation assay (Ames test) is an uncomplicated, quick, and prosperous bacterial assay to determine the mutagenic potentiality of test substances including chemicals, food additives, live probiotic microbes, and metabolic products of probiotics [19]. This test detects point mutation which is supposed to cause numerous

genetic diseases and is responsible for the initiation and development of tumors. In this test histidine auxotrophic mutant of different strains *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA97, TA98, TA100, TA102) and tryptophan auxotrophic mutant of *E. coli* strain (WP2 uvr A, WP2 uvr A,pKM101) with DNA repair deficiency were employed. When the test substance is a mutagen, the mutation occurs in bacteria subjected to undergo Ames test by base pair substitution or by frame shift mutations which in turn retrieve the capability of the bacteria to synthesize the amino acid. The test substance induced reverse mutant bacteria are identified by their potential to grow in the absence of the amino acid deficient media.

The mutagenic potential of probiotic isolates was assessed by bacterial reverse mutation assay (Ames test) in numerous reports. Probiotic *Lactobacillus plantarum*, HK006, HK109, and *Pediococcus pentosaceus* PP31 with or without S9mix revealed the absence of mutagenic property [18]. Assessment of genotoxic activity of vigiis 101 powder from dried *Lactobacillus paracasei* subsp. *paracasei* NTU 101 showed no mutagenic activity [20]. In a similar manner, Dubbert et al. [21] reported that the viable probiotic *Escherichia coli* strain Nissle 1917 (EcN) and its cell-free supernatant exhibited the absence of mutagenic activity assessed by the Ames test. Mutagenic assessment of probiotic by bacterial reverse mutation assay in recent literature is given in Table 1.

2 Materials

2.1 Equipment

- Eppendorf tube.
- Conical flask.
- Beaker.
- Micropipette.
- Disposable tips.
- Water bath.
- Membrane filters (0.22 μm, 0.45 μm).
- Bunsen burner.
- Inoculation loop.
- Digital balance.
- Automatic colony counter.
- Autoclave.
- Incubator.
- Biological safety cabinet class II.
- Magnetic stirrers.
- Refrigerator (4 °C) and freezer (−20 °C).
- Vortex mixer.

Table 1
Mutagenic assessment of probiotic by bacterial reverse mutation assay (Ames test)

Probiotic	Test substance	Amestest strain	Metabolic activation	References
Lactobacillus rhamnosus MP108	Cell dry powder	*S. typhimurium* TA97, TA98, TA100, TA102, and TA1535	Presence and absence of S9mix	Zhang et al. [22]
Akkermansia muciniphila	Pasteurized cell suspension	*S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537	presence and absence of S9mix	Druart et al. [23]
Leptolyngbya sp. KIOST-1 (LK1)	LK1 powder	*S. typhimurium* TA100, TA1535, TA98, and TA1537 *E. coli* WP2 uvrA	–	Lee et al. [24]
Corynebacterium glutamicum	Amino acid additive (L-threonine, L-tryptophan, and L-valine)	*S. typhimurium* TA100, TA1535, TA98, TA1537, TA102, and *E. coli* WP2 uvrA	Presence and absence of S9mix	Kang et al. [25]
Saccharomyces cerevisiae	Yeast cell wall preparation- glycan	*S. typhimurium* TA1535, TA1537, TA98, TA100, and TA102.	Presence and absence of S9mix	Dillon et al. [26]
Escherichia coli Strain Nissle 1917	Cell suspension and cell free supernatant	*S. typhimurium* TA98, TA100, TA1535, TA1537	Presence and absence of 9mix	Dubbert et al. [21]
Enterococcus faecalis UGRA10	Enterocin AS-48	*S. typhimurium* TA97A, TA98, TA100, TA102, TA1535	Presence and absence of S9mix	Cascajosa-Lira et al. [27]
Escherichia coli Strain Nissle 1917	Inactivated cell suspension	*E. coli* WP2 and *S. typhimurium* TA100	Presence of S9 mix	Janosch et al. [28]
Lactobacillus plantarum PS128	PS128 powder (1×10^{11} CFU/g)	*S. typhimurium* TA97, TA98, TA100, TA102, and TA1535	Presence and absence of S9mix	Liao et al. [29]
Bacillus pumilus, *Bacillus megaterium*	Bacterial spores	*S. typhimurium* TA98, TA100, TA1535 TA1537, and *E. coli* WP2 uvrA)	–	Kotowicz et al. [30]
Lactobacillus plantarum	Cell suspension	*S. typhimurium* TA98, TA100	Presence and absence of S9mix	Ahmad et al. [31]

(continued)

Table 1
(continued)

Probiotic	Test substance	Amestest strain	Metabolic activation	References
Lactobacillus mali APS1	Freeze dried cell pellet	*S.typhimurium* TA97a, TA98, TA100, TA102, and TA1535	Presence and absence of S9mix	Lin et al. [32]
Bacillus amyloliquefaciens B-1895 *and Bacillus subtilis* KATMIRA1933.	Cell free supernatants, subtilosin	*S. typhimurium* TA1535	–	AlGburi et al. [33]
Lactobacillus plantarum, HK006, *and* HK109, *Pediococcuspentosaceus* PP31	Probiotic Powder	*S. typhimurium* TA98 *and* TA1535	Presence and absence of S9mix	Tsai et al. [18]

2.2 Media, Reagents, and Recipes

2.2.1 Minimal Glucose Agar Medium

In a 2 L flask, add 15 g of agar, and then add 930 mL of distilled water to it. Dissolve the agar in a water bath and sterilize in an autoclave at 121 °C for 20 min. Cool the flask to around 65 °C and add sterile VB salt solutions 50× followed by the addition of sterile 50 mL of 40% glucose solutions. For probiotic cell suspension, add 2.40 mL of filter-sterilized ampicillin (10 mg/mL) to the medium to get the final concentration of ampicillin 24 μg/mL, aseptically or suitable concentration of any antibiotic to inhibit the growth of probiotic bacteria and not the Ames test strain. Mix the medium by swirling the flask. Aseptically pour approximately 25 mL of the medium into each sterile petri plate. Allow the medium to solidify under a laminar airflow cabinet for about 20–30 min.

2.2.2 Vogel-Bonner Salts (50×)

Ingredients	Quantity
Magnesium sulphate	10 g
Citric acid monohydrate	100 g
Potassium phosphate dibasic	500 g
Sodium ammonium phosphate	175 g
Distilled water warm at 45 °C	1000 mL

Add 700 mL of distilled water in a pertinent glass container. Warm the water to 45 °C in a magnetic stirrer with the hot plate. Add the salts in the above order one by one. Dissolve each salt completely by stirring before the addition of later salts. Bring the volume to 1000 mL with distilled water. Dispense the salt solutions in 200 mL aliquots with a loose cap. Sterilize in an autoclave at 120 °C for 20 min. Leave the solutions to cool. Then tight the caps and store it at room temperature in dark.

2.2.3 Glucose Solution (40% w/v)

Add 40 g of glucose to 70 mL of distilled water. Dissolve it completely by stirring. Make the volume to 100 mL with distilled water. Dispense in 50 mL aliquots with loose caps and sterilize in an autoclave at 120 °C for 20 min and leave the solution to cool. Then tight the caps and store it at 4 °C.

2.2.4 Ampicillin Solution (10 mg/mL)

Dissolve 0.1 g of ampicillin in sufficient distilled water to make a final volume of 10 mL Sterilize it with a 0.45 μm filter. Dispense in 5 mL aliquots and store it at 4 °C.

*2.2.5 Histidine-Biotin Solution 0.5 mM (*S. typhimurium *Strains)*

Ingredients	Quantity
D-Biotin	12.4 mg
L-Histidine	9.6 mg
Distilled water	100 mL

Add specific quantity of biotin and histidine to water boiling in a water bath. After dissolving cool it and sterilize by filtration (0.45 μm) or by autoclave at 121 °C for 20 min. Dispense in 50 mL aliquots and store it at 4 °C in a glass bottle.

*2.2.6 Tryptophan Solution 0.25 mM (*E. coli *Strains)*

Ingredients	Quantity
L-Tryptophan	5.1 mg
Distilled water	100 mL

After adding 5.1 mg of tryptophan to the 200 mL glass beaker, dissolve it in 100 mL of distilled water. sterilize it by membrane filter (0.45 μm) or by autoclave at 121 °C for 20 min. Dispense in 50 mL aliquots and store it at 4 °C in a glass bottle.

2.2.7 Top Agar Supplemented with Histidine-Biotin or Tryptophan

Ingredients	Quantity
Sodium chloride	0.5 g
Agar	0.6 g
Sterile histidine-biotin solution (0.5 mM) (*S. typhimurium* strains) or Sterile tryptophan solution (0.25 mM) (*E. coli* strains)	10 mL or 10 mL
Distilled water	90 mL

Add sodium chloride and agar to 90 mL of distilled water in a 200 mL screw cap bottle. Autoclave it (loosely capped) for 20 min at 120 °C. Then, under a laminar flow hood, add 10 mL of sterile

histidine and biotin solution (0.5 mM) for *S. typhimurium* strains or add 10 mL of sterile tryptophan solution for *E. coli* strains. Mix it well. Dispense 50-mL aliquots in 100 mL sterile screw-cap bottles. Melt the top agar in boiling water at the time of use. Add 120 µL of sterile ampicillin solution to top agar to get a final concentration of 24 µg/mL of ampicillin to inhibit the growth of probiotic bacteria in probiotic cell suspension and not Ames test strain. For the cell-free supernatant use top agar without the antibiotics.

2.2.8 Nutrient Broth (HiMedia)

Accurately weigh 1.3 g of nutrient broth (Himedia) medium as per manufacturer recommendation and add 100 mL of distilled water. Completely dissolve the medium in a hot plate. Sterilize the medium in an autoclave for 15 min at 121 °C. Dispense 20 mL of aliquot in a sterile container.

2.2.9 Phosphate Buffer 0.2 M (pH 7.4)

Solution A: Dissolve 27.6 g sodium phosphate monobasic in 1 L of distilled water.

Solution B: Dissolve 28.4 g Sodium phosphate dibasic in 1 L of distilled water.

Add 120 mL of solution A to a 2 L conical flask followed by the addition of 880 mL of solution B. Mix them well and adjust pH to 7.4 by using solution B. Sterilize in an autoclave at 121 °C for 20 min and store at 4 °C.

2.2.10 Positive Control Mutagen

The recommended strain specific positive control concentrations are given in Table 2 [34].

2.2.11 S9mix

Ingredients	Quantity
D-Glucose-6-phosphate	1.6 g
Nicotinamide adenine dinucleotide phosphate (NADP)	3.5 g
Magnesium chloride (MgCl)	1.8 g
Potassium chloride (KCl)	2.7 g
Sodium phosphate dibasic	12.8 g
Sodium phosphate monobasic	2.8
Distilled water	900 mL

Measure 900 mL of distilled water in a measuring cylinder and transfer to a 2 L flask. Sequentially add each ingredient and dissolve it completely. Sterilize it with the help of 0.45 µm filter. Transfer 9.5 mL of solution to a sterile tube and add 0.5 mL of liver S9 fraction (5%v/v) and store it at −20 °C.

Table 2
Recommended strain specific positive control concentration [34]

	Without S9-mix		With S9-mix	
Bacterial strain	**Chemical**	**Conc. (μg/plate)**	**Chemical**	**Conc. (μg/plate)**
Salmonella typhimurium TA97	9-Aminoacridine (9AC)	50	2-Aminoanthracene (2AA)	2
Salmonella typhimurium TA98	2-Nitrofluorene(2NF) 4-Nitro-o-phenylenediamine (NOPD)	1 2.5	Benz[a]pyrene (BaP)	5
Salmonella typhimurium TA100	Sodium azide (NaAz)	0.5	Benz[a]pyrene (BaP)	5
Salmonella typhimurium TA102	Mitomycin (CMC)	0.5	Danthron (DAN)	25
Salmonella typhimurium TA1535	Sodium azide (NaAz)	0.5	2-Aminoanthracene (2AA)	2
Salmonella typhimurium TA1537	9-Aminoacridine (9 AC)	50	Benz[a]pyrene (BaP)	5
Salmonella typhimurium TA1538	4-Nitro-o-phenylenediamine (NOPD)	2.5	2-Aminoanthracene (2AA)	(2–10)
E. coli WP2 uvrA	4-Nitroquinoline-1-oxide (NQO)	0.5	2-Aminoanthracene (2AA)	20
E. coli WP2uvrA (pKM101)	4-Nitroquinoline-1-oxide (NQO)	0.2	2-Aminoanthracene (2AA)	20

2.3 Microbial Cultures

1. Pure culture of probiotic isolate (*see* **Note 1**).
2. Pure culture of Ames Test Strains (*see* **Notes 2**, **3** and **4**).
   (a) *Salmonella typhimurium* TA97.
   (b) *Salmonella typhimurium* TA98.
   (c) *Salmonella typhimurium* TA100.
   (d) *Salmonella typhimurium* TA102.
   (e) *Salmonella typhimurium* TA1535.
   (f) *Salmonella typhimurium* TA1537.
   (g) *Salmonella typhimurium* TA1538.
   (h) *E. coli* WP2 uvrA.
   (i) *E. coli* WP2 uvrA, pKM101.

3 Methods

Bacterial Reverse Mutation Assay (Ames test) by plate incorporation method (Figs. 1 and 2):

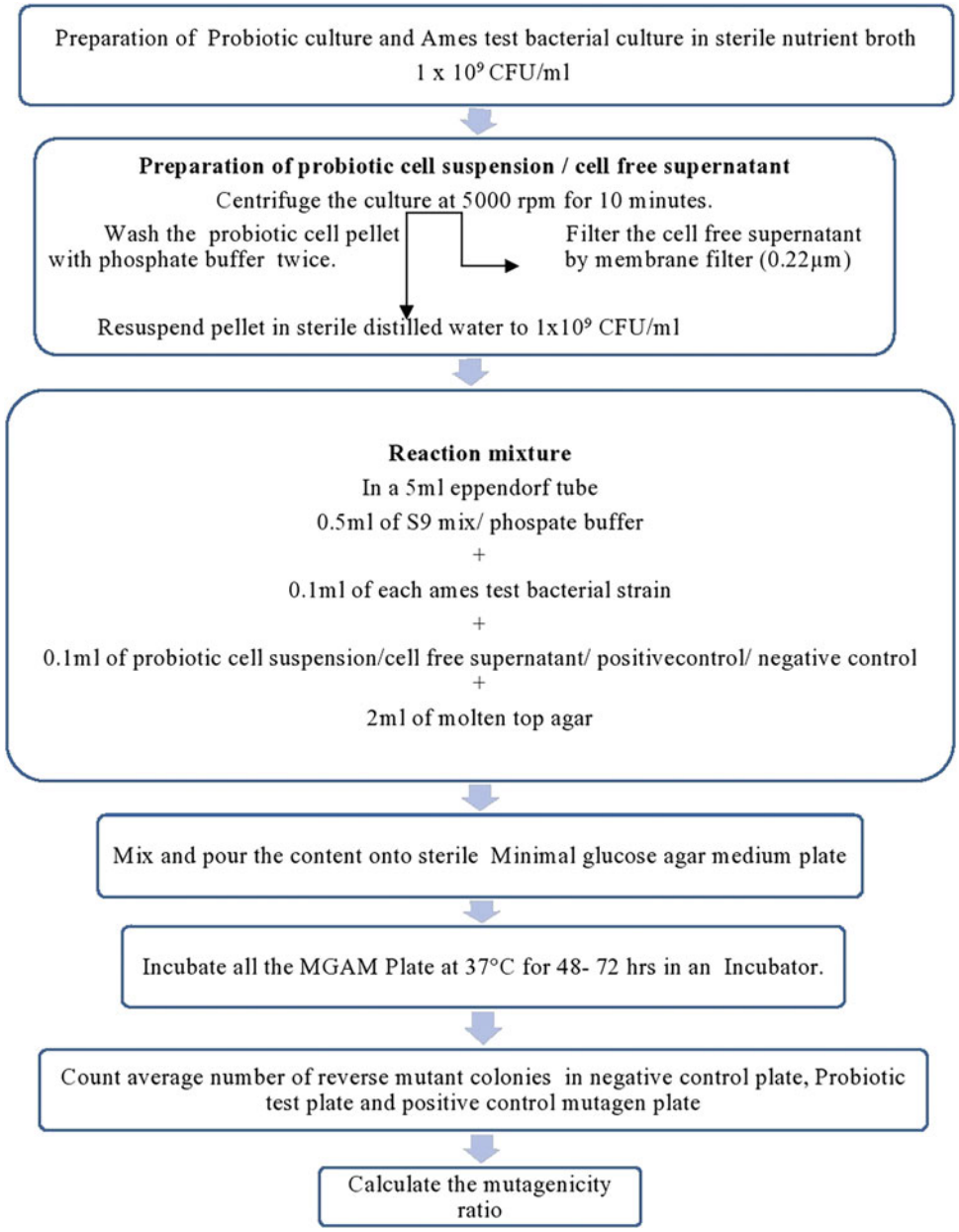

Fig. 1 Steps involved in mutagenicity assessment of probiotic by bacterial reverse mutation test

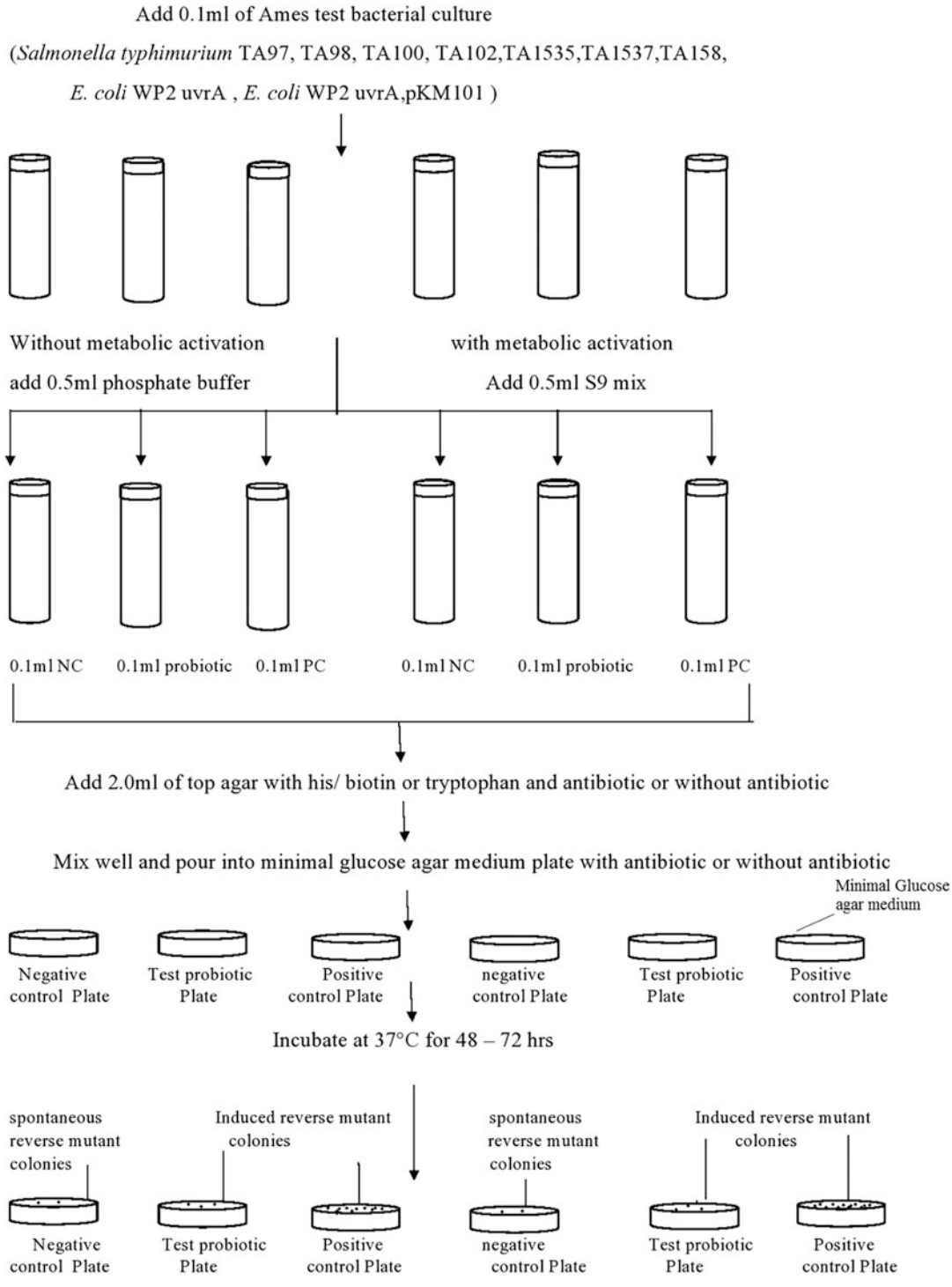

Fig. 2 Schematic representation of assessment of mutagenicity of probiotic by bacterial reverse mutation test (Plate incorporation method)

3.1 Preparation of Probiotic Culture

1. Inoculate the pure culture of probiotic colonies into 20 mL of nutrient broth or any suitable liquid medium to support its growth.
2. Incubate the culture at 37 °C for 16 h to attain 1×10^9 CFU/mL freshly before each test (*see* **Note 1**).

3.2 Preparation of Ames Test Strain

1. Inoculate pure colony of Ames test strains in 10 mL of sterile nutrient broth tube.
2. Incubate the tube 37 °C for 16 h to attain 1×10^9 CFU/mL freshly before each test (*see* **Note 1**).

3.3 Preparation of Probiotic Cell Suspension/Cell-Free Supernatant

1. Centrifuge the medium containing probiotic bacteria at 3075 × *g* for 10 min.
2. Then separate the pellet containing probiotic cells and supernatant aseptically.
3. Wash the probiotic cell pellet with phosphate buffer (0.2 M, pH 7.4) twice.
4. Resuspend the pellet in sterile distilled water adjusted to 1×10^9 CFU/mL [21].
5. Filter the cell-free supernatant containing metabolic products (enzymes) by using a 0.22 μm membrane filter [31].

3.4 Preparation of Reaction Mixture

1. Label three sets of sterile 5.0 mL Eppendorf tubes properly.
2. Add 100 μL of Ames test strain to all the tubes by using a micropipette.
3. To a one set of sterile 5.0 mL Eppendorf tubes, add 100 μL of the probiotic cell suspension or 100 μL of cell-free supernatant separately for test substance.
4. For positive control add 100 μL of mutagen to another set of sterile 5.0 mL Eppendorf tubes (*see* **Note 5**).
5. Add 100 μL sterile distilled water as a negative control to the third set of Eppendorf tubes separately.
6. To the one tube from each set, add 500 μL of S9 mix for metabolic activation.
7. Transfer 500 μL of phosphate buffer (PB) to the remaining three tubes, for without metabolic activation.
8. Transfer 2 mL of sterile molten top agar medium supplemented with histidine/biotin for *salmonella typhimurium* or tryptophan for *E. coli* test strains and ampicillin to all Eppendorf tubes for probiotic cell suspension or without antibiotic for cell-free supernatant.
9. Mix the tube content well in a vortex mixture carefully.

3.5 Plate Incorporation

1. Aseptically pour the Eppendorf tube content onto the minimal glucose agar medium plate with antibiotic for probiotic cell suspension or without an antibiotic medium plate for cell-free supernatant and mark the plate appropriately.
2. Tilt the plate back and forth for a uniform spread of top agar to the entire surface of the MGA medium plate.
3. Keep the MGA medium plate for 15–20 min.
4. Incubate all the plates at 37 °C for 48–72 h in an incubator.
5. Perform the experiment with each Ames test strain in triplicate.
6. Observe each plate for revertant colonies after incubation.
7. Count the average number of revertant colonies by using an automatic colony counter in all the plates including test and control.
8. Calculate the mutagenicity ratio by using the formula described by Wang et al. [35] with modification:

$$\text{Mutagenicity ratio (without metabolic activation)} = \frac{T1 \text{ or } S1}{X1}$$

wherein

*T*1—the average number of induced reverse mutant colonies on the test plate (probiotic cell suspension or cell-free supernatant) without metabolic activation.

*S*1—the average number of induced reverse mutant colonies on positive control plates without metabolic activation.

*X*1—average number of spontaneous reverse mutant colonies in the negative control plates without metabolic activation.

$$\text{Mutagenicity ratio(with metabolic activation)} = \frac{T2 \text{ or } S2}{X2}$$

wherein

*T*2—the average number of induced reverse mutant colonies on the test plate (probiotic cell suspension or cell-free supernatant) with metabolic activation.

*S*2—the average number of induced reverse mutant colonies on positive control plates with metabolic activation.

*X*2—average number of spontaneous reverse mutant colonies in the negative control plates with metabolic activation.

4 Interpretation of Result

When the mutagenicity ratio is greater than ≥ 2 the test is positive. Positive results indicate that the probiotic cell suspension or cell-free supernatant (metabolic products) induces point mutations by base substitutions and/or frame shift in Ames test strain either *Salmonella typhimurium* and/or *E. coli*. When the mutagenicity

Table 3
Observation table for assessment of mutagenicity of probiotic by bacterial reverse mutation assay

Ames test strain	Avg. no. of spontaneous revertant colonies in negative control		Avg. no. of induced revertant colonies				Mutagenicity ratio (M.R)	
			Probiotic cell suspension/cell free supernatant		Positive control (Standard mutagen)			
	Without S9mix *X1*	With S9mix *X2*	Without S9mix *T1*	With S9mix *T2*	Without S9mix *S1*	With S9mix *S2*	Without S9 mix $\frac{T1 \text{ or } S1}{X1}$	With S9mix $\frac{T2 \text{ or } S2}{X2}$
Salmonella typhimurium TA97								
Salmonella typhimurium TA98								
Salmonella typhimurium TA100								
Salmonella typhimurium TA102								
Salmonella typhimurium TA1535								
Salmonella typhimurium TA1537								
Salmonella typhimurium TA1538								
E. coli WP2 uvrA								
E. coli WP2 uvrA (pKM101)								

ratio is less than <2 the test is negative. Negative results indicate that under the test conditions, the probiotic cell suspension or cell-free supernatant (metabolic products) is not mutagenic in the tested species (Table 3).

5 Notes

1. Use fresh bacterial culture at late exponential phase or early stationary phase (1×10^9 cells/mL).
2. Choose the number of Ames test bacterial strains for mutagenicity assessment depending upon the test substance and requirements or a minimum of five bacterial strains as per OECD guidelines.
3. Perform the test to determine the characteristics of Ames test bacterial strains before the experiment by standard procedures for histidine/biotin dependence for *Salmonella typhimurium* strains and tryptophan dependence for *E. coli* strains, rfa mutation, uvrB mutation, ampicillin resistance for the presence of pKM101 plasmid, tetracycline resistance for plasmid pAQ1, viability assay, spontaneous mutation rate, etc.
4. Determine the antibiotic sensitivity pattern of probiotic bacteria and Ames test strain by suitable methods previously.
5. Select the positive control mutagen specific for the bacterial strain with metabolic activation and without metabolic activation as recommended.

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Chapter 22

Assessment of Induction and Destruction of Thrombi

M. Veerapagu, K. R. Jeya, and A. Sankaranarayanan

Abstract

Microbial infection may increase the risk of thrombosis. Infection associated thrombosis is importantly mediated by inflammation. Inflammation induces platelet activation which may accompany damage to the endothelium, resulting in fibrin deposition and thrombus formation. Many lactic acid bacteria (LAB) are exploited in various fermented foods, namely fermented dairy products, fermented meat, fermented fish, fermented vegetables, etc. as starter culture and also used as probiotics. Safety evaluation of probiotic was for thrombi induction was determined by flow cytometry analysis. Human beings suffering from thromboembolic disorders, namely pulmonary emboli, deep vein thrombosis, strokes, and heart attacks are the major causes of morbidity and mortality in both developing and developed countries. Due to the limitations of most of the thrombolytic drugs used for the treatment of thrombosis, probiotic bacteria are evaluated for thrombolytic potential and it is determined by in vitro clot lysis method.

Key words Probiotic, Safety evaluation, Platelet, Thrombosis, Thrombi induction, Flow cytometry, Thrombolytic drug, In vitro clot lysis method

1 Introduction

Thrombus formation portray consequential role on the immune system and human defense mechanism in the hemostasis process. Coagulation cascade and platelets play a major role in thrombus formation [1]. Platelet initiate clot formation and control blood loss from the site of injury. Thrombolytic system clear-outs thrombus by natural processes during recovery. Increased platelet activity may lead to intravascular thrombosis [2]. Intravascular thrombosis are the major cause of mortality and morbidity worldwide [3]. Thrombi formed by infection may occlude blood vessels and result in atherothrombosis. In recent years control and management of atherothrombosis pose a serious problem in most of the developed countries in the globe.

Systemic or localized infections may increase the risk of thrombosis in pneumonia, symptomatic urinary tract, oral, intra-abdominal, and systemic infections [4, 5]. Ischemic stroke is

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_22,

associated with acute infections namely after respiratory or urinary tract infections [6]. Many microbial infections caused by *Helicobacter pylori*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Hemophilus influenzae*, *Streptococcus pneumoniae*, *S. aureus*, *E. coli*, Epstein–Barr virus, herpesvirus, and cytomegalovirus [7] enhance the risk of thrombotic complications such as stroke, acute myocardial infarction, and unstable angina [8].

Any living microbes which provide beneficial health effect to the host are defined as probiotics. Lactic acid bacteria (LAB) are exploited in numerous fermented products such as liquor, pickle, yoghurt, and cheese, and they are generally consumed as probiotics. Lactic acid bacteria (LAB) such as *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Pediococcus*, and *Weissella* have been investigated for platelet aggregation [9–12]. Gastrointestinal tract (GIT) pathogen may foster septicemia through platelet aggregation or formation of platelet-fibrin clot on the endothelial surface when it enters into blood circulation due to injury or surgery [13]. Since many probiotic bacteria are utilized as starter culture in dairy products such as cheese, yoghurt, fermented milks, fermented meat products, fish products, pickled vegetables, and olives, safety evaluation of probiotic bacteria for thrombi induction is an important concern and it is determined by flow cytometry assay method.

Flow cytometry method needs a very small amount of whole blood and an invaluable method to assess platelet activation. The principle of this method is based upon the detection of cell surface proteins expressed on the surface of activated platelets with fluorescent labeled antibodies. It offers several advantages for the evaluation of platelet functions and activation [14]. By this method platelets are examined and observed in their physiological environment in whole blood with RBC and WBC. Whole blood supplemented with agonist may be used to evaluate the platelet reactivity. Most common platelet activation markers used in the flow cytometry are fibrinogen binding site exposed on the activated platelets which can be detected by FITC conjugated anti-fibrinogen antibody [15]. P-selectin a platelet activation dependent granule membrane protein expressed on the platelet membrane surface after activation may be detected by Phycoerythrin conjugated CD26p antibody which interacts specifically with P-selectin [16, 17].

Thrombus (blood clot) obstructs the blood flow by blocking the blood vessel; therefore tissues deprive of normal blood flow and oxygen which results necrosis of the tissue and may leads to acute myocardial infarction and ischemic stroke and leading to death [18]. Myocardial infarction, stroke, transient ischemic attack (TIA), venous thromboembolism (VTE), deep vein thrombosis (DVT), pulmonary embolism (PE), and other cardiovascular diseases results from different types of thrombosis are the principal cause of human death in last few decades. Different thrombolytic

drugs alteplase, streptokinase, urokinase, and tissue plasminogen activator (TPA) are widely used to dissolve clots [19]. But their use is accompaniment with possibility of hemorrhage, anaphylactic reaction and lacks specificity [20]. Hence in recent years probiotic bacteria are evaluated for thrombolytic potential by in vitro clot lysis method.

In vitro clot lysis method involves visualization of thrombolytic activity of probiotic culture or a test drug. When a drug or a metabolite which has ability to dissolve an already preexisting blood clot is said to have thrombolytic properties. In this assay venous blood collected fresh is allowed to form a clot is further incubated or treated with probiotic culture supernatant or test drug in addition to negative control and positive control like streptokinase for a fixed time at 37 °C. Initial clot weight and final clot weight, after lysis are measured [21, 22]. Finally, the percentage of clot lysis is calculated from the difference in weight of initial clot and final clot after lysis [23, 24].

2 Materials

2.1 Determination of Thrombi Induction of Probiotic Bacteria by Flow Cytometry Analysis

- Vacutainer (BD Bioscience).
- 2 mL Eppendorf tubes.
- Centrifuge.
- Pasteur pipette.
- Micropipette.
- FACS Verse flow cytometer (BD-biosciences).
- Incubator.
- PE-CD62P (P-Selectin) Monoclonal Antibody (Invitrogen).
- Microbial culture: Pure culture of probiotic bacteria.
- *MRS broth (for Lactic acid bacteria-LAB)*: Dissolve 5.515 g of MRS broth (Himedia) as per manufacturer recommendation and add 100 mL of distilled water. Completely dissolve the medium on a hot plate. Dispense 20 mL aliquots in a tube and sterilize the medium in an autoclave for 15 min at 121 °C.
- *Phosphate buffer 0.2 M (pH 7.4)*.

  Solution A: Dissolve 27.6 g sodium phosphate monobasic in 1 L of distilled water.

  Solution B: Dissolve 28.4 g Sodium phosphate dibasic in 1 L of distilled water.

  Add 120 mL of solution A to a 2 L conical flask followed by the addition of 880 mL of solution B. Mix them well and adjust pH to 7.4 by using solution B. Sterilize it in autoclave at 121 °C for 20 min and store at 4 °C.

- *HBS buffer* (2×, Sigma-Aldrich): dextrose—2.0 g/L, HEPE—10 g/L, KCl—0.74 g/L, NaCl—16 g/L, $Na_2HPO_4.2H_2O$—0.27 g/L. Dilute the 2× HBS buffer with equal volume of double distilled water to make 1× concentration before use.
- *Thrombin Receptor Activator peptide-6 (TRAP-6)* (Sigma-Aldrich): Add 0.037 g of TRAP- 6 to 1 L of HBS buffer to get final concentration of 50 μM/L.
- *Fixative Buffer*: 0.2% formaldehyde, 154 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2PO_4, 1.15 mM KH_2PO_4, 10.2 mM $NaHPO_4$, and 4 mM EDTA; pH 7.4. Prepare fixative buffer with above composition in double distilled water freshly and store it at 4 °C.

2.2 Determination of Thrombolytic Activity of Probiotic Bacteria (by In Vitro Clot Lysis Method)

- Vacutainer (BD Bioscience).
- Eppendorf tube (Himedia).
- Centrifuge.
- Digital Balance.
- Pasteur pipette.
- Incubator.
- *Streptokinase positive control (Cadila Pharmaceuticals limited)*: Add 5 mL of sterile distilled water to Streptokinase (1,500,000 IU) and mix well.
- *MRS broth* (Himedia): as mentioned in Subheading 2.1.
- *Microbial culture*: Pure culture of probiotic bacteria.

3 Methods

3.1 Determination of Thrombi Induction of Probiotic Bacteria by Flow Cytometry Analysis

- Thrombi induction can be evaluated by Azizpour et al. [25] as mentioned below:

3.1.1 Preparation of Probiotic Bacterial Cell Suspension

1. Inoculate the pure culture of probiotic colonies into to 20 mL of MRS broth or any suitable liquid medium to support its growth.
2. Incubate the culture at 37 °C for 16–18 h in an incubator.
3. Centrifuge the medium containing probiotic culture at 3000 × *g* at 4 °C for 15 min.
4. Then separate the pellet containing probiotic cells and supernatant aseptically.

5. Wash the probiotic cell pellet with phosphate buffer (0.2 M, pH 7.4) twice.
6. Resuspend the pellet in phosphate buffer and adjust concentration of cell suspension to 1×10^9 CFU/mL [26].

3.1.2 Collection of Blood Samples

1. Select healthy blood donors of age 25–40 years, non-smokers, and should not have consumed alcohol for minimum of 2 days prior to blood donation and without any medications for at least 2 weeks.
2. Collect the blood from donor by venipuncture with 20 gauge needle into 0.105 M trisodium citrate vacutainer.

3.1.3 Treatment of Samples

1. To a 2 mL Eppendorf tubes, add 25 μL of HBS buffer, agonist (TRAP-6), sample probiotic cell suspension separately.
2. Add 5 μL of whole blood to each tube.
3. Transfer 25 μL of CD62P (P-Selectin) antibody to each tube.
4. Incubate the tubes at 37 °C for 10 min.
5. Add 500 μL of fixative buffer to each tube to terminate the activation.

3.1.4 Flow Cytometry Analysis

Figure 1 depicts the different steps for determination of thrombi induction of probiotic bacteria by flow cytometry analysis.

1. Dilute the treated blood samples twofolds with HBS buffer.
2. Analyze each sample on FACSVerse flow cytometer.
3. Observe mean fluorescence (MFL) and the percentage of CD62P positive cells.

3.2 Determination of Thrombolytic Activity of Probiotic Bacteria by In Vitro Clot Lysis Method

The thrombolytic activity can be assessed by method described Islam et al. [27]. Figure 2 depicts the different steps for determination of thrombolytic activity of probiotic bacteria using in vitro clot lysis method.

3.2.1 Preparation of Probiotic Culture Supernatant

1. Inoculate the pure culture of probiotic colonies into to 20 mL of MRS broth or any suitable liquid medium to support its growth.
2. Incubate the culture at 37 °C for 16 h to attain 1×10^9 CFU/mL freshly before each test.
3. Centrifuge the medium containing probiotic bacteria at $3075 \times g$ for 10 min.
4. Then separate the pellet containing probiotic cells and supernatant aseptically.

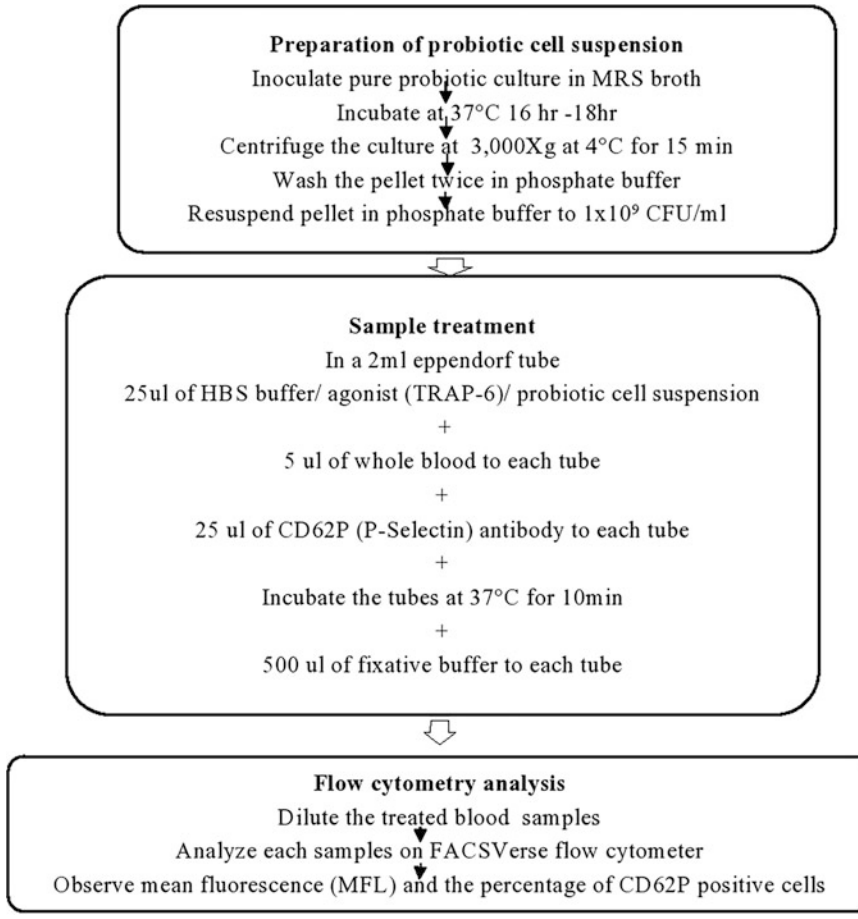

Fig. 1 Different steps for determination of thrombi induction of probiotic bacteria by Flow cytometry analysis

3.2.2 Blood Collection

1. Select healthy blood donors of age 25–40 years, non-smokers, should not have consumed alcohol for minimum of 2 days prior to blood donation and without any medications for at least 2 weeks.
2. Collect the blood from donor by venipuncture without anticoagulant into vacutainer.

3.2.3 In Vitro Clot Lysis

1. Accurately weigh three empty sterile 2 mL microcentrifuge tubes ($W1$).
2. Add 1 mL of venous blood to each microcentrifuge tube separately.
3. Incubate all the three tubes at 37 °C for 45 min. For clot formation.
4. Carefully separate serum from each tube without disturbing the clot.
5. Measure the weight of tube with the clot from each tube ($W2$).

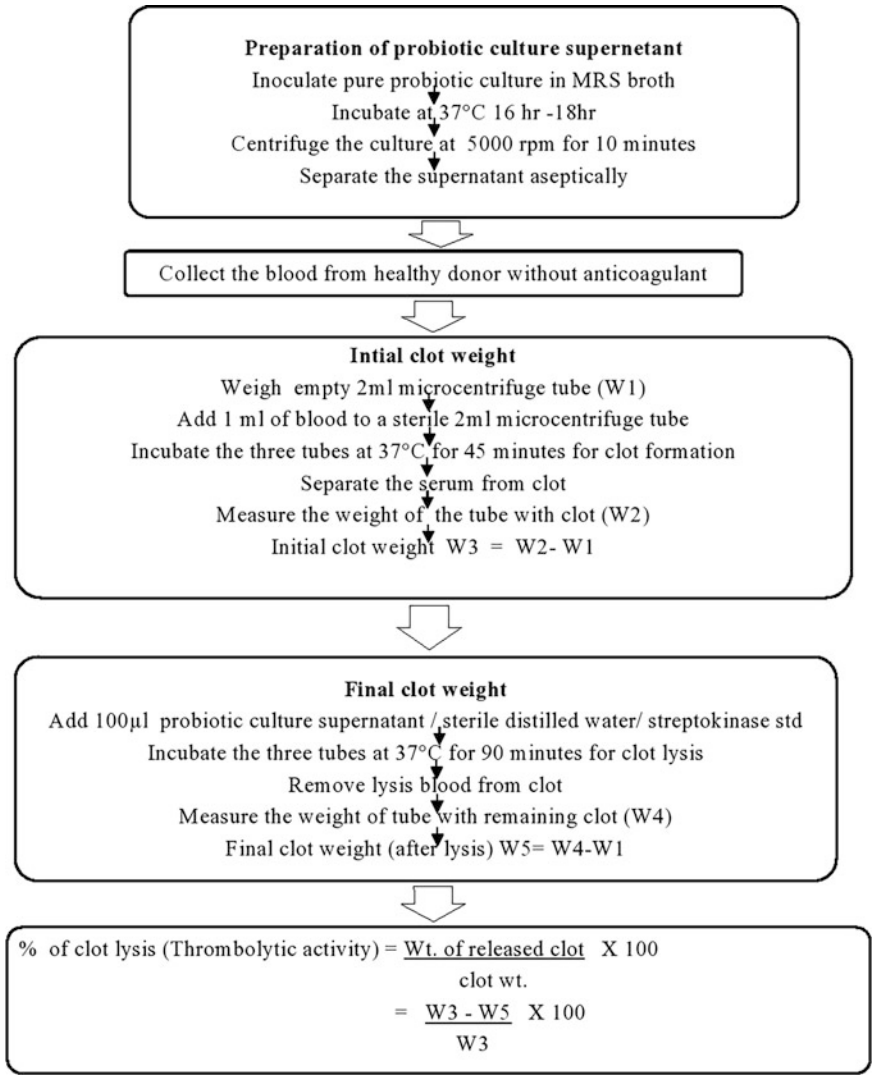

Fig. 2 Steps involved in the determination of thrombolytic activity of probiotic bacteria by in vitro clot lysis method

6. Calculate the Initial clot weight (before lysis) using the below mentioned formula:

   Initial clot weight(before lysis) $W3 = W2 - W1$

   $W2$—weight of the tube with clot (grams).
   $W1$—weight of the empty tube (grams).

