J.Natn.Sci.Foundation Sri Lanka 2023 51 (3): 375 – 386
DOI: http://dx.doi.org/10.4038/jnsfsr.v51i3.11097

RESEARCH ARTICLE

Plant Microbiology

Probiotic potential of bacterial endophytes isolated from leaves of *Murraya koenigii* L.

AC Bandara[1*], CL Abayasekara[1], AM Karunaratne[1] and GJ Panagoda[2]

[1] *Department of Botany, Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka.*
[2] *Division of Microbiology, Department of Oral Medicine and Periodontology, Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka.*

Submitted: 24 February 2022; Revised: 06 September 2022; Accepted: 28 October 2022

Abstract: Probiotics are live microorganisms which confer health benefits to the host when administered in adequate amounts. Endophytes, which live in plant tissues between the plant cells, can act as potential probiotic bacteria. The leaves of *Murraya koenigii* L. is a common cuisine and herbal ingredient used in indigenous medicine. The main objective of the current study was to assess probiotic characteristics of isolated bacterial endophytes of *M. koenigii* leaves. Young and mature leaves of *M. koenigii* were collected from sites in the wet and dry zones of Sri Lanka. The endophytes were isolated using three techniques: placing leaf segments, leaf macerations and preparation of pour plates on three different media *viz*., nutrient agar, Luria-Bertani (LB), and De Man, Rogosa and Sharpe agar (MRS). They were identified from morphological characteristics and preliminary biochemical tests. Probiotic characterization of the isolates was carried out by using a series of standard tests including resistance to low pH, tolerance of bile salts, antimicrobial activity (*Escherichia coli* and *Pseudomonas aeruginosa*), antibiotic resistance (Gentamycine), anti-haemolytic activity, and non-DNase activity. Ten endophytic bacteria (two bacilli, eight cocci including two Gram positive cocci, and eight Gram negative cocci and bacilli) were isolated from the young and mature leaf samples collected. While probiotic characterization tests were positive, four isolates showed antibiotic susceptibility. These results indicate the possibility that *M. koenigii* leaves possess bacterial endophytes with probiotic potential.

Keywords: Antibiotic resistance, antimicrobial activity, endophytes, *Murraya koenigii* L., probiotics.

INTRODUCTION

Probiotics have been referred to as 'live microorganisms, which when administered in adequate amounts confer a health benefit on the host' (Shuhadha *et al*., 2017). Probiotic bacteria can be broadly categorized as plant probiotics and human probiotics depending on their potential host (Oh, 2017), and this study focuses mainly on human probiotics. Human probiotics are mostly Gram-positive microorganisms, which comprise a wide range of the genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus*, including the mostly studied species *Lactobacillus acidophilus, L. casei* group, *L. reuteri*/, *L. fermentum, Bifidobacterium* spp. (*B. animalis*) and *Enterococcus faecium* (Holzapfel *et al*., 2015; Shuhadha *et al*., 2017).

However, there are a few gram-negative bacteria that are considered as probiotics, *viz: Escherichia coli* strain Nissle 1917, *Bacteroides fragilis*, and *Akkermansia muciniphila* (Bhensen *et al*., 2013; Wang *et al*., 2017; Zhai *et al*., 2018).

The most important criteria for a probiotic are that it should have a history of being non-pathogenic, not being associated with diseases such as infective endocarditis or gastrointestinal disorders, ability to survive passage through the digestive tract by exhibiting acid and bile tolerance, ability to proliferate in the gut, and ability to exert their benefits on the host through growth and/or activity in the human body (Kimoto *et al*., 2004; Pineiro & Stanton, 2007). Probiotic characterization is done by considering the major characters of probiotics in the human gastrointestinal tract. Being of human origin, the major criteria

* Corresponding author (adheeshachamodini@gmail.com; https://orcid.org/0009-0009-6379-1405)

T ished under the Creative Commons CC-BY-ND License (http://creativecommons.org/licenses/by-nd/4.0/). This license p ution and reproduction, commercial and non-commercial, provided that the original work is properly cited and is not changed in anyway.

considered for probiotic characterization are antimicrobial activity against pathogenic bacteria, acid tolerance, bile salt tolerance, resistance to antibiotic compounds, anti- haemolytic activity, anti-hydrolysing of DNA, adherence to human epithelial cells, and persistence in the human gastrointestinal tract (Shuhadha *et al*., 2017). Auto-aggregation, hydrophobicity, cholesterol removal, and conjugated linoleic acid (CLA) conversion are some additional probiotic characterization tests (Alkalbani *et al*., 2019)

However, among these criteria, being of human origin has not been a highly required character to be a probiotic because there are probiotics of dairy and plant, which serve as efficient and safe probiotics in humans (Da Sesto, 2008).

Of the microorganisms associated with plants, endophytes are a variety of bacteria and fungi that reside inside the internal tissues of living plants (collectively known as endophytic microorganisms), which do not cause any symptoms and obvious harm to the host plants (Yiing & Ting, 2015). Most of the probiotics extracted from plants live as endophytes though a very few species live as epiphytes and rhizobacteria that are inhabitants of the rhizosphere (Ruiza *et al*., 2011). These plant endophytic probiotics synthesize bioactive compounds, which improve stress tolerance of plants and their medicinal potential (Anand 2023).