7. Label the microcentrifuge tube as sample, negative control and positive control.
8. Add 100 µL of probiotic culture supernatant, sterile distilled water and streptokinase standard (30,000 IU) to the respective tube.

9. Incubate all the three tubes at 37 °C for 90 min for clot lysis.
10. Remove the lysed blood from the clot.
11. Measure the weight of tube with remaining clot from each tube ($W4$).
12. Calculate the final clot weight (after lysis) using the below mentioned formula:

    Final clot weight(after lysis) $W5 = W4 - W1$

    $W4$—weight of the tube with remaining clot after lysis (grams).
    $W1$—weight of the empty tube (grams).
13. Determine the weight of released clot and express the result as percentage of clot lysis using the below mentioned formula:

$$\% \text{ Clot lysis (thrombolytic activity)} = \frac{\text{Wt. of the released clot}}{\text{Clot wt.}} \times 100 = \frac{W3 - W5}{W3} \times 100$$

$W3$—initial weight of the clot (before lysis) in grams.
$W5$—final weight of the clot (after lysis) in grams.

4 Observation

4.1 Determination of Thrombi Induction of Probiotic Bacteria by Flow Cytometry Analysis

1. Interpret and record the results of mean fluorescence (MFL) and the percentage of CD62P positive cells as shown in Table 1.
2. Fluorescence intensity higher than $\log^{10}$ than the control (resting platelet) is considered to be positive for platelet activation (thrombi induction).

4.2 Determination of Thrombolytic Activity of Probiotic Bacteria by In Vitro Clot Lysis Method

1. Measure initial clot weight and final clot weight (after lysis) and record the result as shown in Table 2.
2. Calculate released clot weight and percentage of clot lysis.

Table 1
Determination of thrombi induction of probiotic bacteria by flow cytometry

Sample	Percentage of positive CD62P	Mean fluorescence (MFL) CD26P
Probiotic cell suspension		
Positive control		
Negative control		

Table 2
Thrombolytic activity of probiotic bacteria by in vitro clot lysis method

Sample	Initial clot wt. (before lysis) grams *W3*	Final clot wt. (after treatment/lysis) grams *W5*	% of clot lysis $= \frac{W3-W5}{W3} \times 100$
Probiotic culture supernatant			
Negative control			
Positive control (Streptokinase)			

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Chapter 23

Assessment of Degradation of Mucin

Ruma Raghuvanshi, Archana Chaudhari, and Mitesh Kumar Dwivedi

Abstract

Mucin degradation is an important safety aspect for the identification of probiotics bacteria, as excessive mucin degradation cause damage to intestinal tissue. With the problems associated with culturing gut microbes leads to the advent of methods like 16sRNA sequencing. Here, we describe the methods to determine the mucin degradation by combining anaerobe culturing, 16sRNA profiling, transcriptomics, and untargeted metabolomics.

Key words Mucin degradation, O-glycans, Anaerobe culturing, 16sRNA sequencing, Transcriptomics, Untargeted metabolomics

1 Introduction

The major structural components of mucus layer consist of mucins and glycoproteins, which covers the gastrointestinal tract and protects the underlying mucosal surfaces [1]. Mucins are O-linked glycoproteins which are highly glycosylated and form the intestinal glycocalyx. These glycans are attached to the polypeptide backbone with N-acetylgalactosamine through serine or threonine. Among the eight O-glycan cores, colonic mucins predominantly comprise core 3, 4 (GlcNAcb1,6 (GlcNAcb1,3) GalNAcaSer/Thr) [2–4]. Mucin 2 (MUC2) is the major mucin of the gastrointestinal tract and plays an important role in maintaining the barrier function of gut [5]. Adhesion characteristics of probiotics on the mucosal layer are important for the proliferation of probiotics in lower intestinal tract [6]. Probiotics have special surface proteins that bind with mucin-bound oligosaccharides facilitating the protein–carbohydrate interaction responsible for the adhesion properties of probiotics [7]. Several mucus-binding proteins present in probiotics have regions homologous with binding domains of lectins,

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Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_23,

likewise in endosymbiotic bacteria [8]. Mucin is degraded by the enzymes released by mucolytic taxa involving *Bacteroides* and *A. muciniphila* [9]. Probiotic strains should not be mucolytic as the excessive degradation of mucin may be harmful due to excessive translocation of bacteria within intestinal tissues [10, 11].

To determine the mucin degrading capacity of bacterial isolates from fecal samples, firstly, allow the bacteria isolates to grow in mucin rich media (MM) as described in Raimondi et al. [12]. Subsequently, visualize the mucin degradation by the zone clearance as mentioned in Kurkutia et al. [13]. Thereafter, identify the bacterial isolates using 16sRNA gene profiling. As described in Karav et al. [14], the resulting glycans release during mucin degradation are determine by untargeted metabolomics of extracted and purified glycans from individual bacterial isolates in MM media. Due to the complex structure of intestinal glycans, their degradation based on the accumulative action of couple of genes encoded for proteases, sulfatases, and GHs (Glucoside hydrolases). The list of GHs includes the neuraminidases/sialidases, fucosidases, exo- and endo-β-N-acetylglucosaminidases, β-galactosidases, α-N-acetylglucosaminidases, and α-N-acetylgalactosaminidases [15]. These mucin degrading genes are determined by transcriptomics analysis as described in Liu et al. [16].

This chapter describes the methods for mucin degradation ability of the probiotic isolates by different techniques including the measurement of zone of clearance using dye in anaerobic conditions, 16sRNA sequencing for the determination of mucin degraders, transcriptomics analysis to determine the mucin degrading genes and untargeted metabolomics for identifying the released glycans during mucin degradation.

2 Materials

All solutions and reagents should be made using distilled water and analytical grade chemicals. The prepared reagents are stored at room temperature (unless indicated otherwise). Purchase all chemicals from Sigma-Aldrich.

2.1 Preparation of MM Media

- According to the protocol of Miller and Hoskins [17], purify the mucins from porcine stomach type II mucin (Sigma-Aldrich). Briefly, in batches of 25 g stir the mucins for 20 h at 22 °C in 1 L of 0.1 M NaCl containing 0.02 M phosphate buffer, with a few drops of toluene, pH 7.6.
- Readjust the pH to 7.0–7.4 with 2 N NaOH, after the completion of 1 h. Centrifuge the batches at 10,000 × *g*, then collect the supernatant and cool down to 0° ± 2 °C, add the pre-cooled ethanol in a final concentration of 60% vol/vol.

Table 1
Composition of MM media

Media components	Concentration
Purified Mucin	3.0 g/L
KH_2PO_4	2.0 g/L
NaCl	4.5 g/L
$MgSO_4.7H_2O$	0.5 g/L
$CaCl_2.2H_2O$	0.045 g/L
$FeSO_4.7H_2O$	0.005 g/L
Hemin	0.01 g/L
Bile salts	0.05 g/L
Resazurin	0.6 mg/L
Minerals solution[a]	2.0 mL/L
Vitamins solution[b]	1.4 mL/L
Reducing solution[c]	40 mL/L

[a] Minerals solution (0.010 g/L $ZnSO_4.7H_2O$, 0.5 g/L EDTA, 0.003 g/L $MnCl_2.7H_2O$, 0.02 g/L $CoCl_2.6H_2O$, 0.03 g/L H_3BO_3, 0.001 g/L $CuCl_2.2H_2O$, 0.003 g/L $NaMoO_4.2H_2O$ and 0.002 g/L $NiCl_2.6H_2O$)
[b] Vitamins solution (2.0 g/L biotin, 1.0 g/L menadione, 2.0 g/L calcium pantothenate, 0.5 g/L cyanocobalamin, 10 g/L nicotinamide, 0.5 g/L folic acid, 5 g/L PABA, and 4 g/L thiamine)
[c] Reducing solution (80 g/L $NaHCO_3$ and 12.5 g/L L-cysteine.HCl)

- Dissolve the resulting precipitate in 0.1 M NaCl, then again precipitate with ethanol (60% vol/vol). Thereafter, dissolve and dialyze the precipitated purified mucins against distil water, followed by lyophilization.
- To enrich the growth of mucin degraders anaerobically, use the MM media as described in Raimondi et al. [12]. The composition of MM media is shown in Table 1. Autoclave the components of basal mediaat 121 °C for 20 min, then complement it with minerals, vitamins and reducing solutions, after filter sterilization with 0.2 μm filter.

2.2 Preparation of B Media

- Purify the mucins from the porcine stomach type II mucin (Sigma-Aldrich) as described above and use it for making B media. The components of B media are shown in Table 2. Autoclave the components of basal media at 121 °C for 20 min, then complement it with reducing agent (Cysteine HCl), after filter sterilization with 0.2 μm filter.

Table 2
Components of B media

Ingredients	g/L
Tryptone	7.5
Casein	7.5
Yeast extract	3
Meat extract	5
NaCl	5
$K_2HPO.3H_2O$	3
KH_2PO	0.5
Cysteine HCl	0.5
Resazurin	0.002
Mucin	3
Agar	15
Distilled water	1000 mL

2.3 Preparation of MRS Broth

- DeMan, Rogosa, and Sharpe (MRS)-broth composition (BD Difco, Franklin Lakes, NJ, USA):10 g proteose peptone no. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80 (also known as Tween 80), 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium phosphate in 1 L of distilled water (in case using MRS-broth powder, add 55 g of the powder in 1 L of water). Adjust pH of the MRS-broth as 6.5 ± 0.2.

2.4 Assessment of In Vitro Biosafety Aspects of Isolates for Mucin Degradation by Zone Clearance

- Bacterial Isolates.
- Media B.
- Anaerobic cabinet.
- 0.1% w/v amido black.
- 3.5 M acetic acid.
- 1.2 M acetic acid.
- MRS Broth.

2.5 Genomic DNA Isolation

- DNeasyPowerSoil Kit.
- Microcentrifuge (10,000 × *g*).
- Pipettors (50–500 μL).
- Vortex-Genie 2 Vortex.
- Vortex Adapter for 24 (1.5–2.0 mL) tubes.

2.6 16S rRNA Gene Profiling

- Genomic DNA.
- 515F-926R.
- Thermal Cycler.
- Gel electrophoresis unit.
- Quant-iTPicoGreen dsDNA Assay Kit (Thermo Fischer/Invitrogen, cat. no. P11496).
- MoBioUltraClean PCR Clean-Up Kit (cat. no. 12500).
- Miseq System.

2.7 RNA Isolation

- RNeasy Kit.
- Sterile, RNAse-free pipette tips.
- Microcentrifuge (with rotor for 2 mL tubes).
- 70% ethanol.
- Disposable gloves.
- QIAshredder homogenizer.

2.8 Transcriptomics Analysis for Mucin Degrading Genes

- Ribo-Zero rRNA removal kit (Illumina, San Diego, CA).
- Random hexamer primer.
- M-MuLV Reverse Transcriptase (RNaseH-).
- DNA Polymerase I.
- RNase H.
- NEBNext Adaptor.
- AMPure XP system (Beckman Coulter, Beverly, MA).
- USER Enzyme (New England Biolabs, Ipswich, MA).
- Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA).
- Agilent Bioanalyzer 2100 system (Agilent, Santa Clara, CA).
- HiSeq 2500 sequencing system (Illumina, San Diego, CA).

2.9 Extraction of Free Oligosaccharides

- Enriched Bacterial Culture/Individual Bacterial Isolates.
- Ethanol.
- Centrifuge.
- 1.0 M $NaBH_4$.
- C-8 and graphitized carbon cartridges (GCCs) (Glygen Corp, Columbia, MD).
- 20% acetonitrile/water (v/v).
- 40% acetonitrile/water (v/v).
- 0.05% Trifluoroacetic acid.
- Nanopore water.

2.10 Determination of O-glycans

- Enriched Culture Media/Individual Bacterial Isolates.
- Nano-HPLC-Chip-TOF-mass spectrophotometer.
- Nanopore water.
- Aqueous solvent A [3% acetonitrile/water (v/v) in 0.1% formic acid].
- Organic solvent B [90% acetonitrile/water (v/v) in 0.1% formic acid].

3 Methods

3.1 Assessment of In Vitro Biosafety Aspects of Isolates for Mucin Degradation by Zone of Clearance

1. Grow the bacterial isolates in MRS-broth at 37 °C. Then inoculate 10 μL of viable cultures on the surface of medium B and incubate the plates at 37 °C for 72 h under anaerobic condition.
2. Confirm the mucin degradation upon staining with 0.1% w/v amido black in 3.5 M acetic acid (for 30 min) and washing with 1.2 M acetic acid, resulting into the zone of discoloration around the colony as described in Kurkutia et al. [13].
3. The illustrative example is showing the mucin degradation property by two bacterial isolates SP1M and SP1S with clear zones around colonies on medium B (Fig. 1), using *Pseudomonas* as positive control culture for mucin degradation.

3.2 Identification of Mucin Degrading Bacteria

3.2.1 Genomic DNA Isolation

1. Extract the total DNA from enriched culture using Qiagen PowerSoil DNA Extraction Kit (www.qiagen.com) using the following protocol as described by the manufacturer. Briefly, add the enriched culture to the PowerBead tube provided and vortex.
2. Add 60 μL of solution C1 and vortex briefly. Then place the secure PowerBead tubes horizontally in vortex adapter and vortex for 10 min. Centrifuge tubes at 10,000 × *g* for 30 s and transfer the clear supernatant to 2 mL collection tube.

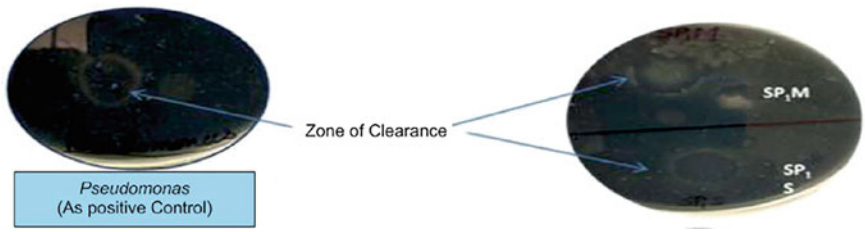

Fig. 1 Mucin degradation by bacterial isolates SP_1M and SP_1S with clear zones around the colonies. *Pseudomonas* is shown as the positive control for mucin degradation

3. Add 250 μL of solution C2 in clear supernatant and vortex for 5 s. Then incubate in 2–8 °C for 5 min, followed by centrifugation for 1 min at 10,000 × *g*. Again, transfer the 600 μL of supernatant to a clean 2 mL collection tube.
4. Add 200 μL of solution C3, then vortex briefly, and incubate at 2–8 °C for 5 min. Centrifuge the tubes for 1 min at 10,000 × *g* and transfer the clear supernatant to 2 mL collection tube.
5. Add 1200 μL of solution C4 after shaking to supernatant and vortex for 5 s. Then load 675 μL onto an MB Spin column, centrifuge at 10,000 × *g* for 1 min, then discard the flow-through and repeat this step twice.
6. Add 500 μL of solution C5, centrifuge at 10,000 × *g* for 30 s, discard the flow-through and then again centrifuge for 1 min at 10,000 × *g*. Place the MB Spin column into 2 mL collection tube, then add 100 μL of solution C6 to the center of the white filter membrane and centrifuge at room temperature at 10,000 × *g* for 30 s. Store the collected DNA at −20 °C to −80 °C.

3.2.2 16S rRNA Gene Profiling

1. All the steps are based on the Earth Microbiome Project (https://earthmicrobiome.org/). Briefly, amplify the V4-V5 region of prokaryotic DNA using 515F-926R primers. Quantify amplicons using Quant-iT PicoGreen dsDNA Assay Kit (ThermoFischer/Invitrogen, cat. no. P11496).
2. Mix 240 ng of each sample together and purify the pool out samples with Mo Bio UltraClean PCR Clean-Up Kit (cat. no. 12500). Submit the samples for illumina Miseq 16sRNA sequencing.
3. Analyze the 16SrRNA sequencing data with QIIME2 (https://doi.org/10.1038/s41587-019-0209-9) [18], which involves series of step. First, demultiplex the imported sequences, followed by sequence quality control with DADA-2. For the generation of the phylogenetic tree apply the align-to tree-mafft-fast tree pipeline of q2-phylogeny plugin of QIIME2. Eventually, to determine the mucin degraders, perform the taxonomic analyses using q2-feature-classifier plugin.

3.3 Identification of Mucin Degrading Genes

3.3.1 RNA Isolation

1. Extract the total RNA from bacterial cells at the early stationary phase using RNeasy Mini kit (www.qiagen.com/resources/RNeasyMin) according to manufactures protocol. Briefly, harvest the cells by centrifuging for 5 min at 300 × *g* and remove the supernatant by aspiration.
2. Add the 350 μL buffer RLT for the cell lysis and vortex or pipet to mix. Homogenize the lysate by loading onto QIAshredder spin column and centrifuge for 2 min at maximum speed. Add 1 volume of 70% ethanol and mix well.

3. Transfer up to 700 μL of the sample to an RNeasy spin column and centrifuge for 15 s at ≥8000 × *g* or ≥10,000 rpm. Discard the flow-through. Add 700 μL buffer RW1 to the RNeasy spin column and centrifuge for 15 s at ≥8000 × *g* or ≥10,000 rpm for the column washing, then discard the flow-through.
4. Add 500 μL buffer RPE to the RNeasy spin column and centrifuge for 15 s at ≥8000 × *g* or ≥10,000 rpm for the column washing, then discard the flow-through. Again add 500 μL buffer RPE to the RNeasy spin column and centrifuge for 2 min at ≥8000 × *g* or ≥10,000 rpm.
5. For the RNA elution, place the RNeasy spin column to new collection tube and add 30–50 μL of RNAse free water to the center of the spin column membrane. Centrifuge at ≥8000 × *g* or ≥10,000 rpm for 1 min.
6. Check the RNA quality using 1% agarose gel electrophoresis and quantify it with NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). To construct library, select the samples with a RIN (RNA integrity number) score greater than 6.

3.3.2 Transcriptomics Analysis for Mucin Degrading Genes

1. Perform the transcriptomics analysis as described in Liu et al. [16]. Briefly, take 3 μg RNA and remove the ribosomal RNA using a Ribo-Zero rRNA removal kit (Illumina, San Diego, CA). Synthesize the first strand of cDNA using random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH-) and for the second strand use DNA Polymerase I and RNase H.
2. Adenylate the 3′ ends of DNA fragments and for hybridization ligate it with NEBNext Adaptor with hairpin loop structure. Purify the library fragments with AMPure XP system (Beckman Coulter, Beverly, MA) to select cDNA fragments of length 150–200 bp.
3. Add 3 μL of USER Enzyme (New England Biolabs, Ipswich, MA) to the size selected cDNA and incubate at 37 °C for 15 min followed by 5 min at 95 °C. Perform the PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), then purify the amplified products with AMPure XP system and assess the library quality on an Agilent Bioanalyzer 2100 system (Agilent, Santa Clara, CA).
4. Sequence the prepared libraries with HiSeq 2500 sequencing system (Illumina, San Diego, CA). Filter the raw reads for adaptor, poly-N and low-quality reads. Then log transform the filter data and perform PCA, Venn diagrams, and heatmaps using DESeq2 (R package, version 1.30.0). Perform GO enrichment analysis of DEGs using the GOseq R package

(version 1.30.0, https://www.r-project.org/) and to test significant enrichment DEGs in KEGG pathways use KOBAS (version 2.0, http://kobas.cbi.pku.edu.cn/).

3.4 Estimation of Mucin O-glycans Released by Mucin Degraders

3.4.1 Extraction of Free Oligosaccharides

1. Extract the free oligosaccharides from the enriched bacterial cultures and individual bacterial isolates using the method described in Davis et al. [19]. Briefly, remove the proteins with ethanol precipitation at −80 °C for 1.5 h, centrifuge the supernatant for 30 min, then collect the supernatant again and allow it to dry.
2. Reduce the resulting glycans to alditol form using 1.0 M $NaBH_4$ at 65 °C for 1.5 h. Removal of salts and purification of reduced glycans is achieve by using both C-8 and graphitized carbon cartridges (GCCs) (Glygen Corp, Columbia, MD) through the solid phase extraction.
3. Load the resulting glycans onto preconditioned C8 cartridges, collect the flow-through and load onto preconditioned GCCs. Elute the resulting purified glycans using 20% acetonitrile/water (v/v) and 40% acetonitrile/water (v/v) in 0.05% trifluoroacetic acid, then evaporate the eluent solvent to dry and dissolve in nanopore water.

3.4.2 Determination of O-glycans

1. Estimate the mucin O-glycans released by mucin degraders in enriched culture media and individual bacterial isolates using nano-HPLC-Chip-TOF mass spectrometer as described in Davis et al. and Karav et al. [14, 19]. Briefly, use the Agilent 1200 series unit, high-performance liquid chromatography (HPLC) system with microfluidic chip coupled with Agilent 6220 series time of flight (TOF) mass spectrometer via chip cube interface.
2. Load the sample onto 40-nL enrichment column with the flow rate of 4 μL/min and injection volume of 1 μL to separate the analytes using nano pump on the analytical column (75 × 43 mm) packed with graphitized carbon.
3. Use the binary gradient of aqueous solvent A[3% acetonitrile/water (v/v) in 0.1% formic acid] and organic solvent B [90% acetonitrile/water (v/v) in 0.1% formic acid] for the separation of analytes.
4. Introduce the samples into TOF mass spectrophotometer through electrospray ionization and calibrate it with dual nebulizer electrospray with ions ranging between m/z 118.086 to 2721.895.
5. Collect the data in positive ion mode and analyze the untargeted spectra with Agilent MassHunter Workstation Data Acquisition software or GNPS.

3.4.3 Analysis of Untargeted Mass Spectra

1. Analyze the untargeted mass spectra using Agilent MassHunter Workstation Data Acquisition software as described in Karav et al. [14]. Determine the mucin glycans with "Find Compounds by Molecular Feature" function of the software. Here, volume of ions count represents the absolute abundances of compounds.
2. Measure the abundance of human colonic mucins glycans such as 1HexNAc-1NeuAc, 1HexNAc-1Hex-NeuAc, 2Hex-NAc-1NeuAc, 2HexNAc-1Hex-1Fuc, 2HexNAc-1Hex-1NeuAc, 2HexNAc-1Hex-2Fuc, 3HexNAc-1Hex-1Fuc, 2HexNAc-1Hex-1Fuc-1NeuAc, 2HexNAc-1Hex-1Fuc-2NeuAc, 3HexNAc-1Hex-2NeuAc, and 3HexNAc-1Hex-2Fuc-1NeuAc. It helps to determine the extent of mucin degradation by mucin degraders after enrichment in MMmedia as well as the mucin degradation capacity of individual bacterial isolates.
3. Alternatively, use the Global Natural Products Social Molecular Networking (GNPS) [20], for the analysis of untargeted mass spectra. Firstly, convert the raw files to the centroid mzXML file format using MSconvert. Then upload the data to GNPS for the generation of molecular networks. Create the feature based molecular networks using feature finding with mzMine v.2.0 software followed by generation of networks in GNPS. To find features through mzMine, chromatograms are first deconvoluted, deisotoped, aligned, then filtered and finally gap fill for further analyses. In inference, mass spectrometry results determine the released glycans associated with particular mucin degradation pattern of specific bacterial isolates.

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Part II

In Vivo Biosafety Assessment of Probiotics: Monitoring In Vivo Toxicity of Probiotics

Chapter 24

Evaluation of General Health Status of the Animals During the In-Life Phase

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Abstract

Probiotics have gained importance in recent years as a viable substitute to antibiotics for boosting livestock performance. Along with enhanced nutrient digestibility and immunomodulation, probiotics have reflected an enormous reduction in gastrointestinal tract infection through in-feed usage. Although, each novel probiotic strain cannot be presumed to share a historical safety perspective with conventional strains. Harmful effects of probiotics can be dependent on prevailing immunological conditions, strain specified, and physiological conditions of the host. The most important consideration is the strain's stability. Probiotics state an effective chance of replacing antibiotics in animals, their safety measures should have been adhered to for safety concerns. The chapter aims to provide the several tests for evaluating the general health of the animals after probiotic treatment such as, Cylinder, Irwin, Wire suspension, and vertical pole tests. These tests are being utilized for past years to enable practical evaluation of animal species in laboratory by following these protocols. They are scientifically evident and experimentally safe as per the requirements to avoid animal suffering during analysis.

Key words Probiotics, Animal health, Nutrition

1 Introduction

Due to the rapid advancement in investigating mice for laboratory analysis for a decade is observed in biomedical research; number of mice maintained and bred to their natural life span with their potentially hampering phenotypes. This has led to monitor and practice the general health guidelines for practical analysis. As consideration of the endpoints is effective because animals might be suffering from any kind of stress conditions that leads to suffering. For which researchers should outline their objectives that complies them to direct their endpoints in order to minimize animal's suffering. For this purpose, a body condition scoring is used for knowing the overall mouse condition [1]. It is simple to perform, as

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_24,

by holding it from tail you can pass your finger to the sacroiliac bones to know the body conditions that are scored 1–5 as follows: 1. Bones are prominent, muscle wasting is new and fat deposits are not observed, in such condition euthanasia (an early intervention) is required. 2. The bones are more obvious because mouse is getting thinner and euthanasia is recommended in this condition. 3. This condition is optimal for mouse as it is good in health. 4. A healthy mouse that is well-fleshed and observing bones is trickier. 5. This is an obese condition of mouse with no chance of feeling bones [2]. In addition to BCS, there are some health conditions that are an obvious problem that would impede to general monitoring like *barbering* in which a mouse during its growing process chews its hair. *Fighting*; that declines animal's ability to proper functioning for a time being, *Malocclusion* in which weight gain is poor after weaning stage. Some health issues are subtle like anemia, diarrhea, icterus, and hypothermia, and additional issues like abnormal mobility and breathing that recommends euthanasia [3]. Probiotics that are known to be living organisms that has many health benefits when consumption is in significant and sufficient quantity, among which some of renowned microbial species as *Bifidobacterium* spp. and *Lactobacillus* spp. are having diverse benefits in health by food supplements [4]. These isolates are safe through in vitro analysis among animals, and its biosafety assessment has suggested probiotics use in future years [5]. The chapter provides the tests which are used to evaluate general health status of an animal after probiotic treatment.

2 Materials

2.1 Administration of Probiotics in Animals

The materials used for administration of probiotics in animal

1. Mice.
2. Binocular lamp.
3. Chemicals.
4. Video camera.

2.2 Cylinder Test

1. Ethanol 70%.
2. Mice.
3. Black curtain.
4. Soap.
5. A cylindrical glass of 20 cm in diameter and 40 cm in height.
6. A 2 L graduated beaker in the lab, formed by borosilicate glass.
7. Three cameras that are qualified to record videos at the 15–25 frames/s, resolution of 1280 × 720 pixel and fulfilled with infra-red (IR) onboard storage and illumination.

8. Any computerized device that is able to run recorded videos.
9. Timer.
10. For video speeding, any VLC device that is able to change playback speed.
11. A GraphPad prism software.

2.3 Irwin Test

1. Elevage Janvier supplies the rats (male Wistar).
2. Testing compounds.
3. Makrolon cages with the wood litter.
4. Binocular lamp, animal balance of 1 g.
5. Thermometer of digital laboratory with a probe of rat rectal with 5 mL Terumo syringes.
6. Gastric probes with oral extremity.
7. Metal forceps for measuring of analgesia.
8. Two vertical supports assisted by double bars for traction measurement.
9. A wire mounted grid that is attracted to wood frame, custom made, for measuring akinesia and grasping [6].

2.4 Wire Suspension Test

1. A 2 mm thick and 55 cm metallic wire is secured to vertical stands. It is coated to the plastic material. It should be tied to frame to avoid any unwanted displacement of wire while the researcher is handling the animals during measurement. Wire maintenance is done at 35 cm diameter above the breeding material. This is done to prevent animal injury.
2. Mice (as young as 4 weeks and 19 months).

2.5 Vertical Pole Test

1. Pole of 50 cm and 10 mm in diameter.
2. Large cage of mouse with bedding.
3. A stopwatch.
4. An adhesive tape for recovering pole surface.
5. A video camera.
6. VCR (optional).

3 Methods

3.1 Administration of Probiotics in Animals

Administration of probiotics in rodents is conventionally achieved by water bottles or the oral gavage that might be compromising animal welfare, dosing accuracy, or ease of administration. Furthermore, their slow consumption for several hours could be a source of varying bacterial stability, or clogging that would be affecting the reliability of this method. In the following way, a 3R principle of

replacement, reducing, and refinement a syringe feeding is known to be a refine method that is accurate in administration of probiotics in animals [7]. Therefore, animals directly consume the probiotic solution through syringe in their cages, which enables controlled dosage into individual animals. Its recent clinical observations have shown the advanced effects of probiotics in showing therapeutic response to inflammatory bowel disease [8]. In mice, an evaluation was regularly administrated of lactic acid bacteria LAB in their digestive tracks, to reflect their safety status, immunomodulation capacity by utilizing *Lactobacillus* spp. Its laboratory analysis undergone by literature survey has shown with no detrimental effects and *L. plantarum* NCIMB8826 as an active probiotic candidate that is utilized for treatment of chronic inflammation [9].

The steps of administration of probiotics in rodents are given below:

1. Divide the mice in several groups. The first one is control group and others are probiotic treated groups.
2. Administer the probiotic by syringe feeding.
3. Carry out the behavioral testing after treatment in all mice as described by the below mentioned tests.
4. Additionally, collect the fecal samples from random selection of mice and perform taxonomical analysis of intestinal microbiota.

3.2 Cylinder Test

Among rodents, cylindrical test is performed to measure its forelimb use that can be utilized for evaluating the sensor-motor function in the relative injury models that leads to forelimb asymmetry. In this test, the cylinder glass is set to place mouse and its rearing on the wall is measured which touches the cylinder (Fig. 1). Its purpose of evaluation is that taps at the wall are consequently scored for the sides of wall as left, right, or even both paws, recorded by the investigator in the gradually recorded videos. The outcomes are represented as the ratio of each paw usage comparative to the sum of all taps. This is the mutual behavior evaluation that is operated to analyze the motor impairment in trail of the Parkinson's disease [10].

This test can be performed for evaluation of preclinical screening anti-Parkinson's therapeutic interventions that are onto the functional recovery of the contralateral paw (Fig. 1). These tests are known to be feasible in terms of performing and are sensitive to detect the motor impairments which are undetected by other tests. While, the analysis of video-type tape record is not feasible to be conducted frequently as they are time-consuming and mice would evade its concern in searching vertical surface of cylinder [11].

The steps of this cylinder test are given below:

1. Carry out the cylindrical experiment during the dark period that is mice's active cycle.

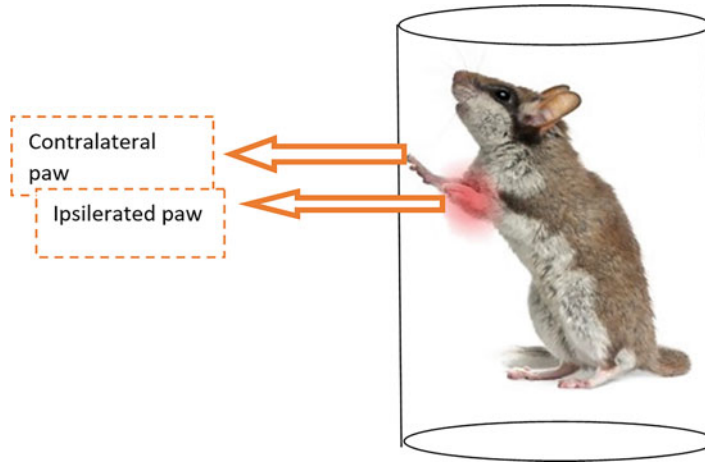

Fig. 1 Cylindrical test in mouse

2. Use a waterproof pen for marking mice trails prior to an experimental day. This will facilitate a simultaneous classification of caged mice along with avoiding the stress at high level simultaneously before testing.
3. Run all the behavioral evaluation at the same time.
4. Set the cylinder at the midpoint of the bench, utilize the black curtain for covering all edges of the cylinder to restrict the ocular distraction of mice.
5. Position the camera above for top-view, it should be to ensure entire diameter of the cylinder.
6. Under the low light condition, test should be performed and the dark surrounding will arouse the reflex of mice, for animal proficiency a ceiling white light can be used as the basis of radiance. This is measured by a dimmer switch that is set to supply above the cylinder 40 lux.
7. Prepare the test cards to analyze every animal during video recording, but do not indicate the experimental groups to assure blind scores.
8. The behavioral trail should not be noticeable to assayed mouse conducted by human experimenter.
9. Before 30 min of testing, acclimate the mice to the testing room and provide light condition and allow the mice to undisturbed rest.
10. In behavior assessment, start the video recording.
11. Tag the test card among the glass cylinder.
12. Do recording for 10 min and at the end of the test return mouse to home cage.
13. Then sanitize the cylinder with water and soap.

14. By the help of 70% ethanol, spray the cylinder for removing any odorant trace. Allow it to dry before the next test.
15. Recur these steps for the mice that are tested and at the closure, return whole of them to the housing compartment [12]. *See* **Note 1** for following this procedure.

3.3 Irwin Test

For Rodents, the Irwin test is used for accessing acute toxicity of testing agents like drugs with its possible impacts. It is also used for estimation of minimum lethal dose of the test substance. The dose ranges for primary effects, CNS response, physiological and behavioral functions. The results of this test are utilized for predicting potential therapeutic activity of the selected dose for the subsequent test for efficacy. Data from these tests are used as the risk linked assessments by the use of this agent. For its procedures, mice and rats are administrated and observed for following few hours and the next day.

3.3.1 Basic Protocol for Rats

This protocol is used to measure the primary effects of the test compounds on rats with their behavioral and physiological functions. Responses are evaluated after administration in 15, 30, 60, 120, 180 min, and 24 h. Measurements at the 48–72 h following administration could be added for agents which display the longer duration of action or for additional evaluation [13].

3.3.2 Preparation of Animals

1. With a free access to food and water the wood litter six per cage rats is set on delivery sustaining facilities with a range of 21 °C ± 2 with illumination facility for 12 h in a dark unreversed cycle initiating from 7 am.
2. Set the selected rat species in the selected Makrolon cages with the wood litters on the preceding experimental day with free access to food and water. Sustain the resting room at 18 °C and 24 °C with the light illumination identical to the acclimatization facilities.
3. On the morning, when experiment starts, remove all water and food for 1 h at least initial to first manipulation.
4. Assign the sampled animals randomly with each phase of three groups of animals having three animals per group; among which one is marked as control neutral group receiving non-active vehicle, and other two groups receiving the test compounds at the specified doses. Each animal in the particular cage should receive the same treatment.
5. Before starting the experimental phase, mark those animals that are on tail, and record pre-administrating values of pupil diameter, body weight, and rectal temperature.
6. Then administer the test compounds in a body weight of 5 mg/kg volume to each animal in a similar cage within that

experimental phase as soon as possible in <10 min. There are four groups in the following order of Dose 1, Dose 2, Dose 3, starting with control group.

7. Note down the timing of first animal in first cage and the last one in last treatment cage for evaluating the relative testing time for the series of rats.
8. Then evaluate the behavioral and physiological changes for each phase, without handling of animals, cage manipulation. Evaluate them group wise with their presence and absence of below mentioned characteristics. Compare this with the neutral group; their observation continues at the initial and after administration for 15 min to know the immediate effects. During the initial period, due to practical and technical reasons only mitigate those that are not including animal handling. Complete all the measurements and observations for the complete phase within 15 min in order to avoid interference with the consequent observation periods [14]. These measurements and observations include the followings:
   (a) Respiration (more or less).
   (b) Lethality (present or absent) with respect to time.
   (c) Jumps (present or absent).
   (d) Convulsions (presence or absence).
   (e) Scratching (presence or absence).
   (f) Motor incoordination.
   (g) Loss of balance.
   (h) Abnormal writhes.
   (i) Stereotypies (presence or absence).
   (j) Excitation (present or absent).
   (k) Piloerection (present or absent).
   (l) Abnormal gait.
   (m) Sedation (present or absent).
   (n) Head twitches (present or absent).
9. Produce the noise for evaluation of fear by snapping the cage.
10. Remove the cover of cage, and after appropriate manipulation note presence or absence of followings:
   (a) Muscle tone (more or less).
   (b) Reactivity to touch (more or less).
   (c) Ptosis (presence or absence).
   (d) Intensity of excitement or sedation.
   (e) Loss of traction.
   (f) Aggressiveness towards experiment.

(g) Akinesia .
(h) Analgesia.
(i) Loss of grasping.
(j) Defecation .
(k) Loss of corneal reflex.
(l) Salivation.

11. At the particular time, repeat the behavioral and physiological changes at 15, 30, 60, 120, 180 min, 1 day, and 2 days.
12. Then start the next experimental phase for group of animals immediately after the completion of 60 min. Duration of the preceding phase.
13. Replace the water and food in the cage after the last measurement of the day [15].

3.4 Wire Suspension Test

This is another test that is used in rats for accessing global sub-acute muscle coordination and function in mice over time. It is mainly based on the latency of mouse to fall off of the wire upon exhaustion [16].

3.4.1 Fall and Reaches Method

1. It is a hanging test subjected to mice for 180 s. During which the reaching and falling is recorded.
2. Increase the score by one when a mouse falls or reaches on the sides of the wire.
3. Create a Kaplan–Meier like curve. Use a 55 cm long wire (as described in Subheading 2). In this method, the length is constant throughout, because it has an influence over the outcomes.
4. Set the timing of 180 s. With falling and reaching score as 10 and 0.
5. It is handled by the tail and bought close to the wire; it is suspended by the forelimbs by the operator after which majority of animals catch the wire by four limbs that is allowed.
   You can follow **step 6** if time reaches to 0 s.
6. If the animal is reported to reach at the end of wire, stop the timer and record the reaching score as 1, then you can follow **step 5**.
7. The timer is stopped if the animal falls; the falling score is diminished by 1.
8. The falling score is more than zero, then the procedure is restarted from the **step 2**, when timer is over you can move to **step 6**.
9. Record the reaching and falling scores and the test is finished.

10. Set the parameters as the timer at 180 s, falling score at 10, and reaching as 0 [5].

3.4.2 Longer Suspension Method

A simple protocol exists for longer suspension time for three trails, for which an unlimited hanging time can be used, and if the fixed time is used, a hanging time of 600 s. Can be used and the starting time can be varied. The steps of this test are given below:

1. Handle the mouse by the tail and enable it to grasp the middle of the wire by the forelimbs and lower it; so the hind paws grasp the wire apart few centimeters from the fore paws.
2. Accompany the mouse gently along the axis of wire when it turns upside down.
3. Release the tail, while the mouse is still grasping four paws and start the timer. It is recorded until the mouse has completely released its grasp and falls.
4. It is reportedly given three trials per session along with a recovery period of 30 s.
5. Record a maximum hanging time when a fixed time of 600 s is used. A mouse reaches to the maximum independent trail number while others are allowed to rest.
6. For those mice that are given an unlimited hanging time, the body weight effect could be diminished by utilizing holding impulse that is equivalent to the body mass in grams and hanging time in seconds. This shows the tension animal is facing during maintaining itself on wire.
7. Record the body weight before and after the experiment [11].

3.5 Vertical Pole Test

The pole test is utilized for accessing basal ganglia concerned movement disorders in the mice. Its purpose is to access the motor dysfunction after stroke [17]. It facilitates the ability of mice to maneuver and grasp the pole in terms of descending to the home cage. They are trained to complete the pole test over training test. They are trained as a natural orientation of the head upward at the top of the pole they are oriented to downward and descend the length of the pole in order to return to the home cage. The time required for animal orientation downwards and descending to the base of the pole is recorded for five trails [18].

The steps of this vertical pole test are given below:

1. One day training is done, for a purpose of pre-testing that is optional and post-operative testing anytime.
2. Place the mice at the 50–60 cm vertical pole with 1 cm diameter that is directed in a triangular base stand and place in the home cage to be preferred by mice to descend towards the cage floor.

3. Start the recording when animals start the turning movement, with the record of descending to the floor and turning downward completely.
4. Repeat this trail if the animal descending is paused. If the animal is observed to be descending with lateral body instead of turning, then the "Ttotal" is known to be "Tturn."
5. The maximum score is accorded when animal falls off the pole suddenly, as 10 s Tturn and 15 s Ttotal.
6. Train the mice to descend the pole by turning around. The surface of the pole should be kept rough to avoid any kind of sliding.
7. It also happens that sometimes animals climb over the tip instead of making a turn; to prevent the climbing a cardboard piece can be placed at the top of the pole.
8. Repeat this test three times per animals and record the data analysis as "Tturn" and "Ttotal."
9. It is feasible to be carried at three different points, with one before surgery and a post-surgery at 24–48 h.
10. It is cleaned prior to experimentation with Kimwipe soaked with water, directed by rescue among each test run between mice [19].
11. Measure the time to turn downward completely for reaching ground.

4 Note

1. The mass of the glass cylinder might differ conferring to the scope of the mouse stress, its active and broader straining can try to escape in order to jump into cylinder's rim. In such cases, taller cylinders could be utilized, the ones utilized in it is C57BL/6 mice. Certain examiners have used mirrors next to the cylinders to enhance view visibility. Prior to the testing, habituation should be avoided. Trial duration is different among the protocols. Most of the studies use eight to ten mice per group [10].

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Chapter 25

Assessment of Bacterial Translocation Through Blood Cultures

Li Hui Ang, Premmala Rangasamy, Wallace Jeng Yang Chee, Shu Yih Chew, and Leslie Thian Lung Than

Abstract

Probiotic bacteria are commonly used to improve the quality of human life, primarily in maintaining good health and well-being. Whilst most probiotics are "generally regarded as safe" (GRAS), there are important concerns that administrated probiotics may translocate into the bloodstream and other internal organs, resulting in rare complications such as bacteraemia, fungaemia, sepsis, and multiple organs failure. It is evident that the safety of probiotic bacteria warrants further investigation, especially on the potential risk of bacterial translocation and infection. Here, we described a safety assessment method for probiotic translocation via blood cultures.

Key words Bloodstream, Blood cultures, GRAS, Probiotic bacteria, Probiotic translocation

1 Introduction

The World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. *Lactobacilli* are lactic acid bacteria (LAB) which are commonly found in the human gastrointestinal and genitourinary tracts [2, 3]. Generally, probiotics play an essential role in maintaining human health in many ways, including improvement of gastrointestinal and vaginal health, as well as modulation of the host immune system [2, 4]. Probiotics are generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) to be utilized as medical treatments, supplements, and fermented foods. However, individuals with underlying medical history or underlying conditions such as immunosuppression, solid organ transplantation, and defective in intestinal barrier are more vulnerable to infections caused by bacterial translocation following probiotic intake [5].

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Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_25,

Bacterial translocation is defined as the transfer of viable bacteria cells travelling from gastrointestinal tract (GIT) to the mesenteric lymph node (MLN) complex and extra intestinal sites [6]. Probiotic translocation might cause infection such as sepsis, bacteraemia, fungaemia, and multiple organ failure [5, 7]. According to a case report, *Lactobacillus* bacteraemia caused by probiotic translocation was reported in an immunosuppressed individual with severe ulcerative colitis (UC) after taking *Lactobacillus rhamnosus* GG [8]. A retrospective study on 200 reported cases of lactobacilli-associated infections reported a mortality rate of approximately 30% [9]. Hence, the safety assessment of probiotics bacteria is crucial as it predicts the potential risk of them to translocate to other organs and tissues.

2 Materials

2.1 Preparation of Probiotic and Animal Model

1. Anaerobic gas generating sachets and anaerobic jar.
2. Forty, BALB/c mice: Female, 7–8 weeks old (20–25 g), acclimatize them under controlled conditions (12/12 h light/dark cycle), 22–25 °C for 7 days before starting of the experiment. Provide the animals with standard rodent diet and water ad libitum [10].
3. De Man, Rogosa, and Sharpe (MRS) agar and broth.
4. Distilled water.
5. Freeze dryer.
6. Ketamine (80 mg/kg).
7. Needle (23–25G) with 5 mL syringe.
8. Oral gavage feeding curved needle (18–20G).
9. Phosphate buffered saline (PBS).
10. Skim milk, 20% (w/v).
11. Xylazine (10 mg/kg).
12. 1.5 mL centrifuge tubes.
13. 2 mL Ethylenediaminetetraacetic acid (EDTA) tubes.
14. 90 × 15 mm Petri dish.

2.2 Bacterial Translocation Assay

1. Anerobic gas generating sachets and jar.
2. Incubator (37 °C).
3. Blood samples.
4. Brain heart infusion (BHI) agar.
5. MRS agar and broth.

2.3 Random Amplified Polymorphic DNA (RAPD)

1. Agarose powder.
2. Bench top centrifuge.
3. Commercially available nucleic acid stain.
4. Commercially available polymerase chain reaction (PCR) kit: Taq DNA polymerase, PCR reaction buffer, magnesium chloride ($MgCl_2$), and dNTPs mixture.
5. Commercially available spin column-based or bacterial genomic DNA purification kit.
6. DNA loading dye (6×).
7. Electrophoresis tank.
8. Gel documentation system.
9. Microwave oven.
10. PCR Thermocycler.
11. Primers (Arbitrary primers: 5′ ACG AGG CAC 3′ and 5′ ACG CGC CCT 3′) [11].
12. UV spectrophotometer.
13. Water bath (60 °C and 70 °C).
14. 1× Tris-Borate-EDTA (TBE) buffer: 0.089 M Tris, 0.089 M borate, 2 mM EDTA, pH 8.3.
15. 0.2 mL PCR tubes.
16. 1.5 mL microcentrifuge tubes.

3 Methods

3.1 Preparation of Probiotics

1. Inoculate probiotic strain, i.e. *Lactobacillus* spp. on MRS agar and incubate the plate anaerobically for 48 h at 37 °C in an anaerobic jar.
2. After 48 h of incubation, transfer two to three colonies of the strain into MRS broth and adjust the culture to OD_{600} of 0.1 (approximately 1×10^5 CFU/mL). Incubate the strain anaerobically for 12 h at 37 °C in an anaerobic jar (stationary phase).
3. After 12 h of incubation, harvest the cells by centrifugation at 9000 × *g* for 15 min.
4. Prepare the cells in three different doses (5×10^8, 5×10^9 and 5×10^{10} CFU/mL). Wash the cells twice with sterile PBS.
5. Reconstitute the cells with skim milk (20% w/v) (cryoprotectant) and mix until it forms a homogeneous suspension.
6. Transfer the suspension into a new sterile centrifuge tube and freeze it for 4 h at −20 °C and overnight at −80 °C.
7. Transfer the frozen suspension into freeze dryer for lyophilization.

8. Upon completion of the freeze-drying process, retrieve the lyophilized probiotic strain and store at 4 °C until further use.
9. Check the viability of the lyophilized strain before starting the animal experiment through viable cell counting on MRS agar. Determine its CFU after 48 h of incubation anaerobically at 37 °C.

3.2 Oral Administration of Probiotic in BALB/c Mice

1. Prepare the lyophilized probiotic strain (5×10^8, 5×10^9 and 5×10^{10} CFU/mL) by reconstituting it in sterile distilled water.
2. Administer 200 μL of the strain suspension (Group 1: 1×10^8, Group 2: 1×10^9 and Group 3: 1×10^{10} CFU/mL) into three experimental mice groups ($n = 10$ for each group) via oral gavage (once daily, for a total of 28 days).
3. Administer 200 μL of skim milk only (Group 4: No probiotic strain) into mice from control group ($n = 10$) via similar route and duration as experimental groups.
4. Monitor the behavior, activity, and general health of the mice daily. Measure and record the body weight (BW) and food intake (FI) on a weekly basis throughout the duration of the experiment.
5. At the end of the experiment (Day 29), anesthetize the mice by intraperitoneal (IP) administration of ketamine (80 mg/kg) and xylazine (10 mg/kg) using 1 mL of syringe.
6. Collect the mice blood (approximately 1–2 mL) via cardiac puncture using a 23–25G needle with 5 mL syringe.
7. Transfer the collected blood into an EDTA tube and store at 4 °C until further use.

3.3 Bacterial Translocation Assay

1. Transfer 50 μL of blood sample from EDTA tube and spread it evenly onto MRS and BHI agars.
2. Incubate both the agar plates for 48 h at 37 °C under anaerobic and aerobic condition, respectively.
3. After 48 h of incubation, count the colonies that are present on both agars (if any).
4. The presence of growth on these agars indicates bacterial translocation.
5. The formula to express the incidence of translocation is:

$$\frac{\text{The number of mice where translocation is detected}}{\text{The total number of mice}}$$

6. If there is growth on both agars, confirm the identity of colonies by RAPD-PCR (as detailed in Subheading 3.4).

Table 1
PCR cycling conditions for RAPD PCR

Steps	Temperature	Time	Cycle
Initial denaturation	94 °C	5 min	–
Denaturation	94 °C	30 s	
Annealing	52 °C	30 s	40 cycles
Extension	72 °C	2 min	
Final extension	72 °C	10 min	–

3.4 RAPD-PCR

1. Inoculate each individual colony found on MRS and BHI agars (from the bacterial translocation assay) into MRS broth and incubate for 24 h at 37 °C anaerobically.
2. After 24 h of incubation, transfer 1 mL of the broth cultures into a sterile 1.5 mL microcentrifuge tube and spin at 1600 × *g* for 1 min to pellet the cells. Perform genomic DNA extraction using commercially available bacterial DNA extraction kit according to the manufacturer's instructions.
3. Prepare a standard 25 μL PCR reaction mixture containing 1× Taq DNA polymerase buffer, 2.0 mM $MgCl_2$, 0.2 mM of dNTPs, 1.0 μM each of the two arbitrary primers, 1 U Taq DNA polymerase and DNA template (*see* **Note 1**).
4. Amplify the reaction mixture in a PCR thermocycler with the following PCR cycling conditions (Table 1).
5. Prepare 1% agarose gel and pre-stain it with commercially available nucleic acid stain. Carefully load a DNA ladder into the first lane of the gel. Subsequently, pre-mix and load 5 μL of PCR sample with 1 μL of DNA loading dye into the remaining sample wells.
6. Run the agarose gel in 1× TBE running buffer for 60 min at 80 V.
7. View the agarose gel under UV light using a gel documentation system and interpret the RAPD results as shown in Fig. 1.

4 Note

1. Perform all procedures with utmost precautions (e.g., in DNA extraction and RAPD-PCR) to avoid contamination. Keep all reagents and samples on ice throughout the preparation.

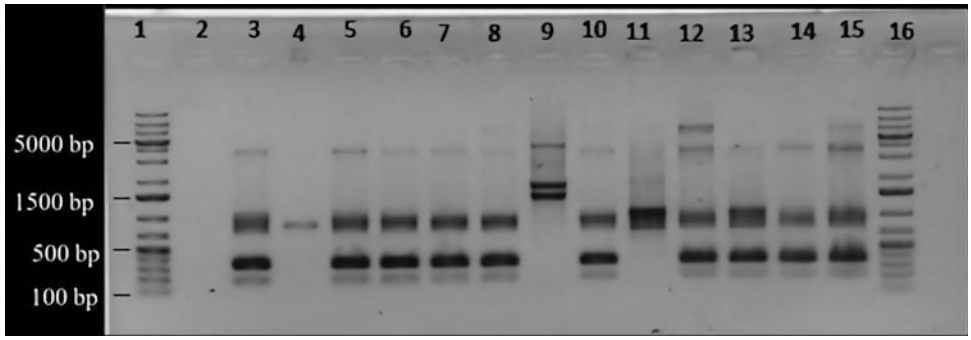

Fig. 1 Comparison of the RAPD patterns of orally-administered probiotic strain and bacterial isolates recovered from blood culture following probiotic intake. Lanes 1 and 16: DNA ladder; Lane 2: no template control, Lanes 3–10: isolates from blood cultures; Lane 9 is orally-administered probiotic probiotic strain; Lanes 11–15: other lactobacilli strains. The RAPD amplicons showed distinct patterns between orally-administered probiotic strain (Lane 9) and isolates recovered from bacterial translocation assay. (Reproduced from ref. 10 with permission from Elsevier)

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Chapter 26

Determination of Splenic Weight Index and Weight-to-Length Ratio

Firdosh Shah and Mitesh Kumar Dwivedi

Abstract

Probiotics are responsible for exhibiting health promoting properties such as modulation of immune responses, inhibition of pathogens, as well as reduction of toxic compounds. The toxicological profiling within the animal models after the administration of probiotics also falls under one of the most indispensable experimentations. Different organs such as spleen, liver, kidney, etc. are surgically removed and analyzed for assessing the effects of probiotics on these organs. Similar to this context, splenectomy is also performed in animal models in order to identify any changes occurred in the splenic weight index of the animal. The body mass index or weight-to-length ratio of an animal also suggests an overall well-being of the animal after the administration of probiotics. The aim of this chapter is to provide the experimental methods adopted for maintaining growth conditions and administration of probiotics within animal model and assessing the splenic weight index and weight-to-length ratio within the animal model.

Key words Probiotics, Spleen, Weight-to-length, Splenic weight index, Splenectomy

1 Introduction

Since past few years there has been an enormous increase in utilizing probiotic bacteria for nutritional and medicinal benefits. The ability of probiotic bacteria to modulate intestinal microbiota has been well established in various animal models and clinical trials with several health promoting effects, most of them include lactic acid bacteria [1]. Few of the health advantages provided by probiotics include anti-bacterial activity [2], ameliorating effects on intestinal inflammation [3], immunomodulatory effect [4], and efficient prevention against allergic conditions [5]. The above-mentioned health benefits of probiotics render them a good representative for incorporation into functional foods or pharmaceutical products with an optimum proportion of newly identified bacterial strains.

In case of selecting new probiotic strains, bacteria are subjected to various safety criteria, among which assessment of pathogenicity

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_26,

is one of the major aspects [6]. Moreover, there are few reports which mark association of probiotic strains with pathological conditions such as bacteraemia [7], abscess, and endocarditis [8]. Although, there are very few studies representing probiotics as a causative agent in the pathological conditions but few reports raise concerns regarding the safety aspects of the bacteria, particularly those which are considered for human dietary consumptions [9, 10]. Therefore, in order to ensure the safety aspects of probiotic strains, the bacterial strains are subjected to several in vivo biochemical and clinical parameters to preclude their deleterious effects.

The spleen is located in the cranial abdomen and it looks dark red to blue-black in color. It is roughly elongated and triangular in cross-section. The major function of spleen is concentrated on the systemic circulation; it filters blood while removing foreign materials and damaged erythrocytes [11–13]. The gross size and shape of spleen is variable and depends on the species. Normally, the ratio of splenic weight to body weight remains constant across the ages, around 0.2%. One of the major evaluating factors in any toxicity studies is measurement of splenic weight after the administration of probiotics. Similarly, weight-to-length ratio is also considered as a crucial factor which measures body proportionality within the study groups after the administration of probiotics [14].

2 Materials

2.1 Probiotics and Growth Conditions

- MRS broth.
- de Mann Rogosa, & Sharpe (MRS) Agar.
- Sterile Petri plates.

2.2 Animal Preparations

- Adult male and female mice (*Mus musculus*).
- Rectangular polyacrylic cages.
- Dust-free paddy husk.
- Standard pellet diet.
- Clean tap water.

2.3 Administration of Probiotics

- Phosphate-buffered saline (PBS).
- 0.2 M $NaHCO_3$ buffer containing 2% glucose.
- Bicarbonate buffer.
- 3 mg Ketamine.
- 46.7 μg of diazepam.
- 15 μg of atropine.
- 3.5 F catheter.

2.4 Specimen Collection

- Scissors (Straight/curved), Forceps (10 cm long with curved ends).
- Sterile 10–10 cm gauze sections, Blotting Paper/Filter paper.
- Microbalance, sample bottles.
- Normal Saline and Formaldehyde fixative.

2.5 Tissue Processing, Staining and Histopathological Evaluation

- Rotary microtome.
- DPX mountant, compound light microscope.
- Glass microscopic slides, glass cover slips.
- 10% neutral buffered formalin, ethyl alcohol.
- Paraffin wax, Harris' hematoxylin.
- Ethanol, Xylene, 1% alcoholic eosin.
- Tap water, distilled water.
- Saturated sodium bicarbonate solution.

2.6 Splenic Weight Index

- Analytical scale, Millimeter scale.
- Slide caliper.
- Ultrasound equipment.
- 3.5-MHz multifrequency sector or convex probe.

2.7 Weight-to-Length Ratio

- Electronic balance.
- Digital caliper.

3 Methods

After the administration of probiotics, the splenic weight index and weight-to-length ratio can be measured in the mice model through the below mentioned steps (Fig. 1).

3.1 Probiotics and Growth Conditions

The probiotic strains of *Lactobacillus*, *Bifidobacteria,* or any other strain exhibiting the beneficial probiotic traits such as acid tolerance, bile tolerance, in vitro adherence to epithelial cells, etc. [15] can be grown by the below mentioned steps [16]:

1. Take an isolated colony of the probiotic strain and inoculate it within MRS (de Mann, Rogosa, & Sharpe) broth at 37 °C for overnight.
2. Take a loop of overnight grown bacterial cell suspension and streak it on MRS agar plate (pH 5.5).

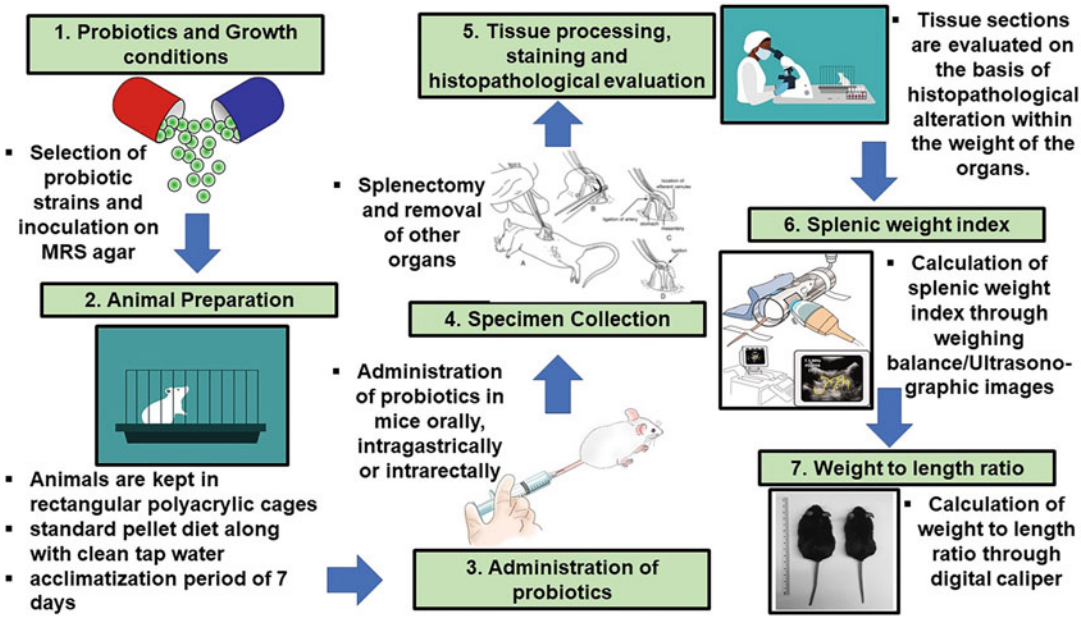

Fig. 1 Steps involved in maintaining growth conditions and administration of probiotics within animal model for measuring splenic weight index and weight-to-length ratio. Step 1. Probiotics and growth conditions; Step 2. Animal Preparation; Step 3. Administration of probiotics; Step 4. Specimen collection; Step 5. Tissue processing, staining and histopathological evaluation; Step 6. Splenic Weight index measurement through weighing balance and ultrasonography; and Step 7. Weight-to-length ratio measurement through digital caliper

3. Incubate the plates anaerobically for the period of 2 days at 37 °C.
4. The well isolated colonies can be observed and can be further used for determining its effects on animal models.

3.2 Animal Preparation

1. Take the 6–7 weeks old adult male and female mice (*Mus musculus*) of weight 20–25 g in which the splenic weight index and weight-to-length ratio will be determined.
2. Keep the animals in rectangular polyacrylic cages with bedding material as dust-free paddy husk (*see* **Note 1**).
3. Allow the animals to take the standard pellet diet along with clean tap water ad libitum except in the case where starvation is needed.
4. Change the water and food regularly and clean the cages along with the fresh husk replacement every 3 days.
5. Set an acclimatization period of 7 days before experimentation, in order to minimize any non-specific stress [17–20].

3.3 Administration of Probiotics

Probiotics can be administered within mice model through below mentioned steps [21]:

1. Grow the probiotic strains exponentially unless the measurement of turbidity reaches 1–2 (absorbance 600 nm).
2. Harvest the bacterial cell suspension through centrifugation at 1008 × *g* for 10 min. With repeated washing with phosphate-buffered saline (PBS) and resuspend them either in PBS or 0.2 M $NaHCO_3$ buffer containing 2% glucose (*see* **Note 2**).
3. In case of oral administration of probiotics in mice, resuspend 10^9 colony forming units (CFUs) in bicarbonate buffer and administer 100 μL of suspension in mice intragastrically; whereas, administer 25 μL of suspension while delivering through mouth.
4. For intrarectal administration, administer 10^9 CFU of the probiotic within anaesthetized mice (anaesthetized with 3 mg of Ketamine, 46.7 μg of diazepam, and 15 μg of atropine) through 4 cm insertion, proximal to the anus using 3.5 F catheter.

3.4 Specimen Collection

1. At the end of the experiments and after the blood collection, sacrifice all the mice in the treated and control groups by cervical dislocation [22, 23].
2. Open the abdominal cavity and remove the different organs such as spleen, kidney, and liver from each animal and blot them with filter paper.
3. Weigh the organs immediately on a semi-microbalance (*see* **Note 3**).
4. Rinse the organs in normal saline. Make sections from each of the harvested organs, including spleen.
5. Remove two coronal halves of the right kidney, as well as dissect other tissue samples out in block from the spleen.
6. Place the spleen the pre-labelled sample bottles containing fixative for further histopathological studies.

3.5 Tissue Processing, Staining and Histopathological Evaluation

The tissue samples taken during autopsy can be processed for histopathological studies under the light microscope [22, 23].

1. Immerse the tissue samples taken from each organs including spleen and fix it separately in 10% neutral buffered formalin at room temperature.
2. Keep the specimens in fixatives for 24 h.
3. After fixation, rinse the tissues with running water, and dehydrate them by immersing it in ascending grades of ethyl alcohol.
4. Impregnate the tissues with molten paraffin wax in hot oven and embed it in paraffin blocks at room temperature.

5. Use the Rotary microtome for sectioning the paraffin blocks at 5–6 μm thickness.
6. Collect each eighth to tenth sections and float them gently on a flotation bath at 40 °C and finally pick them up on glass microscopic slides.
7. Dehydrate the tissue sections with ethanol and deparaffin them using xylene before staining.
8. Rinse the slides with distilled water and stain the sections regressively for 10 min with Harris' hematoxylin (*see* **Note 4**).
9. Rinse the sections in running tap water in order to remove excess acid and halt destain.
10. Place the slides in saturated sodium bicarbonate solution for 3 min and counter stain them for 1 min within 1% alcoholic eosin.
11. Dehydrate the H- & E-stained sections by increasing concentration of xylene and ethanol and mount it using glass cover slip and DPX mountant.
12. Examine the microscopic slides under compound light microscope using different optical lenses of magnification 40× and 100×.
13. Evaluate the tissue sections of treated groups and controls is on the basis of histopathological alteration and changes observed within the weight of the organs (Spleen).
14. Use the digital photo camera mounted on binocular compound microscope for taking photomicrographs of selected slides of each organ.

3.6 Determination of Splenic Weight Index

The splenic weight index in the mice can be measured through below mentioned methods:

3.6.1 Splenic Weight Calculation Through Weighing Balance

1. Measure the weight of the mice after the mice is injected with Ketamine IM.
2. Open the abdominal cavity, take out the spleen and weigh it using an analytical scale.
3. Measure the length and width of the spleen using a ruler of a millimeter scale or through slide caliper or ruler.
4. Calculate the splenic index using the below mentioned equation [24].
5. The splenic weight index = weight of spleen of mice/body weight of mice.

3.6.2 Splenic Weight Calculation Through Ultrasonography

The spleen size can be routinely monitored through ultrasound examinations as well [25].

1. Record the size of spleen as it appears in the abdominal ultrasound.
2. Assess the size of spleen as spleen bipolar diameter (crossing the spleen hilum) using last-generation equipment with a 3.5-MHz multifrequency sector or convex probe.

3.7 Determination of Weight-to-Length Ratio

The weight-to-length ratio of the mice can be determined by the below mentioned steps:

1. Record the animal's (mice) weight before its death by cervical dislocation.
2. Immediately after the death, measure the nose-to-anus length/weight-to-length ratio of the mice.
3. Dissect the uterine fat pads within female mice and epididymal fat pads within male mice and weigh them on an electronic balance.
4. Harvest the right hind limb, dissect the tibialis anterior, soleus, gastrocnemius, and extensor digitorum longus muscle and weigh them to the nearest hundredth of a milligram.
5. Clean the tibia and femur and store them at −20 °C for future studies and thaw the bones at ambient temperature.
6. Measure the femoral length and width by using a digital caliper at the center of the diaphysis in both sagittal and coronal planes along with epiphyseal width in coronal plane.
7. Measure the femoral neck and head diameter through digital caliper. In particular, measure the proximal, distal, and epiphyseal width [14].
8. Measure the tibial lengths of mice using a PIXImus system (GE-Lunar) [26].
9. Calculate the weight-to-length ratio using the formula: weight (kg)/height $(\mathrm{m})^2$.

3.8 Inferences

In mice, the average normal range of the splenic weight index is 0.2–0.6 g. The test bacterial strains or probiotics exhibiting splenic weight index beyond the above mentioned are considered toxic. We recommend that each weight-to-length ratio in animal subjects should establish its own normal range by measuring normal weight-to-length ratio in control (healthy) animal subjects in comparison with the test animals. The probiotic strains which exhibit toxicity have been shown to increase weight-to-length ratio in animals as compared to that of the healthy controls [27].

4 Notes

1. During the study period, the male and female mice are kept in separate cages to avoid breeding under a constant laboratory condition with 12-h light/dark cycle at temperature of 22 ± 2 °C.
2. For each experiment a fresh bacterial suspension should be prepared.
3. Each organ is weighed on a semi-microbalance sensitive to 0.001 g and in order to obtain relative weight of organ, body weight is normalized and expressed as per 100 g body weight.
4. In order to remove excess stain, the sections are washed in tap water and dipped into 1% acid alcohol for differentiation.

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Chapter 27

Determination of Total Liver Glutathione and Plasma Malondialdehyde Concentrations

Firdosh Shah and Mitesh Kumar Dwivedi

Abstract

Probiotics are recommended to improve human and animal health; however, the assessment of safety aspects of probiotics is an important concern to consider prior to the administration of probiotics into the human and animal subjects. One of the crucial parameters of toxicological profiling of probiotics is oxidative stress which may be induced upon administration. Determination of glutathione, glutathione disulfide, and other related intermediates help in assessing the metabolic status of biological systems in vivo and in vitro. Similarly, lipid peroxidation results into a range of intermediate products such as aldehydes and malondialdehyde. The assessment of malondialdehyde levels indicates the level of oxidative stress induced by probiotics. In this chapter, we describe the methods and techniques used to estimate the levels of glutathione and lipid peroxidation in animal subjects upon administration of probiotics, which act as biomarkers for antioxidant status and oxidative stress.

Key words Probiotics, Glutathione, Glutathione disulfide, Malondialdehyde, Oxidative stress, Lipid peroxidation

1 Introduction

Probiotics are living microorganisms which are intended to confer health benefit within the host upon administrating them at an adequate proportion [1]. Although, several studies suggest role of probiotics (e.g., lactic acid bacteria and *Bifidobacterium*) in improving the human health; however, assessment of safety aspects of such bacteria is needed to prevent any undesired effects upon their administration into the host. Several factors of probiotics affect host health including nature of microbe being used, level of exposure, physiological conditions, method of administration, and health status of the host. Therefore, it also becomes necessary to assess the adverse effects of probiotic strains prior to their administration into the host. One of the key parameters of toxicological profiling of probiotics is to assess the oxidative stress which may be induced upon administration. The antioxidant status can be

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_27,

assessed by determining the level of glutathione and glutathione disulfide which is a crucial tripeptide thiol antioxidant and its reduced intracellular concentration marks an indication of oxidative stress [2]. Glutathione exists in two different forms within the cells: oxidized form—glutathione disulfide, and the reduced-sulfhydryl form. Oxidative stress greatly impacts the cellular thiol balance which results into declination in glutathione and glutathione disulfide ratio in many of the body organs [3]. Moreover, glutathione takes active participation within signal transduction, apoptosis, gene expression, and cellular reactions of the host. Therefore, glutathione and glutathione disulfide status are mostly measured for physiological and patho-physiological conditions [4]. Two different methods are reported for determining the levels of glutathione in complex biological samples and plasma. The 5,5′-dithio-bis (2-nitrobenzoic acid) DTNB enzyme recycling method and glutathione reductase recycling method, which is sensitive and form yellow derivative of 5′-thio-bis (2-nitrobenzoic acid) TNB (measurable at 412 nm). The HPLC methods are also utilized in the cases, where there is a limitation of small sample volume [5]. Similarly, lipid peroxidation is also considered as one of the most important indices of oxidative stress and is implicated as contributing factor in a vast range of diseases such as diabetes [6], Alzheimer's disease, Parkinson's disease, cardiovascular diseases [7], and psychiatric disorders [8]. The process of lipid peroxidation exhibits range of end products and intermediates such as aldehydes, lipid hydroperoxides, and malondialdehydes. These aldehydes result into single and double strand breaks which leads to the formation of DNA adducts [9]. These end products and intermediates which are produced during lipid peroxidation cascade are often utilized for assessment of lipid peroxidation, but among these most popular approaches is thiobarbituric acid (TBA) test [10]. TBA reacts with malondialdehyde to produce a pink pigment with an absorption maximum at 532 nm [11] and mass ion at 323 amu [12], which acts as a true indicator of lipid peroxidation. The adoption of HPLC techniques helps in overcoming issues observed regarding specificity and the sensitivity of malondialdehyde determination through spectrophotometry and fluorescence assays [13]. In this chapter, we provide the methods and techniques used for determining the levels of glutathione and lipid peroxidation within animal subjects upon administration of probiotics.

2 Materials

2.1 Probiotics and Growth Conditions

- MRS broth.
- de Mann Rogosa & Sharpe (MRS) Agar.
- Sterile Petri plates.
- Probiotic strains.

2.2 Animal Preparations

- Adult male and female mice (*Mus musculus*).
- Rectangular polyacrylic cages.
- Dust-free paddy husk.
- Standard pellet diet.
- Clean tap water.

2.3 Administration of Probiotics

- Phosphate-buffered saline (PBS).
- 0.2 M $NaHCO_3$ buffer containing 2% glucose.
- Bicarbonate buffer.
- 3 mg Ketamine.
- 46.7 μg of diazepam.
- 15 μg of atropine.
- 3.5 F catheter.

2.4 Specimen Collection

- Scissors (Straight/curved), Forceps (10 cm long with curved ends).
- Sterile 10–10 cm gauze sections, Blotting Paper/Filter paper.
- Microbalance, Sample bottles.
- Normal Saline and Formaldehyde Fixative.

2.5 Preparation of Samples

- Plasma buffer.
- PBS.
- Liquid nitrogen.
- Mortar & Pestle.

2.6 Estimation of Glutathione and Glutathione Disulfide Concentration Through DTNB and Glutathione Reductase Recycling Method

2.6.1 Preparation of Assay Buffer Solution

- Dissolve 14.196 g of sodium phosphate dibasic into 800 mL of double distilled water. Add 1.86 g EDTA and dissolve completely.
- Dissolve 3.45 g sodium phosphate monobasic within 250 mL of double distilled water and add 0.47 g EDTA and dissolve completely.
- Add monobasic solution into dibasic solution (# 2 to # 1) in order to achieve pH 7.4.
- Make final volume to 1000 mL by adding double distilled water after achieving pH of 7.4.

2.6.2 Preparation of DTNB Stock Solution

- Dissolve 99.1 mg DTNB within 20 mL of assay buffer.
- Make final volume up to 25 mL by adding assay buffer into volumetric flask.
- The DTNB stock solution can be stored in refrigerator for a period of 1 month.

2.6.3 Preparation of N-ethylmaleimide (NEM) Stock Solution

- Dissolve 125.13 mg of NEM within 1 mL of acetonitrile. Store it in refrigerator.

2.6.4 Preparation of Glutathione Disulfide Standard Stock Solution

- Dissolve 15.3 mg glutathione (oxidized form) into 5 mL assay buffer. Prepare an aliquot of 25 μL of the solution and store it in −20 °C. Prepare fresh tube for each use.

2.6.5 Preparation of Glutathione Disulfide Working Standard

- Add 10 μL of glutathione disulfide standard within 990 μL of assay buffer for concentration of 0.1 mM.

2.6.6 Preparation of Reaction Mixture #1

- Add 4.38 mL assay buffer in 5 mL of the tube and place 313 μL of 10 Mm DTNB stock solution. Also add 50 μL glutathione reductase and store it in refrigerator (2–8 °C).

2.6.7 Preparation of Reaction Mixture #2

- Add 5 mg NADPH in 5 mL assay buffer and store it in ice.

2.7 Estimation of Glutathione and Glutathione Disulfide Concentration Through HPLC

2.7.1 Preparation of Plasma Buffer Solution

- 5 mg sodium heparin.
- 10 mg BPDS (bathophenanthrolinedisulfonate).
- 20 mg iodoacetic acid.
- 8 mL 100 mM boric acid (0.62 g/100 mL).
- 2 mL 100 mM sodium tetraborate (3.81 g/100 mL).
- 105 mg L-serine.

2.7.2 Preparation of Sample Buffer

- Dissolve the sample in a total volume of 500 mL of double distilled water.
- Iodoacetic acid (prepare it fresh; add 14.8 mg in 2 mL of distilled water).
- KOH/tetraborate: Within a plastic bottle add 5 g $K_2B_4O_7{\cdot}4H_2O$ and 5.6 g KOH to 100 mL distilled water and mix thoroughly. Allow it to stand for overnight and remove the supernatant while discarding precipitant.
- Dansyl chloride (20 mg/mL in acetone).
- Chloroform.
- Mobile phase:
  - 80% MeOH, 20% water.
  - Acetate-buffered MeOH, pH 4.6 [640 mL MeOH, 200 mL acetate stock*, 125 mL glacial acetic acid, 50 mL water].
    [*Composition of acetate stock: 272 g Na-acetate trihydrate, 122 mL water, 378 mL glacial acetic acid].

2.7.3 Preparation of Standards

- HPLC column Glutathione (IC1800rp).
- Reconstitution solution.

For glutathione measurement, standards are made by dissolving in sample buffer with concentrations ranging from 10 to 40 nmol/assay, whereas for glutathione disulfide determination the concentration ranges from 1 to 4 nmol/assay. Since thiols are unstable, they should be made fresh each time.

2.8 Estimation of Lipid Peroxidation (Spectrophotometrically)

- Aqueous lipid sample.
- 4× thiobarbituric acid reagent.
- 2% (w/v) butylated hydroxyl toluene in ethanol.
- 1,1,3,3-Tetramethoxypropane (optional).
- Boiling water bath.
- Spectrophotometer or spectrofluorometer.

2.9 Estimation of Malondialdehyde Concentration Through HPLC

- HPLC with HP3395 integrator.
- C18 reversed-phase column (25 cm, 4.6 mm; 5 μm particles).
- 1,1,3,3-tetramethoxypropane.
- Butylated hydroxytoluene.
- Methanol.
- KOH.
- KH_2PO_4.
- $HClO_4$.
- 0.1 M HCl.
- Working stock solution of malondialdehyde.

3 Methods

3.1 Probiotics and Growth Conditions

The probiotic strains of *Lactobacillus*, *Bifidobacteria*, or any other strain exhibiting the beneficial probiotic traits such as acid tolerance, bile tolerance, antimicrobial activity, in vitro adherence to epithelial cells, etc. [14] can be grown by the below mentioned steps [15]:

1. Take an isolated colony of the probiotic strain and inoculate it within MRS (de Mann Rogosa & Sharpe) broth at 37 °C for overnight.
2. Take a loop of overnight grown bacterial cell suspension and streak it on MRS agar plate (pH 5.5).
3. Incubate the plates anaerobically for 2 days at 37 °C.
4. The well-isolated colonies can be observed and can be further used for determining its effects on animal models.

3.2 Animal Preparation

1. Take the 6–7 weeks old adult male and female mice (*Mus musculus*) of weight 20–25 g.
2. Keep the animals in rectangular polyacrylic cages with bedding material as dust-free paddy husk (*see* **Note 1**).
3. Allow the animals to take the standard pellet diet along with clean tap water ad libitum except in the case where starvation is needed.
4. Change the water and food regularly and clean the cages along with the fresh husk replacement every 3 days.
5. Set an acclimatization period of 7 days before experimentation in order to minimize any non-specific stress [16–19].

3.3 Administration of Probiotics

The probiotics can be administered within mice model through below mentioned steps [20]:

1. Grow the probiotic strains exponentially unless the measurement of turbidity reaches 1–2 (absorbance 600 nm).
2. Harvest the bacterial cell suspension through centrifugation at 3000 rpm for 10 min. With repeated washing with phosphate-buffered saline (PBS) and resuspend them either in PBS or 0.2 M $NaHCO_3$ buffer containing 2% glucose (*see* **Note 2**).
3. In case of oral administration of probiotics in mice, resuspend 10^9 colony forming units (CFUs) in bicarbonate buffer and administer 100 μL of suspension in mice intragastrically, whereas administer 25 μL of suspension while delivering through mouth.
4. For intrarectal administration, administer 10^9 CFU of the probiotic within anesthetized mice (anesthetized with 3 mg of Ketamine, 46.7 μg of diazepam, and 15 μg of atropine) through 4 cm insertion, proximal to the anus using 3.5 F catheter.
5. Control mice received phosphate-buffered saline (PBS) diluted in sterile water and were maintained under identical conditions as the test group.

3.4 Preparation of Samples

The following steps are followed for preparation of plasma, cells, and tissues during determination of total glutathione and plasma malondialdehyde concentration [21]:

3.4.1 Plasma Samples

1. Separate the plasma from blood cells using plasma buffer.
2. Once separated, add 200 mL of sample buffer into 200 mL of the plasma/buffer mixture and mix it thoroughly.
3. Centrifuge the mixture at 5000 rpm for 5 min in order to precipitate the proteins and plasma supernatant and store it to −80 °C for further analysis.

3.4.2 Cells Preparation

1. In case of cells, first remove the media and wash the cells at least thrice with PBS.
2. Before harvesting the cells, place the cells directly into the buffer. (The acidity of buffer causes cells to get thicker and granular due to precipitations of the proteins.)
3. Scrape off the mixture from the plate and place it in the tube and centrifuge to precipitate out the proteins.

3.4.3 Tissue Preparation

1. Harvest the tissues and snap-froze it prior to analysis.
2. In 0.5–1.0 mL of sample buffer, homogenize approximately 10 mg of the tissue. Homogenization of the sample must be done in cold condition.
3. Centrifuge the homogenate at 5000 rpm for 5 min. The proteins get precipitated while centrifuging the homogenate. In case where sample size exceeds 50 mg, the tissue is grounded under liquid nitrogen using cool mortar & pestle and the homogenate mixture is further transferred in sample buffer solution.

3.5 Estimation of Glutathione and Glutathione Disulfide Concentration Through DTNB and Glutathione Reductase Recycling Method

The DTNB and glutathione reductase recycling method are two methods utilized for measuring glutathione and glutathione disulfide concentration [5]. The detailed assay procedures for these methods are stated below:

3.5.1 Glutathione Assay Procedure

1. Prepare tissue samples by homogenizing 0.1 g tissue sample within 900 mL of assay buffer. Centrifuge homogenate at 12,000 rpm for 20 min. Remove supernatant and place it in a new tube.
2. Prepare plasma samples by adding 250 μL of blood into equal volume of ice-cold 50 mM potassium phosphate, and 50 mM serine borate containing 17.5 mM EDTA (pH 7.4). Mix the sample gently by inverting the tube thrice. Immediately separate the plasma by centrifugation and further use it for analysis. The samples can be stored at −80 °C until further analysis.
3. Add 30 μL standard or tissue sample in wells in duplicate.
4. Add 120 μL of assay buffer in each well.
5. Add 50 μL of reaction mixture #1 in each well.
6. Add 50 μL of reaction mixture #2 in each well.
7. Take absorbance at 30 s of intervals at 415 nm for continuous 3 min in a plate reader.

Glutathione Data Analysis

1. Plot a standard curve using the absorbance reading obtained for each standard well.
2. Measure the concentration of glutathione for each test sample.
3. Calculate the total glutathione concentration of sample through following formula:

$$\text{Total Glutathione(reduced glutathione + oxidized glutathione) (μM)} = \left[\frac{\text{O.D.at 415 nm/ min} - (y - \text{intercept})}{\text{Slope}}\right] \times \text{sample dilution} \times 2^{*}$$

*The equation is multiplied by 2 as 1 oxidized glutathione = 2 reduced glutathione.

3.5.2 Glutathione Disulfide Assay Procedure [5]

1. In 890 μL assay buffer, homogenize 0.1 g tissue sample with 10 μL NEM. Centrifuge at 12,000 rpm for 20 min and remove the supernatant and place it in a fresh tube.
2. Preparation of plasma samples: Add 250 μL blood to an equal volume of ice-cold 50 mM serine borate, 50 mM potassium phosphate buffer containing 17.5 mM EDTA and 10 mM NEM, pH 7.4. Gently mix the sample by capping and inverting the tube 3 times. Immediately centrifuge the samples, separate the plasma, and analyze. The samples can be stored at −80 °C until analysis.
3. Follow the below steps for preparation of Sep-pak tissue samples and standards:
   (a) Wash new column with 3 mL of double distilled water and subsequently with 3 mL of methanol and assay buffer (1 drop/s).
   (b) Flush column with air and insert 1 mL of syringe after placing column into microcentrifuge.
   (c) Add 200 μL of standard/tissue supernatant through syringe and add 800 μL of assay buffer through syringe and flush the column with air and vortex the tube.
   (d) Clean column while washing with 3 mL methanol followed by 3 mL assay buffer and flush the column with air.
   (e) Repeat the above steps for any additional samples (*see* **Note 3**).
4. Add 200 μL of Sep-pak eluent in each well (in duplicates).
5. Add 50 μL of reaction mixture #1 & #2 in each well.
6. Take absorbance every 30 s of intervals at 415 nm for continuous 3 min in a plate reader.

7. Calculate the total glutathione disulfide concentration of sample through following formula:

$$\text{Glutathione disulfide (Oxidized glutathione)}(\mu M) = \text{Total Glutathione} - \text{Glutathione concentration (Reduced glutathione)}$$

3.6 Estimation of Glutathione and Glutathione Disulfide Concentration Through HPLC [5]

1. Reconstitute the calibrator (CAL) in 0.25 mL reconstitution solution (RECON). The calibrator is for single use only; discard the rest of the material. The concentration of glutathione might have minor changes from lot-to-lot.
2. Reconstitute the reduction solution (RED) in 1.2 mL reconstitution solution. The solution is then stable for 3 months at 2–8 °C.
3. Before loading samples into either auto-sampler vials or manually injecting into HPLC, make sure to centrifuge the samples for 2 min. Typical injection volume of prepared sample is 25–35 μL.
4. Keep the gradient conditions as follows:
   (a) Initial conditions: 80% A, 20% B at 1 mL/min.
   (b) Hold at initial conditions for 10 min.
   (c) Linear gradient to 20% A, 80% B from 10 to 30 min.
   (d) Hold at final conditions for 15 min.
   (e) Return to initial conditions for column re-equilibration, at least 15 min.
5. Detection: Peaks are detected by fluorescence using an emission wavelength of 541 nm and excitation wavelength of 328 nm.
6. Quantification: The sample concentration is assessed by experimentally derived standard curves. Calculate the total glutathione disulfide concentration of sample using the following formula:

Glutathione total

$$\text{Concentration of total glutathione disulfide } (\mu\text{Mol/L}) = \left[\frac{\text{Peak area of test sample} \times \text{concentration of calibrator}}{\text{peak area of internal standard sample}}\right] \times F$$

$$\text{where, } F = \left[\frac{\text{peak area internal standard of the calibrator}}{\text{peak area of the calibrator}}\right]$$

Glutathione reduced:

$$\text{Concentration of reduced glutathione}(\mu\text{Mol/L}) = \left[\frac{\text{Peak area of test sample} \times \text{concentration of calibrator}}{\text{peak area of calibrator}}\right]$$

3.7 Estimation of Lipid Peroxidation Using Spectrophotometer

Malondialdehyde is a product of lipid peroxidation which is produced after the breakdown of polyunsaturated fatty acids. In vitro quantification of malondialdehyde can be considered as a good indicator of lipid peroxidation [22].