Murraya koenigii L. or curry leaf plant [Karapincha (Sinhala), Karuvapillai (Tamil)] of the family Rutaceae is a small deciduous tree with highly pungent aromatic leaves and characteristic taste (Yankuzo *et al*., 2011). The plant is recorded to have various ethnobotanical, pharmacognostic, phytochemical and pharmacological properties (Gahlawat *et al*., 2014). A scrutiny of specific literature reveals some notable pharmacological activities of the plant such as on the heart through cholesterol reducing properties, and in addition it is recorded to have antimicrobial activity, and anti- cancer activity (Balakrishnan *et al*., 2020). Its medicinal values include, antioxidative properties, cytotoxic and anti-diarrhoea activity (Chaudhary, 2020; Rautela & Katiyar, 2023), and additionally it is considered to be anti-diabetic (Habbu *et al*., 2014). The plant possess various health beneficial properties due to the presence of secondary metabolites such as polyphenols (reduce oxidative stress and prevent degenerative diseases such as cardiovascular disease and cancers), flavonoids (regulation of cholesterol levels), flavonoid compounds such as myricetin, quercetin and epicatechin (effective in the growth inhibition of breast cancer cells), minerals (maintenance of normoglycemia or normal glucose content of blood), and antioxidants as well as presence of beneficial probiotic endophytic microorganisms (Abeysinghe *et al*., 2021). It has been found that the leaves, stems and roots of *M. koenigii* L. have both bacterial and fungal endophytes including endophytic Bacilli (Khan et *al*., 2022). Fungal endophytes isolated from *M. koenigii* L. includes *Aspergillus niger, Aspergillus flavus, Candida albicans, Penicillium sublateritium* and *Phoma hedericola* (Soundappan *et al*., 2018). Since *M. koenigii* has various medicinal properties viz., anti- cancer, anti- diabetic ability etc., the presence of potential probiotic endophytes in *M. koenigii* leaves would be an added feature that could be exploited either for the leaves to be consumed raw (paste) or produce probiotics as a commercial product. Therefore, the objectives of the current study were to isolate bacterial endophytes from leaves of *M. koenigii* and to investigate their probiotic potential.

MATERIALS AND METHODS

Sample collection

Samples of leaves were collected from Gampaha, Kandy and Jaffna districts covering the wet and dry zones in Sri Lanka, for the isolation of endophytic bacteria. Young and mature leaves were used separately for testing. Young leaves and mature leaves were distinguished as young leaves with pale green colour occurred at the distal end of the branch while mature leaves with dark green colour located below the distal end of branches.

Surface sterilization of leaves

The leaves were sterilized by washing with running tap water, followed by soaking in 70% ethanol for 30 s, and sodium hypochlorite solution (5 %) for 3 min, and finally rinsing thrice with distilled water (Habbu *et*

al., 2014).

Isolation of endophytic bacteria from leaves of *M. koenigii*

The bacteria were isolated by placing cut leaf sections (1 cm^2) on the solid medium (Mohamad *et al*., 2020), placing leaf macerations on the solid medium (Habbu *et al*., 2014), and preparing pour plates with leaf extract (Costa *et al*., 2012). nutrient agar (NA), Lauria-Bertani (LB) and de Man, Rogosa and Sharpe (MRS) media were used for isolation of non-fastidious endophytes, endophytic bacteria belonging to the family Enterobacteraceae, and bacterial endophytes of the genus *Lactobacillus*, respectively.

Preliminary identification and characterization of pure cultures

Morphological identification of isolated bacterial endophytes was carried out by Gram staining and endospore staining, followed by motility test and catalase test for biochemical identification (Cullimore, 2019).

Gram staining

Bacterial smears were prepared on clean microscopic slides. The heat fixed smear was then flooded with crystal violet (primary stain) and allowed to stand for 1 min. Then the excess dye was removed and the smear was flooded with Gram's iodine solution, and allowed to stand for 1 min. The smear was again washed with running tap water and dipped in 95% ethanol until the smear became decolorized. The smear was then flooded with safranin (secondary stain) and allowed to stand for 1 min. The slide was again washed with running tap water to remove excess stain and observed under the microscope using an oil immersion lens.

Endospore staining

Bacterial smears were prepared on clean microscope slides, malachite green (primary stain) was applied, and heat fixed with a steam bath. The slides were then removed from the flame, cooled, and rinsed with running tap water to decolorize bacterial cells. The slides were flooded with the counter stain (safranin) for 30 s and rinsed with water. Subsequently, the slide was air dried and observed under the light microscope.

Motility test

The hanging drop method was used to determine the motility of bacterial isolates. Using the inoculating loop a small drop of the bacterial sub culture was aseptically placed in the centre of a clean coverslip. The cavity slide was lowered, with the concavity facing down, onto the coverslip to allow the drop to protrude into the centre of the concavity of the slide. It was pressed gently to form a seal. The drop was examined under the light microscope.

Catalase test

A drop of sterile distilled water was placed on the middle of a glass slide and a small amount of the culture was picked up with a sterile inoculating loop. One or two colonies were emulsified on each drop to make a smooth suspension. Then, one drop of 3% peroxide was placed over the test smear using a Pasteur pipette. The smears were observed for rapid gas bubble formation.

Probiotic characterization of endophytic bacteria

Determination of resistance of bacterial isolates to low pH

The resistance of bacterial isolates to low pH was determined using three methods. In the first method, the pH of the nutrient broth was adjusted to pH 3. In the fasted state, the median gastric pH is 1.7 and when the meal is administered the gastric pH climbs briefly to a median peak value of 6.7 (Dressman *et al*., 1990). PH was adjusted to 3 in the current experiment as a middle value between these two states. The survival rate of each

bacterial isolate was measured by enumeration of bacterial colonies on nutrient agar (NA) plates at hourly intervals, up to 6 h (Both *et al*., 2010). In the second method, spread plates were prepared on NA plates which were adjusted to pH 3 and the survival rate was measured by enumeration of bacterial colonies on NA plates at hourly intervals up to 6 h. In the third method, a mixture of cooked rice, saliva and leaf macerations of *Murraya koenigii* L. was added to normal nutrient broth, adjusted to pH 3. The survival rate of bacterial isolates was measured by determining the absorbance of each broth inoculated with bacteria culture, using UV-VIS spectrophotometer (Camspec M02, Spectronic Campsec Ltd., UK).