1. Prepare 1× working solution of thiobarbituric acid reagent by diluting the stock solution with fourfold water. Add butylated hydroxytoluene to a final concentration of 0.03%, while continuously stirring the solution with a magnetic stir bar. These solutions must be continually stirred and should be prepared fresh.
2. Combine the blank (absence of lipid) and the aqueous lipid suspensions with thiobarbituric acid reagent at a reagent:sample ratio of 2:1 (v/v).
3. After mixing the suspensions thoroughly, place the samples in boiling water bath for 15 min.
4. Cool down the samples at room temperature and further centrifuge it at 1000 rpm for 10 min.
5. Determine the absorbance of the solution at 535 nm against the blank (Blank contains all reagents except lipid) and the thiobarbituric acid-malondialdehyde can be quantified [23].
6. Calculate the concentration of malondialdehyde by using extinction coefficient $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ or prepare standard curve by using 1,1,3,3-tetramethoxypropane as the standard ranging from 0 to 50 nmol malondialdehyde/sample.
7. Calculation can be done according to the slope, calculated from the standard graph of 1,1,3,3-tetramethoxypropane:

$$\text{Concentration of malondialdehyde}(\mu M) = \left[\frac{\text{O.D.of the Test}}{\text{Slope}}\right] \times 100$$

3.8 Estimation of Malondialdehyde Concentration Through HPLC

The chromatographic estimation of malondialdehyde is performed using high performance liquid chromatography by utilizing 1100 series pump and a UV absorbance detector. In order to record retention times, chromatograms and evaluate peak heights, HP3395 integrator can be employed.

3.8.1 Preparation of Malondialdehyde Standards

1. Take 10 μL of 1,1,3,3-tetramethoxypropane and dilute it with 10 mL of 0.1 M HCl in a screw-cap tube.
2. Place all the tubes in boiling water bath for 5 min and then rapidly allow it to cool.
3. The working stock solution of malondialdehyde is prepared by pipetting 1 mL of the hydrolyzed acetal into a 100 mL calibrated flask and finally dilute it with water.

4. The working stock solution of malondialdehyde is 4.05×10^{-5} M acetal or 2.92 μg/mL malondialdehyde.
5. The stock solution is diluted and used for the calibration graph [24].

3.8.2 Assay Procedure for Determination of Malondialdehyde Concentration Through HPLC [25]

1. Add 50 μL of serum sample into 250 μL of 0.1 M $HClO_4$ and 700 μL of distilled water.
2. Centrifuge the sample for 5 min at 4500 rpm for HPLC analysis.
3. Use the mobile phase of 30 mM KH_2PO_4-methanol (65 + 35, v/v%), and keep the flow rate of 1.5 mL/min.
4. Monitor the chromatograms at 254 nm and use injection volume of 20 μL.
5. Keep the retention time of malondialdehyde around 1.55–1.60 min.

4 Inferences

- Tissue levels of both glutathione and glutathione disulfide fall into a range between 1–10 mM and 0.01–0.05 mM, respectively.
- The lowest detection for glutathione and glutathione disulfide is 0.103 nm in a 96-well plate [21].
- Probiotic strain which results in a significant increase in glutathione and glutathione disulfide concentrations is considered safe for human and animal applications [26].
- Probiotics strains that exhibit significant decrease in malondialdehyde concentration as compared with the control groups could be administered as safe for human consumption [27].

5 Notes

1. During the study period, the male and female mice are kept in separate cages to avoid breeding under a constant laboratory condition with 12-h light/dark cycle at temperature of 22 ± 2 °C.
2. For each experiment, a fresh bacterial suspension should be prepared.
3. Columns should not be reused more than 3 times for samples and more than 2 times for tissue supernatants.

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Chapter 28

Determination of Serum Lactate and Fecal Calprotectin for Assessing the Intestinal Inflammation

Firdosh Shah and Mitesh Kumar Dwivedi

Abstract

Since past decades probiotics have been consistently reported to exhibit various health benefits. Probiotics are considered to stabilize the intestinal barrier and epithelial tight junction by modulating the immune functions and further hampering increased permeability disorder observed in inflammatory diseases. Several serological biomarkers such as serum lactate are utilized for determining the conditions of clinical sepsis and intestinal inflammation. Similarly, calprotectin which is also a abundant neutrophil protein found in fecal and plasma sample is responsible for elevating infectious and inflammatory conditions within the patients and rodents. The fecal calprotectin is also used for determining the underlying inflammatory response within the host upon probiotic administration. Both serum lactate and calprotectin serve as markers for the intestinal inflammation for assessing the safety of the probiotic use in the host. The main objective of this chapter is to provide the detailed experimental methods which can be considered while maintaining growth conditions and administration of probiotics within animal models and assessing the serum lactate and calprotectin levels through spectrophotometer, HPLC, and ELISA.

Key words Probiotics, Serum lactate, Fecal calprotectin, Biomarker, Intestinal inflammation

1 Introduction

Probiotic bacteria have become increasingly popular due to the expanding scientific evidence which points out the beneficial effects on human health. Probiotics are considered of an utmost importance due to interplay between genetic susceptibility and environmental factors and also contribute significantly for improvement of the intestinal inflammatory diseases such as inflammatory bowel disease [1]. Abnormal bacterial interaction leads to the alteration within immunological function and triggers the inflammatory response [2]. In inflammatory bowel disease patients, the luminal microflora exhibited lack in anti-inflammatory function than in normal condition due to reduction in number of anaerobic bacteria and *Lactobacillus* spp. Administration of probiotic can help in

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_28,

restoring microbial homeostasis within gut, downregulate intestinal inflammation, and also ameliorate the diseases [3].

While proceeding for initial screening of new probiotic strains, bacteria are subjected to various anti-inflammatory activities among which assessment of clinical sepsis through serum lactate and calprotectin level is one of the major aspects [4]. Therefore, in order to ensure the safety aspects of probiotic strains, the bacterial strains are subjected to several in vivo serum lactate and calprotectin levels estimation parameters.

Serum lactate level acts as anti-inflammatory marker for sepsis condition which is a highly fatal systemic illness which results into some serious clinical manifestations such as excessive inflammation, pyrexia, multiple organ dysfunction, etc. Serological biomarkers are considered crucial for the diagnosis and treatment of clinical sepsis in spite the fact that evaluation of biomarkers is complex and unclear and it varies between rodent, CLP models and human sepsis [5]. Similarly, calprotectin is also a biomarker of inflammation and is found in both plasma and stool. Calprotectin is elevated in infectious and inflammatory conditions, including inflammatory bowel disease. There are numerous studies which suggest reference range of elevated levels of calprotectin and inflammation in patients with inflammatory bowel diseases [6–8]. In this chapter, we are providing methods for determination of serum lactate and calprotectin levels within rodents through spectrophotometer, HPLC, and ELISA respectively.

2 Materials

2.1 Probiotics and Growth Conditions

- MRS broth.
- de Mann Rogosa & Sharpe (MRS) Agar.
- Sterile Petri plates.

2.2 Animal Preparations

- Adult male and female mice (*Mus musculus*).
- Rectangular polyacrylic cages.
- Dust-free paddy husk.
- Standard pellet diet.
- Clean tap water.

2.3 Administration of Probiotics

- Phosphate-buffered saline (PBS).
- 0.2 M $NaHCO_3$ buffer containing 2% glucose.
- Bicarbonate buffer.
- 3 mg Ketamine.

- 46.7 μg of diazepam.
- 15 μg of atropine.
- 3.5 F catheter.

2.4 Preparation of Serum Samples

- PE-50 catheter.
- 5% sheep blood agar.
- Incubator.
- ELISA Kits.
- Biochemistry automatic analyzer.

2.5 Assessment of Serum Lactate Levels Through Spectrophotometer

- Test tubes.
- Buffered substrate (0.2 mL lactic acid, 0.4 M Tris-HCl, pH 8.2).
- Buffered control (0.2 g potassium oxalate, 0.2 g ethylenediaminetetraacetic acid, disodium dihydrate dissolved in 100 mL 0.4 M Tris-HCl, pH 8.2).
- Color reagent [prepared by mixing 5 mL Meldola Blue (0.25 mg/mL), 4 mL NAD^+, and 10 mL INT (5 mg/mL)]. The color reagent is stored in low actinic container at 5 °C.
- In order to dissolve INT, sonicate the solution for 1 min within a Branson Model 185 Sonifier Cell Disruptor.
- 0.1 N Hydrochloric acid (HCl).

2.6 Chromatographic Measurement of Serum Lactate Through High-Performance Liquid Chromatography

- High-performance liquid chromatography (HPLC).
- Micro vacuum degasser.
- LPG system.
- UV-vis Detector.
- Methanol: 0.1 M phosphate buffer.

2.7 Preparation of Stool Sample

- Sterile container.
- Eppendorf tubes.

2.8 Detection of Fecal Calprotectin Through ELISA

- Fecal sample collection tube.
- Pipettes (50 μL, 100 μL, 500 μL, etc.).
- Aluminum foil.
- Deionized or distilled water.
- Plastic microtiter well covers or polyethylene film.
- ELISA multichannel wash bottle or automatic washing system.
- Spectrophotometric microplate reader.

3 Methods

3.1 Probiotics and Growth Conditions

The probiotic strains belonging to *Lactobacillus*, *Bifidobacteria*, or any other strain exhibiting beneficial probiotic traits such as acid tolerance, bile tolerance, in vitro adherence to epithelial cells [9] can be grown by following the below mentioned steps [10]:

1. Take an isolated colony of the probiotic strain and inoculate it within MRS (de Mann Rogosa & Sharpe) broth and incubate it at 37 °C for overnight.
2. Take a loop full of overnight grown bacterial cell suspension and streak it on MRS agar plate (pH 5.5).
3. Incubate the plates in anaerobic condition for the period of 2 days at 37 °C.
4. The well-isolated colonies can be observed and are further used for determining its effects on systemic models.

3.2 Animal Preparation

1. Take 6–7 weeks old adult male and female mice (*Mus musculus*) of weight 20–25 g.
2. Keep the animals in rectangular polyacrylic cages with bedding material as dust-free paddy husk.
3. During the study period, keep the male and female mice in separate cages to avoid breeding under a constant laboratory condition with 12-h light/dark cycle at temperature of 22 ± 2 °C.
4. Animals are allowed free access to standard pellet diet along with clean tap water ad libitum except in the case where starvation is needed.
5. Change the water and food regularly and the clean the cages along with the fresh husk replacement every 3 days.
6. In order to minimize any non-specific stress, an acclimatization period of 7 days is allowed before experimentation [11, 12].

3.3 Administration of Probiotics

Probiotics can be administered within mice model through below mentioned steps [13]:

1. Grow the probiotic strains exponentially unless the measurement of turbidity reaches 1–2 (absorbance 600 nm).
2. Harvest the bacterial cell suspension through centrifugation at 3000 rpm for 10 min. With repeated washing with phosphate-buffered saline (PBS) and resuspend them either in PBS or 0.2 M $NaHCO_3$ buffer containing 2% glucose (*see* **Note 1**).
3. Resuspend 10^9 colony forming units (CFUs) of probiotics in bicarbonate buffer and administer 100 μL of suspension in mice intragastrically, whereas administer 25 μL of suspension while delivering through its mouth.

4. For intrarectal administration, administer 10^9 CFU of probiotic in anesthetized mice (anesthetized with 3 mg of Ketamine, 46.7 μg of diazepam, and 15 μg of atropine) by insertion of 4 cm proximal to the anus using 3.5 F catheter.

3.4 Preparation of Serum Samples

The serum samples are prepared through the below mentioned steps [14]:

1. Withdraw the blood samples through sterile technique using PE-50 catheter.
2. Place 10 μL of blood samples on 5% sheep blood agar plate under aerobic conditions and incubate it at 37 °C for 24 h in order to determine the bacterial load.
3. Enumerate the colony forming units by manual counting.
4. Collect 0.6 mL of blood sample for biochemical assays.
5. Remove the serum through centrifugation at 1000 rpm for 15 min and store it at −80 °C for further analysis.
6. The serum is further used for the determination of lactate levels through spectrophotometer or high-performance liquid chromatography.

3.5 Assessment of Serum Lactate Levels Through Spectrophotometer

The serum lactate level is determined at 25 °C by measuring conversion of lactate to pyruvate through the below mentioned steps [15].

1. Add 0.05 mL of serum in two tubes. In one tube add 0.25 mL buffered substrate and in another tube, add 0.25 mL buffered control.
2. Properly mix the tubes and place them in the incubator at 37 °C.
3. After 20 s, add 0.25 mL of color reagent to the tubes.
4. Stop the reaction after 12 min by adding 5 mL of 0.1 N HCl.
5. Mix the tubes well and measure the absorbance by spectrophotometer at 510 nm (*see* **Note 2**).
6. Derive the final reading by subtracting the absorbance reading of the buffered substrate reaction from the absorbance of the buffered control reaction.
7. Also, a standard curve must be plotted for lactate from which the unknown concentration of lactate in sample can be derived.
8. Meldola Blue of 0.025 mg/mL concentration is used with varied concentration over 20- to 60-folds range for plotting standard curve.

9. Serum lactate concentration can be determined by using the below mentioned formula:

$$\text{Concentration of Serum Lactate(mg/mL)} = \frac{\text{Absorbance buffered substrate reaction} - \text{Absorbance buffered control reaction}}{1000}$$

3.6 Chromatographic Measurement of Serum Lactate Through High-Performance Liquid Chromatography (HPLC)

The serum lactate is measured through HPLC [16] which is equipped with micro vacuum degasser, LPG system, UV-vis Detector (2550: is set at 220 nm) (*see* **Note 3**). The method involves the following steps:

1. Measure the lactate at the optimum separation condition by HPLC with flow rate of 1 mL/min within isocratic binary mobile phase consisting of methanol: 0.1 M phosphate buffer.
2. Determine the applicability, accuracy and precision of extraction and determination of lactate through investigating serum along with below mentioned standard addition method.
3. The method is validated in regard to limits of detection (LOD), limits of quantification (LOQ), linearity, precision, accuracy, and specificity. Measure the LOD at a signal/noise (S/N) ratio of 3.
4. Adjust the LOQ at S/N ratio of 10 and measure the noise by calculating the area under the curve (AUC) of a blank sample.
5. Evaluate the accuracy and precision by assaying samples in triplicates. The precision is expressed as relative standard deviation (RSD).
6. Determine the accuracy through comparing calculated concentration from the standard curves to the theoretical concentration.
7. Perform the chromatographic calculations through EZCHROM elite system.
8. Calculate the serum lactate concentration using the below mentioned formula:

$$\text{Concentration of Serum Lactate}(\mu g/mL) = \frac{\text{Amount}_{IS}}{\text{Sample Amount}_{U} \times \text{Dilution Factor} \times \text{Amount Ratio}_{unknown}}$$

wherein,

- Amount_{IS} = Amount of the Internal Standard
- Sample Amount_{U} = Amount of the unknown sample
- $\text{Amount Ratio}_{unknown}$ = Amount ratio value taken from the calibration curve at the given area ratio for the unknown sample.

3.7 Preparation of Stool Sample

1. Collect the stool sample from the animal cage and keep it in the sterile airtight collection tube pre-filled with sample extraction buffer (*see* **Notes 4** and **5**).
2. Dilute the collected sample in two steps with 1:40 and 1:9, before the measurement.
3. Weigh 50–100 mg of stool sample using the inoculation loop by placing it into the pre-tared tube.
4. Note down the net amount of sample and break down the inoculation loop and leave it in the tube itself.
5. Immediately add extraction buffer [2 mL extraction buffer; stool sample weight (mg) = 50–54] into the tube and dissolve it through vortexing.
6. Centrifuge the sample at 3000 rpm for 5 min. Transfer 0.15 mL of clear supernatant (no particles) within a tube containing 1.2 mL of extraction buffer.
7. Mix the sample gently through vortexing and use it further for detecting fecal calprotectin levels.

3.8 Detection of Fecal Calprotectin Through ELISA

The below mentioned steps for fecal calprotectin determination can be performed by the method described by Eagle Bioscience Inc. diagnostic manual for fecal calprotectin estimation (Catalog Number: CAL35-K01).

1. Reconstitute all standard assay level 1–7 by adding 0.5 mL of demineralized water in each vial including controls.
2. Place all the standards and controls undisturbed for 5 min and then mix well through gentle inversions or vortexing.
3. Place sufficient number of calprotectin-coated microwell strips in a holder to run human calprotectin standards, controls, and unknown samples in duplicate.
4. Prepare antibody working solution by diluting 1:21 fold dilution of the calprotectin tracer antibody through tracer antibody diluent.
5. Add 50 μL of assay buffer into the designated microwells and gently tap the plate to coat the wells evenly.
6. Add 50 μL of standards, controls, and extracted fecal samples into the wells. Seal the plate wells securely and protect it from light while covering through aluminum foil.
7. Rotate it on ELISA plate shaker at 400–450 rpm for 1 h.
8. Wash each well 5 times by dispensing 350 μL of working solution into each tube and completely aspirate the contents.
9. Add ELISA HRP substrate of 100 μL in each well and cover it with aluminum foil in order to prevent exposure to light and incubate it for 12 min at room temperature.

10. After removing foil, read the absorbance immediately at 620 nm.
11. Immediately add 100 μL of ELISA Stop solution into each of the wells, mix well and take absorbance at 450 nm with a reference filter at 620 nm or 650 nm.
12. Calculate the average absorbance for each pair of duplicate test results.
13. The standard curve is generated through the average absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using log-log paper or the calculation is made through computer-assisted data reduction programs.
14. The concentration of calprotectin in μg/g can be read from the calibration curve for the test sample. Calculate the calprotectin concentration by using the below mentioned formula:

$$\text{Concentration of Calprotectin } (\mu g/g) = \text{Calprotectin concentration calculated through calibration curve } (\mu g/g) \times 2.78$$

4 Inferences

The normal range for serum lactate levels in mice determined through spectrophotometer is in the range of <2 mmol/L [17]. The recommended normal cut-off for fecal calprotectin concentration by using ELISA and sample collection system is 120 ng/mL or 43.2 μg/g directly read from assay standard curve. We strongly suggest that each clinical laboratory should establish its own normal cut-off levels by measuring normal stool samples with this ELISA along with test sample. In mice, if the serum lactate levels exceed more than 2 mmol/L, it is considered as an indicative of intestinal inflammation. The calprotectin concentrations of 120.1 μg/g and higher are suggestive of an active inflammatory process within the gastrointestinal system [18].

5 Notes

1. For each experiment, a fresh bacterial suspension should be prepared.
2. Absorbance can also be measured through blood analyzer colorimeter using green calibration scale.
3. Utilize MZ ODS-C18 (250 mm 4.6 mm, 5 mm) column for high-performance liquid chromatography (HPLC).

4. Stool sample should be collected either at the end of the day or in the following morning.
5. The collected fecal sample may be transported at ambient temperature or else can be stored at 2–8 °C if the testing needs to be conducted within 3 days. Fecal sample may be stored below −20 °C for a longer storage period. Avoid freeze-thaw cycle for each specimen more than 3 times.

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Part III

In Vivo Biosafety Assessment of Probiotics: Measuring Infectivity in Animal Models

Chapter 29

In Vivo Evaluation of Adhesion Properties of Probiotics

Nabendu Debnath, Pooja Yadav, Ashish Tyagi, and Ashok Kumar Yadav

Abstract

Probiotic microorganisms induce several health promoting functions in the host such as pathogen exclusion, maintenance of microbial homeostasis, immunomodulation, stimulation of intestinal barrier function, and other metabolic functions. The capability of probiotic bacteria to adhere to intestinal epithelium is a prerequisite for bestowing the beneficial effects and is one of the primary criteria for selecting a potential probiotic strain. A higher adhesion capability would result in temporary colonization in the intestine. This ensures an increased transit time in the gut and more time for bacteria to exert its beneficial effects. In vivo methods used for the evaluation of adhesion activities of probiotics are much superior as compared to in vitro models. In vivo models simulate human intestinal conditions and provide better understanding of mechanistic functions of probiotics. Additionally, safety and efficacy of probiotic bacteria can be addressed in in vivo models than in vitro methods. In this chapter we describe a rapid in vivo screening method of adhesion capability of probiotic bacteria using *Caenorhabditis elegans* (*C. elegans*).

Key words Probiotics, Extra-cellular matrix, Fibrinogen, Fibronectin, Collagen, Mucus, *Caenorhabditis elegans* (*C. elegans*)

1 Introduction

Probiotics are defined as "live non-pathogenic microorganisms which when administered in adequate amounts confer a health benefit on the host" [1, 2]. Consumption of probiotics induces several health benefits to the host such as maintaining a homeostatic intestinal microbiota population, inhibiting growth of the pathogens by secretion of inhibitory organic acids, bacteriocins, inducing lactose tolerance, producing metabolites, and inducing mucosal immunity [3]. Probiotics also compete against other microorganisms for adhesion to the mucosal surfaces, thus limiting the capability of other bacteria for binding [4]. Adhesion of probiotics to gastrointestinal (GI) epithelium increases their time of colonization along with their interaction with GI immune cells, hence increasing their effectiveness. Colonization of probiotics directly depends on their adhesion capabilities to gut components

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_29,

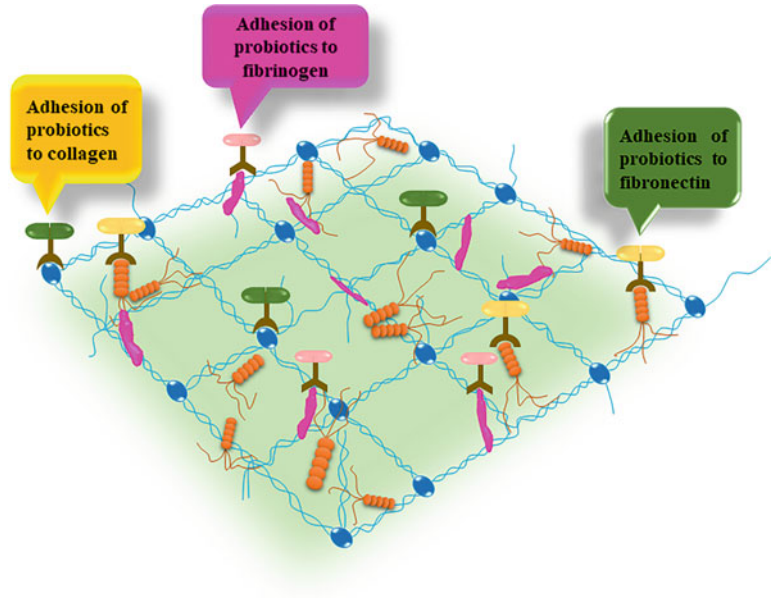

Fig. 1 Adhesion of probiotic bacteria with ECM components through adhesin proteins found in the surface of the bacteria

such as extra-cellular matrix (ECM) proteins and mucus components [5]. The four major classes of molecules play an important role in the formation of ECM. These molecules are collagens, proteoglycans, structural glycoproteins (laminin, fibronectin, vitronectin, and entactin), and elastin (Fig. 1) [6]. Additionally, GI tract is covered by a mucus layer that prevents the lower epithelium from damage and infections. This mucus layer is a gel-like structure consisting of mucins and secreted by goblet cells and mucosal glands [7, 8]. Mucus layer is the outermost layer that serves as the first point of interaction with probiotics. Hence, various studies have demonstrated the binding of probiotic strains to mucus layer as a measure of their adhesion capability [9, 10]. Adhesion is a complicated process and various factors influence the completion of adhesion. Nevertheless, adhesion of probiotics to epithelium of the gut is thus considered to be the most important characteristic and is frequently considered the most important selection criteria for potential probiotic strain [11]. Several studies with different probiotic bacteria have evaluated the adhesion abilities [12–19]. Although various *in vitro* models have been developed to demonstrate the adhesion capabilities of probiotics, additional in vivo approaches are required to validate in vivo findings. Presently, several in vivo models are available to evaluate adhesion abilities of probiotics but they are often difficult to carry out, require more time and often expensive.

C. elegans is a non-parasitic nematode that has recently gained attention as a relevant candidate as an in vivo model for interactions studies between bacteria and host. The in vivo screening model is particularly suitable for evaluating adhesion capabilities of a probiotic bacteria as compared to others due to the similarities of intestinal cells of *C. elegans* with human intestinal cells [20]. Along with the similarity in morphology of intestinal cells, the phenomena of endocytosis and exocytosis are also similar to humans. Owing to the ease of use and similarities to human microflora, *C. elegans* is considered as an efficient in vivo model for identifying potential probiotics. In this chapter, we briefly describe the method of in vivo adhesion assay with *C. elegans*.

2 Materials

Prepare the required buffers and other reagents in ultrapure water (18 MΩ cm at 25 °C). Prepared reagents should be stored at room temperature until mentioned separately.

2.1 De Man, Rogosa and Sharpe (MRS) Broth

55.15 g of MRS broth is dissolved in 1 L of distilled water. To dissolve the medium, heat can be applied prior to the sterilization. Sterilization is done by autoclaving the medium at 15 lbs pressure (121 °C) for 15 min.

2.2 Nematode Growth Medium (NGM)

Weigh 15 g agar, 2.4 g Sodium Chloride (NaCl), 2 g Tryptone, and 2.72 g Potassium dihydrogen phosphate (KH_2PO_4) in 2 L conical flask, make volume up to 1 L with water, autoclave and allow it to cool to 60 °C in a water bath. Add 0.8 ml each of 1 M Calcium chloride ($CaCl_2$), Cholesterol (5 mg/ml in ethanol), and 1 M Magnesium sulfate ($MgSO_4$) (*see* **Note 1**).

2.3 M9 Buffer

Dissolve 3 g ofKH_2PO_4, 6 g of Disodium hydrogen phosphate (Na_2HPO_4), and 5 g of NaCl in 1 L of H_2O. Autoclave it for 20 min and then add 1 ml of 1 M $MgSO_4$ (*see* **Note 2**).

2.4 Triton X-100

100 µl of triton x-100 in 9.9 ml of water.

2.5 Phosphate Buffer (PBS)

To prepare 1 L of PBS buffer, add 8 g of NaCl, 200 mg of Potassium Chloride (KCl), 1.44 g of Na_2HPO_4, and 245 mg of KH_2PO_4 to 800 ml of distilled water and adjust the pH to 7.

2.6 Acridine Orange

Make a 2 mg/ml solution of Acridine orange (Invitrogen, A1301) in distilled water and dilute it to 1:100.

3 Method

3.1 Determination of In vivo Adhesion Properties of Probiotics

The in vivo adhesion properties of probiotics can be assessed in *C. Elegans* through the following steps [21, 22]:

1. Culture the probiotic microorganisms in MRS broth for 18 h at 37 °C.
2. Prepare fresh bacterial culture (10^8 CFU/ml) by adding PBS.
3. Expose *C. elegans* to probiotic candidates (10^8 CFU/ml) on NGM plates for five days.
4. After completion of five days, take out ten *C. elegans* and wash them two times with M9 buffer.
5. Place the washed *C. elegans* on brain-heart infusion plates (*see* **Note 3**).
6. Wash *C. elegans* five times with M9 buffer and transfer into a microcentrifuge tube containing M9 buffer with 1% Triton X-100 and disrupt mechanically to get bacterial suspensions.
7. Dilute this suspension by performing serial dilution (ten-fold) and spread this on MRS agar plates and incubate at 37 ° C for 24 h.
8. After 24 h, count the colonies and compare with positive control (*see* **Note 4**) [21, 22].

$$\text{CFU/ml} = \frac{(\text{No. of colonies} \times \text{dilution factor})}{\text{Volume of the culture plate}}$$

9. Additionally, to determine the adhesion of probiotic bacteria with ECM components and mucus, transmission electron microscopy can be used (*see* **Note 5**) [22].
10. On the other hand, after washing *C. elegans* with M9 buffer, staining method can be applied to determine adhesion abilities of probiotics. Acridine orange is used for staining for 15 min. Excessive stain is removed by additional wash with M9 buffer and adhesion of probiotics is observed under fluorescent microscope (Fig. 2) (*see* **Note 6**) [23, 24].

4 Inferences

The in vivo models that are available in the present time for the evaluation of adhesion abilities of probiotic microorganisms include mostly multicellular organisms such as mice. However, several limitations have been observed during experimental procedures such as ethical issues and their maintenance. Here, we have described a simple in vivo model which includes *C. elegans*, a

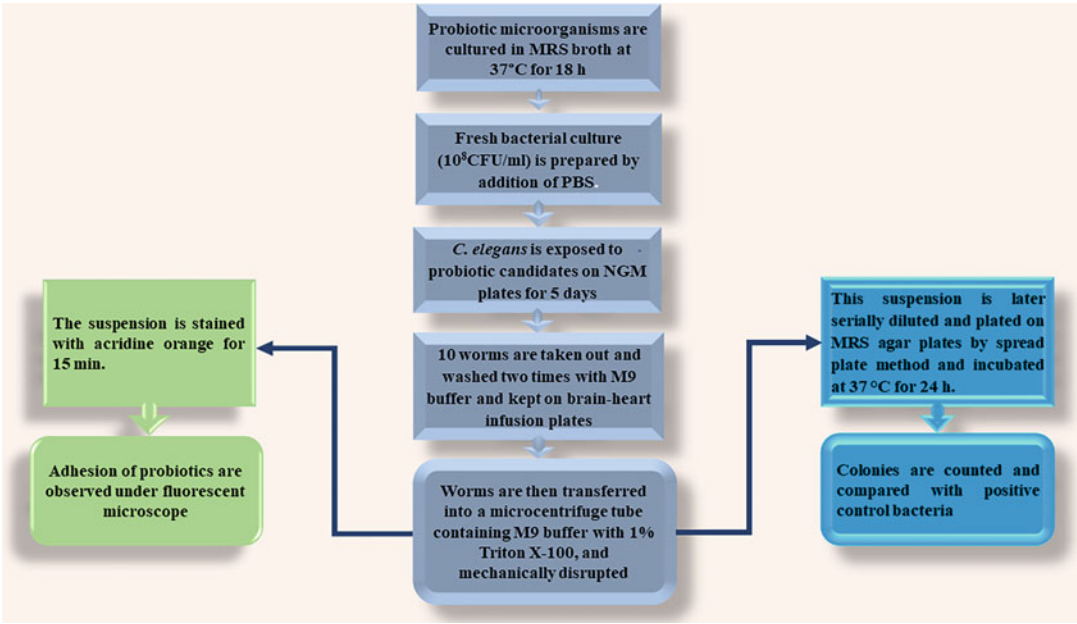

Fig. 2 Flowchart summarizing the in vivo model for probiotic adhesion using *C. elegans*

multicellular worm. This model provides several advantages such as it is easy to handle, has a transparent body and more importantly the absence of ethical issues. Therefore, *C. elegans* based in vivo study provides a better platform with high accuracy.

5 Notes

1. To prevent fungal and bacterial contamination, 1 ml streptomycin (100 mg/ml) and 1 ml nystatin (10 mg/ml) can be added to each liter of medium.
2. Store at room temperature and regularly check for visible contamination before use.
3. Kanamycin (100 μg/ml) and streptomycin (100 μg/ml) can be added for the prevention of contamination.
4. Positive controls should be used carefully. Only choose probiotic bacteria that have established adhesion abilities.
5. For each observation, at least five cross sections should be evaluated.
6. The intensity of fluorescence is directly proportional to the adhesion of probiotics.

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Chapter 30

Determination of *Streptomyces* Probiotics Oral Administration in Broiler Chicken

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Abstract

Reducing antibiotic intake and eliminating antibiotic residues in food animal agriculture has become one priority in food safety and public health. Probiotics are a potential replacement for antibiotics in animal feed. A novel *Streptomyces fradiae* isolated from soil effectively inhibits the growth of *Salmonella gallinarum*, which is a causing agent of poultry typhoid. Also *Streptomyces* sp. can produce potential antagonistic and antimicrobial compounds and secretes the exo-enzymes which may promote feed utilization and digestion once they colonize the host intestine in aquaculture and poultry. This protocol illustrates the effects of oral administration of probiotics in chicken through feed, water, litter application, oral gavage on growth performance and safety of probiotics on blood profiles, relative organ weight, and meat quality in control and experimental broiler chicken (*Gallus gallus domesticus*). The safety of oral administration of *Streptomyces* probiotic in chicken is measured by average feed intake, feed conversion ratio, mortality, hematological analysis, internal organs weight, gut morphological measurements using histopathology of comparison with control and experimental broiler chicken.

Key words *Streptomyces*, Probiotics, Intestinal microflora, Poultry, Broilers

1 Introduction

Poultry is one of the fastest growing segments of agriculture and veterinary sector. Several feed additives are used as growth promoter like synthetic hormone and antibiotics for enhancing poultry production. But it causes adverse effects by promoting antibiotic resistant bacterial strains and residual effects of these feed additives in eggs and meat, they lead to various health hazards to consumers [1]. About 20–50% fresh or frozen broilers were antibiotic-residue positive, due to the administration of antibiotics in broiler feed. Thus, the best of all above to the use of probiotics as feed additives for better and safe production in livestock in general and specific in poultry [2]. Lilly and Stillwell coined the term "Probiotics" in

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_30,

1965. The word probiotics originates from Greek language, "Pro" means "for" and "Bios" means "life," that is, "for life." The Food and Agriculture Organization of United Nations (FAO) and World Health Organization (WHO) define probiotics used in food as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [3]. Inoculating one-day-old chick with probiotics serves as an effective model for determining the modes of action and efficacy of these microorganisms on their growth and gut microflora. As the susceptibility of one-day-old chicks to infection [4]. There are many different methods for administering probiotics preparations to broiler chickens: through feed, water, gavage (including droplet or inoculations), spray or litter, vent lip method, and *in ovo* injection technique [4, 5] Beneficial effects of probiotics on broiler can be: (a) increased growth performance, (b) gut health and modulation of intestinal microflora, (c) pathogen inhibition, and (d) immune modulation and gut mucosal immunity [6]. Microorganisms belonging to the following genera: *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus* as well as some fungi and yeast strains of *Saccharomyces cerevisiae* and *Kluyveromyces* species are used as probiotics (Table 1) [7].

The genus *Streptomyces* (Phylum: *Actinobacteria*) are Gram positive, high G + C (70%) genome content, soil-living bacteria with characterized branching filamentous morphology. The production of a variety of wide-spectrum chemical compounds as demonstrated by *Streptomyces* has the advantage of producing potential antagonistic and antimicrobial compounds that can be valuable as probiotics in aquaculture [8]. Probiotic *Streptomyces* sp. are isolated from feces of country and broiler chicken (*Gallus gallus domesticus*) gut system [9, 10]. The *in vitro* probiotic properties like acid, bile resistance, pepsin and pancreatin [11], proficient adhesion properties, auto-aggregation, co-aggregation, susceptibility to antibiotics and non-hemolytic activity [10, 12], and heavy metal tolerance [13] are analyzed in *Streptomyces* sp. JD9 [9]. Mass production of *Streptomyces* is optimized in conventional and new medium formulation for large-scale operation [13]. A novel *Streptomyces fradiae* WR isolated from soil effectively inhibits the growth of *Salmonella gallinarum*, which is a causing bacteria of fowl typhoid, and harmful microorganisms, thereby being used for preventing and treating fowl typhoid in poultry [14]. This procedure demonstrates the effects of oral administration of probiotics in chicken through feed, water, litter application, oral gavage on growth performance and safety of probiotics on blood profiles, relative organ weight, and meat quality in control and experimental broiler chicken (*Gallus gallus domesticus*).

Table 1
Experimental trials regarding the probiotic administration of different species in chicken

Sr. no.	Name of the probiotic organism	Animals used in experiment	Period of experiment	Route of administration	References
1	*Streptomyces fradiae*	140 (7 and 8 week) chickens	4–12 days	Feed delivery	[14]
2	*Enterococcus spp., Pediococcus spp., Bifidobacterium spp., Lactobacillus spp., Bacillus subtilis*	252 (Ross 708) 1-day-old Broiler chicken	45 days	Feed delivery	[15]
3	*L. Johnsonii*	366 cobb 1-day-old Broiler chicken	5 weeks	Feed delivery, Oral delivery, Litter delivery, spray solution water delivery	[16]
4	*Lactobacillus plantarum, L. bulgaricus, L. acidophilus, Bifidobacterium bifidum, Streptococcus thermophilus, S. faecium, aspergillus oryzae,* and *Torulopsis bovina*	198 broiler 1-day-old Broiler chicken	4 weeks	Feed delivery	[17]
5	*Aspergillus oryzae, lactobacillus acidophilus, L. rhamnosus, L. plantarum, L. bulgaricus, Bifidobacterium bifidum, Enterococcus faecium, Streptococcus thermophilus* and *Candida pintolopesii*	150 (Ross 308) 1-day-old Broiler chicken	10 days	Oral gavage Spray solution In ovo injection Vent lip	[18]
6	*Aspergillus oryzae, lactobacillus acidophilus, L. rhamnosus, L. plantarum, L. bulgaricus, Bifidobacterium bifidum, Enterococcus faecium, Streptococcus thermophilus,* and *Candida pintolopesii*	360 male 1-day-old Broiler chicken	42 days	Feed delivery Water delivery	[19]
7	*L. Plantarum* and *L. salivarius*	432 broiler breeder eggs	42 days	In ovo injection	[20]
8	*Pediococcus acidilactici Enterococcus faecium Bacillus subtilis*	480 fertile cob eggs	42 days	In ovo injection	[21]
9	*Streptomyces* spp.	400 ornamental fish (*Xiphophorus helleri)*	50 days	Feed delivery	[22]
10	*Streptomyces* sp. RL8 *Streptomyces* sp.N7 *Bacillus tequilensis* YC5–2 *Bacillus endophyticus*C2–2	1500 white shrimp (juvenile) *Litopenaeus vannamei*	30 days	Feed delivery	[23]

2 Materials

2.1 Preparation of Streptomyces Probiotics

1. *Streptomyces fradiae*.
2. *Streptomyces* sp. JD9.
3. 1 L conical flask.
4. Test tubes.
5. SCA (starch casein agar).
6. SCB (starch casein broth).
7. Distilled water.
8. Rotary shaker.

2.2 Procurement and Rearing of Broiler Chicks in Experimental House

1. 40 healthy 1-day-old, unsexed broiler chicks.
2. 10% formaldehyde.
3. Wire mesh.
4. Coconut pith bedding.
5. Non-medicated feed.
6. Ranikhet "LaSota" strain vaccine.
7. IBD vaccine.

2.3 Formulation of Experimental Probiotic Diet

1. *Streptomyces* sp. JD9 (lyophilized powder).
2. *Lactobacillus subtilis*.
3. *Bacillus plantarum*.
4. Beta-glucan (β-Glucan).
5. Fructooligosaccharides.
6. Maize-soybean meal.
7. Drinking water.
8. Saw dust.
9. MRS Agar.
10. PBS Solution.

3 Methods

3.1 Preparation of Streptomyces Probiotics [13]

1. The mass cultivation of actinobacterial probiont *Streptomyces* sp. JD9 is carried out in SCB through conventional batch fermentation.
2. For that aseptically inoculate a 7-day-old culture into 500 mL of SCB in a 1 L conical flask and incubate on a rotary shaker at 200 rpm for 7 days under 41 ± 2 °C.
3. Prepare several batches of mass culture to obtain an adequate quantity of probiotic cell mass.

4. Harvest the culture which developed as a mat on the broth surface by filtration.
5. Wash three times with sterile distilled water.
6. After that, lyophilize the cells.
7. Determine the CFU in SCA by serial dilution technique before storing at −20 °C.

3.2 Procurement and Rearing of Broiler Chicks in Experimental House

1. Conduct in vivo trial at the Experimental Shed, Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India with the permission of Institutional Animal Ethics Committee (IAEC) supported by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (BDU/IAEC/30/2013/09.04.2013).
2. Procure a total of 40 healthy 1-day-old, unsexed broiler chicks of Vencobb-400 with an average initial body weight of 56 ± 1 g from a commercial hatchery (Sun India Hatcheries Pvt. Ltd., Namakkal, Tamil Nadu, India).
3. Rear at the experimental house for 42 days under hygienic conditions with the guidelines approved by IAEC was followed to take care and for management of the birds.
4. Wash and disinfect the experimental house (concrete floor) thoroughly with 10% formaldehyde before the arrival of birds.
5. Divide into 4 pens (1.5 × 2.0 m) using wire mesh and keep as an open house under natural conditions.
6. Cover all the pens with a 5 cm coconut pith bedding (litter was kept dry forever by replacing the spoiled litter) and equip them with individual feeders, drinkers, and electrical brooders.
7. Further clean the pens daily to avoid direct contact of birds with their excrements.
8. During the experiment, provide *ad libitum* access to non-medicated feed (administered twice a day particularly at early morning and late evening) and water together to the birds.
9. Besides, vaccinate the birds against Ranikhet Disease (Primary: Ranikhet "F" strain vaccine at the age of 5–7 days and Booster: Ranikhet "LaSota" strain vaccine at the age of 19–21 days) as well as Infectious Bursal Disease (IBD vaccine at the age of 10–14 days) by ocular route of administration according to the recommendations of Cobb Broiler Management Guide (2010).

3.3 Formulation of an Experimental Probiotic Diet

1. Formulated a typical maize-soybean meal-based diet in mash form (purchased from Feed Manufacturing Unit, Department of Animal Nutrition, VCRI, Namakkal, Tamil Nadu, India) as a basal diet (free from coccidiostats, antibiotics, and other growth promoters) for the rearing of broiler chicks.
2. It should meet or exceed the National Research Council (NRC, 1994) recommended levels of critical nutrients during the feeding period.
3. The feeding program of broilers consists of 3 basal diets, namely pre-starter (1–14 days), starter (15–28 days), and finisher (29–42 days).
4. Formulation of the experimental probiotic diet consists a mixture of biomass of *Streptomyces* sp. JD9 (lyophilized powder without any support) to the basal diets at the concentration of 9.6 log CFU kg^{-1} (i.e., 4×10^9 CFU kg^{-1} or 325 mg kg^{-1}).
5. Incorporated the probiotic strain into feed daily to sustain the viable cells throughout the study period.
6. Moreover, check the viability weekly to ensure the concentration of viable cells which remains at 9 log CFU kg^{-1} of feed.