Determination of bile salt tolerance of bacterial isolates

One milliliter of each overnight culture, which was adjusted similar to McFarland 0.5 turbidity were aseptically transferred to 10 mL of 0.3% bile containing broth, and streak plates were prepared on NA plates at hourly intervals for 4 h (Bassyouni *et al*., 2012). Semi-quantitative screening streak method was used to prepare streak plates to obtain isolated colonies (Furgason *et al*., 1995; Croxatto *et al*., 2015). Plates were incubated at 37 °C for 24 h.

Determination of antimicrobial activity of bacterial isolates

Antimicrobial activity of the bacterial strains was determined using the disc diffusion method on Muller Hinton agar (MHA) against the pathogenic strains of *Pseudomonas aeruginosa* and *Escherichia coli*. The pathogenic strains, which were adjusted similar to McFarland 0.5 turbidity, were inoculated on MHA plates using sterilized cotton swabs. Sterile filter paper discs of 9 mm diameter were dipped in fresh overnight cultures of endophytic bacterial isolates and placed on the surface of an agar plate. The petri plates were kept at 4 °C for 30 min to permit diffusion on the assay material, and incubated at 37 °C for 24 h, and the zone of inhibition was measured in millimetres. The assay was repeated 3 times. Discs dipped in sterile distilled water and gentamycin discs (10 mg) served as negative and positive controls respectively (Abeysinghe *et al*., 2021).

Determination of antibiotic resistance

Fresh overnight endophytic bacterial cultures, which were adjusted similar to McFarland 0.5 turbidity were spread evenly on the surface of MHA plates using sterile cotton swabs. One Petri plate was used for spreading one endophytic bacterial isolate and each Petri plate was divided into two equal halves. In one half, a 6 mm diameter Gentamicin disc (10 mg/mL) was placed, and in other half, a filter paper impregnated with distilled water was placed that served as the negative control. The plates were then incubated at 37 °C for 24 h, and the diameter of the zone of inhibition was measured (Fallah *et al*., 2013).

Determination of haemolytic activity of bacterial isolates

Haemolytic activity of endophytic bacterial isolates was tested using blood agar base with 10% human blood. A loop full of each fresh overnight culture was aseptically transferred to the blood agar plates separately and incubated at 37 °C for 24 h. After incubation, agar plates were observed for clear zones around the colonies. Freshly sub cultured *Streptococcus* sp. was used as the positive control and *Lactobacillus* sp. was used as the negative control (Bassyouni *et al*., 2012).

Determination of DNAse activity of bacterial isolates

DNase activity of bacterial isolates was tested by aseptically transferring a loop full of fresh overnight broth cultures on DNase agar plates separately. Plates were incubated at 37 °C for 24 h. After incubation, DNase agar plates were flooded with conc. HCl and the excess was removed. After 5 min, agar plates were observed for the appearance of a 'halo' surrounding the strains. Freshly sub cultured *Staphylococcus aureus* was used as the positive control and *Lactobacillus* sp. was used as the negative control (Gündoğan *et al*., 2006).

RESULTS AND DISCUSSION

Sample collection and isolation of bacterial endophytes

Endophytes are microorganisms living inter or intra cellularly in most of plant tissues without causing any disease symptoms while producing plant growth promoting compounds (Nair & Padmawathy, 2014). Ten morphologically different endophytic bacterial strains were isolated on NA and LB media and none were isolated from leaves inoculated on MRS Medium (Table 1). In this study, samples of leaves were collected from different maturity stages and environments. According to Ullah *et al*., (2018), abundance and composition of endophytic communities can vary depending on plant genotype, colonizing tissue type, plant developmental stage, soil type and environmental conditions. Due to this reason both mature and young leaf samples were taken from three districts covering the wet and dry zones. Out of the ten isolates, eight were isolated from leaf samples taken from the wet zone and six were isolated from young leaves (Table 1).

Plants that are adapted to live in dry climatic conditions (i.e., xerophytes) usually have a high number of plant endophytes (Yadav & Meena, 2021). However, in the current study, there was a high number of endophyte isolates from leaves sampled from the wet zone. In the current study, young leaves had a higher number of endophytes than mature leaves (Table 1).

Probable reasons could be that young leaves are more prone for herbivory than mature leaves because of their lower fragility due to thinner cell walls, and some endophytic bacteria secrete allelopathic chemical compounds, which reduce competition from pathogenic bacteria for colonization of intercellular tissues of plants and reduce herbivore attacks (Milute *et al*., 2015). Further, nutritional composition is higher in young leaves when compared with mature leaves due to nutrient sequestration, which probably facilitates an abundant endophytic bacteria community when compared to mature leaves (Kandel *et al*., 2017).

MRS medium was specifically used for isolation of *Lactobacillus* spp. (Shuhadha *et al*., 2017). The isolated bacteria did not grow on MRS medium although they grew on NA and LB media (Table 1) probably indicating the isolated strains do not belong to the genus *Lactobacillus*. Out of the three methods used to isolate endophytes from leaves, pour plates with the addition of leaf extracts resulted in most isolations, probably as all the endophytic bacteria had an equal chance of being released from the leaf tissue, which were thoroughly macerated.

Table 1: Isolate, location, maturity status of the leaf sample, isolated medium and inoculation method of isolates.

Isolate	Location (Dry zone/wet zone)	Maturity status of the leaf sample	Isolated medium	Inoculation method
1	Jaffna (DZ)	Mature	LB	C
2	Jaffna (DZ)	Mature	NA	C
3	Gampaha (WZ)	Young	LB	A
4	Gampaha (WZ)	Young	LB	A
5	Kandy (WZ)	Young	NA	A
6	Kandy (WZ)	Young	NA	A
7	Kandy (WZ)	Mature	LB	B
8	Kandy (WZ)	Young	LB	B
9	Kandy (WZ)	Young	NA	B
10	Kandy (WZ)	Mature	NA	B

NA - nutrient agar; LB – Lauria-Bertani medium; A - placing 1 cm^2 square particles of leaves on the medium; B - placing leaf macerations on the medium; C -preparing pour plate with leaf extraction; WZ - wet zone; DZ - dry zone.