3.4 Experimental Design

1. At the beginning of in vivo trial, weigh the experimental birds individually and provide wing-bands, and randomly assign into 4 groups (10 birds/pen).
2. Categorized experimental groups as follows based on broilers feeding program.
3. Control group (C) = Basal diet (without probiotic supplement).
4. Treatment groups such as:
   - T1 = Probiotic diet for 14 days (addition of probiotic to the pre-starter basal diet).
   - T2 = Probiotic diet for 28 days (addition of probiotic to the pre-starter and starter basal diets).
   - T3 = Probiotic diet for 42 days (addition of probiotic to the pre-starter, starter, and finisher basal diets).
5. Feed T1 and T2 treatment groups with the basal diet after 14 days (starter and finisher basal diets) and 28 days (finisher basal diet), respectively, and extend for 42 days.

3.5 Probiotics Administration Using Feed [14]

1. Culture the *Streptomyces fradiae* in a liquid medium containing 0.4% glucose, 1% malt extract, and 0.4% yeast extract medium at 30 °C for 5 days.
2. Incubate, allow the culture to dry with hot air at 40 °C for 3 days.

3. Pulverize the dry mixture and make into powder form. From this mixture, two types of feed prepared.
   (a) Mix lactic acid bacteria *Lactobacillus subtilis* 1.0×10^7 cfu/g, *Bacillus plantarum* 2.0×10^7 cfu/g, and *Streptomyces fradiae* wr 2×10^8 cfu/g.
   (b) Mix the *Streptomyces fradiae* wr with beta-glucan (-β-glucan, as an immunopotentiator) and fructooligosaccharides ("fos"), which promote the growth of enteric beneficial microbiota and the absorption of nutrients.
      - General feed (control).
      - 2.500 g of fructooligosaccharide + probiotics (*Streptomyces fradiae* wr) 0.5% + β-glucan 100 g.
      - 3.500 g of fructooligosaccharide + probiotics (*Streptomyces fradiae* wr) 0.5% + β-glucan 200 g.
      - 4.500 g of fructooligosaccharide + probiotics (*Streptomyces fradiae* wr) 1% + β-glucan 100 g.
      - 5.500 g of fructooligosaccharide + probiotics (*Streptomyces fradiae* wr) 1% + β-glucan 200 g.
4. During the test period, check feeding amount daily and check the weight gain once a week.
5. Clean the mixer equipment thoroughly between the mixing of different treatments by using a vacuum cleaner and a wash diet (basal feed).

3.6 Probiotics Administration Using Drinking Water [16]

1. For the first three weeks, drinking water supplies through pipes (nipples drinker installed) connects to a 20^{-1} drum.
2. Install and constantly agitate the water using small pump (low power, aqua one maxi-series power head).
3. Culture the *Streptomyces* strains under shaking conditions at 30 °C for 7 days.
4. Prepare the water containing the probiotic daily and supply for the first three weeks in probiotic water treatment groups.
5. Confirm the bacterial load in the water by plate count using TSA plates for *Streptomyces*.
6. Control diet receives an equivalent amount of sterile water.
7. Transfer the birds after three weeks to slide-in cages and drinking water supplies in troughs places outside the cages.

3.7 Probiotics Administration Using Litter Application [16]

1. For this experiment the sawdust from commercial products produces from litters.
2. Determine the lactic acid bacterial concentration using an MRS agar plate display.

3. Before use the sawdust contains a low number of lactic acid bacteria (10^2 cfu/g of sawdust).
4. Spray the probiotic solution (PBS, pH 7.4 containing $>10^6$ cfu/mL of *L. johnsonii*) on litter daily for the first three weeks for the litter treatment groups.

3.8 Probiotics Administration Using Oral Gavage [16]

1. Resuspend the *L. johnsonii* cultures into PBS solution (pH 7.4) which contains 10^8 cfu/mL.
2. Give 1 mL of PBS mixed probiotic solution on day 1, 2, 4, 6, and 14 to each bird.
3. Give 1 mL of PBS solution (pH 7.4) on the same days to the negative control group birds.

4 Observation

4.1 Sample Collection, Processing, and Probiotic Safety Assessment

The safety of oral administration of *Streptomyces* probiotic in chicken is measured by average feed intake, feed conversion ratio, mortality, hematological analysis, internal organs weight, gut morphological measurements using histopathology of comparison with control and experimental broiler chicken as mentioned below:

1. Calculate the average feed intake (fi) and body weight for every week by taking the weight of the feed leftovers and birds.
2. Record the mortality when it occurs and feed conversion ratio (FCR; feed intake/weight gain) is corrected for mortality**.**
3. Randomly select three birds on day 7 and two birds on day 21, from each cage and kill by cervical dislocation.
4. Collect the blood from birds in sterile tube for hematological analysis as indicated in Table 2.
5. Open the abdominal cavity and weigh the visceral organs. Record the weights of the empty gizzard, the duodenum, jejunum, and ileum individually. Also record the weights of the pancreas, liver, spleen, and bursa individually (Tables 3 and 4).
6. Collect the contents of the gizzard, ileum, and caeca in plastic containers, and store them at −20 °C until performing the volatile fatty acids (VFA) analysis.
7. Flush a 2 cm piece of the proximal ileum with ice-cold phosphate-buffers saline (PBS) at pH 7.4 and fix in 10% formalin for gut morphological measurements using histopathology.
8. Transfer one gram (approximately) each of ileal and caecal fresh digesta individually into 15 mL McCartney bottles containing 10 mL of anaerobic broth for bacterial enumeration using the methods.

Table 2
Internal organs weight of experimental chickens administrated with *Streptomyces* probiotic [16, 24]

Sr. no.	Traits	C	T_1	T_2	T_3
1.	Head (g)				
2.	Heart (g)				
3.	Liver (g)				
4.	Spleen (g)				
5.	Bursa of Fabricius (g)				
6.	Gizzard (g)				
7.	Kidney (g)				
8.	Abdominal fat pad (g)				
9.	Intestine length (cm)				

[Control, T1 = Probiotic diet for 14 days (addition of probiotic to the pre-starter basal diet), T2 = Probiotic diet for 28 days (addition of probiotic to the pre-starter and starter basal diets), T3 = Probiotic diet for 42 days (addition of probiotic to the pre-starter, starter, and finisher basal diets)]

Table 3
Effects of different route of probiotic administration on internal organs weight of chickens [16, 24]

		Day-7					Day-21				
Sr. no.	Traits	Control	Oral	Feed	Water	Litter	Control	Oral	Feed	Water	Litter
1.	Liver (g)										
2.	Spleen (g)										
3.	Pancreas (g)										
4.	Bursa of Fabricius (g)										
5.	Gizzard (g)										
6.	Duodenum (cm)										
7.	Jejunum (cm)										
8.	Ileum (cm)										

Table 4
Hematological profile of control and probiotic fed chickens [25]

Sr. no.	Parameters	C	T_1	T_2	T_3
(a) Erythrogram					
1.	Hb (g/dl)				
2.	RBC count ($\times 10^6/mm^3$)				
3.	PCV (%)				
4.	MCV (μm^3)				
5.	MCH (pg)				
6.	MCHC (%)				
(b) Leucogram					
1.	WBC count ($\times 10^3/mm^3$)				
2.	Leucogram Lymphocytes (%) Heterophils (%) Monocytes (%) Basophils (%) Eosinophils (%)				

[Control, T1 = Probiotic diet for 14 days (addition of probiotic to the pre-starter basal diet), T2 = Probiotic diet for 28 days (addition of probiotic to the pre-starter and starter basal diets), T3 = Probiotic diet for 42 days (addition of probiotic to the pre-starter, starter, and finisher basal diets)]

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Chapter 31

Determination of Infectivity of Probiotics Using Animal Model

Shabari Girish and Lokesh Ravi

Abstract

Endocarditis is an inflammatory disease that occurs at the interior padding of the heart valves. Inducing endocarditis determines to be beneficial in many ways to analyze probiotic infectivity as they provide exact checkpoints to monitor infections in multiple models (female Wistar rats in this case) and examine infectivity with other probiotic bacteria. While infectivity in rabbits can be ascertained by various other methods such as the burn wound method, and so on, a simple and effective alternative has been provided that uses bacteriological, biochemical approaches in the following protocol. In both cases, this particular protocol uses biostatistical methods such as Fischer's exact test and INSTANT to get reliable outcomes.

Key words Probiotic infectivity, Lactic acid bacteria (LAB), Fischer's exact test, Colony-forming unit (CFU), ANOVA

1 Introduction

Probiotics can be defined as non-pathogenic microbes that are existing in foods and can be administered to increase, boost, and balance microbial flora, most vitally in the gut and intestine of various organisms [1]. These microorganisms also have been used to treat various medical illnesses, some being effectively supported by extensive research and others without enough evidence [2]. Probiotics that are supplied to aid diet are generally available in capsules, tablets powders, or commonly even as fermented foods such as yogurt or drinks, these probiotics are mainly of the *Lactobacillus* and *Bifidobacterium* [3]. The infectivity and potency of probiotics are determined by how they survive the gastrointestinal region and battle in a definite site, to make these probiotics resistant to intestinal juices and other body chemical mechanisms they are oftentimes enteric-coated or micro-capsulated [4]. There are multiple species under the family *Lactobacillaceae* such as *L. acidophilus*, *L. bulgaricus*, *L. casei*, etc., and under genera *Bifidobacterium*,

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_31,

there are various species alike *B. animalis*, *B. adolescentis*, *B. lactis*, etc. [5]. These bacteria produce various chemicals like lactic acid and propionic acid which contain the increase of harmful bacteria, thus instituting the microbial flora. The unique feature of these probiotics is such that their impact is bound based on a distinct strain making the effects noticeably different from one strain to another even if they belong to the same species [6]. While probiotics ought to be extensively safe, some studies show an indication that probiotics could be serious or infective in unique cases [7]. These diseases created by probiotics are frequently witnessed in vulnerable people with grave conditions. These infectivities include the transference of antimicrobial-resistant genes from probiotics to the bacteria in varied components of the body such as the gastrointestinal region [8]. As mentioned earlier, endocarditis is a condition that is correlated with inflammation and damage of inner valves of the heart [9]. Inducing endocarditis in animals such as Wistar rats in these cases makes it easy to find checkpoints and follow through the infectivity models of the probiotic which implies our interest [10]. The current experimental protocol involves using an animal such as rabbits and Wistar rats to study the infectivity of probiotics which includes experimental endocarditis in rats, etc. by comparing them along with pathogenic bacteria that are employed as control while this protocol also exploits the application of biostatistical tools and methods to provide many efficient results.

2 Materials

All animal models utilized in this protocol are required to be disease-free, mature, and as per the requirements mentioned in the following steps; the bacterial isolates are expected to be cultured to sustain a pure culture form. All the reagents are reckoned to be prepared using pure water (generally prepared by reverse osmosis and is devoid of any chlorides, sulfates, ammonia, and other heavy metals).

2.1 Bacterial Isolation and Growth of Probiotics

1. The strains of probiotics, which are of our interest, are recommended to be isolated according to the PROSAFE bacterial isolation methods (*see* **Note 1**).
2. Another strain could be isolated from food products that are expected to be non-pathogenic to use as a test when administered to animals.
3. Selection of potential bacteria based on Fluorescent Amplified Fragment Length Polymorphism (FAFLP) or protein profiling (*see* **Note 2**) [11].

4. To culture and grow bacteria we require Petri plates, incubators, flask, measuring cylinders, oven, autoclave, and MRS agar/broth which contains peptose 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, dextrose 20 g/L, polysorbate 80 1 mg/L, Ammonium citrate 2 g/L, Sodium acetate 5 g/L, Magnesium sulfate 0.5 g/L, Manganese sulfate 0.05 g/L, esculin 3 g/L, ferric ammonium sulfate 0.2 g/L, agar 15 g/L (if solid medium), and 10 μg of chloramphenicol (*see* **Note 3**).

2.2 Animals Utilized and Safety Equipment to Be Used in Animal Colony Rooms/Farms

1. Wistar rats and Male Dutch Rabbits/White New Zealand rabbits are employed in the current protocol.
2. The Wistar rats are maintained in separate animal colony rooms at the temperature of 21 °C (±2) under the 12:12 reversed light-dark cycle [12].
3. Rabbits employed in this experiment can be male Dutch rabbits or new Zealand rabbits.
4. All individuals entering the animal culture lab/farms are required to have Personal Protective Equipment (PPE).
5. Head caps, gloves, and footwear covers are required in the laboratory or colony rooms.
6. Respiratory protection such as masks are also advised to protect oneself from inhaling hazardous microbes.
7. Specials bins for disposing of hazardous waste are required [13].

2.3 Experimental Endocarditis in Rats to Determine Infectivity of Probiotics

1. Poly-ethylene catheters.
2. Injections.
3. Phosphate Buffer Saline (add 8 g of NaCl, 200 mg of KCl, 1.44 g of Na_2HPO_4, and 245 mg of KH_2PO_4 800 mL of distilled water in measuring cylinder).
4. Titration apparatus such as flasks, pipettes, burettes, and stands.
5. MRS agar (as per **item 4** of Subheading 2.1).
6. Fischer exact test (Mathematical technique to estimate statistical significance in contingency tablets) [14].

2.4 Infectivity of Probiotics in Rabbits

1. Set of white New Zealand rabbits with bodyweight around 0.80–1.2 kg kept at room temperature under proper housing conditions with proper diet.
2. Probiotics are deemed to be chosen per the PROSAFE method (as discussed in **item 1** of Subheading 2.1).
3. Control strains such as *Escherichia coli* 0157 H7 strain (*see* **Note 4**).

4. Nutrient agar medium, Eosin Methylene Blue (EMB) media are required for the following protocol (*see* **Note 5**).
5. pH meter.
6. Injections, Petri plates, flasks, measuring cylinders, Eppendorf tubes, etc.

3 Methods

3.1 Experimental Endocarditis in Rats to Determine Infectivity of Probiotics

1. Induce 180–200 g of sterilized blood clots in a faction of female Wistar rats using a poly-ethylene catheter across the aortic valve of the rat's heart.
2. Monitor the above setup for about 24 h and administer the rats with phosphate buffer saline in increasing order of organisms 10^4–10^8 CFU (*see* **Note 6**) [15].
3. Bacterial inoculum is supposed to be selected, and isolated based on points mentioned under Subheading 2.1 in liquid culture and plated onto MRS agar and incubated at 37 °C for 18 h.
4. For each strain, it is advised to use a minimum of 8–13 animal models (female Wistar rats in this case).
5. Inject 0.5 μL strains into the female Wistar rats.
6. The animals are left for about 72 h for the bacterial challenge.
7. Sacrifice the Wistar rats and dissect the heart under sterile conditions (*see* **Note 7**).
8. After dissection, the vegetative portions of the blood clots are selected, serially diluted and plated onto MRS agar, and then incubated for 48 h at 37 °C.
9. Test the minimal infective dose for that of aortic blood clots for each of the strains against control strains (*see* **Note 5**) [16].
10. Measure the bacterial density in $\log_{10}$ CFU and these densities can be studied using biostatistical means such as Fischer's exact test that employs mathematics (for infectivity of two different bacterial strains in this case) [17] (*see* **Note 8**).

3.2 Infectivity of Probiotics in Rabbits

1. Culture the selected probiotic strains and *E. coli* 0157 H7 strain on the nutrient medium, and EMB medium before injecting them to the rabbits.
2. Use a set of white New Zealand rabbits (65) as mentioned by Dimerdash et al. [18].
3. From the above set, sacrifice five rabbits and subject them to bacterial isolation (*see* **Note 9**).

4. Divide the rest 60 rabbits into four groups namely **C**, $\mathbf{G_A}$, $\mathbf{G_B}$, and $\mathbf{G_C}$, where **C** is the control, $\mathbf{G_A}$ are the rabbits that are deemed to be infected with *E. coli* strain 0157 H7, $\mathbf{G_B}$ are the rabbits that are fed with probiotic bacteria of interest while $\mathbf{G_C}$ are the rabbits that are inoculated with *E. coli* strain 0157 H7 and are also fed with the probiotic bacteria of interest.
5. Feed probiotics to White New Zealand rabbits at the proportion of 1 g/3 L of water) constantly for 17 days [19].
6. Sacrifice three rabbits from each group after the seventeenth day and collect the blood and intestinal samples.
7. Store the collected blood samples in Eppendorf tubes by freezing them at −20 °C (*see* **Note 10**).
8. Subject the intestinal samples to bacteriological, biostatistical investigations and subject the collected blood sample to biochemical investigations.
   (a) *Bacteriological investigation*: The colon section of the collected intestinal sample of each rabbit from their particular sample is taken and weighed for 1 g and coalesced with 10 mL of distilled water and is serially diluted for bacterial colony count, these counts are measured in CFU units and are inoculated in nutrient agar medium and EMB medium (to identify *E. coli* predominantly) [20]. The above cultured bacterial colonies and the counts are measured as CFU.
   (b) *Biostatistical investigation*: From the above information, calculate the bacterial density using ANOVA statistical method (*see* **Note 11**).
   (c) *Biochemical investigations*: The acquired bacterial strains from the blood samples (*see* Subheading 3.2, **step 7**) are cultured at 30 °C for 24 h and are subjected to biochemical studies as proposed by Nazzaro et al. [21]. These studies involve testing these strains for the creation of short-chain fatty acids such as acetic acid, propionic acid, and butyric acid by refining the secondary metabolites presented by the microbial culture. The abovementioned sample is subjected to HPLC (*see* **Note 12**) and is eluted with 0.005 M sulfuric acid for 40 min [22]. It is also to be heeded that the flow rate is to be set at 0.6 mL/min and the exposure wavelength is to be set at 210 nm [23].

4 Notes

1. PROSAFE strains as mentioned by Vankerckhoven et al. are a European Union-funded collection of strains that imply often probiotics which are from 907 genera belonging to both from nutritional and human isolates, predominantly comprising the

genera *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Lactococcus*, etc., this makes it straightforward for us to obtain our desired strains for the current study [24].

2. FAFLP is constructed on the platform of PCR amplification that can digest total genomic DNA which makes it effortless to distinguish closely related species simultaneously [25] while protein profiling is performed in the lack of profoundly high-level FAFLP. It depends on the sort of proteins and their estimates which are checked using the SDS-PAGE technique [10].
3. Man Rogosa Sharpe (MRS) agar/broth is a typical culture medium used for culturing lactic acid bacteria and numerous other probiotics. It is widely recognized to replace tomato juice as a medium for bacterial growth while chloramphenicol was employed to check any kind of fungal contamination [26]. The different bacterial culture media used in this particular experiment are mentioned in Table 1.
4. Control is used to compare our strains of interest with other organisms. In this case, the control is supposed to be pathogenic bacteria. In this case, we see the usage of *E. coli* strain 0157 H7.
5. EMB agar is a selective media that is used to differentiate *E. coli* from other gram-negative pathogenic bacteria [27] while nutrient agar medium is a general agar that is utilized for bacterial isolation and to characterize bacterial colonies [28].
6. At this point, the female Wistar rats are not inoculated with bacterial strains but are expected to be distributed on what concentrations they would be injected. CFU stands for Colony-Forming Unit which estimates the fraction of viable bacteria in a given inoculum or colony [29].
7. The bacterial challenge is the process in which a bacteria in a given time reproduces and colonizes a particular medium [30].
8. Fischer exact test is a biostatistical tool to determine if there are some nonrandom associations between two categorical variables, in this case, control and test rats.
9. These five rabbits are sacrificed to know the general microbial flora present in the animal which would be important to study infectivity in them.
10. Storage of blood vials in −20 °C is always preferred because at this temperature it prevents any kind of contamination or fungal growth since blood can act as a base for microbial growth [31].

Table 1
Different bacterial culture media used in this protocol

Bacterial medium	Composition	Specificity	Utilization of the medium in the current protocol
Nutrient agar Nutrient broth	28 g of Nutrient agar by Sigma aldrich in 1 L of distilled water 25 g of Nutrient broth by Sigma aldrich in 1 L of distilled water	It is a general media used to culture bacteria, yeast, and microscopic fungi	In the current protocol, the nutrient medium is used to culture a strain of *E. coli* 0157 H7 to obtain a pure culture
Eosin Methylene Blue agar (EMB)	37.5 g of EMB agar by Sigma aldrich in 1 L of distilled water	A selective media that is used to differentiate *E. coli* from other gram-negative pathogenic bacteria	The cultured *E. coli* strain 0157 H7 is also cultured using EMB to identify if there are any other gram-negative bacterial strains along with our strain of interest
Man Rogosa Sharpe (MRS) agar/ broth	Peptose 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, dextrose 20 g/L, polysorbate 80 1 mg/L, Ammonium citrate 2 g/L, Sodium acetate 5 g/L, Magnesium sulfate 0.5 g/L, Manganese sulfate 0.05 g/L, esculin 3 g/L, ferric ammonium sulfate 0.2 g/L, agar 15 g/L (if solid medium)	A typical culture medium used for culturing lactic acid bacteria and numerous other probiotics	Exclusively used to culture probiotic bacteria selected in accordance with PROSAFE

11. ANOVA is a statistical tool used to check if multiple factors influence the means of different samples [32]. In the above case, it is noticed that C, G_A, G_B, and G_C act as different conditions of factors while the grouped rabbits are the samples used in this case.
12. High-performance Liquid Chromatography (HPLC) is a system in chemistry that is used to ascertain, separate, quantify, and evaluate products and extracts. In the current context, the secondary metabolites given out by the microbial culture are estimated using the above methods to determine short-chain fatty acids as a biochemical test [33].

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Chapter 32

Determination of Infectivity Using Immunosuppressed Hosts

Sumana Chatterjee and Tamalika Chakraborty

Abstract

People suffering from the diseases or conditions like acquired immune-deficiency syndrome (AIDS), cancer, diabetes, malnutrition, and certain genetic disorders have weakened immune system. Organ transplantation and controlling of inflammatory disorders also may cause immunocompromised condition. Immunosuppression may lead to interaction of the hosts with different microbial infection including opportunistic bacteria, fungi, viruses, and parasite infection causing acute disease. Although probiotics can reduce infectivity of common bacteria, but under severe immune-compromised condition, the probiotic strain can lead to opportunistic infection, thus infectivity of probiotic strain in immunocompromised host need to be assessed. The present protocol chapter describes the animal models that can be used to monitor the infectivity of probiotic strains in immunocompromised hosts. Various techniques of induction of immunosuppression in animal model, assessment of immunosuppression, in vitro toxicity studies after administration of probiotics in animal model, and determination of potential of probiotic bacteria to colonize and infect in animal model are described in this protocol.

Key words Infection, Immunosuppression, Opportunistic infection, Probiotics, Animal model

1 Introduction

Probiotic is one of the most essential microorganisms, namely bacteria and yeast which are responsible for various health benefits and are recommended during various types of illness; however, there are published report of rare infection associated with *Lactobacillus* sp. and *Bifidobacterium*; hence an evidence-based report need to be developed to check their infectivity in an immunosuppressed (severe debilitated) host. There are various sources of exposure for these probiotics such as *Lactobacillus* sp. and *Bifidobacterium*. These sources are application of probiotics to patients, fermented food and one of the most important and neglected sources are host's own microbial flora [1]. In a healthy individual, *Lactobacilli* are normal resident of oral cavity, colon, ileum, and dominant organism of female vagina [2]. Evidences suggest for

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_32,

cases of infection caused by *Bifidobacterium* and some strains of *Lactobacillus* sp. with an estimate of 0.05–0.4% cases of infective endocarditis and bacteraemia [3, 4]. Most of the severe infection caused by *Lactobacillus* sp. usually occurs in patient with underlying comorbidities and immune suppression [5]. Immunocompromised individual is more susceptible to infections and exhibits a higher incidence of opportunistic infections. However, there are very few evidences that consumption of probiotics such as *Lactobacillus* sp. and *Bifidobacterium* can lead to increased risk of opportunistic infections in such patients. Two such studies have been conducted for determination of safety of probiotics given to a small group of severely immunocompromised population suffering from AIDS; however, no such safety issues were associated with the application of probiotics [6, 7]. Thus, most probiotic species including *Bifidobacterium*, *Lactobacilli*, and some strains of yeast are considered as GRAS (Generally Recognized as Safe) [8]. However, there are some microorganisms like *Streptococcus* sp., *Bacillus* sp., and *Enterococci* sp. which are used as probiotics and these are not classified as GARS. Therefore, for such probiotics strains safety assessment must be checked in immunocompromised animal models.

2 Materials

2.1 Preparation of Immunodeficient Mouse [9]

1. Foxn1^{nu} nude mice.
2. Animal house facility.
3. Sterile food and water.
4. Female-specific pathogen-free BALB/c mice (body weight: 20.0 ± 2.0 g).

2.2 Strains of Probiotics

1. *Enterococus* sp.
2. *Bacillus lactis.*
3. *L. paracasei.*

2.3 In Vitro Toxicity Tests

2.3.1 Mucin Degradation Test

1. MRS basal medium (with or without application of 0.3% purified mucin and 1% glucose).
2. Aerobic chamber (jar).
3. Microplate photometer.
4. SDS-PAGE reagents and equipment.

2.3.2 Platelet Aggregation Test

1. Immunocompromised nude mice (weight: 2–3 kg).
2. Probiotic strains.
3. 1% pentobarbital sodium solution.
4. Arachidonic acid (10 μg/mL).

5. 3.2% Sodium citrate solution.
6. Sterile syringe.
7. Centrifuge.
8. Microcentrifuge tubes.
9. Platelet aggregator.
10. Colorimetric cup.

2.3.3 Antibiotic Susceptibility Testing

1. Probiotic strain (1.5×10^8 CFU/mL).
2. MRS agar medium.
3. Petri plates.
4. Antibiotic disk.
5. Sterile forceps.
6. Incubator (37 °C).
7. Vernier caliper.

2.3.4 Assessment of Cytotoxicity

1. A549 (adenocarcinomic human alveolar basal epithelial cell line; 1.2×10^5 cells/mL).
2. HUVECs (human umbilical vein endothelial cells; 1×10^4 cells/mL).
3. HaCaT (spontaneously transformed aneuploid immortalized keratinocyte derived from adult human skin).
4. Probiotic strains.
5. 96-Well microtiter plate.
6. RPMI medium.
7. MTS/PMS mixture.
8. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide solution (5 mg/mL).
9. Dimethyl sulfoxide (DMSO).
10. Microplate reader.

2.4 Alternative Method of Preparation of Immunodeficient Mouse and Assessment of Immunity

1. Female-specific pathogen-free BALB/c mice (body weight: 20.0 ± 2.0 g).
2. Plastic cages (with an ambient temperature of 23 ± 1 °C and $50 \pm 10\%$ humidity).
3. Standard laboratory pellets.
4. Sterile water.
5. Probiotic strains (at different concentrations: 5×10^7 CFU/mL; 5×10^8 CFU/mL; 5×10^9 CFU/mL).
6. CTX (80 mg/kg/day).
7. Sterile Phosphate buffer saline (PBS; pH 7.4).

2.4.1 Analysis of Parameters for Establishment of Immunosuppression

Assay of Splenocyte Proliferation

1. Splenocytes (1×10 cells/mL).
2. 0.l M cold Phosphate Buffer Solution.
3. Homogenizer.
4. 200-Mesh sieve.
5. RPMI 1640 medium supplemented with 10% FBS.
6. Serum-free RPMI 1640 medium.
7. 96-Well flat-bottomed microplates.
8. conA.
9. CO_2 incubator.
10. Cell Counting Kit (CCK)-8.
11. Microplate reader.

Assay of NK Cell Activity

1. RPMI 1640.
2. Splenocytes (1×10 cells/mL).
3. YAC-1 cells (1×10 cells/mL) as the target cells into the wells.
4. CO_2 Incubator.
5. CCK-8.
6. Microplate reader.

Determination of Pinocytosis of Peritoneal Macrophages

1. Peritoneal lavage.
2. RPMI 1640 medium.
3. 10% heat-inactivated FBS.
4. 0.072% neutral red.
5. $1\times$ PBS.
6. 50% ethanol containing 1% glacial acetic acid.
7. Blotting papers.
8. Centrifuge.
9. 96-Well plates.
10. CO_2 Incubator..

Cytokine Quantification

1. Diethyl ether.
2. Blood collection tubes.
3. Centrifuge.
4. Cryovials.
5. Deep freezer ($-40\ ^\circ C$).
6. ELISA assay kits (to estimate mouse IL-2, IL-6, and IFN-γ).

2.4.2 Assessment of Hematological Parameters (Blood Cell Count)

1. BALB/c female mice (6- to 10-week-old).
2. Capillary tubes.
3. K_3EDTA vacutainers.
4. 1.5 mL microcentrifuge tubes.
5. Blood Sample.
6. Glass slide.
7. Compound light microscope.
8. Flow Cytometer.
9. Phosphate Buffer Solution (PBS): The composition of PBS is 0.01 M sodium phosphate and 0.15 M sodium chloride.
10. Lysis Solution: Add 1 mM EDTA, 11.3 mM $KHCO_3$, and 150 mM NH_4Cl in sterile water. Filter the solution through a 0.2 μm mesh. Prepare the working solution freshly by diluting the stock, one in ten part of sterile water.
11. Antibody for Flow Cytometry: 1A8 antibody is specific to a Ly6G epitope on the surface of mouse neutrophils and therefore, injections of the 1A8 antibody selectively deplete neutrophils in mice [10, 11].
12. Staining Reagent: Anti-mouse Ly6GFITC.
13. Isotype Control: Use Rat IgG2a, K-FITC cas Isotype control, which acts as primary antibodies without having specificity to the target.

2.5 Assessment of Potential of Probiotic Bacteria to Colonize and Infect Animal Model

2.5.1 Preparation of Animal Model

1. Congenitally immunodeficient germfree (GF) beige-athymic (bg/bg-nu/nu) mice.
2. Congenitally immunodeficient germfree (GF) beige-euthymic (bg/bgnu/+) mice.
3. Animal house facility.
4. Sterile food and water.

2.5.2 Inoculation of Probiotic Bacterial Culture

1. Sterile flexible-film isolators.
2. Sterile food and water.
3. GF mice.
4. Sterile swabbing.
5. Pure culture of probiotic strains.
6. De Man, Rogosa and Sharpe (MRS) agar plates.
7. Anaerobic chamber (jars).

2.5.3 Assay of Colonization of Probiotics Species in the Gastrointestinal Tracts of GF Mice

1. Sterile water.
2. MRS agar plates.
3. Anaerobic chamber (jars).
4. Aluminum weighing dish.
5. pH meter.
6. Standard pH solutions (pH 2.0, 7.0, 8.0).

2.5.4 Determination of Number of Viable Bacteria in the Internal Organs

1. Excised spleen, liver, and kidney from the sacrificed GF mice and probiotic colonized mice.
2. Homogenizer.
3. Sterile distilled water.
4. MRS agar plates.
5. Anaerobic chamber (jars).
6. Aluminum weighing dish.
7. Oven dryer (with 80 °C).

2.5.5 Histological Evaluations

1. Excised alimentary tract and the major internal organs of the sacrificed mice.
2. 10% formaldehyde (fixative).
3. Phosphate-buffered saline (PBS; pH 7.4).
4. Paraffin.
5. Microtome.
6. Slides.
7. Stains: Hematoxylin, Eosin, and Gram's stains.
8. High resolution microscope (along with an automatic camera and imaging software).

2.5.6 Determination of Immune Response to Probiotics

1. MRS broth.
2. 1× PBS.
3. Anaerobic chamber (jar).
4. Centrifuge.
5. French pressure cell (15,000 lb/in.^{2}).
6. Western blot analysis kit.
7. Radial immunodiffusion assay kit (for IgG, IgA, and IgM levels).
8. Bicinchoninic acid protein assay kit.
9. Lymphocyte proliferation assays.

3 Methods

3.1 Preparation of Immunodeficient Mouse [9]

Foxn1 (Fork head box N1) which is formerly known as Hfn11 and Foxn1^{nu} mutation is commonly known as nude. Mice who are homozygous mutants usually lack Thymus and are therefore termed as T-cell deficient and thus they show poor response to thymus-dependent antigens. They are found to be unable to reject xenogeneic and allogenic grafts, and have greatly increased susceptibility to infections. Hence, nude mouse is prepared for the assessment study.

3.2 In Vitro Toxicity Tests

3.2.1 Mucin Degradation Test [12]

1. Inoculate the strains of *Enterococcus* sp., *Bacillus lactis*, and *L. paracasei* in MRS basal medium with or without application of 0.3% purified mucin and 1% glucose and incubate it at 37 °C in aerobic conditions for 48 h.
2. After the incubation for 12, 24, 36, and 48 h, evaluate the bacterial growth using the microplate photometer at 600 nm.
3. Confirm the mucin degradation by SDS-PAGE.

3.2.2 Platelet Aggregation Test [12]

1. Inject 1% pentobarbital sodium solution at the auricular border (dose: 3 mL/kg) in the healthy and immunocompromised nude mice (weight: 2–3 kg).
2. Anesthetize the mice completely and collect the blood by intubation in both the tracheal arteries.
3. Do the anticoagulation of the blood at the ratio of (anticoagulant: blood) by adding 3.2% Sodium citrate solution.
4. Centrifuge the whole blood at 93 × *g* for a time interval of 12 min and collect the supernatant containing "platelet rich plasma (PRP)."
5. Centrifuge the rest of the blood at 2325 × *g* for 12 min and collect the supernatant which is termed as platelet poor plasma (PPP).
6. PPP is further used to adjust the concentration of PRP to 3×10^8 cells/mL.
7. Take 400 μL of PPP for the base test and set the transmittance of light of the platelet aggregator to 0%.
8. Take 400 μL PRP into colorimetric cup and add 50 μL of 10 μg/mL agonist arachidonic acid and 50 μL test (probiotic) strain which is equivalent to 10^7 CFU/mL or negative control (PBS) and incubate it at 37 °C for 5 min.
9. Keep the tubes at room temperature (RT) for reactions.
10. Test the rate of platelet aggregation after stirring the content within 5 min.

11. Calculate the rate of platelet aggregation using the below given formula:

$$\text{Aggregation rate} = \frac{(\text{Maximum aggregation rate of test group} - \text{Maximum aggregation of blank group})}{\text{Maximum aggregation rate of blank group}} \times 100 \quad (1)$$

3.2.3 Antibiotic Susceptibility Testing [12]

The antibiotic susceptibility of the probiotic microorganisms is assessed on MRS plates using the method of disk diffusion and further determination of MIC (minimum inhibitory concentration) and minimum bactericidal concentration (MBC).

1. 1 mL of probiotic strain (1.5×10^8 CFU/mL) is absorbed in 15 mL MRS medium respectively, mixed thoroughly and poured into the petri dish.
2. After the solidification of the medium, antibiotic disk is attached to the medium surface with the help of sterile forceps.
3. The tested strain is placed in the incubator at 37 °C.
4. After 48 h, measure the diameter of the zone of inhibition and record it with vernier caliper with an accuracy of 0.02 mm.
5. Antibiotics of different groups are tested against eight antibiotics according to CLSI guidelines.
6. Both MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) are calculated for determination of drug resistance, if any.

3.2.4 Assessment of Cytotoxicity [12]

1. Take A549 cell line (adenocarcinomic human alveolar basal epithelial cell) at concentration of 1.2×10^5 cells/mL, HUVECs (human umbilical vein endothelial cells at 1×10^4 cells/mL), and HaCaT (spontaneously transformed aneuploid immortalized keratinocyte derived from adult human skin).
2. Seed the cells in 96-well microtiter plate.
3. When the cell reaches a confluency of 70–80%, replace the medium by 100 μL of medium containing test microorganisms.
4. Measure the cell viability at 490 nm using a microplate reader after addition of 20 μL of MTS/PMS mixture.
5. Add 10 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide solution (5 mg/mL) to the HaCaT cell.
6. Discard the supernatant in each well and dissolve the formed crystals in 100 μL dimethyl sulfoxide (DMSO).
7. After shaking for 15 min, measure the viability of the cell at 590 nm.

3.3 Alternative Method of Preparation of Immunodeficient Mouse and Assessment of Immunity

In this alternative method of preparation of immunodeficient mouse, female-specific pathogen-free BALB/c mice are used with a body weight of 20.0 ± 2.0 g. The below is the experimental design for the same.

3.3.1 Experimental Design

(a) *Group Division*: Ninety female-specific pathogen-free BALB/c mice are randomly divided into six groups:

- A normal control (NC) group.
- Probiotic groups with four different doses (5×10^7 CFU/mL; 5×10^8 CFU/mL; 5×10^9 CFU/mL, 0.2 mL/day).
- A positive control (PC) group.

(b) *Housing and Acclimatization*:

- House the mice in plastic cages at an ambient temperature of 23 ± 1 °C and 50 ± 10% humidity.
- Maintain 12/12 h light–dark cycle and the mice with standard laboratory pellets, and allow water ad libitum.
- Acclimatize animals to laboratory conditions for 1 week before commencement of the animal experiment.

(c) Apply all experimental protocols approved by the Animal Care Review Committee.

(d) *Induction of Immunosuppression*:

In order to induce immunosuppression, inject CTX 80 mg/kg/day of body weight in sterile saline intraperitoneally for three consecutive days to all groups, except the normal control (NC) group.

(e) *Route of Administration and Treatment Schedule*:

- Treat all groups of mice excepting the NC group with 10 mL/kg body weight by oral administration once daily for 20 days.
- Inject the mice of NC group with an equivalent volume of sterile PBS as the immunosuppression group.

3.3.2 Analysis of Parameters for Establishment of Immunosuppression

Analysis of Body Weight

1. Body weight is used as a parameter for measuring immunosuppression effect [13].
2. Monitor the body weight of the animal every 4 days throughout the experiment.
3. The bodyweight is expected to be decreased significantly in all five immunosuppressed groups after administration of CTX 80.
4. Increase in body weight after probiotic administration indicates immunoenhancement.

Analysis of Immune Organ Index

1. After 20 days of oral administration, weigh the animals.
2. Sacrifice the animals cautiously and excise the thymus and spleen surgically and weigh the excised thymus and spleen.
3. Calculate the immune organ index according to the following formula:

$$\text{Spleen or Thymus indices}\left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{spleen/thymus weight mg}}{\text{body weight g}} \quad (2)$$

4. Higher value of immune organ index indicates enhancement of immune function in mice.

Assay of Splenocyte Proliferation Induced by T-Cell Mitogen conA [14]

1. Aseptically remove the mouse spleens.
2. Place the spleen in 0.1 M cold Phosphate Buffer Solution (PBS).
3. Homogenize gently and pass through a 200-mesh sieve to generate single-cell suspensions.
4. Wash erythrocytes rapidly by hypo-osmotic hemolysis.
5. Suspend the cells at a final density of 1×10 cells/mL in RPMI 1640 medium supplemented with 10% FBS.
6. Place splenocytes into 96-well flat-bottomed microplate in triplicates at concentration of 2×10 cells/well.
7. Add 2.5 μg/well of conA to the wells.
8. Culture the cells at a total volume of 200 μL/well at 37 °C in 5% CO_2 using Serum-free RPMI 1640 medium as the control.
9. Incubate for 48 h of incubation and then add 20 μL Cell Counting Kit (CCK)-8 to each well.
10. Incubate the plate for another 2.5 h.
11. Finally, measure the absorbance at 450 nm using a microplate reader.

Assay of NK Cell Activity

1. Prepare the splenocytes as described in the assay of splenocyte proliferation induced by T-cell mitogen conA.
2. Add blank control (RPMI 1640) and spleen cells (1×10 cells/mL) at the level of 0.1 mL per well.
3. Add 100 μL of 1×10 cells/mL YAC-1 cells as the target cells into the wells.
4. Add RPMI 1640 and spleen cells at 0.1 mL per well (used as the effector cells).
5. Incubate the plates at 37 °C in 5% CO_2 for 20 h.
6. Add 20 μL of CCK-8.

7. Following another 4 h of co-culture, measure the optical density (O.D.) of each well using an XD711 microplate reader.
8. Measure the absorbance for the target cell control, blank control, and effector cell control.
9. The percentage of NK cell activity was determined by the following equation:

$$\%\ \text{of NK cell activity} = \left[1 - \frac{\text{O.D. of Test Samples} - \text{Optical Density of Effector Control}}{\text{Optical Density of target cell control}}\right] \times 100 \quad (3)$$

Determination of Pinocytosis of Peritoneal Macrophages [14, 15]

1. Harvest the peritoneal cells after sacrificing the mice by peritoneal lavage with 4 mL of RPMI 1640 medium.
2. Supplement RPMI 1640 medium with 10% heat-inactivated FBS.
3. Aspirate 3 mL of cell-rich lavage fluid and centrifuge at 1500 × *g* for 5 min.
4. Resuspend the pellet at 1 × 10 cells/mL in RPMI 1640 medium.
5. Place the cells in 96-well plates at 200 μL/well.
6. Incubate the plates for 3 h at 37 °C in 5% CO_2.
7. Wash the cells thrice and remove the non-adherent cells by aspiration.
8. Use the attached cells as peritoneal macrophages.
9. Resuspend the cells in 200 μL RPMI 1640 containing 10% FBS.
10. After 24 h, culture the cells at 37 °C under 5% CO_2.
11. Discard the culture medium and add 100 μL of 0.072% neutral red to each well.
12. Culture the cells for another 0.5 h and then discard the mixed solution.
13. Wash each well thrice with PBS buffer to remove the excess dye and dry them by blotting.
14. Resuspend the cells in 50% ethanol containing 1% glacial acetic acid (lysis solution) and keep it overnight at 4 °C.
15. Measure the optical densities (O.D.) at 540 nm.
16. Determine the Phagocytic index using the following formula:

$$\begin{aligned}\text{Phagocytic index} = {} & (\text{total number of engulfed cells}/\text{number of macrophages containing engulfed cells}) \\ & \times (\text{number of macrophages containing engulfed cells}/\text{total number of counted macrophages}) \\ & \times 100\end{aligned} \quad (4)$$

Cytokine Quantification

1. Collect blood from orbital cavity of each mouse under diethyl ether anesthesia.
2. Keep the fresh blood standing for 10 min at 37 °C and then for 15 min at 4 °C.
3. Centrifuge at 3000 × *g* for 10 min to obtain serum and store the serum at −40 °C until use.
4. Use ELISA assay kits to measure IL-2, IL-6, and IFN-γ.
5. Express the results as the concentration of cytokines per milliliter of mouse serum by standard cytokines provided in the kits.