Preliminary identification and characterization of pure cultures

The ten bacterial isolates were categorized based on their morphological characteristics such as cell shape, Gram staining results, presence/absence of spores, motility and catalase activity (Table 2, Figure 1). All the isolates were non- motile, catalase negative, non- endospore forming bacteria including eight cocci strains and two bacilli strains. According to the Gram staining results, two isolates were Gram positive while the other eight isolates were Gram negative. Previous studies done on endophytic bacteria isolated from plant parts of *M. koenigii* L. reveal that there are Gram positive endophytes in the plant, and according to literature Gram positive bacilli isolated from leaves could be *Bacillus* sp. (Khan *et al*., 2022). Furthermore, Habbu *et al*., 2014 states that Gram positive *Exiguobacterium indicum* endophytes are present in various plant parts of *M. koenigii*. As the 10 isolates were Gram positive, there is a possibility for that *Exiguobacterium indicum* is also included among them.

Table 2: Results obtained from preliminary identification tests.

Isolate	Gram stain	Shape/ arrangement	Catalase test	Endospore staining	Motility test
1	Gram negative	Cocci	-	-	-
2	Gram positive	Cocci	-	-	-
3	Gram negative	Cocci	-	-	-
4	Gram negative	Bacilli	-	-	-
5	Gram negative	Streptococci	-	-	-
6	Gram negative	Cocci	-	-	-
7	Gram negative	Cocci	-	-	-
8	Gram negative	Cocci	-	-	-
9	Gram negative	Bacilli	-	-	-
10	Gram negative	Cocci	-	-	-

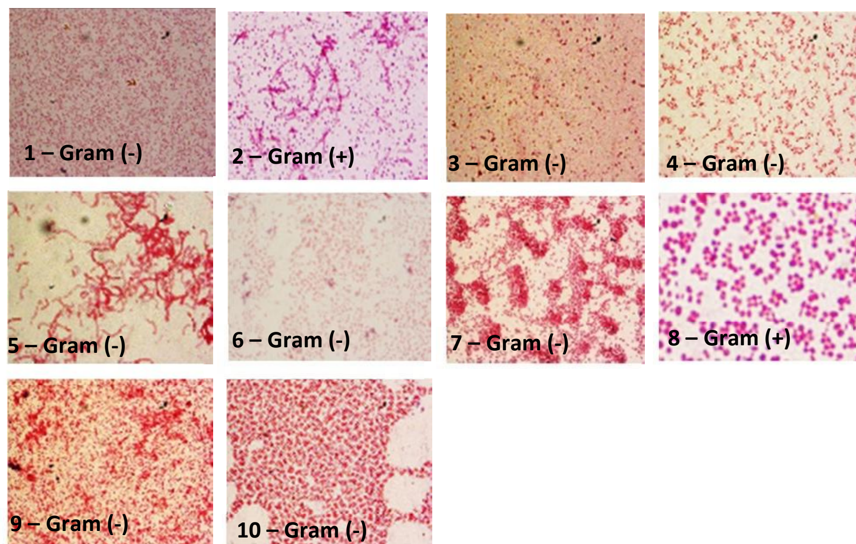

Figure 1: Microscopic images of isolated bacterial endophytes (×10 ×100).

Probiotic characterization of endophytic bacteria

Probiotics are viable, non-pathogenic microorganisms (bacteria or yeast) that, when ingested, are able to reach the intestines in sufficient numbers to confer health benefits to the host (Wong *et al*., 2015). Probiotic characterization tests, which were carried out with isolated endophytic bacteria reveal their potential as probiotics.

Determination of resistance of isolated bacteria to low pH

The ability of a certain isolate to survive in an acidic pH and high levels of bile enable their probiotic potential by ensuring their transit through the stomach to reach the intestine. The results showed that no colonies could survive at pH 3, either on nutrient broth or NA plates (Figures 2, 3 and 4). Absorbance in nutrient broth cultures containing additional nutrients was slightly increased six hours after inoculation (Figures 2, 3 and 4), in each set of isolates indicating their survival in low pH. Nutrient Broth treated with a mixture of macerated *M. koenigii* leaves, rice and saliva showed a higher increase in absorbance (higher survival rate of isolates) than nutrient agar broth without the mixture (Figures 2, 3 and 4). Tolerance to acidic pH conditions was measured using three protocols (Hassanzadazar, 2012; Shuhadha *et al*., 2017); the most effective method, which indicated the survival of isolates in low pH, was nutrient broth at pH 3 with the additional components of rice, saliva and *M. koenigii* leaf macerations (Figure 1, 2 and 3) to simulate the environmental conditions ~~as~~ inside the gut. In the first two methods, the non-appearance of bacterial colonies at any of the time intervals on the NA plates may be due to the very low survival in an acidic environment, which were not sufficient to make a visible colony on the medium. This test was carried out for all 10 isolates as 3 different sets. Therefore, controls are different for each set of isolates. Hence, it is difficult to do a comparison of values for all 10 isolates at once and decide on the isolates with the highest and lowest pH tolerance. But, as an overall result, isolates 1-10 were susceptible to low pH.