Assessment of Hematological Parameters (Blood Cell Count)

Abnormal count of certain components of blood along with blood smear examination indicates certain diseases and immunodeficiency states. Neutropenia is commonly associated with encapsulated, Gram-positive, Gram-negative bacteria, Herpesviruses (*Herpes Simplex* Virus, *Varicella Zoster* Virus, Cytomegalo Virus), Mycobacteria, *Candida*, *Aspergillus*, Cryptococcus infection. Therefore, abnormal neutrophil count is one important indicator of infectivity with these microorganisms in immunosuppressed subjects [10]. Immunosuppression can be associated with Thrombocytopenia, a secondary manifestation, which is a risk factor for infection [11, 16].

T cells and B cells occupy about 50–70% and 5–15% of peripheral blood lymphocytes, respectively. Therefore, lymphopenia can indicate T-cell or combined immunodeficiency disorders such as severe combined immunodeficiency disease or DiGeorge syndrome [17]. The routine microscopic method of neutrophil count is suffering from low reliability as this method depends on the quality of the blood smear, skill of the analyst and is time-consuming [10]. Optical measurements based automated hematology analyzers could not replace the manual method because of its inaccuracy of assignment of monocytes, eosinophils, and basophils from many animal species [11]. Flow cytometric (FCM) assay can be used to measure absolute neutrophil count in a short time for large number of samples. The steps of flow cytometric (FCM) based method are given below:

Sample Preparation

1. Allow bleeding of 6- to 10-week-old BALB/c female mice through the retro-orbital lobe.
2. With the help of capillary tubes, collect an average of 200 μL of peripheral blood into a mini-collection tubes containing K_3EDTA as anticoagulant.
3. Invert the blood samples gently to ensure proper mixing with anticoagulant, and store it at room temperature before processing and data acquisition.

Blood Cell Count Through Flow Cytometry

Within 1 h of collection, process all the blood samples as follows:

1. Add 50 μL of peripheral whole blood into 1.5 mL tubes.
2. Stain the samples with saturating concentrations of anti-mouse Ly6GFITC (clone 1A8) for 15 min at room temperature in dark within 1 h of collection.
3. Add Rat IgG2a, K-FITC, serving as isotype control.
4. Incubate it for 15 min and then add 450 μL of NH_4Cl lysis solution into the tubes.
5. Incubate the samples for 20–40 min. With gentle inversions before the data acquisition on flow cytometer.
6. For obtaining best signal to noise ratio, the event detection threshold is to be set at 330,000 on flow cytometer.
7. Record the acquired volume and acquired Ly6G count.
8. To stop event acquisition, 50 μL volume trigger is used.
9. Calculate the absolute neutrophil count in original whole blood, as shown in Eq. (5),

$$\mathrm{ANC}_{\mathrm{Cells}/\mu\mathrm{L}} = \frac{\mathrm{Number\,of\,Ly6G1\,events}}{V_{\mathrm{Acquired\ on\ Cytometer}} \times V_{\mathrm{total\ in\ acquisition\,tube}}} V_{\mathrm{Processed\ blood}} \quad (5)$$

where all volumes (V) are expressed in μL.

3.4 Assessment of Potential of Probiotic Bacteria to Colonize and Infect Animal Model [18, 19]

Another important aspect is probiotic translocation which is difficult to be induced in healthy humans. Even if the translocation occurs, the detrimental effect is rare. However, reports are there where probiotic translocation has resulted in detrimental effect in the immunocompromised patient. Since the high degree of safety of probiotics, they were usually overlooked as contaminants and they are least suspected as pathogens, but increased emergence of antibiotic resistance in some strains has led to enhancement of complexity of their eradication. Thus, further investigation is needed for probiotic translocation and infection. Moreover, it should become the facet of safety assessment such that benefit of probiotics should always outweigh the risk.

3.4.1 Preparation of Animal Model

1. Use congenitally immunodeficient germfree (GF) beige-athymic (bg/bg-nu/nu) and beige-euthymic (bg/bgnu/+) mice to establish the infectivity capacity of probiotic bacteria in immune-compromised hosts.
2. The beige-athymic (bg/bg-nu/nu) mice lack a thymus and thus result in T-cell deficiency. It makes the nude mouse immune-deficient.

3.4.2 Inoculation of Probiotic Bacterial Culture

1. House the mice in sterile flexible-film isolators and mate them to obtain litters of approximately equal numbers of nude and heterozygous mice.
2. Keep all the mice on sterile food and water ad libitum.
3. Inoculate the GF mice by swabbing their oral cavity and anal area with a pure culture of probiotic species. The culture should contain approximately 10^8 viable bacteria per mL.
4. Colonize additional GF mice and newborn mice by being exposed to feces, feed, and bedding from mono-associated mice.
5. Monitor the colonization of the mice by enumeration of viable bacteria in the feces of gnotobiotic mice.
6. Inoculate dilutions of feces onto De Man, Rogosa and Sharpe (MRS) agar plates and incubate at 37 °C in anaerobe jars.
7. After incubation, count the colonies of viable bacteria (CFU/mL).

3.4.3 Survival and Growth of Immunodeficient Mice Colonized with Probiotics Species

1. Record the body weights and survival at 4th, 8th, and 12th weeks after bacterial colonization.
2. Compare the body weights of the adult mice and the growth rates of newborn mice between 4, 8, and 12 weeks of age with those of GF control mice.
3. Evaluate the differences in the survival of GF mice and probiotic colonized mice by Kaplan–Meier survival analysis with log rank probability statistics.

3.4.4 Assay of Colonization of Probiotics Species in the Gastrointestinal Tracts of GF Mice

Quantitative estimation of viable bacteria in the contents of the stomach, small intestine, cecum, and colon from mice that had been sacrificed indicates the potential of the probiotic species to colonize specific portions of the gastrointestinal tract. The assay of colonization of bacteria involves the following steps:

1. Wash the stomachs, small intestines, cecum, and colons of the sacrificed mice with sterile water.
2. Dilute the washout serially.
3. Inoculate 50 μL aliquots of the diluted washout onto MRS agar plates.
4. After brief drying, incubate the plates at 37 °C overnight in anaerobic jars and count the colonies of viable probiotic bacteria (CFU/mL).
5. Dry 1 mL aliquot of undiluted suspension of intestinal contents overnight at 80 °C in a tared aluminum weighing dish.
6. Weigh the dried dishes after cooling to room temperature.

7. Record the numbers of probiotic bacteria as log10 CFU/gram (dry weight) of contents.
8. Determine the pH values of alimentary tract washings with a pH meter (use different standard pH solutions to calibrate the pH meter).

3.4.5 Determination of Number of Viable Bacteria in the Internal Organs

The quantitative determination of viable probiotic bacteria cultured from the internal organs of the probiotic-colonized mice indicates translocation capacities of probiotic species from the gastrointestinal tract to internal organs. This method involves the following steps:

1. Aseptically excise spleen, liver, and kidney from the sacrificed GF mice and probiotic colonized mice.
2. Combine half of each excised organ and homogenize in a glass tissue grinder with 5 mL of sterile distilled water.
3. Dilute this homogenate serially and culture it on anaerobic MRS agar plates overnight at 37 °C to detect and quantify the systemic dissemination of the probiotic bacteria.
4. Put 1 mL aliquot of undiluted suspension of homogenized tissue suspension in a tared aluminum weighing dish.
5. Dry the aluminum dish overnight at 80 °C.
6. Weigh the dried dishes after cooling to the room temperature.
7. Record the number of viable bacteria in the internal organs as CFU per gram (dry weight) of tissue.

3.4.6 Histological Evaluations

1. Collect the alimentary tract and the major internal organs of the sacrificed mice.
2. Fix the tissues in 10% formaldehyde prepared in phosphate-buffered saline (PBS; pH 7.4).
3. Dissect the fixed tissues, embed it in paraffin, and section it into 5 mm sections onto slides.
4. Do the staining of these sections with haematoxylin, eosin, and Gram's stains.
5. The tissue sections of the alimentary tract and the major internal organs can be evaluated by a pathologist for any evidence of infection and inflammation.
6. The evidence of infection can be scored by the pathologists using the following criteria:
   (a) Score 1: 1–10 microorganisms/high power field at a magnification of 3400 (HPF).
   (b) Score 2: 10–50 microorganisms/HPF.
   (c) Score 3: 50–100 microorganisms/HPF.

(d) Score 4: Confluent microorganisms/HPF.

(e) Score 5: Confluent microorganisms/HPF.

7. Capture the photomicrographs with a high resolution microscope along with an automatic camera attached to imaging software.

3.4.7 Determination of Immune Response to Probiotics

Western immunoblots are used to evaluate serum antibody responses to antigens.

1. Grow the probiotic bacterial species in MRS broth for 48 h under anaerobic condition.
2. The antigens are prepared from such 48 h incubated anaerobic cultures, as follows:
   (a) Centrifuge the 500 mL of the anaerobic culture at 2000 × *g* for 15 min.
   (b) Wash the bacterial pellets three times with an equal volume of PBS and centrifuge again.
   (c) Resuspend the final bacterial pellet in 10 mL of PBS and pass it through a French pressure cell at 15,000 lb/in.^{2} to disrupt the bacteria.
   (d) Centrifuge the disrupted bacteria at 2000 × *g* for 10 min.
   (e) Estimate the protein content of the supernatant by the bicinchoninic acid protein assay.
   (f) Use this protein as the antigen for Western blot analysis and lymphocyte proliferation assays.
3. In addition, determine the serum concentrations of IgG, IgA, and IgM immunoglobulins by radial immunodiffusion assays.

4 Inference

Since literature depicts that patient with neutropenia should avoid probiotics [19, 20], hence, it is expected that severely immunocompromised patient infected with probiotic bacteria may show considerable neutropenia in blood count examination. Many studies have highlighted that probiotic bacterium has an immunomodulatory role; hence, it is expected that thrombocytopenia or lymphopenia can be overcome by the application of probiotics. However, further investigations are needed to find a correlation of T-cell depletion with the application of probiotics in immunocompromised patient.

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Part IV

In Vivo Biosafety Assessment of Probiotics: Measuring Reproductive and Developmental Toxicity in Animal Models

Chapter 33

Assessment of Reproductive Toxicity

Firdosh Shah and Mitesh Kumar Dwivedi

Abstract

The utilization of animal models in order to assess hazard and risk to humans from exogenous substances continues to be the standard for protecting the human health. For this purpose, the animal tests are employed to predict any reproductive harm by probiotics and bacterial isolates to be considered as probiotics. Animal reproductive toxicity is designed in such a manner where the examination of the entire reproductive cycle is either as a series of single tests that assesses specific stages of the reproductive cycle (fertility, pre- and post-natal development), or a two-generation test. These tests determine structural and functional parameters from gametogenesis through embryonic and post-natal development to adulthood in animal. Many guidelines have been issued from the various international organizations where standardized protocols have been laid for testing potential hazardous drugs or chemicals for assessing human hazards and risks which can be eliminated. The goal of reproductive toxicity testing in animals is to identify possible adverse effects resulting from exposure of probiotics and to develop dose-response relationships that will allow evaluation of responses and extrapolation to human reproductive toxicity. The chapter aims to provide detailed methods for evaluating reproductive toxicity of probiotics while addressing series of tests that could be performed to evaluate specific stages of the reproductive cycle such as fertility, reproduction, pre-natal development, and post-natal development.

Key words Probiotics, Reproductive toxicity, Fertility, Pre-natal development, Post-natal development

1 Introduction

The utilization of probiotics can enhance the productivity of animals from 10% to 20%, and also increases the effectiveness of gastrointestinal diseases' treatment around 25–40% [1]. The use of probiotics in animal husbandry has become an integral part of the process, since the growth and health of animals are largely dependent on the work of the intestines [2]. Probiotics have gained enormous amount of popularity as an alternate to antibiotic in order to control and prevent intestinal pathogens within animals. In spite of the fact that probiotics are considered beneficial, the results are highly variable and the knowledge regarding their mode of action is limited [3]. There is pile of evidence in the literature

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_33,

which suggests that probiotics prevents dysbacteriosis, normalizes metabolism, stimulates regeneration processes in the body, increases non-specific resistance of the body and promotes the stimulation of cellular and humoral factors of immunity [4–8]. Assessment of the reproductive toxicity of probiotics in laboratory animals makes it possible to identify harmless doses of drugs and their side effects. The key objective of reproductive toxicity within animals is to determine the effect of probiotic administration on the physiological state, mortality rate of rats and their offspring and reproductive functions. This makes easier to determine the toxic properties of the probiotics and to assume the degree of safety in utilization of veterinary medicine. This chapter provides the detailed methods for evaluating reproductive toxicity within animal models while subjecting towards series of tests which could be performed to evaluate specific stages of the reproductive cycle such as fertility, reproduction, pre-natal development, and post-natal development.

2 Materials

2.1 Probiotics and Its Culture

- MRS broth.
- de Man Rogosa & Sharpe (MRS) Agar.
- Sterile Petri plates.

2.2 Animal Preparations

- Adult male and female mice (*Mus musculus;* 6–7 weeks old).
- Rectangular polyacrylic cages.
- Dust-free paddy husk.
- Standard pellet diet.
- Clean tap water.

2.3 Administration of Probiotics

- Phosphate-buffered saline (PBS).
- 0.2 M $NaHCO_3$ buffer containing 2% glucose.
- Bicarbonate buffer.
- 3 mg Ketamine.
- 46.7 μg of diazepam.
- 15 μg of atropine.
- 3.5 F catheter.

3 Methods

3.1 Probiotics and Growth Conditions

The probiotic strains belonging to *Lactobacillus*, *Bifidobacteria*, or any other strain exhibiting beneficial probiotic traits such as acid tolerance, bile tolerance, antimicrobial activity, and in vitro adherence to epithelial cells [9] can be grown by following the below mentioned steps [10]:

1. Take an isolated colony of the probiotic strain and inoculate it in MRS broth and incubate it at 37 °C for overnight.
2. Take a loop full of overnight grown bacterial cell suspension and streak it on MRS agar plate (pH 5.5).
3. Incubate the plates anaerobically for the period of 2 days at 37 °C.
4. The well isolated colonies can be observed and are further used for determining its effects on animal models.

3.2 Selection of Animal Species and Strain

1. The rat is normally the preferred species. Appropriate modifications and justification should be given to the protocol, if another species is used (*see* **Note 1**).
2. The experiments are carried out on 2 months old white rats (males and females; live weight 150.1 ± 13.1 g) which are placed on a normal diet in a vivarium in accordance with GOST 33044-2014 "Principles of good laboratory practice" [11].
3. Prior the experiments, animals are placed in 14 days quarantine. The experimental animals are then divided into two equal groups.
4. First group with three males and six females as a control group and the second group with again three males and six females, daily injected with 1 mL of test suspension, termed as experimental group.

3.3 Age, Body Weight, and Inclusion Criteria

1. Healthy animals which aren't subjected to any previous experimental procedures should be utilized.
2. Both male and female rats should be considered while experimentation. Female rats should be non-pregnant and nulliparous.
3. Along with sexually mature rats, similar weight of rats should be ensured at initiation of dosing and similar age should be maintained at mating (approximately 90 days).
4. At the 8–10 weeks of age, deliver male and female rats in the test facility. After arrival animals should be acclimated for at least 5 days.

5. Animals are randomly assigned into experimental and control groups.

3.4 Animal Preparation

1. Take 6–7 weeks old adult male and female mice (*Mus musculus*) of weight 20–25 g.
2. Keep the animals in rectangular polyacrylic cages with bedding material as dust-free paddy husk.
3. During the study period, keep the male and female mice in separate cages to avoid breeding under a constant laboratory condition with 12-h light/dark cycle at temperature of 22 ± 2 °C.
4. Animals are allowed free access to standard pellet diet along with clean tap water ad libitum except in the case where starvation is needed.
5. Change the water and food regularly and the clean the cages along with the fresh husk replacement every 3 days.
6. In order to minimize any non-specific stress, an acclimatization period of 7 days is allowed before the experimentation [12, 13].

3.5 Administration of Probiotics

Probiotics can be administered within mice model through below mentioned steps [14]:

1. Grow the probiotic strains exponentially unless the measurement of turbidity reaches 1–2 (absorbance at 600 nm).
2. Harvest the bacterial cell suspension through centrifugation at $1008 \times g$ for 10 min. With repeated washing with phosphate-buffered saline (PBS) and resuspend the cells either in PBS or 0.2 M $NaHCO_3$ buffer containing 2% glucose (*see* **Note 2**).
3. Resuspend 10^9 colony forming units (CFUs) of probiotics in bicarbonate buffer and administer 100 μL of suspension in mice intragastrically whereas administer 25 μL of suspension while delivering through its mouth.
4. For intrarectal administration, administer 10^9 CFU of probiotic in anaesthetized mice (anaesthetized with 3 mg of Ketamine, 46.7 μg of diazepam, and 15 μg of atropine) by insertion of 4 cm proximal to the anus using 3.5 F catheter.
5. The control mice receive phosphate-buffered saline diluted in sterile water and are maintained under identical conditions as the test group.

3.6 Dosing Schedule and Administration of Doses

1. In case where substances are administered via the diet or drinking water, it is necessary to ensure that the test substance doesn't interfere with the normal water balance or nutrition.

2. In order to administer test probiotic substance in the diet, either a constant dose level or dietary concentration (ppm) in terms of body weight of the animal should be employed and the chosen option should be specified [15].
3. While administrating test probiotic substance through gavage, the volume of liquid administered at one time should not exceed 1 mL/100 g body weight (*see* **Note 3**).
4. The treatment should be given each day at the same time duration.
5. The dose to be administered for each animal should normally be decided on the basis of recent body weight determination and adjusted at least weekly in adult males and females.
6. In case of pregnant females, dose should be decided on the basis of every two days' body weight determination.
7. However, during the last week of pregnancy, the gavage dose should be adjusted to prevent administration of an excessively toxic dose to the dam.
8. On the day of parturition, females should not be treated by gavage or any other route of treatment. Omission of test probiotic substance on that day is preferable to avoid disturbance in the birth process.

3.7 Housing and Feeding Conditions

1. The experimental animal room temperature should be 22 °C (±3°) and relative humidity should be sustained between 30% and 70% with an ideal range of 50–60%.
2. Artificial lighting should be set at 12 h dark and light period.
3. Conventional laboratory diets could be supplied with an unlimited supply of drinking water.
4. Standardized, open-formula diets with reduced estrogenic substances are recommended [16].
5. The feed and drinking water should be regularly analyzed for contaminants. Samples of the diet should be retained until finalization of the report.
6. Animals of same sex and treatment group should be caged within small groups. They are housed individually to avoid possible injuries.
7. Mating procedures should be carried out in separate cages. After copulation, females are separately placed in the maternity or parturition cages where they are provided with defined nesting materials (gestation period of 16–18 days).

3.8 Matting and Pregnancy

1. Place each female with a single, randomly selected, unrelated male from the same dose group (1:1 pairing) until 2 weeks have elapsed or evidence of copulation is observed.

2. Once mating evidence is confirmed through an observation of vaginal plug or sperm discharge, that day is considered as the day 0 of pregnancy [15].
3. Place the animals separately immediately after there is observation of copulation.
4. If there are no signs of mating even after 2 weeks, the animals should be separated without further opportunity of mating. In this case, mating pairs should be clearly identified.

3.9 Litter Size

1. In order to remove any possible effect on litter size parameters such as survival, body weight, growth, and acquisition of developmental landmarks, standardization of litter size is recommended to be 10.
2. After standardization is performed, on fourth day after birth, adjust the size of each litter by eliminating extra pups by random selection to yield, five males and five females per litter.

3.9.1 Assessment of Offspring Parameters

1. Examine each litter immediately after parturition to establish sex and number of pups, livebirths, stillbirths, and the presence of gross anomalies (externally visible abnormalities, subcutaneous hemorrhages, abnormal skin color or texture, including cleft palate, presence of umbilical cord, etc.).
2. The first clinical examination of the neonates includes qualitative assessment of body temperature, state of activity and reaction to handling.
3. Pups found dead on post-natal day 0 or at a later time should be examined for finding possible reason of death or possible defects.
4. On post-natal day 0 or 1, weigh the live pups and count them individually, and this step has to be done regularly at least on post-natal day 4, 7, 14, and 21.
5. Maintain a record for change in gait, posture, presence of clonic or tonic movements and stereotypy or bizarre behavior.
6. Measure the anogenital distance of each pup between post-natal day 0 and post-natal day 4 (Table 1). Measure the body weight on the same day of anogenital distance measured and anogenital distance should be normalized to a measure of pup size, preferably the cube root of body weight [17]. The presence of nipples/areolae in male pups should be checked on post-natal day 12 or 13.
7. Observe any physical or behavioral abnormalities in offspring. The physical parameters include auditory canal and eye opening, hair growth, incisor eruption, pinna detachment, surface righting reflex, attainment of hearing ability, etc.

Table 1
Indices for fertility and reproductive function

Index	Calculation
Female mating index	(No. estrus cycles with copulation ÷ No. cycles required for conception) × 100
Female fertility index	(No. females presumed pregnant ÷ No. of females cohabited) × 100 Or (No. females inseminated ÷ No. animals paired) × 100
Female fecundity index (also, called the conception or pregnancy index)	(No. confirmed pregnant ÷ No. with copulatory plug or sperm) × 100
Male mating index	(No. of males with pregnant females ÷ No. males) × 100
Parturition index	(No. parturitions ÷ No. females confirmed pregnant) × 100
Gestation index	(No. females with pups born alive ÷ No. females confirmed pregnant) × 100
Live litter size	(No. of live offspring ÷ No. of females with copulatory plug or sperm) × 100
Live birth index	Mean pups per litter born alive ÷ mean pups per litter delivered
Viability index	Mean pups per litter alive day 4 ÷ mean pups per litter born alive
Lactation index	Mean pups per litter alive day 21 ÷ mean pups per litter alive day 4

8. Evaluate at least three females per litter daily for the early detection of the vaginal patency. If any abnormalities such as vaginal thread are observed, then it should be noted down.
9. Evaluate all selected males daily for balano-preputial separation commencing before the expected day of balano-preputial separation.
10. Compare the sexual maturity of males and females to physical development by determining age and body weight at vaginal opening or balano-preputial separation, respectively [18].

4 Inferences

- Low mortality rate among rat pups is observed upon administration of probiotics.

- Normal hematological parameters are observed after the administration of probiotics in the blood of rats.
- However, comparison must be made between the probiotic treated animals group with the control group for any reproductive toxicity as determined by the indices for fertility and reproductive function (Table 1).

5 Notes

1. Strains with a well-known high incidence of spontaneous developmental defects or with low fecundity should not be used.
2. For each experiment, a fresh bacterial suspension should be prepared.
3. In exceptional cases, the aqueous solutions up to 2 mL/100 g volume of body weight can be administered.

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Chapter 34

Assessment of Developmental Toxicity in Zebrafish Model

Tamalika Chakraborty, Sumana Roy, Dipanjan Mandal, and Jeenatara Begum

Abstract

According to the international guidelines, the drug administered to a woman of childbearing potential must undergo developmental toxicity testing in non-rodent and rodent species. For successful development of a new drug, large number of rodents is used for the teratogenic assessment. However, the present animal intensive process is not in accordance with 3R principle of human research (replacement, reduction, and refinement). Thus, the high cost and the requirement of long-term assessment of development toxicity has led to an alternative selection of vertebrate Zebrafish as the ideal model organism used for toxicity testing. Zebrafish supports the 3R perspective of toxicology but the variation in the methodologies used by the various groups of researchers is posing challenges to integrative analysis. The increased use of probiotics to combat and prevent various disease demands its safety assessment in various in vivo models thus determination of any types of developmental toxicity, teratogenicity which will determine the dose and duration and frequency of application. The present protocol focuses on the application of various probiotics and determination of biosafety assessment by assessing the morphological abnormalities on Zebrafish model and its future direction.

Key words Developmental toxicity, Teratogenic assessment, Replacement, Reduction, Refinement, Zebrafish

1 Introduction

Probiotics are defined as live microorganisms which are used for various health benefits when it is consumed in correct sufficient quantities [1]. Lactic acid bacteria are the natural inhabitant of human gut, skin, mouth, and urinary tract and are beneficial to both human and animals and have a positive impact on ecosystem too. Presently both yeast such as *Saccharomyces cerevisiae* and bacteria are used in probiotics formulation. The probiotic bacteria include various lactic acid bacteria such as *Lactobacillus*, *Enterococcus* and *Streptococcus*, *Propionibacterium*, *Bifidobacterium* and *Escherichia coli*. Instances of adverse effects and consumption of probiotics are few with certain exceptions of adverse effects being

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_34,

reported primarily in patients with underlying medical conditions [2]. It is difficult to ascertain the relation between health risk and the probiotic microorganisms as they are usually non-pathogenic in nature [3]. For instance, the rate of *Lactobacillus* infection has been estimated about one in ten million people within a century of probiotic consumption. There are many clinical studies where various probiotics have been safely administered in premature infants, immune-compromised hosts, patient with Crohn's disease, and elderly individuals [4, 5]. However, investigation is further required for probiotic use in neonates, severely immunocompromised subjects, and hospitalized patients. Thus, for using probiotics in sensitive populations, it is mandatory to determine the safety assessments by various in vitro assays or animal models for the assessments of infectivity. The safety assessments must take into account the nature of microbes in use, level of exposure, administration methods, and various physiological functions.

Zebrafish (*Danio rerio*) is a sub-tropical carp which belongs to cyprinidae family which belongs to the order cypriniformes. Since Zebrafish shares high genetic homology with the humans, it has been used as an efficient model organism for determination of toxicological study, behavioral assays, etc. Due to its small size, optical clarity, high fecundity, and external development, Zebrafish can be used as an efficient model for developmental toxicity.

1.1 Principle of the Method to Assess Developmental Toxicity

The basic principle includes the exposure of newly fertilized eggs till 96 h post-fertilization, with an observation of every 24 h till all the four different apical observations include the key indicators of lethality, as mentioned below:

(a) Coagulation of fertilized egg.

(b) Lack of somites formation.

(c) Detachment of tail-bud from the yolk-sac.

(d) Lack of heartbeat.

The end of the experiment is marked by the determination of acute toxicity based on positive results followed by calculation of LC_{50} value.

2 Materials

1. Zebrafish eggs.
2. Fish tank: It should be made up of chemically inert material like glass and should have a definite suitable capacity related to the recommendation to loading designed for the brood fish maintenance.
3. Binocular microscope (with minimum 80-fold magnification).

4. Thermometer: Temperature of the room must be maintained to 26 ± 1 °C.
5. 24-Well standard plates with approximately 20 mm depth.
6. Oxygen meter.
7. pH-meter.
8. Spawn trap setup: The trays of the instrument are made up of stainless steel, glass, and other inert materials having wire-type mesh (size of the grid: 2 ± 0.5 mm) for the protection of the laid eggs; one can use certain spawning substrate (plant analogs of inert materials) [OECD 229, Annex 4a (23)] [6, 7].
9. Micropipettes having wider opening for collection of eggs.
10. Glass vessels for preparation of control, standard, and test solutions.
11. 3,4-Dichloroaniline (for positive control).
12. Crystallization dishes for collection of Zebrafish eggs.
13. Probiotic microorganisms (*Lactobacillus plantarum*, *Bifidobacterium breve*, *Enterococcus faecalis*, *Clostridium butyricum*, *Bacillus mesentericus*, and *Lactobacillus casei*).

3 Methods [8–14]

3.1 Administration of Probiotics in Zebrafish Eggs

1. Prepare the broth culture of the test microorganisms (probiotic strains) with the microbial load of 10^{10} cells/mL.
2. Expose the Zebrafish eggs to various concentrations of probiotic microorganisms (10^2, 10^4, 10^6, 10^8, and 10^{10} cells/mL).
3. Expose 20 embryos of Zebrafish to each concentration from 24 to 120 HPF.

3.2 Exposure Conditions

1. Expose, per concentration 20 embryos (one embryo per well) to a specific concentration (as mentioned in Subheading 3.1).
2. Maintain the exposure of embryos in such a way that ±20% of the nominal chemical concentration must be maintained throughout the test.
3. If there is difficulty in maintenance of the above concentrations, all concentrations must be maintained at the beginning and at the end of each interval of exposure.
4. Maintain the chambers for the test, for at least a time period of 24 h with the test solution before initiation of the test.

3.3 Test Concentration [15, 16]

1. Usually, five different concentrations of the test organisms are kept between the constant factor without exceeding 2.2.
2. If a test requires less than five embryos, justification must be provided. The highest concentration should bring about 100% lethality for Zebrafish embryo and the lowest concentration should not result into any visible effect.
3. Test for finding range can give a proper insight to the concentration. Test for finding range is usually done using ten embryos per concentration.

3.4 Controls

1. Prepare both negative control and inner plate control using the dilution water. If it is observed that there is greater than one dead embryo, then the above plate must be rejected.
2. Prepare positive control of concentration of 4 mg/L, 3,4-dichloroaniline with each set of eggs going to be used for the test.
3. Prepare an extra plate having an exposure of 20 embryos to the solvent if any solvent is used for the test.

3.5 Initiation of Exposure and Duration of Test [7, 17–19]

1. Conduct the test just after the fertilization of the eggs followed by its determination after the exposure of 96 h.
2. Place the test embryos in the test solution during its 16 celled condition.
3. Immerse, twice the number of eggs into control and test solution randomly. This is done within 90 min post-fertilization.
4. Keep the fertilized viable eggs separate from unfertilized eggs and transfer them to 24-well plate which is subjected to pre-conditioning for 24 h and is further refilled (2 mL/well) with solution freshly prepared within 180 min post-fertilization.

3.6 Eggs Distribution Over 24-Well Plates

Distribute the eggs collected from the Zebrafish to the wells of the plates as per the following numbers:

(a) A single plate may contain 20 eggs for each test concentration.
(b) A plate with solvent content distributed with 20 eggs.
(c) A plate with positive control distributed with 20 eggs.
(d) Internal plate control with four eggs.
(e) Negative control with dilution water distributed with 24 eggs.

3.7 Validity of the Test

There are few criteria for the validity of the test result.

1. The rate of overall fertilization of all the eggs should be $\geq$70% in the set subjected to testing.
2. The temperature of the residing water for the fish must be maintained at 26 $\pm$ 1 °C within the test chamber at all time during the experimentation.

3. The rate of overall survival of the embryo in the control (diluent-water) as well as the solvent control preferably should be ≥90% until the end of exposure of 96 h post-fertilization.
4. Exposure of 4.0 mg/L 3,4-dichloroaniline (for Zebrafish) should result in minimum mortality of about 30% during the end of 96 h exposure post-fertilization.
5. The hatching rate in the negative control should be ≥80% during the end of 96 h exposure post-fertilization.
6. End of 96 h exposure is marked by the dissolved oxygen concentration with highest concentration in the negative control should be ≥80% of saturation.

3.8 Observation [20, 21, 22]

The apical observation for each tested embryo usually includes:

1. Embryocoagulation.
2. Tails detachment.
3. Lack of somite formation.
4. Lack of the heartbeat.

These observations are used for lethality and any positive observation may indicate the death of Zebrafish embryo. Further hatching must be recorded in test and control groups regularly, starting from 48 h post-fertilization. Ideally observations must be recorded every 24 h, until the end point of the test. The apical observation indicates acute toxicity in Zebrafish embryo 24–96 h post-fertilization (Table 1).

3.8.1 Embryo Coagulation

The embryos which are coagulated appear milky white and dark under the microscope. Number of embryos which are coagulated must be determined after 24, 48, 72, and 96 h post-fertilization.

3.8.2 Somite Formation

At the temperature 26 ± 1 °C, about 20 numbers of somites are formed after 24 h in the Zebrafish embryo developing normally. The embryo developing normally usually shows spontaneous

Table 1
Observation table showing parameters of toxicity with respect to exposure time

Sr. no	Parameters of toxicity	Exposure time (h)			
		24	48	72	96
1.	Coagulated embryos	+	+	+	+
2.	Non-detachment of tails	+	+	+	+
3.	Lack of somite formation	+	+	+	+
4.	Lack of heartbeat		+	+	+

movement and it reveals the presence of somites. The absence of somites is usually recorded after 24, 48, 72, and 96 h. Retardation of development is indicated by the lack of somites.

3.8.3 Non-Detachment of Tails

In a Zebrafish embryo with normal development, tail detachment occurring from the yolk is observed following the elongation of posterior part of the embryonic body. Absence of detachment of tail is usually recorded after 24, 48, 72, and 96 h.

3.8.4 Lack of Heartbeat

In Zebrafish embryo with normal development at 26 ± 1 °C, the heartbeat can be observed after 48 h. Observation must be taken with care as erratic and irregular heartbeat may not be an indicator of lethality. Moreover, visibility of heartbeat without any positive circulation in aorta abdominalis is usually considered as non-lethal. Embryos showing no visible heartbeats are observed in 80× magnification for minimum of 1 min. The presence or absence of heartbeat is usually recorded at 48, 72, and 96 h.

3.8.5 Hatching Rate

Hatching rates for all the experimental and control groups are recorded from 48 h.

3.8.6 Lethal Concentration 50 (LC_{50})

1. Depending upon 50% death of the embryo, the LC_{50} is determined and analyzed by Probit Analysis method.
2. Effective concentration 50 (EC_{50}) is further determined by calculating the sum of deformities in terms of time of exposure and analyzed by Probit analysis method.
3. Teratogenicity Index (TI) is further calculated for the overall test.

4 Expected Results [23, 24, 25]

In the following, the test wells are taken as independent replicates used for the statistical analysis. The test embryo percentage for which minimum one of the apical observations is positive in either 48 or 96 h are plotted against the test concentration of the compound. Calculation of the slope of the curve can be done by calculating LC_{50} value with the confidence limit of 95%.

1. LC_{50} = 50% of lethal concentration.
2. EC_{50} = Concentration where 50% of Zebrafish embryos shows developmental abnormalities.
3. Teratogenicity Index (TI) = LC_{50}/EC_{50}.

5 Inference

Since the literature shows low sensitivity of Zebrafish embryo to the exposure of bacteriocins isolated from probiotics making them effective for application, very few literatures are there regarding the developmental toxicity of probiotics on Zebrafish model. Hence, a partial inference can be drawn that bacteriocins are non-toxic to Zebrafish with low teratogenicity and therefore, the probiotic microorganisms can also be assumed to have negligible toxicity and teratogenicity on Zebrafish as many researches are conducted on behavioral assays associated with the psychobiotics, which are probiotics having an effect on gut-brain axis. However, this area need to be further nurtured and researched upon as probiotics have therapeutic application irrespective of the different ages.

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Part V

In Vivo Biosafety Assessment of Probiotics: Measuring Immunological Parameters in Animal Models for Safety

Chapter 35

Assessment of Inflammation in Animal Models (Macroscopic or Histological Inflammation in the Ileum or in the Colon)

Sumana Roy, Tamalika Chakraborty, Prerona Saha, and Sriparna Kundu Sen

Abstract

Inflammation is the basic indication of any type of infection responsible for development of any disorder. Probiotics which are applied as a part of health benefits can induce pro-inflammatory and anti-inflammatory cytokines. A few strains are associated with such immunomodulatory effects; hence, those probiotic strains need to be screened for their inflammatory activity keeping in mind the efficacy and safety assessment of probiotics and its applications. Assessment of inflammation in animal model is a key parameter required to be evaluated to measure degree of infection as well as extent and effectivity of any therapeutic agents. Histochemical analysis of inflamed section of tissues and scoring parameter measure the intensity of infection. Various macroscopic animal models with various incusing agents are used to induce inflammation. Reduction in paw volume and thickness of paw are the parameters evaluated to measure the intensity of inflammation. Measurement of total and differential leucocytes is carried out to measure pleurisy. Difference in weight is the parameters used for measurement of ear edema.

Key words Inflammation, Paw edema, Histological inflammation, Acute inflammation, Colitis

1 Introduction

Inflammation is defined as conserved immunological process which results in the repair and recovery of damaged tissue having a potential to cause more damage if it is insufficiently regulated. Usually in the locations such as intestine there is always a requirement of controlled inflammation for proper immunological function as the various regulatory immune cells are always in interaction with food particles and intestinal bacteria in order to regulate the pro-inflammatory effector cell facilitating the anti-inflammatory pathway [1]. There are many events such as epithelial barrier disruption, stimulation of unregulated effector cell, uncontrolled colonization of bacteria, and improper regulation of homeostatic balance can

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_35,

contribute to the disease onset process. These events of inflammation can manifest anywhere in the location of large intestine and small intestine. Although the diseases related to inflammation of the intestine are often referenced with respect to temporal and localized inflammatory effects in the parts of large and small intestine, the uncontrolled inflammations always result in a systemic impact on the body [2, 3]. Probiotics are usually incorporated for health benefits. Data on clinical and experimental studies revealed that many probiotic *Lactobacillus* sp. have immunomodulatory activity showing decrease in inflammatory response under various pathological conditions including necrotizing enterocolitis and allergic response in children. However, in vitro studies revealed that some strains of probiotics induce pro-inflammatory cytokines such as IL-8, IL-12, and TNF-α [4, 5]. Study conducted by Lopez et al. showed that UV inactivated *Lactobacillus rhamnosus* GG are equally effective in decreasing the concentration of IL-8 in the intestinal epithelium. In addition, Li et al. [6] suggested that both live and heat killed *Lactobacillus* sp. are able to induce both pro- and anti-inflammatory chemokines and cytokines when they are incorporated into the diet of infant rats, but these effects are limited to only some strains of *Lactobacillus* sp. while other strains should not be assumed to have the same effect [7]. However, the etiology of both chronic and acute intestinal disease caused due to application of probiotic is often difficult to understand therefore compromising the correct treatment choice and efficacy of the treatment [1, 2]. Chronic inflammatory disease of the intestine such as Irritable Bowel Disease (IBD) is associated with the previous acute inflammatory disease induced by bacteria, virus and various parasites, improper regulation of intestinal immune response, and certain autoimmune disorders [3]. However, the impact of probiotics in inducing inflammation of intestine lies a major area of research. Thus, the use of an appropriate animal model is required to ascertain the actual etiology of inflammatory disease of the intestine, if any, induced by the application of probiotics and is advantageous in the elucidation of the onset and progression of both chronic and acute disease. There are different animal models which can be used to study various acute and chronic inflammation. Mice can be considered as a good animal model as their development of intestine is very similar to the human intestine as they have similar kinds of immune response and genes ortholog to the human [1]. Rat models are more advantageous than mice models as they are larger in size which may result in acquisition of large sample size [2]. Many invertebrates like Drosophila and nematodes have been used for various immunological studies in order to investigate mechanism of innate immune response. Zebrafish model has been increasingly used for both innate and adaptive immune response [3]. Higher animals like pig have been used as a single monogastric model as their functions of intestine and

morphology are similar to human being. Although no single model are perfect for studying all the components associated with intestinal inflammation, thus each model possess unique feature to target various aspects of inflammation and inflammatory diseases.

2 Materials

2.1 Administration of Probiotic in Animal Model

1. Mice (male or female, 8 weeks old preferably).
2. Probiotic strains (*see* **Note 1**: List of probiotic strains).
3. Water ad libitum.

2.2 Dextran Sulfate Sodium (DSS) Induced Colitis Model [8]

1. Dextran Sulfate Sodium Salt (Molecular wt. 36–50 kDa).
2. Fecal occult blood Hemoccult.
3. Serum separating tubes.
4. Dextran and brominated deoxy uridine to measure epithelial barrier permeability, epithelial cell proliferation and migration.
5. 10% PBS-buffered formalin.
6. Hematoxylin and eosin.
7. Ear Punch.
8. Animal Balance.
9. Sterile Forceps.
10. Fluorescence spectrophotometer.
11. Homogenizer.
12. Refrigerated centrifuge.

3 Methods [9, 10]

3.1 Determination of Inflammation Through Dextran Sulfate Sodium Induced Colitis Model

3.1.1 Preparation of Animal

1. On the day of experiments place healthy animals in three different groups.
   (a) Group 1: Negative Control (treated with plan water).
   (b) Group 2: Positive Control (treated with DSS).
   (c) Group 3: Test Group (treated with Probiotic Strain).
   - Number of animals in each group: 06 (minimum).
2. Determine the weight and average weight of Group to overcome any significant difference in weight in all experimental groups.
3. Preparation and optimization of test and standard solution: Prepare the Probiotic and DSS solution by weighing certain amount of powder and mixing until a clear solution is achieved.

Table 1
Scoring system for comparative analysis of intestinal bleeding

Nature of sample	Scoring
Normal stool consistency with negative hemoccult	0
Soft stools with positive hemoccult	1
Very soft stool with traces of blood	2
Watery stool with visible rectal bleeding	3

Table 2
Scoring system for analysis of endoscopic damage

Parameter	Scoring
Colon translucency	0–3
Presence of fibrin attached to the bowel wall	0–3
Granular aspect of the mucosa	0–3
Morphology of the vascular pattern	0–3
Stool characteristics: normal to diarrhea	0–3
Presence of blood in the lumen	0–3

4. Fill the cage water bottle with 100 mL of plain water, DSS solution, and Probiotic Solution. The amount should be sufficient for Six Mice for 2–3 days.
5. Measure the water intake by each group by comparing leftover water for the respective group.
6. Measure the body weight and occult blood regularly for each group.
7. Collect the fecal samples from each animal with sterile forceps into microcentrifuge tubes (*see* **Note 1**).
8. Monitor the occult blood on Hemoccult.
9. Monitor the intestinal bleeding and analyze it through the scoring system, as mentioned in Table 1.
10. Direct in vivo observation of DSS-induced colonic mucosal damage in coloview system: Inflate the colon with air after anesthetizing the animal to observe 3 cm of the proximal colon.
11. Analyze the endoscopic damage by the scoring system as mentioned in Table 2.

3.1.2 Determination of Intestinal and Spleen Inflammation

1. Monitor the intestinal epithelial cell proliferation and migration through histological staining. Administer BrdU through i.p route at 4 and 24 h before sacrifice.
2. On the day of sacrifice, withdraw all animals from food for 4 h.
3. Administer FITC-dextran tracer in 0.1 mL phosphate buffer intragastrically (0.6 mg/g body weight).
4. Collect the hemolysis-free serum after 3 h.
5. Prepare FITC-dextran standard curve by serial dilution.
6. Measure the correlation of intestinal permeability with fluorescence intensity using Fluorescence Spectrophotometer (at Excitation wave length: 488 nm; Emission wave length: 520 nm).
7. On the day of termination, carry out the terminal bleeding and euthanasia of mice, as per the institutional ethical protocol.
8. To generate a quantitative measurement of intestinal inflammation, measure serum keratinocyte derived chemokine (KC) and/or lipocalin 2, which correlates with disease activity via Duoset ELISA kits (R&D Systems) following manufacturer instructions. For this purpose, dilute control serum samples 1:2 or 1:4 for KC and 1:200 for lipocalin 2. Samples from DSS-treated mice require a much higher dilution.
9. Spray the 70% Ethanol and open the animal through ventral midline incision. Remove the spleen and weigh it (*see* **Note 2**).
10. Lift the collected colon with the help of a forceps and pull carefully until the cecum is visible (*see* **Note 3**).
11. Isolate the colon and cecum by separating them from the small intestine at the ileocecal junction and from the anus at the distal end of rectum. Take the gross picture of intestine of all the groups and compare.
12. Measure the length of the colon (straighten but do not stretch). Separate the colon from the cecum (at ileocecal junction) and quickly flush it using phosphate buffer (use 5–10 mL syringe with feeding needle) to remove the feces and blood (*see* **Note 4**).
13. Cut colons into pieces to compare the same region of colon from three different groups. Proximal colon may be investigated for MPO and rectal region fixed in 10% formalin cassette for histology.