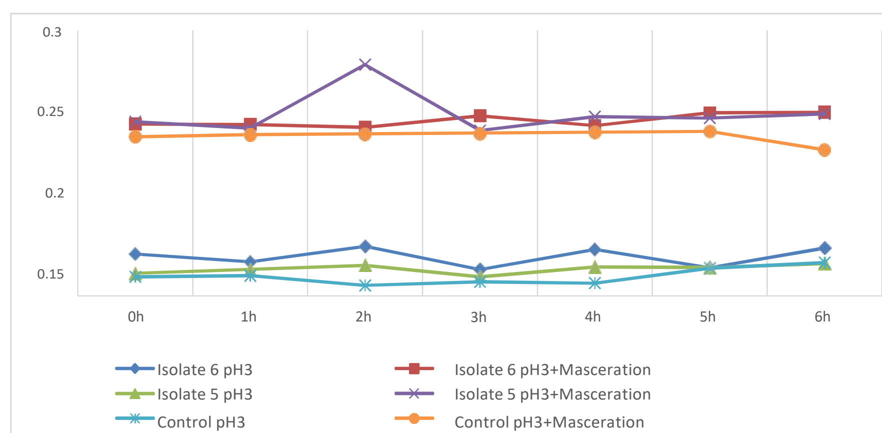

Figure 2: Survival in acidic media. Absorbance (λ = 600 nm) vs time for isolates 5 and 6

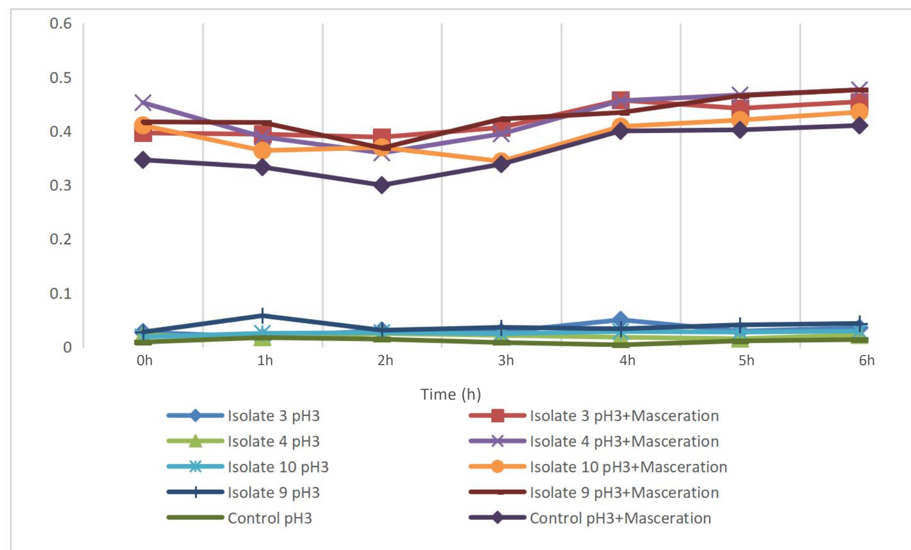

Figure 3: Absorbance (λ = 600 nm) vs time for isolates 3, 4, 9 and 10

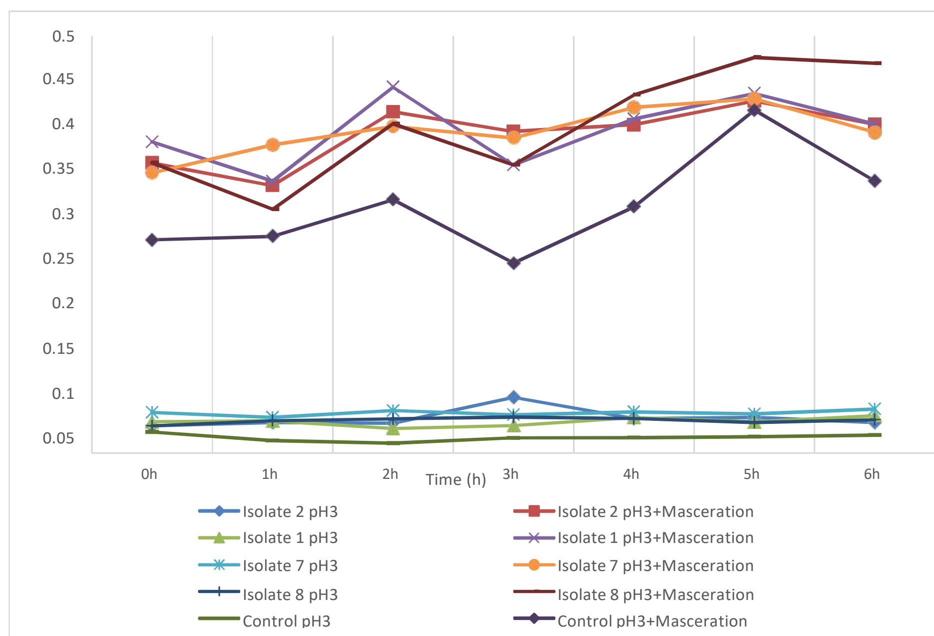

Figure 4: Absorbance (λ = 600 nm) vs time for isolates 1, 2, 7 and 8

Determination of bile salt tolerance

After bacterial exposure to bile salts, disruptions of cellular homeostasis cause the dissociation of the lipid bilayer and integral protein of their cell membranes, resulting in leakage of bacterial contents and finally cell death (Hassanzadazar, 2012). Even four hours after inoculation, considerable number of colonies were observed onthe solid medium indicating their ability to survive in the bile salt medium. Isolates 2, 3, 6 and 7 showed a considerable increase in colony count three hours after inoculation (Table 3). According to the number of colonies formed after four hours of incubation, isolates 4 and 5 showed the highest ability to survive in the presence of bile salts and the lowest bile salt tolerance was detected in isolate 9. When compared with the initial values, bacterial growth was drastically decreased after four hours; however, they survived even after this time period.