3.1.3 Myeloperoxidase (MPO) Assay

The MPO assay involves the weighed and the frozen colon tissue. The extent of neutrophil infiltration correlates with its concentration in the tissue.

1. Weigh colonic tissue (50–100 mg) and wash thoroughly in PBS until it is free of fecal matter and store it at −80 °C until analyzed.

2. Homogenize the colon tissue in 0.5% hexadecyltrimethylammonium bromide (Sigma, Ref. H6269) in 50 mM PBS, pH 6.0.
3. Freeze-thaw three times and sonicate it for 10 s to get a homogenous tissue suspension and centrifuge it at high speed at 4 °C.
4. Assay MPO in the clear supernatant in a 96-well plate by adding 1 mg/mL of dianisidine dihydrochloride (Sigma, Ref. D3252) and $0.5 \times 10^{-4}\%$ H_2O_2, and measure optical density (O.D.) at 450 nm (*see* **Note 5**).
5. A brownish yellow color should develop slowly over a period of 10–20 min. If color development is too rapid, particularly in DSS-treated colonic samples, dilute and repeat the assay. Express MPO as U/mg protein or U/g tissue.

3.1.4 RT-PCR Analysis

1. Place a piece of colon (50 mg) in RNA later solution for RNA extraction. RNA later should be frozen at −20 °C.
2. On the day of RNA extraction, remove the colon from RNA later solution and extract the RNA by Trizol method [11].
3. Remove all traces of polysaccharides including DSS via lithium chloride method according to Chassaing et al. [12].

3.1.5 Histological Staining [13–15]

1. Clean the slide and make it free from wax.
2. Cut each piece of colon longitudinally and wrap it around a toothpick wetted with phosphate buffer.
3. Place it in a cassette, fix it with 10% buffered formalin, and transfer it to 70% ethanol.
4. Add haematoxylin or eosin stain in the fixed tissue.
5. For detection of epithelial cells, incubate the sections with primary antibody followed by secondary antibody (rabbit anti-rat secondary antibody, Dianova).
6. For negative control, omit the primary antibodies and use hematoxylin as counterstain for nuclei.
7. Histologic scoring: Perform the blinded histologic scoring on H&E stained colonic tissue as follows:
   (a) Assign each section, four scores based on the degree of epithelial damage and inflammatory infiltration into the mucosa, submucosa, and muscularis/serosa.
   (b) Each of the four scores is multiplied by 1 if the change is focal, by 2, if it is patchy and by 3 if it is diffused.
   (c) Add the four individual scores per colon, which results in a total scoring range of 0–36 per mouse.
   (d) Tabulate the average scores for control and DSS-treated groups.

3.1.6 Determination of Severity of Inflammation

1. Cut longitudinally 1 cm of colon. Wash it three times in HBSS with 1.0% antibiotics (penicillin and streptomycin).
2. Place the washed colon in a 24-well plate containing 1 mL serum-free RPMI 1640 medium with 1% antibiotic.
3. Incubate it at 37 ° C for 24 h under 5% CO_2.
4. Collect the supernatants and centrifuge it for 10 min at 4 °C.
5. Store it at −80 °C to analyze pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6, etc.).
6. Measure the severity of inflammation ex vivo for all the three groups.

4 Inferences

Literature shows the evidence that effect of probiotics is mediated by short-chain fatty acids (SCFAs; metabolites generated by probiotics). These SCFAs have anti-inflammatory activity [16]. However, few studies also showed evidence of development of inflammation with some specific strain of microorganisms [17]. Any bacterial strain considered to be used as probiotic, should be tested for inflammatory activity in reference with a standard inflammatory agent (DSS). If the administration of particular strain shows evidence of inflammation in comparison to the standard inflammatory agents, then the particular bacterial strain cannot be considered as safe and should not be used as probiotic.

5 Notes

1. The frozen stools/fecal samples can be preserved for the measurement of inflammatory markers at −20 °C.
2. Increased spleen weight correlates with the extent of inflammation and anemia.
3. Dissecting colons from severely inflamed DSS-treated animals is tricky as this tissue thins, shortens and becomes attached to extra-intestinal tissues.
4. After flushing with PBS, colon weights can be taken. In accordance with observed tissue wasting, severely inflamed colons exhibit reduced weight as both correlate with the severity of acute inflammation. In chronic models of colitis, unlike acute DSS-induced colitis, inflammation is associated with increased colon weight due to the granulomatic nature of inflammation.
5. Human neutrophil MPO (Sigma, Ref = M6908) can be used as a standard (Range: 0.5–0.015 U/mL). One unit of MPO activity is defined as the amount needed to degrade 1.0 μmol of peroxide/min at 25 °C.

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Chapter 36

Assessment of Inflammation in Animal Models (Quantification of *TNFA*, *IFNG*, *IL4*, and *IL10* mRNAs by Real-Time PCR)

Sachin Kumar, H. M. Rashmi, and Brijesh Kumar

Abstract

Cytokines are known to play a key regulatory function in immune responses. The commencement or progression of immunopathology in different disorders is often associated with the anomalous production of one or more cytokines. Quantitative reverse transcription PCR (qRT-PCR) analysis is an important tool to monitor changes in gene expression in animal models. The qRT-PCR is currently the most reliable method of quantifying low-level transcripts such as cytokine mRNAs. Here we have discussed a qRT-PCR protocol to assay pro-inflammatory and anti-inflammatory cytokines genes such as *TNFA*, *IL6*, *IFNG*, *IL4*, *IL10*, and *IL13* in the mouse model. This method enables normalization against several housekeeping genes, e.g., *HPRT1*, *TBP1*, *PPIA*, *YWHAZ*, *PGK1*, *GUSB*, *ACTB*, and *GAPDH*. The method includes collection and storage of animal tissue material, isolation of RNA using TRIzol or TRI reagent, synthesis of cDNA from RNA by reverse transcription followed by reverse transcriptase-quantitative PCR (RT-qPCR) approach for quantification of pro- and anti-inflammatory cytokines. The relative expression ratios of mRNA can be calculated by a mathematical model, which induced an efficiency correction for real-time PCR efficiency of the individual transcripts. PCR efficiency and sensitivity allow the assessment of mRNA levels from very small samples.

Key words Inflammatory cytokines, Animal model, mRNA, Transcript, cDNA, qRT-PCR, *TNFA*, *IFNG*, *IL4*, *IL10*, Real-time PCR

1 Introduction

Cytokines are known to play a key regulatory role in immune responses. The onset or progression of immunopathology in various diseases is often associated with the aberrant production of one or more cytokines. It is therefore of considerable interest to characterize cytokine "profiles" associated with disease processes. Many methods are employed for the identification and quantification of cytokines produced by different cell types during inflammation in animal models. Such bioassays are technically restrictive

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_36,

owing to the time required for performance and because of sensitivity and specificity problems.

Enzyme-linked immunosorbent assays (ELISAs), on the other hand, detect both biologically active and inactive cytokines without discrimination. These assays are easy to use, but the commercial kits are usually expensive. Both bioassays and ELISAs are unable to identify actual cytokine production and do not account for cytokines consumed by cells. Although cells producing cytokine protein may be detected by immunocyto/histochemistry, only a limited number of antibodies with good performance are available [1], and the possibility of confusing synthesis with cellular uptake of cytokines exists. As an alternative (or supplement) to cytokine protein measurement by ELISA, cytokine production can be quantified at the mRNA level. For this, reverse transcription-quantitative PCR (RT-qPCR) is the method of choice. PCR is widely used and has proven to be the molecular technique of choice for the detection and analysis of minute amounts of DNA [2]. It is an in vitro method for enzymatically driven synthesis of defined sequences of DNA. The reaction employs two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified. A repetitive series of cycles involving template denaturation, primer annealing, and extension or elongation of the annealed primers by a thermostable DNA polymerase results in the exponential accumulation of a specific DNA fragment. The length of the products generated during PCR is equal to the sum of the lengths of the two primers plus the distance in the target DNA between the primers and the amount of PCR product synthesized can be quantified (qPCR) on a real-time basis using DNA-binding-fluorescent-dyes (SYBR Green I), fluorescent PCR primer, and probe-based chemistries [3].

Over the past two to three decades, qPCR has become one of the most widely used methods of gene quantization and its popularity has grown immensely with the publication of more than 85,000 papers in diverse fields of science (https://pubmed.ncbi.nlm.nih.gov/?term=qPCR). With wide acceptance, this application of qPCR has become one of the most widely used methods for gene quantitation because it has a large dynamic range, boasts tremendous sensitivity, can be highly sequence-specific, has little to no post-amplification processing, and has high throughput [4]. However, in gene expression studies, RNA cannot serve as a template for PCR, so amplification of RNA molecules is performed by a method that combines reverse transcriptase (RT) to turn RNA into a complementary DNA (cDNA) strand with PCR colloquially referred to as RT-qPCR (reverse transcription followed by quantitative polymerase chain reaction analysis or qRT-PCR). However, systematic errors in the application of RT-qPCR (inappropriate choice of reference genes for normalizing transcript levels of test

genes before comparative analysis of different biological samples) and small differences in any of the variables (cDNA yield and concentration used in the RT-qPCR reaction) that control the reaction rate can dramatically affect the yield of the PCR products and can compromise the interpretation of results at the gene expression level. Hence, 11 golden rules of quantitative RT-PCR have been published for the relative quantification of gene expression [5]. Besides, MIQE (minimum information for publication of quantitative real-time PCR experiments) was introduced to the scientific fraternity to encourage better experimental practice, allowing more reliable and unequivocal interpretation of RT-qPCR results [6].

Normalization of target gene expression data, by choosing the appropriate housekeeping/reference genes (RGs), is fundamental for obtaining reliable and accurate results in reverse transcription-quantitative PCR (RT-qPCR). The selection of appropriate genes is based on the principle that the expression of reference genes should be stable under all experimental conditions with all test components. Hence, a minimum of seven housekeeping genes is generally selected for stability analysis. Besides, their specificity should be confirmed either by in silico BLAST search or with in vitro PCR assays with optimization of annealing temperature through gradient PCR. Cytokines play a key important role in the regulation of immune responses and the inflammatory process. The main group of cytokines involved in this process includes tumor necrosis factors (TNFs), interferons, interleukins (ILs), and colony stimulatory factors (CSFs). In the group of cytokines, pro-inflammatory cytokines are those immunoregulatory cytokines that favor the inflammation of tissues. IL-6, TNF-α IL-1α, and IL-1β are the major pro-inflammatory cytokines responsible for early responses in the inflammation process. However, the anti-inflammatory cytokines neutralize various aspects of inflammation including the synthesis of pro-inflammatory cytokines and IL-4, IL-10, and IL-13 are most widely used as anti-inflammatory markers. Hence, both pro-inflammatory and anti-inflammatory cytokines are used as markers for the assessment of inflammation in animal models.

2 Materials

2.1 Collection and Storage of Animal Tissue Material

1. Mice or rat tissues (colon, liver, heart, etc.) under normal or diseased (inflammatory) or after probiotic or bacterial isolates treatment conditions can be collected for assessment of inflammation through RT-qPCR studies. For storage, please *see* **Note 1**.

2.2 Isolation of RNA

1. TRIzol reagent.
2. DEPC (diethylpyrocarbonate) (0.01%).
3. Isopropanol.
4. Sterile KimWipes.
5. 75% Ethanol.

2.3 Synthesis of cDNA from RNA by Reverse Transcription

1. Use commercially available cDNA synthesis kits (RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific or First Strand cDNA Synthesis Kit from Roche Life Science reagents, and PrimeScript first strand cDNA Synthesis Kit from Takara).
2. PCR tubes or PCR strips.
3. Thermocycler.

2.4 Reverse Transcriptase-Quantitative PCR (RT-qPCR)

1. 50–100 ng of template each cDNA.
2. 2× SYBR Green I Master Mix buffer (5.0 μL).
3. 0.5 μL of each 10 μM primer.
4. Use the required amount of RNase/DNase-free sterile water to make the reaction volume 10 μL.
5. PCR tubes or PCR strips.
6. Real-time PCR machine.

2.5 Selection of Housekeeping Genes

The list of housekeeping genes and their respective primer sequences most commonly used in RT-qPCR expression studies related to inflammation in animal models are given in Table 1.

2.6 Selection of Genes as Inflammation Markers

After the selection of target genes as markers of inflammation, download the respective gene sequences from the nucleotide databases and design the RT-qPCR primers with freely available primer designing tools. The specificity of each primer set can be systematically analyzed using a bioinformatics workflow developed from publicly available resources (NCBI Primer-BLAST, in silico PCR in UCSC genome browser, Ensembl DNA database) [12]. The sequences of target (inflammation marker) gene primer and their respective amplified product lengths widely used in assessing the inflammation in animal models are listed in Table 2.

3 Methods

3.1 Isolation of RNA Using TRIzol or TRI Reagent

RNA may be prepared from tissue or cells in several ways. Here, we have described total RNA extraction by TRIzol/TRI reagent method that is widely used for the extraction of RNA from animal tissues [17]. Numerous commercial kits are also available for the successful extraction of RNA, e.g., RNAzol (Biogenesis, Poole, UK) and RNeasy (Qiagen, Hilden, Germany).

Table 1
Primers sequences of eight housekeeping genes of mouse mRNA and their respective amplified product lengths for use in animal studies

Gene name	Primer sequence	Size (bp)	Annealing temperature (°C)	Reference
HPRT1	F: 5′-GGCCAGACTTTGTTGGATTT-3′ R: 5′-CAGATTCAACTTGCGCTCAT-3′	155	58	[7]
TBP1	F: 5′-ATCAACATCTCAGCAACCCA-3′ R: 5′-TTGAAGCTGCGGTACAATTC-3′	187	58	[7]
PPIA	F: 5′-GAGCTGTTTGCAGA CAAAGTTC-3′ R: 5′-CCCTGGCACATGAATCCTGG-3′	125	60	[8]
YWHAZ	F: 5′-GAAAAGTTCTTGATCCC CAATGC-3′ R: 5′-TGTGACTGGTCCA CAATTCCTT-3′	134	60	[8]
PGK1	F: 5′-ATGTCGCTTTCCAACAAGCTG-3′ R: 5′-GCTCCATTGTCCAAGCAGAAT-3′	164	60	[9]
GUSB	F: 5′-GGCTGGTGACCTACTGGATTT-3′ R: 5′-TTGGCACTGGGAACCTGAAGT-3′	134	60	[10]
ACTB	F: 5′-GTGACGTTGACATCCG TAAAGA-3′ R: 5′-GCCGGACTCATCGTACTCC-3′	245	60	[8]
GAPDH	F: 5′-ATG GTG AAG GTC GGT GTG AA-3′ R: 5′-GAG TGG AGT CAT ACT GGA AC-3′	151	60	[11]

TRIzol solubilization and extraction is a widely used method for deproteinizing RNA. This method is most useful in situations where cells or tissues are enriched for endogenous RNases or when the separation of cytoplasmic RNA from nuclear RNA is ineffective. TRIzol (TRI Reagent) is a monophasic solution of phenol and guanidinium-iso-thiocyanate that simultaneously solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation, where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Therefore, RNA, DNA, and protein can be purified from a single sample (hence, the name TRIzol). TRIzol extraction is also an effective method for isolating small RNAs, such as microRNAs, piwi-associated RNAs, or endogenous, small interfering RNAs.

Table 2
Primers sequences of target (inflammation marker) genes of mouse mRNA and their respective amplified product lengths for use in animal studies

Gene	Primer sequence	Product size (bp)	Reference
TNFA	F: 5′-GGCAGGTCTACTTTGGAGTCA-3′ R: 5′-CACTGTCCCAGCCATCTTGTG-3′	223	[13]
IL6	F: 5′-GTTCTCTGGGAAATCGTGGA-3′ R: 5′-GCATTGGAAATTGGGGTAGG-3′	141	[13]
IFNG	F: 5′-AGACATCTCCTCCCATCAGCAG-3′ R: 5′-TAGCCAAGACTGTGATTGCGG-3′	158	[14]
IL4	F: 5′-GAG ATC ATC GGC ATT TTG AA-3′ R: 5′-GCA GCT TAT CGA TGA ATC CAG-3′	249	[15]
IL10	F: 5′-CTCGTTTGTACCTCTCTCCG-3′ R: 5′-ATCTCCCTGGTTTCTCTTCC-3′	263	[13]
IL13	F: 5′-TGT TTC GCC ACG GCC CCT TC-3′ R: 5′-GCT CAA GCT GCT GCC TGC CT-3′	312	[16]

The brief protocol for extraction of RNA from collected tissues by the TRIzol method is as follows:

1. Remove frozen tissue specimens from liquid nitrogen and homogenize tissue samples in 1 mL of TRI reagent per 50–100 mg of tissue using a glass Dounce homogenizer (Kontes, Vineland, NJ) or using mortar pestle treated with DEPC (0.01%).
2. Following homogenization, transfer the homogenized samples to 2 mL microcentrifuge tubes (RNase- and DNase-free).
3. Incubate the homogenized samples for 5 min at room temperature (25–30 °C) to permit the complete dissociation of nucleoprotein complexes.
4. Add 200 μL of chloroform per 1 mL of TRIzol reagent and shake tubes vigorously by hand for 15 s and incubate them at room temperature for 10–15 min.
5. Centrifuge the samples at no more than 12,000 × *g* for 15 min at 4 °C and following centrifugation, and observe for the separation of the mixture into a lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase.
6. Transfer the aqueous phase to a fresh tube and precipitate the RNA from the aqueous phase by adding an equal amount of isopropanol, mixing the resulting solution, and incubating the mixture at room temperature for 5 min.
7. Then centrifuge the contents at 12,000 × *g* for 15 min at 4 °C.

8. After centrifugation, the RNA will form a pellet on the side or bottom of the tube. Decant the supernatant and drain the sample on several layers of sterile KimWipes.
9. Then, wash the RNA pellet with ice-cold 75% ethanol by adding at least 1 mL of 75% ethanol.
10. Mix the sample by vortexing and centrifuge at 10,000 × *g* for 10 min at 4 °C.
11. Repeat the washing step one more time, decant the supernatant, and drain the tube by inversion on several layers of sterile KimWipes for approximately 5 min.
12. Re-suspend the washed RNA pellet in DEPC (diethylpyrocarbonate) and proceed on to quantitation of total RNA.
13. Determine the yield and purity of the RNA by measuring the OD_{260}:OD_{280} ratio. RNA purified by this method should result in an OD_{260}:OD_{280} ratio of 2 (>1.7). At this point, keep the isolated RNA on ice when being handled, or store at −70 °C.

3.2 Synthesis of cDNA from RNA by Reverse Transcription

1. Use commercially available cDNA synthesis kits for the synthesis of cDNA by following the manufacturers protocol for RT-qPCR-based gene expression studies.
2. For example, reverse transcriptase reactions can be carried out using the RNA PCR Core Kit (PE Applied Biosystems, Foster City, CA, USA) [18]. In this kit, prepare each reaction tube containing 10 μg of total RNA in a volume of 150 μL containing: 5 mmol/L $MgCl_2$, 1× PCR Buffer II, 500 μmol/L of each dNTP, 0.6 U/μL of RNase inhibitor, 2.5 U/μL of MuLV Reverse Transcriptase, 2.5 μmol/L of random hexamers and DEPC-treated water (to make up the volume up to 150 μL).
3. Reverse transcriptase reactions can be carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Branchburg, NJ, USA) at 42 °C for 20 min and 99 °C for 5 min.
4. The cDNA is then stored at −20 °C.

3.3 Reverse Transcriptase-Quantitative PCR (RT-qPCR) Approach for Quantification of Pro- and Anti-Inflammatory Cytokines

Widely studied MIQE guidelines provide an accurate quantitative gene expression analysis [6]. The following are the steps to be followed for studying the expression of target genes after selecting a stable reference gene.

1. Quantify cDNA synthesized from RNA extracted from the tissues of the animals used in inflammation response studies and subsequently dilute it to a concentration of 50 ng/μL for use as a template in RT-qPCR.
2. Conduct the RT-qPCR expression study by using the available RT-PCR machine with recommended chemicals.

3. Briefly, prepare the RT-qPCR reactions in a total volume of 10 μl containing 50–100 ng of template each cDNA, 2× SYBR Green I Master Mix buffer (5.0 μL), and 0.5 μL of each 10 μM primer and use the required amount of RNase/DNase-free sterile water to make the reaction volume 10 μL.
4. Conduct the reactions with optimized thermal cycler conditions, e.g., denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s and repeat it for 40 cycles with melt curve analysis at a temperature range of 60–95 °C (*see* **Note 2**).
5. Conduct all reactions including the reactions for generation of standard curves with different concentrations of cDNA (0.625 ng/μL to 100 ng/μL) for selection of stable housekeeping genes under test and control conditions, no template controls (NTC), and plate controls in duplicate.
6. Measure the fluorescence once every cycle after the extension step using filters for SYBR Green (excitation at 492 nm and emission at 530 nm) and analyze the Cq data to select stable reference housekeeping genes for normalization in RT-q PCR gene expression data using different algorithms (BestKeeper, NormFinder, and geNorm) and also note the C_q values of the target genes for all test samples.

3.4 Statistical Analysis of Relative Target Gene Expression

The generation of quantitative data by real-time PCR is based on the number of cycles required for optimal amplification generated fluorescence to reach a specific threshold of detection (the Quantification cycle; C_q value) [6].

1. Calculate the relative expression ratios by a mathematical model, which induced an efficiency correction for real-time PCR efficiency of the individual transcripts [19], as follows:

$$\text{Ratio} = \left(E_{\text{target}}\right)^{\Delta Cq\ \text{target}}(\text{control} - \text{sample}) / \left(E_{\text{ref}}\right)^{\Delta Cq\ \text{ref}}(\text{control} - \text{sample})$$

2. The relative expression ratio of a target gene can be computed based on its real-time PCR efficiencies (E) and the crossing point difference (ΔC_q) for an unknown sample versus a control.
3. Besides, REST 2009, the software tool developed [20] can also be used for the expression analysis of respective inflammation marker genes.

4 Inference

The qRT-PCR is currently the best and quick way to investigate inflammatory cytokine production. The investigations can be completed by the analysis of genes regulated by cytokines or involved in cytokine signaling, providing indirect information on cytokine

protein expression. The PCR efficiency and sensitivity allow the assessment of mRNA levels from very small samples. The bacterial isolates (for probiotic consideration) which lead to increased pro-inflammatory (such as *TNFA*, *IFNG*, *IL6*, etc.) and decreased anti-inflammatory (such as *IL10*) cytokines' mRNA expression in animal model cannot be considered safe as per the biosafety aspects.

5 Notes

1. Since RNA is a highly unstable molecule, it is recommended to flash-freeze the samples in liquid nitrogen to preserve them at –70 °C or lower. Avoid freeze-thaw cycles of the samples to prevent RNA degradation [21].
2. The optimum annealing temperature of primers needs to be optimized with gradient PCR assay.

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Chapter 37

Assessment of Inflammation in Animal Models (Quantification of TNF-α, IFN-γ, IL-4, and IL-10 Proteins by ELISA)

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Abstract

Cytokines trigger a variety of pro- and anti-inflammatory effects in the body, which makes them useful biomarkers for inflammation. Enzyme-linked immunosorbent assay (ELISA) is a sensitive and highly versatile method for quantification of cytokines. This chapter describes protocols for the quantification of cytokines found in blood and tissue homogenates for probiotic safety assessment in animal model through the use of sandwich (direct/indirect) ELISA. The goal of this chapter is to provide readers with a baseline on how the above-mentioned formats of ELISA work in studying inflammation in animal models through quantification of tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), interleukin-4 (IL-4), and interleukin-10 (IL-10) proteins.

Key words Animal models, Cytokines, Enzyme-linked immunosorbent assay (ELISA), Inflammation, Probiotics, TNF-α, IFN-γ, IL-4, IL-10

1 Introduction

Inflammation is one of the host's immune system responses towards potentially harmful stimuli such as invading pathogens. It serves to protect the host from harm by removing the threat and initiating healing. However, uncurbed inflammation can lead to a variety of acute and chronic diseases such as rheumatoid arthritis (RA), multiple sclerosis, diabetes, Crohn's disease, inflammatory bowel disease (IBD), Alzheimer's disease, cancer, psoriasis, atherosclerosis, asthma, obesity, immune-inflammatory ailments, neoplastic transformations, and cardiovascular diseases [1–3]. Cytokines are a group of signaling protein molecules enrolled by the

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_37,

host for the immune defense and immunoregulation to prevent diseases [4]. Identification and quantification of cytokines have been used for the diagnosis, risk assessment, prevention, and treatment of a wide range of immunological disorders [5].

Probiotics have been extensively studied for their role in regulating host immunological activities and enhancing immunological barrier. They reportedly modulate the host innate and adaptive immune response by maintaining a balance between T helper cells type 1/2 (Th1/Th2). Probiotic exogenous antigens activate the host innate immune system and induce polarization of Th cells, which can be distinguished by the cytokines produced and immune responses [6]. Technically, probiotics alter the cytokines production by modulating cellular signal transduction. Probiotic administration induces Th1 profile response (↑TNF-α and ↑IFN-γ) while Th2 cytokines such as IL-4 stimulate the production of antibodies against the exogenous substances. Subsequently, the crosstalk induces expression of regulatory T cell anti-inflammatory cytokine such as IL-10 [7, 8]. A similar finding was reported, whereby the immune regulation associated with probiotic administration inhibited allergic response through a modulation of Th1/Th2 balance and an increase of regulatory T cells [9]. Hence, this describes the significance of studying immunomodulatory effects of probiotic strains in the treatment of various diseases.

Accurate quantification of cytokines is a powerful approach in the study of inflammation, in both in vitro cell culture system and in vivo animal model. Animals are well-accepted models to conduct preclinical testing of new probiotic to determine their immunological profile in terms of pro- and anti-inflammatory effects as well as safety in relation to strain-specific adverse effects, systemic infections, deleterious metabolic activities, gene transfer and excessive immune stimulation [10–13]. To date, enzyme-linked immunosorbent assay (ELISA) is deemed to be the gold standard as it provides the specificity and sensitivity required for cytokines detection [14]. Detection is established by complexing antibodies and antigen to produce quantitative data in the 96-well microtiter plate. In this chapter, the cytokines of interest consist of TNF-α, IFN-γ, IL-4, and IL-10. Categorically, TNF-α and IFN-γ are the pro-inflammatory cytokines, while IL-4 and IL-10 are the anti-inflammatory cytokines. Hypothetically, overexpression of pro-inflammatory cytokines may lead to unruly systemic inflammation. Therefore, anti-inflammatory cytokines are important to maintain the balance in the immune system [15]. Here, we provide a protocol for probiotic safety assessment via quantification of TNF-α, IFN-γ, IL-4, and IL-10 in probiotic administered BALB/c mice.

2 Materials

2.1 Preparation of Animal

1. BALB/c mice (female, $n = 20$).
2. Pellet diet.
3. Water.
4. Cage.
5. Animal house facility.

2.2 Preparation of Probiotic Strain

1. Probiotic strains.
2. Glycerol (Sigma).
3. MRS agar and broth (Sigma).
4. Anaerobic jar (chamber).
5. Sterile phosphate buffer saline (PBS).
6. Skim milk (20% w/v; cryoprotectant).
7. Spectrophotometer.
8. Deep freezer (−20 and −80 °C).
9. Lyophilizer.
10. Centrifuge.

2.3 Administration of Probiotic Bacteria

1. Probiotic bacteria.
2. Feeding gavage (oral gavage).
3. Sterile distilled water.
4. Skim milk.

2.4 Collection and Preparation of Animal Samples

1. Blood.
2. Ketamine.
3. Xylazine.
4. Tissues (heart, lung, liver, brain, and spleen).
5. Sterile 1× PBS.
6. Cell lysis buffer (100 mg/mL): 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, and 5 mM EDTA (*see* **Note 1**).
7. Protease inhibitor cocktail (pH 7.2).
8. PBS solution containing 0.05% Sodium azide and 0.5% Triton X-100.
9. Deep freezer (−80 °C).
10. Centrifuge.
11. Syringe.
12. K_3EDTA Vacutainers.

13. Tissue homogenizer.
14. Weighing machine.
15. Microcentrifuge tubes.

2.5 Materials and Equipment for ELISA Assay

1. *Coating buffer*: 0.05 M carbonate-bicarbonate, pH of 9.6; 2.88 g sodium bicarbonate ($NaHCO_3$) and 1.67 g sodium carbonate (Na_2CO_3) anhydrous in 1 L of distilled water.
2. *Washing buffer*: PBS-T which consists of 1× PBS [PBS; 120 mM sodium chloride (NaCl), 1.2 mM sodium phosphate monobasic (NaH_2PO_4), 2.8 mM potassium chloride (KCl), 8.8 mM sodium phosphate dibasic (Na_2HPO_4)] with 0.05% Tween-20, pH 7.4.
3. *Blocking buffer*: 2% bovine serum albumin (BSA), in 1× PBS, pH 7.4.
4. *Test antigen*: Animal samples specific to the protein of interest to be detected, i.e., plasma, serum, tissue homogenate, etc.
5. *Matched pair of antibodies* (can be monoclonal or polyclonal antibodies):

   Capture antibody: It should be from the host species of test antigen. For example, if the host of test antigen is a mouse, choose mouse TNF-α or IFN-γ or IL-4 or IL-10 antibody.

   Detection antibody: It should be from the host species of capture antibody. For example, if the host for capture antibody is mouse, choose mouse TNF-α or IFN-γ or IL-4 or IL-10 antibody (*see* **Note 2**).
6. *Enzyme-conjugated secondary antibody*: Horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibody.

   Secondary antibody: It should be against the host species antibody. For example, if the host is a mouse, choose rabbit anti-mouse TNF-α or IFN-γ or IL-4 or IL-10 antibody.
7. *Substrate*: 3,3′,5,5′-Tetramethylbenzidine (TMB) is highly recommended for HRP. 1% TMB dissolved in dimethyl sulfoxide (DMSO), diluted 1:100 in 0.1 M sodium acetate (pH 6.0) and 0.005% hydrogen peroxide (H_2O_2). Do not add in hydrogen peroxide (H_2O_2) until just prior to the use of the substrate. It is suggested to freeze aliquots of TMB (WARNING: TMB is a known carcinogen and light sensitive).
8. *Stop Solution*: Ready-to-use 0.2 M sulfuric acid (H_2SO_4). Store it at room temperature (Molecular weight of H_2SO_4 is 98.079 g/mol). 1 M stock solution: 98.079 g of H_2SO_4 in 1 L sterile water. 0.2 M solution: 19.616 g of H_2SO_4 in 1 L sterile water.
9. Recombinant proteins, i.e., TNF-α, IFN-γ, IL-4, and IL-10 as standards.
10. Test tube for serial dilution of recombinant protein.

11. 96-Well flat-surface polystyrene microtiter plates.
12. Multichannel pipette and single-channel pipette.
13. Incubator.
14. Automatic plate washer. If not available, blot the plate on tissue paper to remove excess or unbound materials.
15. Microplate reader.

3 Methods

3.1 Preparation of Animal Model

1. Prior to administration of probiotic strain, acclimatize BALB/c mice (female, $n = 20$) for 7 days under controlled conditions (temperature of 25 ± 2 °C, humidity at $60 \pm 2\%$ and a 12/12 h light-dark cycle) with unrestricted access to pellet diet and water.
2. Samples (i.e., blood) are collected from the mice at Day 0 (zero) after the acclimatization and on the same day prior to probiotic administration for continuous assessment of cytokines expression.

3.2 Preparation of Probiotic Strain

1. For the preparation of probiotic strain, inoculate it from glycerol stock on MRS agar and incubate the plate anaerobically for 48 h at 37 °C in an anaerobic jar.
2. After 48 h of incubation, transfer two to three single colonies of freshly cultured strain into MRS broth and adjust the culture to OD_{600} of 0.1 (approximately 1×10^5 CFU/mL).
3. Incubate the probiotic culture anaerobically for 12 h at 37 °C in an anaerobic jar (stationary phase).
4. After 12 h of incubation, harvest the cells by centrifugation at ~$9000 \times g$ for 15 min. Wash the cells twice with sterile PBS.
5. Reconstitute the pelleted cells (5×10^8 CFU/mL) in skim milk (20% w/v; cryoprotectant) and mix until it forms a homogeneous suspension.
6. Transfer the mixture into a new sterile centrifuge tube and freeze it for 4 h at −20 °C and overnight at −80 °C.
7. Transfer the frozen probiotic in skim milk into the freeze dryer for lyophilization.
8. Upon completion of the freeze-drying process, retrieve the lyophilised probiotic and store at 4 °C until further use (*see* **Note 3**).

3.3 Administration of Probiotic Bacteria

1. Determine the suitable route of administration based on the purpose of the research and the type of strain.
2. Reconstitute the lyophilized probiotic by dissolving it in sterile distilled water.
3. Administer 200 μL of the probiotic suspension (5×10^8 CFU/mL) and skim milk into the experimental mice group ($n = 10$) and control mice group ($n = 10$), respectively via oral gavage.
4. Lift the mice by scruffing or gently holding the head and body between thumb and index finger, and insert gavage through the mouth till it reaches the esophagus (*see* **Note 4**).
5. Slowly inject the probiotic and gently withdraw the gavage once done. Check the condition and activity of the mice to ensure the administration has been successfully performed.
6. With regard to safety assessment of probiotic, the frequency of administration may vary in accordance with the organization for economic co-operation and development (OECD) guidelines.
7. Single dose of probiotic shall be administered for acute oral toxicity assessment [16] while daily doses shall be administered up to 28 days for sub-acute oral toxicity assessment [17].

3.4 Collection and Preparation of Animal Samples

3.4.1 Blood

1. Collect blood from the appropriate region of mice according to one's timeline for continuous study (*see* **Note 5**).
2. Anesthetize the mice by delivering 100 μL of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) through intra-peritoneal injection followed by terminal bleeding to humanely sacrifice them.
3. For continuous blood collection, consider other collection routes such as retro-orbital plexus, sub-mandibular, saphenous, or tail vein [18].
4. Collect the blood samples in blood tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuge at 956 × *g* for 10 min at room temperature.
5. Collect the plasma and store it at 4 °C for further analyses (*see* **Note 6**).

3.4.2 Tissue Homogenates

1. Upon sacrifice, collect tissues from the heart, lung, liver, brain, spleen and rinse with 1× PBS to remove excess blood.
2. Immediately freeze the freshly harvested tissues at −80 °C for 1 h (*see* **Note 7**).
3. Weigh the required frozen tissues before the addition of cell lysis buffer (100 mg/mL).
4. Add protease inhibitor cocktail (pH 7.2) and leave the tissues overnight at 4 °C.

5. Homogenize the tissues in PBS containing 0.05% sodium azide and 0.5% Triton X-100.
6. Subsequently, centrifuge the homogenate at 10,621 × *g* for 5 min and transfer the supernatant to a new centrifuge tube, aliquot and store the supernatant at −80 °C until further use.

3.5 Direct Sandwich ELISA

This chapter describes two formats of ELISA for quantification of cytokines, namely direct and indirect sandwich ELISA as illustrated in Fig. 1a, b).

1. Coat the wells of polystyrene microtiter plate with 100 μL of the capture antibody (2 μg/mL) diluted with coating buffer.
2. Seal the plate and incubate overnight at 4 °C or 2 h at room temperature.
3. Discard the capture antibody suspension.
4. Wash the plate thrice with 200 μL 1× PBS-T.
5. Add 300 μL of blocking buffer to block the remaining protein-binding sites of the coated wells.
6. Seal the plate and incubate overnight at 4 °C or 2 h at room temperature.
7. Discard the blocking buffer.
8. Wash the plate twice with 200 μL 1× PBS-T.
9. Add 100 μL of test antigen into each sample wells and recombinant protein into standard wells, respectively (diluted with blocking buffer). For an accurate quantitative outcome, a standard curve is compulsory (Fig. 3). Run the standards and background control (blocking buffer ONLY) with each plate to ensure accuracy.
10. Seal the plate and incubate for 90 min at 37 °C.

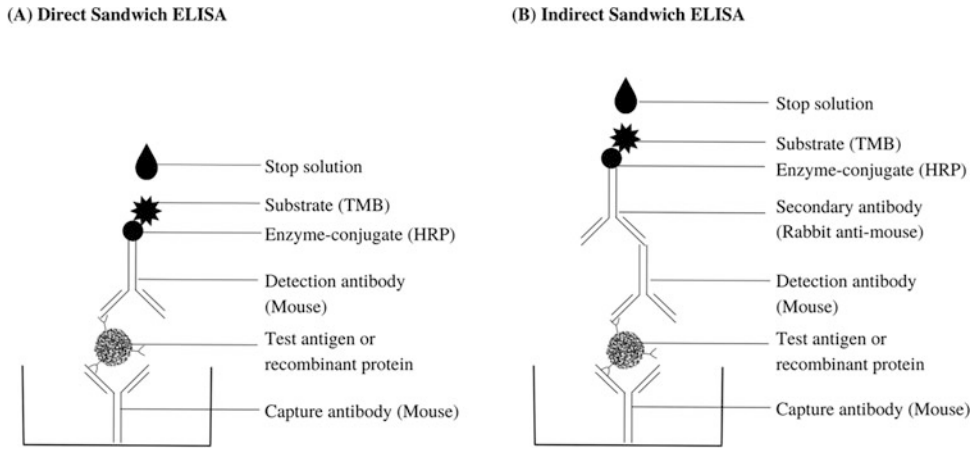

Fig. 1 Direct and indirect sandwich ELISA formats

11. Discard the test antigen and recombinant suspensions, respectively.
12. Wash the plate twice with 200 μL 1× PBS-T.
13. Add 100 μL of enzyme-conjugated detection antibody (for direct sandwich ELISA) while unconjugated detection antibody (for indirect sandwich ELISA) at a concentration of 2 μg/mL (dilute with blocking buffer) into each well.
14. Seal the plate and incubate for 2 h at room temperature.
15. Discard the contents from the well.
16. Wash the plate four times with 200 μL 1× PBS-T.

3.6 Indirect Sandwich ELISA

The following steps (**steps 17–20**) are intended for indirect sandwich ELISA, proceed to **step 21** for direct sandwich ELISA.

17. Add 100 μL of enzyme-conjugated secondary antibody diluted with blocking buffer (2 μg/mL) to each well.
18. Seal the plate and incubate for 2 h at room temperature.
19. Discard the enzyme-conjugated secondary antibody suspension.
20. Wash the plate five times with 200 μL 1× PBS-T.
21. Add 100 μL of H_2O_2 to 100 μL substrate solution prior to adding into the wells. Incubate the plate in the dark for 15–20 min to attain desired color intensity. A blue green color intensity indicates the cytokines level.
22. Add 50 μL of stop solution into the wells to stabilize color development for accurate cytokines measurement.
23. Read the plate using microplate reader of 450 nm wavelength. This offers a yellow product.

3.7 Preparation of Standard Curve Solution

The ELISA standard curve is prepared by making serial dilutions (Fig. 2) of standard, i.e., recombinant protein with known concentration followed by construction of standard curve plotting concentration against absorbance (OD value). Preparations of standard curve are described below:

1. Reconstitute 1 μg recombinant protein with 1 mL blocking buffer to yield a concentration 1 μg/mL of stock solution that is equivalent to 1,000,000 pg/mL.
2. Add 10 μL of reconstituted recombinant protein to a tube containing 990 μL blocking buffer to yield a concentration of 10,000 pg/mL.
3. Transfer 50 μL of the mixture in previous step to another tube containing 450 μL blocking buffer to yield concentration of 1000 pg/mL.

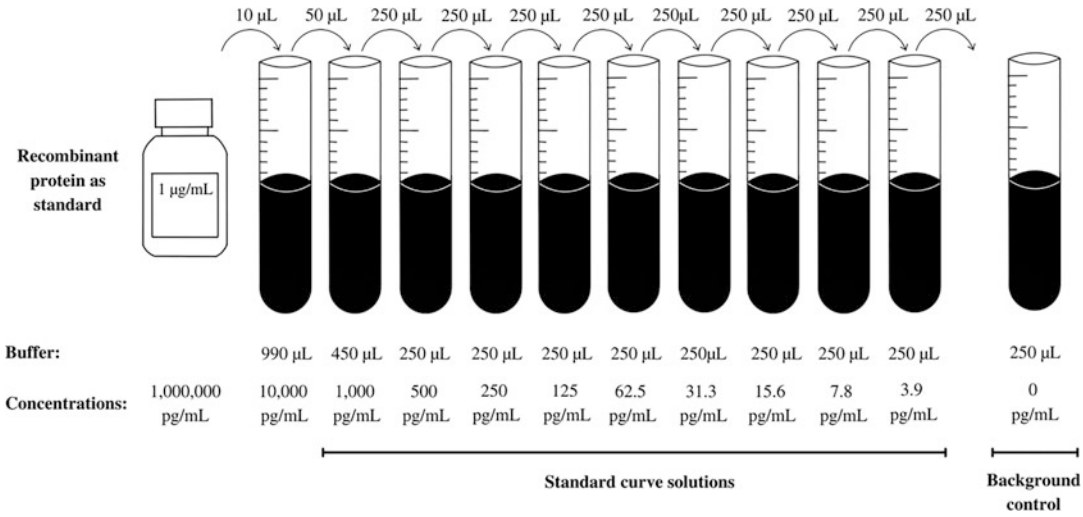

Fig. 2 Preparation of standard curve solutions

4. Transfer 250 µL of the mixture in previous step to another tube containing 250 µL blocking buffer to yield concentration of 500 pg/mL. Repeat the process to generate remaining points of standard curve (250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 pg/mL) (*see* **Note 8**). Discard 250 µL of mixture from the last dilution tube to standardize the final volume of the diluent at 250 µL.
5. Use 250 µL of blocking buffer as negative control.

3.8 Quantification of Cytokines (TNF-α, IFN-γ, IL-4, and IL-10)

The concentration of cytokines in each sample is determined by comparing the absorbance values of test antigen to the absorbance values of a standard, with known concentration in standard curve (Fig. 3).

1. Measure the absorbance values for individual set of duplicates, i.e., test antigen and recombinant protein.
2. Calculate the average of absorbance values. This value should be within 20% of the mean value.
3. Generate a standard curve by plotting the mean absorbance for each recombinant protein concentration on the ordinate (Y-axis) against the recombinant protein concentration on the abscissa (X-axis).
4. Draw a best fit curve through the points of the graph. A five-parameter curve fit is recommended.
5. After deriving a standard curve, calculate the mean absorbance value of the test antigen and find the mean value on the Y-axis. Then, extend a horizontal line to the standard curve.
6. At the point of intersection, extend a vertical line to the X-axis and read the corresponding value as the concentration of the test antigen.