Table 3: Number of colonies of bacterial isolates counted at 1-hour intervals for 4 hours

Number of colonies × 1000 (CFU/mL)	T1			T2			T3			T4		
	R1	R2	Average	R1	R2	Average	R1	R2	Average	R1	R2	Average
1	TNTC	TNTC	-	TNTC	TNTC	-	216	187	201.5	126	143	134.5
2	TNTC	TNTC	-	TNTC	TNTC	-	94	87	90.5	112	131	121.5
3	TNTC	TNTC	-	TNTC	TNTC	-	114	123	118.5	143	152	147.5
4	TNTC	260	-	TNTC	TNTC	-	272	264	268	250	274	262
5	TNTC	TNTC	-	TNTC	TNTC	-	204	212	208	182	171	176.5
6	TNTC	TNTC	-	TNTC	TNTC	-	108	121	114.5	119	131	125
7	TNTC	TNTC	-	TNTC	TNTC		103	136	119.5	121	127	124
8	TNTC	TNTC	-	155	133	144	126	109	117.5	91	96	93.5
9	TNTC	TNTC	-	TNTC	TNTC	-	84	93	88.5	86	81	83.5
10	TNTC	TNTC	-	164	173	168.5	127	134	130.5	95	89	92

T1 - one hour after inoculation; T2 - two hours after inoculation; T3 - three hours after inoculation; T4 - four hours after inoculation; R1 - replicate one for taking colony count; R2 - replicate two for taking colony count; TNTC- too numerous to count

Determination of antimicrobial activity

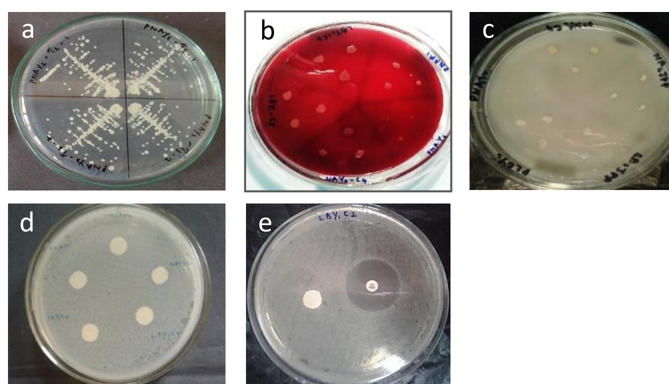

Figure 5: Isolated bacterial endophytes showing (a) Bile salt tolerance with colonies grown on NA medium after 4 hours incubation in bile salt; (b) non-haemolytic activity without forming clear zones around isolates; (c) non-DNase activity without forming clear zones around isolates; (d) negative antimicrobial activity with no inhibition zones around the filter paper discs impregnated with the isolates; (e) antibiotic susceptibility with isolate forming inhibition zone around gentamycin disc.

Antimicrobial activity was tested against two potential pathogenic strains in the gut microbiota, *E. coli* and *P. aeruginosa*. None of the bacterial isolates (1-10) formed inhibition zones in the spread plates of *E. coli* and *P. aeruginosa*, although Gentamicin discs formed inhibition zones of 20.12 mm and 16.31 mm, respectively with these pathogenic strains (Figure 5), indicating that the endophytic bacterial isolates do not possess antimicrobial activity. The ability of the isolates to inhibit the colonization of gut epithelial cells by pathogenic bacteria through mechanisms other than antagonism (i.e., blocking the colonization sites on epithelial cells, inhibiting the ability of pathogens to adhere to the epithelial cells) cannot be ruled out (Candela *et al*., 2008). However, the results obtained in this *in vitro* study may not completely reflect their performance *in situ*. This is because there are number of other physiological conditions, which may possibly affect the survival of the bacteria (Hassanzadazar, 2012). The current research did not look into the anti-adherence property. It is suggested that further testing should be done to assess the isolates for anti-adherence property, as according to literature there are some microorganisms which have the ability to block the adherence of pathogenic microorganisms to gut epithelial cells.

Determination of antibiotic resistance

According to recent studies, some probiotics in dietary supplements possess antibiotic resistant properties (Wong *et al*., 2017) while most of the potential probiotics do not contribute to the spread of antibiotic resistance and may even reduce it (Ouwehand *et al*., 2016). There is a negative aspect of antibiotic resistance strains because horizontal gene transfer can deliver the antibiotic resistance genes to pathogenic bacteria (Imperial & Ibana, 2016), which can act as Multidrug Resistant Strains that are resistant to more than one common antibiotic (Gueimunde *et al*., 2013). Isolates 1, 2, 7, 8, 9 and 10 showed antibiotic resistance while isolates 3, 4, 5 and 6 showed antibiotic susceptibility. The highest antibiotic susceptibility (lowest antibiotic resistance) was showed by isolate 3. However, the potential of horizontal gene transfer of the antibiotic resistance genes of bacteria strains, which were antibiotic resistant (bacterial isolates 1, 2, 7, 8, 9, 10) should be studied before considering them as safe probiotics.

Table 4: The antibiotic resistance and diameters of zones of inhibition for antibiotic susceptible bacterial isolates.

Name of the isolate	Antibiotic resistant / susceptible	Diameter of the zone of inhibition (mm)
1	Resistant	-
2	Resistant	-
3	Susceptible	28 ± 0.57
4	Susceptible	10 ± 1.25
5	Susceptible	24 ± 0.57
6	Susceptible	24 ± 0.57
7	Resistant	-
8	Resistant	-
9	Resistant	-
10	Resistant	-

Determination of haemolytic activity and DNase activity

Detection of non-haemolytic activity and non-DNase activity is considered as a safety prerequisite for characterizing a certain bacterium as a probiotic organism (Shuhadha *et al*., 2017). According to the results obtained, none of the bacterial isolates (1-10) showed either haemolytic or DNase activity (Figure 5), which concludes that they have potential probiotic properties.

CONCLUSIONS

Composition and abundance of endophytic bacteria colonizing *M. koenigii* vary depending on climatic conditions and the maturity of leaves. Leaves of *M. koenigii* possessed Gram positive and Gram-negative endophytic bacteria. All the endophytic bacteria isolated showed most of the potential probiotic characters, while four isolates did not show antibiotic resistance. None of the isolates exhibited antimicrobial activity. Future studies include testing of anti- adherence ability of isolates on pathogenic bacteria and further identification of isolates using molecular techniques.