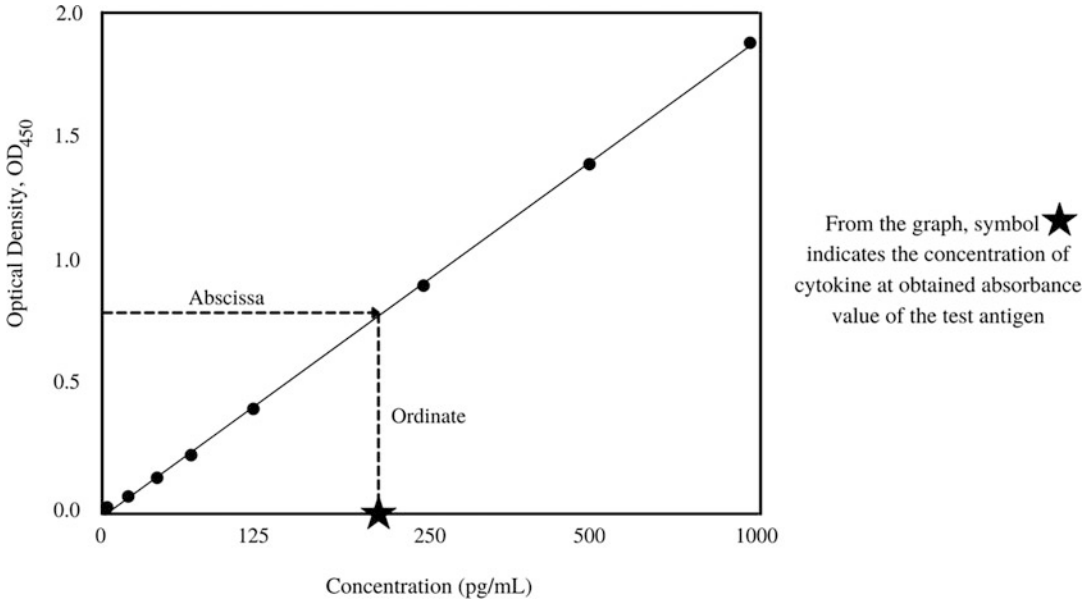

Fig. 3 Representative standard curve of ELISA

4 Notes

1. Protein yields may vary depending on the type of lysis buffer used. It is important to use a lysis buffer that does not contain sodium dodecyl sulfate (SDS) as this may affect stability of cytokines.
2. For direct sandwich ELISA, use enzyme-conjugated detection antibody.
3. Check for the viability of the lyophilised probiotic through viable cell counts on MRS agar before starting the animal experiment. Determine the colony forming unit (CFU) of the lyophilised probiotic strain upon 48 h of anaerobic incubation at 37 °C.
4. Little to no resistance should be observed while inserting the oral gavage. If you notice resistance in animal, you may have accidentally entered the trachea. If you notice fluid bubbling from the nose, stop administration immediately. For visual observation, insert blue food colouring with the probiotic. If the procedure is successful, the stomach of the mouse will have a visible blue hue. If the blue dye is found outside the stomach (neck, chest or axillary region), the animal should be humanely euthanized (in accordance with the animal care rules), as this indicates a rupture of the oesophagus or aspiration.
5. For continuous blood collection from a similar animal model, allocate at least three days gap between collection times to allow the animal to replenish their blood cells and prevent death, which is an unfavourable event for animal study.

6. There are a significant number of cytokines present in the serum, including TNF-α, IFN-γ, IL-4 and IL-10. As for serum collection, clotted blood is used (avoid keeping the sample with anticoagulant). Centrifuge at 1278 x g for 10 min at room temperature to collect serum then store at 4°C until further analysis.
7. Keep the harvested tissues on ice throughout the protein isolation process.
8. The typical ELISA standard curve may span concentrations from 0 to 1000 pg/mL or as high as 3000 pg/mL depending on the expected amount of cytokine in the sample. Consider a trial run of sample and adjust the concentration range of standard curve, if the absorbance of sample is higher than the concentration range of standard curve. For preparation of standard solution, use fresh tips after each dilution and use the solution no later than 2 h.

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Chapter 38

Detection of Myeloperoxidase Activity by Enzyme Linked Immunosorbent Assay

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Abstract

Myeloperoxidase is a heme-containing peroxidase enzyme, abundantly synthesized by neutrophils in diseased host. MPO synthesis indicates the state of inflammation. Myeloperoxidase reacts with hypochlorous acid (HOCl) and oxidizes chloride ion in the presence of H_2O_2. This chapter describes Enzyme Linked Immunosorbent Assay (ELISA) technique utilized to measure activity of myeloperoxidase enzyme. The monoclonal anti-myeloperoxidase antibody is coated on wells of an ELISA plate and attachment of MPO is detected with the help of suitable substrate.

Key words Myeloperoxidase, Antigen, Monoclonal antibody, ELISA, ADHP

Abbreviations

ADHP	10-Acetyl-3,7-dihydroxyphenoxazine
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CTAB	Cetyltrimethyl ammonium bromide
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
FBS	Fetal bovine serum
HBSS	Hanks balanced salt solution
K_2HPO_4	Potassium phosphate dibasic
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
$MgCl_2{\cdot}6H_2O$	Magnesium sulfate heptahydrate
$MgSO_4{\cdot}7H_2O$	Magnesium sulfate heptahydrate
$Na_2HPO_4{\cdot}2H_2O$	Disodium hydrogen phosphate dehydrate
$NaHCO_3$	Sodium bicarbonate
PBS	Phosphate-buffered Saline
TNBS	2,4,6-Trinitrobenzene Sulfonic acid

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_38,

1 Introduction

Myeloperoxidase (MPO) is a member of peroxidase superfamily that is primarily found in neutrophils; however in monocytes, a low amount can also be detected [1, 2]. Other cells such as macrophages, lymphocytes, microglia, etc. also exhibit MPO activity. MPO remains in azurophilic granules of neutrophils and is released only when neutrophil is active. Neutrophils are the most abundantly found granulocytes which is about 40–70% of total human white blood cells [3]. They are the first line of defense against microbial and other pathogenic agents. Neutrophilic polymorphonuclear leukocytes (PMNs) play an important role in defending the host [4]. On neutrophil activation, these azurophilic granules are excreted in extracellular space and phagolysosomal spaces [5].

Host cells recruit neutrophils during any foreign invasion followed by MPO release. Once neutrophil gets activated, NADPH oxidase is acquired inside the phagosomal membrane and produces superoxide radicals that are further converted to H_2O_2 by superoxide dismutase. H_2O_2 acts as an electron acceptor and in its presence, MPO plays a cationic role leading to the first step of the cycle which is electronic oxidation of native compound (Fe^{3+}MPO) to ferryl π cation radical, compound I (MPO–$Fe^{4+} = O^{\bullet +\pi}$) and water [6–8]. Native compound can be restored through electron reduction by halides leading to the formation of hypohalous (HOCl, HOBr, and HOI) [6]. Among other hypohalous, HOCl is formed significantly in higher amounts as the concentration of Cl^- is very high in human plasma. Later in peroxidase cycle, Compound II (MPO–Fe^{4+}-OH) is formed by Compound I through oxidation of organic compounds. Last redox form of MPO is complex III (MPO–Fe^{2+}-O_2) formed either directly by native enzyme or by complex II reacting with H_2O_2. This MPO-HOCl system has major role in pathogen elimination by neutrophils.

The increased levels of MPO in systemic circulation are generally linked with oxidative stress and inflammation and thus drive the crucial innate immune response leading to killing of microbes and host defense against foreign invasions [9]. MPO-knockout mice are used to determine the MPO activity in inflammatory pathways and studies have demonstrated the various role of MPO in inflammatory diseases such as atherosclerosis, heart-related diseases [10–12], multiple sclerosis, kidney diseases, Alzheimer's disease [13], lung cancer [2], neuronal diseases, pulmonary infection, transplant rejection [14], dermal inflammations, etc. In usual circumstances, HOCl derived by MPO helps to protect against bacteria and pathogens; however, in higher pathogenic loads, excessive release of these oxidizers leads to cellular damage in host cells.

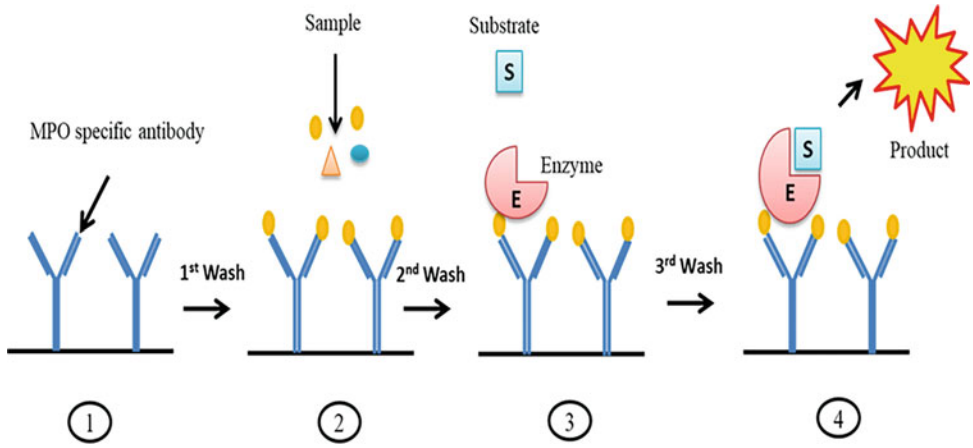

Fig. 1 Schematic representation of principle of ELISA. (**1**) ELISA plate is coated with MPO specific antibody, (**2**) antigen (MPO) is added to the wells that bind to the MPO-specific antibody, (**3**) enzyme (H_2O_2) is added to the wells, (**4**) substrate (ADHP) is added, catalyzed by enzyme, and forms a fluorescent product (Resorufin)

Probiotics are defined as "live non-pathogenic microorganisms which when administered in adequate amounts confer a health benefit on the host" [15–17]. Several studies have proved that altering gut microbiota with probiotics can be an approach to treat several diseases [18]. The extent of disease severity as well as probiotic potency in reducing them can be determined using some factors such as clinical symptoms, body weight, changes in cell morphology, cytokines level, and MPO activity. MPO is an indicator of neutrophil infiltration that shows the local immune response. Therefore, MPO detection is an important factor in diagnosis as well as in finding treatment of chronic diseases. Several methods are available for the detection of MPO activity including the use of probes, for example, guaiacol, o-dianisidine, 3,3′5,5′-Tetramethylbenzidine [19–22]. However, these detection methods are not very specific to MPO. Moreover, presence of inhibitors in tissues can also hinder the assays [23]. MPO expression levels are indicators of inflammatory load on cells. While interpreting the results obtained by mouse models, either wild-type or MPO-knockout (MPO-KO) mice, it is very important to keep in consideration that mice do not have defensins; therefore, estimated MPO level in mice is only about 10–20% of the present neutrophils [1]. Here, we have provided a detailed protocol which states the detection of intracellular as well as extracellular MPO. It is a highly specific, reliable method based on antibody capture activity assay. The principle of this method is based on antigen-antibody interaction that is further being quantified (Fig. 1).

2 Materials

Prepare all solutions in ultrapure water and stored at room temperature for higher sensitivity.

1. Sucrose: To prepare 1 L of 0.32 M sucrose, add 109.53 g of sucrose in 1 L of deionized water.
2. $CaCl_2$: To prepare 1 L of 1 mM $CaCl_2$, add 0.111 g of $CaCl_2$ in 1 L of deionized water.
3. Heparin Sodium 1000 USP Units/mL.
4. Phosphate buffer saline (PBS): To prepare 1 L of PBS buffer, add 8 g of Sodium Chloride (NaCl), 200 mg of Potassium Chloride (Kill), 1.44 g of Disodium hydrogen phosphate (Na_2HPO_4), and 245 mg of Potassium dihydrogen phosphate (KH_2PO_4) to 800 mL of distilled water and adjust pH 7.
5. Acetone (200 mL).
6. HBSS: To prepare 1 L of HBSS, add 8 g of NaCl, 400 mg of KCl, 140 mg of $CaCl_2$, 100 mg of $MgSO_4{\cdot}7H_2O$, 100 mg of $MgCl_2{\cdot}6H_2O$, 60 mg of $Na_2HPO_4{\cdot}2H_2O$, 60 mg of KH_2PO_4, 1 g of D-Glucose (Dextrose), 350 mg of $NaHCO_3$ in 800 mL of distilled water and volume it up to 1 L.
7. DMSO.
8. MPO ELISA dilution buffer.
9. CTAB buffer: To prepare CTAB buffer, add 50 mM potassium phosphate (pH 6.0) to 50 mM CTAB and store it at room temperature (RT).
10. Extraction buffer: To prepare extraction buffer, add 0.32 M sucrose, 1 mM $CaCl_2$, and 10 U/mL Heparin in HBSS and store it at RT.
11. DPBS: To prepare 1 L of DPBS, add 100 mg of $CaCl_2$, 200 mg of KCl, 100 mg of $MgCl_2{\cdot}6H_2O$, and 8 g of NaCl in 800 mL of distilled water and volume it up to 1 L. Filter-sterilize and store it at 25 °C.
12. Fetal bovine serum (FBS).
13. Bovine serum albumin (BSA).
14. ADHP stock solution: 200 mM ADHP in DMSO; prepare 15 μL aliquots and store it at −20 °C for up to 1 year.
15. ADHP working solution: Prepare right before assay, dilute ADHP stock solution (1:1000) in PBS.
16. H_2O_2 working solution: Prepare right before assay, dilute 3% H_2O_2 (1:100) in PBS.
17. Washing buffer: Add 500 μl of Tween-20 in 1 L of PBS.

3 Methods

3.1 Sample Extraction from the Animals Treated with the Probiotic Strains

1. Divide animals into three groups: (1) disease control, (2) PBS control, and (3) probiotic dose receivers.
2. Keep the animals in standard conditions of alternate light and dark cycles (12 h each) at 25 ± 2 °C and with free access of food and water ad libitum.
3. Induce disease in disease control and probiotic group by administrating certain compounds (e.g., TNBS in case of colitis model).
4. After induction of disease, feed second set of mice with probiotics dose, whereas administer PBS to disease control and PBS control for certain period of time as per experimental design.
5. Sacrifice the animals at the end of test period; extract and weigh tissue strips from diseased cells as per method described by Pradhan et al. [24].
6. Suspend the tissue strips in PBS buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0).
7. Homogenize the tissue and centrifuge at 13,400 × *g* for 6 min. at 4 °C.
8. Collect the supernatant for MPO activity as described below with ADHP as substrate.

3.2 Determination of MPO Activity

The MPO activity is determined from extracellular as well as intracellular protein fractions from the organ of interest as per method previously described by Pulli et al. [19] (Fig. 2). Collect the organ of interest from anaesthetized mouse in petri plate and wash it properly with PBS buffer (*see* **Note 1**).

3.2.1 Extraction of Extracellular Protein

1. Wash the collected tissues or organ and incubate it in extraction buffer for 2 h on ice to extract extracellular proteins (*see* **Note 2**).
2. Spin the extraction buffer containing extracellular proteins at 500 × *g* for 5 min at 4 °C. Discard debris and collect supernatant in a fresh falcon tube.
3. Add pre-chilled acetone approximately four times of supernatant and incubate it at 20 °C for 1 h for precipitation of extracellular proteins. Centrifuge at 3500 × *g* for 15 min at 4 °C and collect precipitated proteins (*see* **Note 3**).
4. Remove the acetone completely by air drying and resuspend the pellet in PBS by pipetting without making bubbles (*see* **Note 4**).

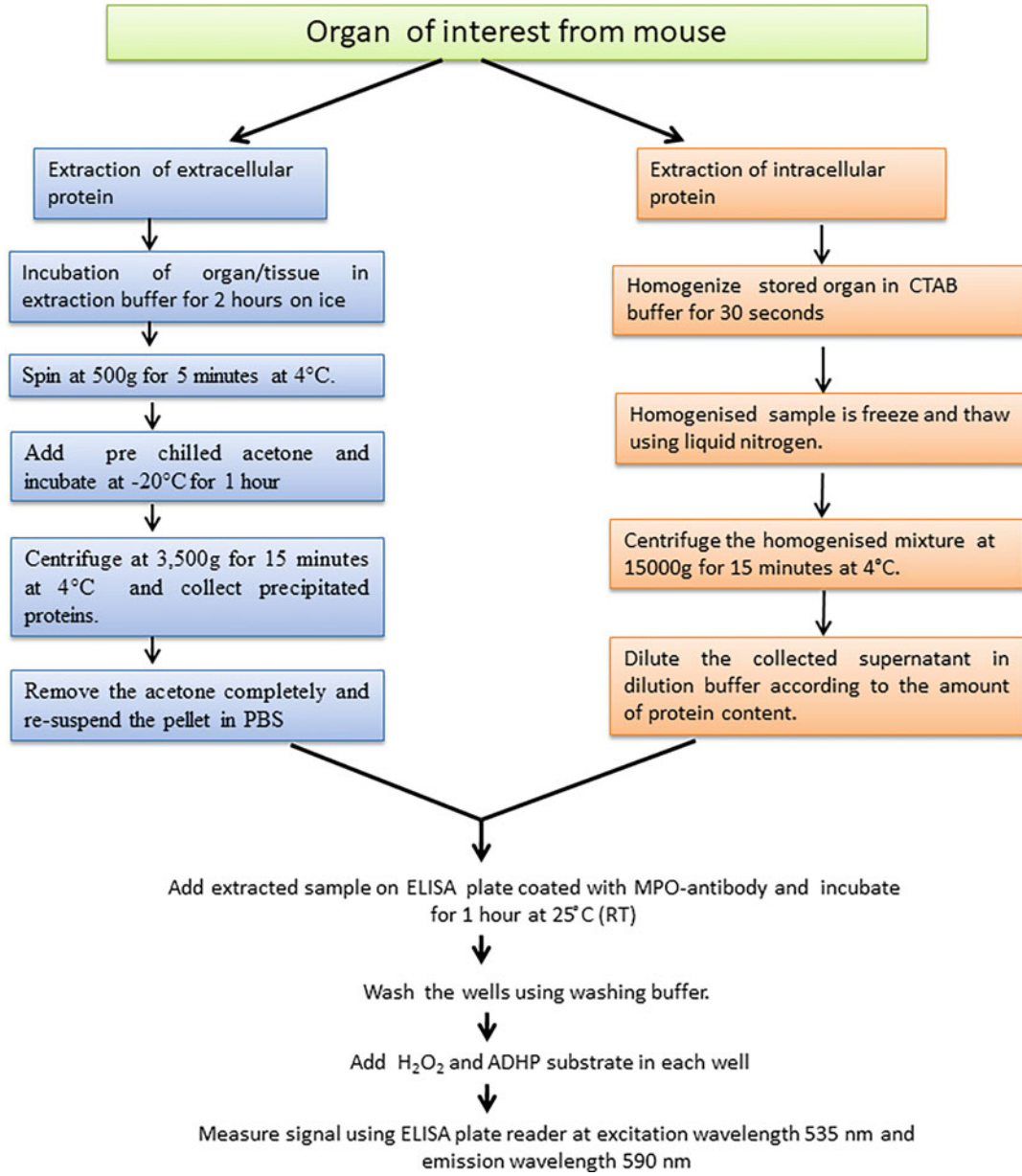

Fig. 2 Protocol for measuring MPO activity in biological samples by using ADHP

3.2.2 Extraction of Intracellular Proteins and Determination of MPO Activity

1. Homogenize the stored organ for 30 s in CTAB buffer. Homogenized sample is frozen and thawed using liquid nitrogen.
2. Centrifuge the homogenized mixture at 15,000 × *g* for 15 min at 4 °C. Supernatant is collected for MPO activity assay (*see* **Note 5**).
3. Dilute the collected protein (resuspended pellet of extracellular protein and supernatant from extraction of intracellular protein step) in ELISA dilution buffer according to the amount of protein content.

4. Coat the flat bottom 96-well ELISA plate with MPO-antibody. Add 100 μl of diluted samples in each well and incubate it for 1 h at room temperature.
5. After 1 h of incubation, remove samples and wash the wells by using 300 μL of washing buffer. Further, add 49 μL PBS to each well and 1 μL of H_2O_2 working solution to each well.
6. Add 50 μL of ADHP working solution to each well immediately, so that quick reaction can occur, which converts ADHP to fluorescent compound known as "resorufin" (*see* **Note 6**). Acquire fluorescence in kinetic mode for 5–10 min (excitation at 535 nm and emission at 590 nm) using plate reader (a linear increase should be observed).
7. Analyze the data by subtracting PBS as negative control and plot it by using $y = mx + c$ equation, where y is the value on y axis, x is the value on x axis, and c is vertical intercept of the line and m is the slope.
8. The slope of the graph will represent MPO activity (RFU/mg of MPO protein). MPO activity is expressed as units per milligram of wet tissue, 1 U represents the conversion of 1 μM of H_2O_2 to water in 1 min at room temperature.

3.3 Positive Control

For the MPO positive control, supernatant is collected by isolating neutrophils and processed in the same way as mentioned above using ADHP. The protocol for neutrophil isolation and harvesting supernatant is given below [19].

1. Harvest red blood cells (RBCs) from bone marrow by flushing it with staining buffer (DPBS, 0.5% BSA, 1% FBS). Centrifuge the RBCs at 400 × *g* for 7 min.
2. Lyse the RBCs using lysis buffer. Further wash cells and centrifuge on 0–62% discontinuous percoll gradient at 1000 × *g* for 30 min.
3. Decant the supernatant and collect the neutrophils containing pellet. Cells are further washed, homogenized by tissue homogenizer and sonicated.
4. Freeze and thaw the homogenized sample repeatedly three times using liquid nitrogen and centrifuge at 15,000 × *g* for 20 min and collect supernatant for MPO activity as described above with ADHP as substrate (*see* **Note 7**).

4 Inference

In the aforementioned protocol, MPO activity is quantified in biological samples by using ADHP, which has a wider assay range and sensitivity. Extracellular MPO activity implies to the oxidative stress and intracellular MPO activity relates to neutrophil content in

the tissues. This intracellular MPO can be used as a biomarker to evaluate neutrophil percolation. This protocol briefly describes the most efficient method for the detection of intracellular MPO activity; hence, it can be used as a diagnostic tool. MPO activity determined from set of mice receiving probiotic doses should not exceed from that of disease control indicating that they can be given to reduce diseased condition. The bacterial strains are not considered for probiotic use, if the MPO activity is 100 RFU/mg.

5 Notes

1. If blood remains in the organ, possible reason could be poor perfusion technique. The possible solution is venepuncture only once in left ventricle using 25 G needle and increase the volume of perfusate.
2. To avoid cell death, possible solution is to use fresh ice during incubation.
3. If protein pellet is not visible, possible reason could be the low amount of protein content which can be solved by increasing the centrifugal force or time of centrifugation.
4. If the dried pellet is not going in solution reason can be either pellet is under-dried, over-dried, or poor handling; solution is to increase or decrease drying time and use pipette to mix PBS to the pellet.
5. If supernatant is not clear, increase homogenization time or centrifugation time.
6. Immediately add ADHP to the wells and record the data in order to receive precise MPO activity.
7. If MPO activity is not shown in neutrophils standards, possible reason can be unsuccessful neutrophil collection which can be solved by using flow cytometry for neutrophil collection [25].

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Chapter 39

Assessment of Bacterial Translocation Through Mesenteric Lymph Nodes [MLN] and Spleen Cultures

Fida Fathima and P. D. Rekha

Abstract

Bacterial translocation is a phenomenon in which the gut bacteria cross to extraintestinal sites and are primarily studied using in vivo model system. Microbiological analysis of mesenteric lymph nodal and spleen tissues are the most commonly used specimen to demonstrate the bacterial translocation in the animal models. Histopathological analyses also help to detect and visualize the bacteria in the tissues using appropriate staining technique. More recently, detection of microbial DNA isolated from the blood by polymerase chain reaction (PCR) using 16S rRNA genes is proved to have higher sensitivity.

Key words Bacterial translocation, Microbiological analysis, Mesentric lymph node, 16S rRNA sequencing

1 Introduction

Bacterial translocation is the passage of viable indigenous bacteria from the gastrointestinal (GI) tract to the mesenteric lymph node complex [MLN] and other extraintestinal sites, including the spleen, liver, kidney, peritoneal cavity, and bloodstream [1, 2]. Gram-negative, facultatively anaerobic Enterobacteriaceae, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, translocate at a greater rate from the GI tract to the MLN compared to the other bacteria of the indigenous GI microflora [2, 3]. Bacterial translocation is more common in conditions including hemorrhagic shock, intestinal obstruction, major burn injury, and acute trauma, which are associated with a high risk of gram-negative bacteria infections and multiple organ failure [4].

Primary mechanisms that promote bacterial translocation include intestinal bacterial overgrowth, increased intestinal permeability, and host immunodeficiency (Fig. 1). Intestinal bacterial overgrowth is common in patients receiving antibiotic therapy. This condition can be mimicked in an animal model, by giving

Mitesh Kumar Dwivedi et al. (eds.), *Biosafety Assessment of Probiotic Potential*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-2509-5_39,

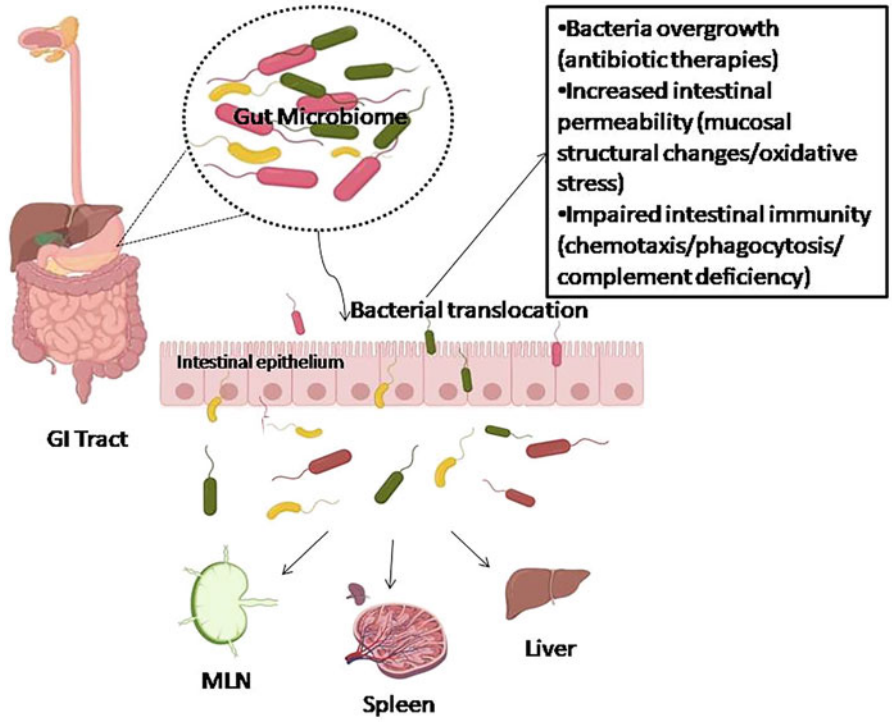

Fig. 1 Bacterial translocation from gastrointestinal tract to extraintestinal sites and its possible mechanisms

them oral antibiotics against indigenous obligate anaerobes, i.e., penicillin, clindamycin, or metronidazole. In increased intestinal permeability, the bacteria engulfed by the intestinal epithelial cells and transported intra-cellularly across the intestinal barrier. Physical damage to the intestinal mucosa or increased intestinal permeability also leads to the movement of intestinal bacteria through the epithelial barrier. For example, ricinoleic acid (12-hydroxy-9-octadecenoic acid), an active constituent of castor oil, given once to mice severely damages the intestinal mucosa and promotes bacterial translocation [5]. Damage created by shock with ischemia/reperfusion to the intestinal mucosa also promotes bacterial translocation from the GI tract [2, 6].

1.1 Measures of Bacterial Translocation

Several methods have been used to identify bacterial translocation, including direct and indirect methods. The identification of intestinal bacteria in sterile MLN is considered as the direct method. Sampling of MLN is a method mostly used in experimental and clinical studies. Data using radioactively labeled bacteria is another direct method to measure bacterial translocation, indicating that translocation can occur even if the culture of MLN failed to identify any microbe. Detection of intestinal bacteria in cultures of the portal or peripheral blood is an indirect method of measuring bacterial translocation [6, 7]. Further, polymerase chain reaction

(PCR) based techniques have been also introduced for detecting microbial DNA in the blood. These methods are known to have higher sensitivity than blood cultures for assessing the bacterial translocation from the intestine [6, 8]. Here, a few methods are explained that are used for studying the bacterial translocation.

2 Materials

2.1 Reagent Preparation

2.1.1 Phosphate Buffered Saline (PBS) (1×, pH 7.4)

To prepare 1 L of 1×, dissolve the reagents listed below in 800 mL of sterile distilled water. Adjust the pH to 7.4 and make final volume to 1 L.

Reagent	Quantity (g)	Final concentration mM (1×)
NaCl	8.0	137.0
KCl	0.2	2.7
Na_2HPO_4	1.4	10.0
KH_2PO_4	0.2	1.8

Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi or by filter sterilization. Store the prepared PBS solution at room temperature (RT) in an airtight glass bottle.

2.2 Reagents for Gram Staining

2.2.1 Gram Crystal Violet Solution

- Dissolve 20 g of crystal violet in 100 mL of 95% ethanol to make the stock solution.
- Dissolve 1 g of ammonium oxalate in 100 mL of water to make an oxalate stock solution.
- Working solution is obtained by mixing 1 mL of the crystal violet stock solution with 10 mL of water and 40 mL of the oxalate stock solution. Store the working solution in a drop bottle.

2.2.2 Gram Iodine Solution

Dissolve 1 g of iodine, 2 g of potassium iodide, and 3 g of sodium bicarbonate in 300 mL of water.

2.2.3 Gram Decolorizer Solution

Mix equal volumes of 95% ethanol and acetone.

2.2.4 Gram Safranin Solution

- Dissolve 2.5 g of safranin in 100 mL of 95% ethanol to make a stock solution.

- Working solution is obtained by diluting one part of the stock solution with five parts of water.

2.3 Preparation of Alcohol Gradient (for 1 L)

Percentage (%)	Volume of ethanol (mL)	Volume of distilled water (mL)
90	900	100
70	700	300
50	500	500

2.4 Bacteriological Media Preparation

Selective culture media are used for the isolation of bacteria. The commonly used media include blood agar, phenylethyl alcohol agar, brain-heart infusion agar, and MacConkey agar. Media are to be prepared according to the manufacturer's instructions.

2.5 Preparation of Buffers for DNA Isolation (Using QIAamp DNA Mini Kit)

- Buffer ATL:

  Before starting the procedure, check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70 °C with gentle agitation.
- Buffer AL:

  Before starting the procedure, check whether precipitate has formed in Buffer AL. If necessary, dissolve by heating to 70 °C with gentle agitation.
- Buffer AW1:

  Add 25 mL ethanol (96–100%) to the bottle containing 19 mL Buffer AW1 concentrate.
- Buffer AW2:

  Add 30 mL ethanol (96–100%) to the bottle containing 13 mL Buffer AW2 concentrate [9].

2.6 Primer Designing

For 16S rRNA gene amplicon sequencing, we use primers flanking two commonly used variable regions, the V1, V2 and V3, V4 regions.

Primers for 16S rRNA gene are designed using Primer3 plus online tool. The primer sequences used are given below:

Forward primer-27F	5′-AGAGTTTGATCCTGGCTCAG-3′
Reverse primer-1492R	5′-GGTTACCTTGTTACGACTT-3′

2.7 Composition of PCR Reaction Mix

Prepare the PCR reaction mix, as shown in Table 1.

Table 1
Preparation of PCR reaction mix

Components		Volume (μL)
Template		10
Primer [16S Universal]	Forward	1
	Reverse	1
Master mix		12
Milli Q water		1
Total volume		25

3 Methods

All surgical procedures are performed under strict sterile conditions with sterile instruments. Surgical procedures for microbial analysis from blood and tissues are provided in the following sections.

3.1 Animal Preparation

Use male Wistar rats, with an initial weight between 200 and 250 g in the experiment. Animals should be housed in appropriate cages and allowed free access to water and feed until the time of the study. Experiments should be performed under ketamine anesthesia (100 mg/kg body weight intramuscularly).

Bacterial translocation of animals can be created in the laboratory by either of the below methods:

1. To induce translocation by intestinal bacterial overgrowth, the animals are treated with penicillin G (500 U/mL), clindamycin (0.5 mg/mL), or metronidazole (1.0 mg/mL) for 4 days along with drinking water when they are 8 weeks old.
2. Translocation by increased intestinal permeability is attained by oral administration of castor oil (2 mL) to each rat which is being starved for 12 h before the experiment, but accessible to drinking water [2, 5, 9, 10].

3.2 Microbiological Analysis from Blood

1. Shave the abdominal skin of the rat and sterilize with iodine.
2. Open the cavity under anesthesia through a midline incision using strict sterile technique using a sharp scalpel.
3. Carefully expose the abdominal viscera and exsanguinate from a systemic vein.
4. The blood collected will be used for bacterial culturing.
5. Inoculate 5 mL of blood into aerobic culture bottles and incubate at 35 °C for 5 days. (Allow the remaining blood to clot at room temperature.)

6. Subculture the bacteria onto selective agar plates (blood agar, phenylethyl alcohol agar, brain-heart infusion agar, or MacConkey agar) and perform gram staining and other biochemical tests for identification.
7. 16S rRNA-based identification can also be performed by isolating the DNA from the bacterial cultures.

3.3 Microbiological Analysis from Tissue

1. From the dissected animals, harvest the tissues carefully aseptically. Remove and weigh the caudal and cranial MLNs and spleen.
2. Homogenize a portion of MLNs and spleen in PBS. Fix the remaining portions in 4% neutral buffered formaldehyde.
3. Plate aliquots of 0.1–0.2 mL homogenate onto agar plates (blood agar, phenylethyl alcohol agar, brain-heart infusion agar, or MacConkey agar) for aerobic and anaerobic bacterial identification.
4. A mesophilic aerobic plate count is made for the total aerobic colony count by placing the samples on agar media and incubating them at 35–37 °C for 1–3 days. Count the number of colonies formed.
5. After incubation, counts of colonies are presented as colony-forming units (CFU) per mL of organ homogenate and the colonies are identified by studying their morphological and biochemical properties [1, 3, 4, 7, 11].
6. Standard biochemical tests are used for microbial identification. The commonly used biochemical tests include catalase, coagulase, oxidase, indole, sulfur, urease, triple sugar iron test, nitrate test, starch hydrolysis test, carbohydrate fermentation test, methyl red test, Voges–Proskauer test, citric acid utilization test, and bile esculin agar test [11–13].
7. Inference: Positive MLN cultures are considered as indicative of bacterial translocation from the intestinal lumen. Positive blood, spleen, cultures are considered as indicative of the passage of bacteria to the portal and/or systemic circulation [4].

3.4 Histological Analysis

Subject the portion of tissue sample fixed in formalin for standard histological analysis. Embed the mesenteric lymph nodes and spleen tissues in paraffin and stain by using Gram's method to identify the presence of Gram-positive bacteria by light microscope. The detailed method is given below.

3.4.1 Paraffin Embedding and Sectioning

1. Dehydration: Transfer the block of tissue through a series of alcohol-water solutions (begin with 50% and run up to water-free or absolute alcohol).
2. Clearing: Use an organic solvent such as xylene to remove the alcohol and allow infiltration with paraffin wax.

3. Embedding:
   (a) Open the tissue cassette, to ensure the correctly identified tissue pieces are present.
   (b) Select the mold and fill with paraffin wax. (There should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax.)
   (c) Using warm forceps select the tissue, taking care that it does not cool in the air at the same time.
   (d) Place the tissue in the mold according to the side to be sectioned. This side should be facing down against the mold. A small amount of pressure may be used in order to have more even embedding.
   (e) Chill the mold on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.
   (f) Insert the identifying label or place the labeled embedding ring or cassette base onto the mold.
   (g) Add more paraffin into the mold to fill the cassette and mold.
   (h) Cool the block on the cold plate and remove from the mold.
4. Tissue sectioning and slide preparation:
   (a) Cut the paraffin sections into thin slices of 5 μm using a microtome.
   (b) Allow them to float in a water bath at 56 °C.
   (c) Mount the sections onto histological glass slides carefully.
   (d) Dry the slides overnight at room temperature.
   (e) Slides with paraffin-embedded sections can be stored either at room temperature or at 2–8 °C for several years in slide storage boxes.
5. Staining:
   (a) Before staining, deparaffinization is done. For removing the paraffin, wash the section with xylene for 5 min three times. Rehydrate with alcohol gradient of 100%, 90%, 70%, and 50% ethanol for 10 min each. Finally, give two washes using deionized water for 5 min.
   (b) Stain the slides initially with crystal violet (1%) to the tissue sections for 5 min at room temperature.
   (c) Rinse the slides under running tap water to remove excess crystal violet.

(d) Add Gram iodine mordant for 2 min to the tissue sections and wash in tap water.

(e) Wash with alcohol for 30 s, then quickly rinse under running tap water until the water runs clear.

(f) Stain with Gram Safranin for 1 min and 40 s and followed by dehydration through a series of alcohols (95–100%) to xylene and then place the coverslip [14, 15].

(g) Finally, the presence of Gram-positive and negative bacteria is identified by light microscope. High magnification images are obtained using a 100× objective under oil immersion.

3.5 Detection and Identification of Bacterial DNA

3.5.1 DNA Isolation

1. DNA is extracted using the QIAamp DNA Mini/Micro Kit (Qiagen) as per the manufacturer's protocol. The steps are given below:

(a) Cut tissue (≤25 mg) into small pieces and place in a 1.5 mL microcentrifuge tube.

(b) Add 180 µL Buffer ATL and 20 µL Proteinase K, mix by vortexing and incubate at 56 °C until completely lysed (1–3 h). Vortex occasionally during incubation.

(c) Add 200 µL Buffer AL. Mix thoroughly by vortexing for 15 s.

(d) Incubate at 70 °C for 10 min. Briefly centrifuge the tube to remove drops from the lid.

(e) Add 200 µL ethanol (96–100%). Vortex for 15 s. Briefly centrifuge the tube to remove drops from the lid.

(f) Pipet the mixture onto the QIAamp Mini spin column (in a 2 mL collection tube).

(g) Centrifuge at 6000 × *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.

(h) Place the QIAamp Mini spin column in a new 2 mL collection tube and add 500 µL Buffer AW1.

(i) Centrifuge at 6000 × *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.

(j) Place the QIAamp Mini spin column in a new 2 mL collection tube and add 500 µL Buffer AW2. Centrifuge at full speed (20,000 × *g*; 14,000 rpm) for 3 min. Discard the flow-through and collection tube.

(k) Place the QIAamp Mini spin column in a new 2 mL collection tube and centrifuge at full speed for 1 min. This eliminates the chance of possible Buffer AW2 carryover.

Table 2
PCR conditions

Steps	Temperature (°C)	Time
1. Initial denaturation	95	5 min
2. Denaturing	95	45 s
3. Annealing	54	45 s
4. Extension	72	50 s
(Steps 2, 3, and 4) No. of cycles = 35		
5. Final extension	72	10 min
6. Cooling	4	∞

(l) Place the QIAamp Mini spin column in a new 1.5 mL microcentrifuge tube and add 200 μL Buffer AE or distilled water and incubate at room temperature for 1 min.

(m) Centrifuge at 6000 × *g* (8000 rpm) for 1 min. to elute the DNA.

(Note: DNA isolation protocol will vary based on the manufacturing companies of the kit.)

2. Measure the yield and purity of DNA by reading A_{260} and A_{260}/A_{280} using Nanodrop.

3.5.2 DNA Amplification

The DNA amplification is carried out using the PCR conditions as shown in Table 2.

3.5.3 16S rRNA Sequencing and Data Analysis

The 16S rRNA sequencing enables the identification of specific microorganisms that are translocated rather than just detecting them by microbiological and histological assessments. The below mentioned steps are given for *16S rRNA* sequencing and data analysis (Fig. 2).

1. Samples are normalized to the desired library concentration using nuclease-free water, pooled, and sequenced on the MiSeq platform using a 500-v2 cartridge (Illumina).
2. The gene sequences are aligned and compared with available standard sequences of bacterial lineage in the National Centre for Biotechnology Information (NCBI), using Basic Local Alignment Search Tool (BLAST).
3. *16S rRNA* sequences are aligned according to the GenBank specifications using the software Sequin [9, 16].

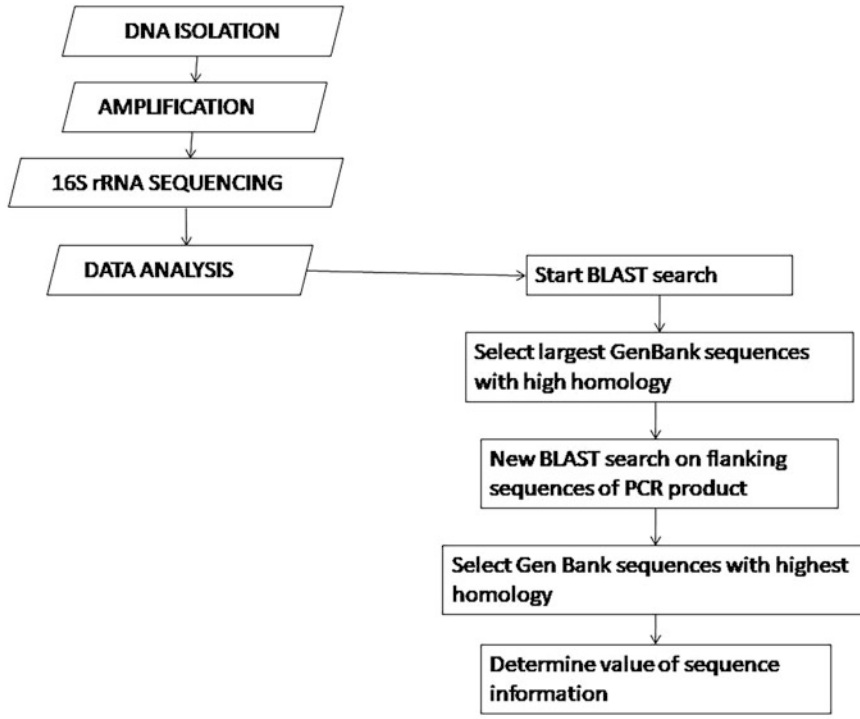

Fig. 2 Pipeline and bioinformatic tools for the analysis of *16S rRNA* data

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