REFERENCES

Abeysinghe D.T., Kumara K.A.H., Kaushalya K.A.D., Chandrika U.G. & Alwis D.D.D.H. (2021). Phytochemical screening, total polyphenol, flavonoid content, in vitro antioxidant and antibacterial activities of Sri Lankan varieties of *Murraya koenigii* and *Micromelum minutum* leaves. *Heliyon* **7**(7): e07449.
DOI: https://doi.org/10.1016/j.heliyon.2021.e07449

AlKalbani N.S., Turner M.S. & Ayyash M.M. (2019). Isolation, identification, and potential probiotic characterization of isolated lactic acid bacteria and in vitro investigation of the cytotoxicity, antioxidant, and antidiabetic activities in fermented sausage. *Microbial Cell Factories* **18**(1): 1–12.
DOI: https://doi.org/10.1186/s12934-019-1239-1

Anand U. *et al*. (13 authors) (2023). Current scenario and future prospects of endophytic microbes: promising candidates for abiotic and biotic stress management for agricultural and environmental sustainability. *Microbial Ecology* 1–32.
DOI: https://doi.org/10.1007/s00248-023-02190-1

Balakrishnan R., Vijayraja D., Jo S.H., Ganesan P., Su-Kim I. & Choi D.K. (2020). Medicinal profile, phytochemistry, and pharmacological activities of *Murraya koenigii* and its primary bioactive compounds. *Antioxidants* **9**(2): 101.
DOI: https://doi.org/10.3390/antiox9020101

Bassyouni R.H., Abdel-all W.S., Abdel-all M.G.F.S. & Kamel Z. (2012). Characterization of lactic acid bacteria isolated from dairy products in Egypt as a probiotic. *Life Science Journal* **9**(4): 2924–2933.

Behnsen J., Deriu E., Sassone-Corsi M. & Raffatellu M. (2013). Probiotics: properties, examples, and specific applications. *Cold Spring Harbor Perspectives in Medicine* **3**(3): a010074.
DOI: https://doi.org/10.1101/cshperspect.a010074

Both E., Gyorgy E., Kibedi-Szabo C.Z., Tamas E., Abraham B., Miklossy I. & Lanyi S. (2010). Acid and bile tolerance, adhesion to epithelial cells of probiotic microorganisms. *UPB Buletin Stiintific, Series B: Chemistry and Materials Science* **72**(2): 37–44.

Candela M., Perna F., Carnevali P., Vitali B., Ciati R., Gionchetti P. & Brigidi P. (2008). Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. *International Journal of Food Microbiology* **125**(3): 286–292.
DOI: https://doi.org/10.1016/j.ijfoodmicro.2008.04.012 s

Chaudhary A. (2020). A review on the culinary uses and therapeutic properties of *Murraya koenigii*. *Journal of Advancement in Pharmacognosy* **1**(1): 1–8.

Chow Y. & Ting A.S. (2015). Endophytic L-asparaginase-producing fungi from plants associated with anticancer properties. *Journal of Advanced Research* **6**(6): 869–876.
DOI: https://doi.org/10.1016/j.jare.2014.07.005

Costa L.E.D.O., Queiroz M.V.D., Borges A.C., Moraes C.A.D. & Araújo E.F.D. (2012). Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris). *Brazilian Journal of Microbiology* **43**(4): 1562–1575.
DOI: https://doi.org/10.1590/S1517-83822012000400041

Croxatto A., Dijkstra K., Prod'hom G. & Greub G. (2015). Comparison of the InoqulA and the WASP automated systems with manual inoculation. *Journal of Clinical Microbiology* **53**(7): %P 2298–2307.
DOI: https://doi.org/10.1128/JCM.03076-14

Cullimore D.R. (2019). *Practical Atlas for Bacterial Identification*. CRC Press, USA.

da sesto VC. (2008). The (non-) sense of human origin of probiotics. In *Prebiotics/probiotics* pp. 8-10. Milano, Italy.

Dressman J.B., Berardi R.R., Dermentzoglou L.C., Russell T.L., Schmaltz S.P., Barnett J.L. & Jarvenpaa K.M. (1990). Upper gastrointestinal (GI) pH in young, healthy men and women. *Pharmaceutical Research* **7**(7): 756–761.
DOI: https://doi.org/10.1023/A:1015827908309

Fallah S.H., Asgharpour F., Naderian Z. & Moulana Z. (2013). Isolation and determination of antibiotic resistance patterns in nontyphoid *Salmonella* spp. isolated from chicken. *International Journal of Enteric Pathogens* **1**(1): 17–21.
DOI: https://doi.org/10.17795/ijep9416

Ferguson J., Tanner J. & Miller J.M. (1995). Evaluation of a new, semi quantitative screening culture device for urine specimens. *Journal of Clinical Microbiology* **33**(5): 1351–1353.
DOI: https://doi.org/10.1128/jcm.33.5.1351-1353.1995

Gahlawat D.K., Jakhar S. & Dahiya P. (2014). *Murraya koenigii* (L.) Spreng: an ethnobotanical, phytochemical and pharmacological review. *Journal of Pharmacognosy and Phytochemistry* **3**(3): 109-119.

Gueimonde M., Sánchez B., de los Reyes-Gavilán C.G. & Margolles A. (2013). Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology* **4**: 202.
DOI: https://doi.org/10.3389/fmicb.2013.00202

Gündoğan N., Citak S.U.M.R.U. & Turan E. (2006). Slime production, DNase activity and antibiotic resistance of Staphylococcus aureus isolated from raw milk, pasteurised milk and ice cream samples. *Food Control* **17**(5): 389–392.
DOI: https://doi.org/10.1016/j.foodcont.2005.01.006

Habbu P.V., Patil B.S., Salagare M.V., Madagundi S.D., Vankudri R.V., Shukla S.T. & Kulkarni V.H. (2014). Antidiabetic potential of endophytic bacterial fraction of *Murraya koenigii* (L.) Spreng. in rats. *Spatula DD* **4**(3): 139–150.

Hassanzadazar H., Ehsani A., Mardani K. & Hesari J. (2012). Investigation of antibacterial, acid and bile tolerance properties of *lactobacilli* isolated from Koozeh cheese. *Veterinary Research Forum* **3**(3): 181.

Holzapfel W.H., Haberer P., Geisen R., Björkroth J. & Schillinger U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition* **73**(2): 365s–373s.
DOI: https://doi.org/10.1093/ajcn/73.2.365s

Imperial I.C. & Ibana J.A. (2016). Addressing the antibiotic resistance problem with probiotics: reducing the risk of its double-edged sword effect. *Frontiers in Microbiology* **7**: 1983.
DOI: https://doi.org/10.3389/fmicb.2016.01983

Kandel S., Joubert P. & Doty S. (2017). Bacterial endophyte colonization and distribution within plants. *Microorganisms* **5**(4): 77.
DOI: https://doi.org/10.3390/microorganisms5040077

Khan S.S., Zargar S.A., Gupta V.K., Verma V. & Rasool S. (2022). Isolation and identification of bacterial and fungal endophytes from selected plants of Western Himalayas in prospect for bioactivities of economic importance. *Biology Bulletin* **49**(5): 444–457.
DOI: https://doi.org/10.1134/S1062359022050090

Kimoto H., Nomura M., Kobayashi M., Okamoto T. & Ohmomo S. (2004). Identification and probiotic characteristics of *Lactococcus* strains from plant materials. *Japan Agricultural Research Quarterly* **38**(2): 111–117.
DOI: https://doi.org/10.6090/jarq.38.111

Miliute I., Buzaite O., Baniulis D. & Stanys V. (2015). Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. *Zemdirbyste-Agriculture* **102**(4): 465–478.
DOI: https://doi.org/10.13080/z-a.2015.102.060

Mohamad A.O.A., Ma J.B., Liu Y.H., Zhang D., Hua S., Bhute S. & Li L. (2020). Beneficial endophytic bacterial populations associated with medicinal plant *Thymus vulgaris* alleviate salt stress and confer resistance to *Fusarium oxysporum*. *Frontiers in plant Science* **11**: 47.
DOI: https://doi.org/10.3389/fpls.2020.00047

Nair D.N. & Padmavathy S. (2014). Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World Journal* **2014**: 250693.
DOI: https://doi.org/10.1155/2014/250693

Oh S. (2017). Animal- origin probiotics vs plant- origin probiotics. *Food and Beverages* **8**(8): 30.
DOI: https://doi.org/10.4172/2157-7110-C1-065

Ouwehand A.C., Forssten S., Hibberd A.A., Lyra A. & Stahl B. (2016). Probiotic approach to prevent antibiotic resistance. *Annals of Medicine* **48**(4): 246–255.
DOI: https://doi.org/10.3109/07853890.2016.1161232

Pineiro M. & Stanton C. (2007). Probiotic bacteria: legislative framework—requirements to evidence basis. *The Journal of Nutrition* **137**(3): 850S–853S.
DOI: https://doi.org/10.1093/jn/137.3.850S

Rautela R. & Katiyar R. (2023). *Aegle marmelos* and *Murraya koenigii*: The wonder plants against bovine infertility. *Indian Journal of Animal Sciences* **93**(1): 6.
DOI: https://doi.org/10.56093/ijans.v93i1.115512

Ruiza D., Agaras B., de Werrab P., Wall L.G. & Valverde C. (2011). Characterization and screening of plant probiotic traits of bacteria isolated from rice seeds cultivated in Argentina. *The Journal of Microbiology* **49**(6): 902–912.
DOI: http://doi.org/10.1007/s12275-011-1073-6

Soundappan K., Bajaj R., Vaidya B.N. & Joshee N. (2018). Anatomical and biochemical investigations on medicinal tree *Murraya koenigii* Spreng. *Journal of Biotech Research* **9**: 70–78.

Shuhadha M.F.F., Panagoda G.J., Madhujith T. & Jayawardana N.W.I.A. (2017). Evaluation of probiotic attributes of *Lactobacillus* sp. isolated from cow and buffalo curd samples collected from Kandy. *Ceylon Medical Journal* **62**(3): 159–166.
DOI: http://doi.org/10.4038/cmj.v62i3.8519

Wang Y., Deng H., Li Z., Tan Y., Han Y., Wang X. & Bi Y. (2017). Safety evaluation of a novel strain of *Bacteroides fragilis*. *Frontiers in Microbiology* **8**: 435.
DOI: https://doi.org/10.3389/fmicb.2017.00435

Wong A., Saint Ngu Dan D.Y., Ooi L.A. & Lim R L.H. (2015). Detection of antibiotic resistance in probiotics of dietary supplements. *Nutrition Journal* **14**(1): 1–6.
DOI: https://doi.org/10.1186/s12937-015-0084-2

Yadav G. & Meena M. (2021). Bioprospecting of endophytes in medicinal plants of Thar Desert: An attractive resource for biopharmaceuticals. *Biotechnology Reports* **30**: e00629.
DOI: https://doi.org/10.1016/j.btre.2021.e00629

Yankuzo H., Ahmed Q.U., Santosa R.I., Akter S.F.U. & Talib N.A. (2011). Beneficial effect of the leaves of *Murraya koenigii* (Linn.) Spreng (Rutaceae) on diabetes-induced renal damage in vivo. *Journal of Ethnopharmacology* **135**(1): 88–94.

Zhai Q., Feng S., Arjan N. & Chen W. (2018). A next generation probiotic, *Akkermansia muciniphila*. *Critical Reviews in Food Science and Nutrition* **59**: 3227–3236.
DOI: https://doi.org/10.1080/10408398.2018.1517725