ANTIMICROBIAL ASSAY OF *LACTOBACILLUS* SPECIES AGAINST MULTIDRUG RESISTANCE BACTERIA

Submitted to

Institute of Science and Technology

Dean's office, Tribhuvan University

Submitted by

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Institute of Science and Technology

Padma Kanya Multiple Campus

Bagbazar, Kathmandu, Nepal

2024

RECOMMENDATION

This is to recommend that Ms. **Rama Khadka**, has carried out project work entitled **"Antimicrobial Assay of *Lactobacillus* species against Multidrug Resistance Bacteria"**, for the mini research project work under my supervision of Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

She has fulfilled the entire requirement laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the submission of the mini research project work.

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Mentor
Central Department of Microbiology
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DECLARATION

This project work entitled **''Antimicrobial Assay of *Lactobacillus* Species against Multidrug Resistance Bacteria''** is being submitted to the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for carried out mini research project work. This work is original and has not been submitted for any other research work.

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2024

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ABSTRACT

Lactic acid bacteria are the group of bacteria which are commonly found in soil and in the guts of animals and they are favorable for both human and animal health. In this study, *Lactobacillus* species were isolated and identify from soil of a cow farm and perform its antimicrobial activity against MDR bacteria from October 2024 to March 2024 in the microbiology laboratory of Padma Kanya Multiple Campus, Bagbazar, Nepal. A total of 30 soil samples were collected from 3 locations of farm of Kathmandu valley for enumeration and isolation of *Lactobacillus* species on selective media (MRS). Pure cultures were obtained through sub culturing on MRS media and further morphological as well as biochemical test was be carried to identify the isolated strains. For the confirmation of *Lactobacillus* species, Gram staining, motility test and catalase test were done. For the further analysis, fermentation test, growth on different temperature, different salt concentration and different pH were also done. Antibiotic susceptibility test (AST) of *Lactobacillus* species and test bacteria was done by a modified Kirby- Bauer method. The screening test for bacteriocin was done by dot plate technique method. Then, the extraction of bacteriocin from isolated *Lactobacillus* spp. was done by precipitation method and antimicrobial assay was done against MDR test bacteria. From this study, 10 *Lactobacillus* species were identified. In this study, bacteriocin like compound were extracted from *Lactobacillus* spp. were from 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5days) and 120hrs (6days). However, antimicrobial activity showed against MDR bacteria only after 72hrs (4day), 96hrs (5days) and 120hrs (6days). From 96hrs (5days) of extraction of antimicrobial substance bacteriocin like compound was able to inhibit all test bacteria which were *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The highest zone of inhibition was showed by BL10 isolate against *B. cereus* (30mm) followed by KL1 isolate against *E. coli* (21mm) and KL5 isolate against *B. cereus* (20mm). From this study, antimicrobial activity of bacteriocin like compound showed the antimicrobial activity against all MDR bacteria. So, next generation antibiotics can be prepared from bacteriocin like compound extracted from *Lactobacillus* spp. for targeting the multi drug resistant bacteria.

***Keywords*:** Cow farms, soil, *Lactobacillus* species, AST, Bacteriocin.

शोधशार

ल्याक्टिक एसिड व्याक्टेरिया (Lactic acid bacteria), व्याक्टेरिया कै एक प्रजाती हो । यो विशेषगरी माटो र जनवारहरुको पाचननलीमा पाइन्छ र यो प्रजातीको व्याक्टेरियाले मानव र पशुको स्वास्थ्यमा अनुकुल प्रभाव पारेको हुन्छ। यस अध्ययनमा गाइपालन क्षेत्रहरुमा रहेको माटोको नमुनालाई लिएर ल्याक्टोव्यासिलस प्रजातीलाई (*Lactobacillus* species) पहिचान गरिएको छ। यसपछि सो नमुनाहरुलाई सन् २०२४ सालको मार्च देखी अक्टोवरसम्म पद्मकन्या बहुमुखी क्याम्पस, बाघवजारमा रहेको माइक्रोबायोलोजी प्रयोगशालामा एन्टीमाइक्रोवियल परिक्षणहरु गर्‍यौं। काठमाडौं उपत्यकाका विभिन्न ३ वटा क्षेत्रहरुमा रहेको फार्महरुबाट कुल ३० वटा नमूनाहरु संकलन र अलग गर्‍यौं । यसपछि ती नमुनाहरुमा निश्चित मिडियाको (MRS) प्रयोग गरी ल्याक्टोव्यासिलसको गणना गरियो । ल्याक्टोव्यासिलस (*Lactobacillus* spp.) अलग गर्न एमआरएस मिडिया साथै सो प्रजातीको बाह्य स्वरुप पहिचान गर्न अन्य बायोकेमिकल परिक्षणहरु गरियो । ल्याक्टोव्यासिलसको पुष्टिकरण गर्न ग्राम स्टेनिंग, गतिशिलता परिक्षण र क्याटालेज परिक्षणहरु गरियो । थप विश्लेषणको लागी फर्मेन्टेशन परिक्षण र यसको बृद्धि मापनको लागी विभिन्न तापमात्रा तथा साल्ट घनत्व परिक्षणहरु पनि गरियो । यसपछि ल्याक्टोव्यासिलस प्रजातीबाट प्रेसिपिटेशन विधिद्धारा व्याक्टेरियोसिन लाई अलग गरियो । यस अध्ययनबाट १० ल्याक्टोव्यासिलस प्रजाती पहिचान गरियो । अलग गरिएको व्याक्टेरियोसिन जस्तै कम्पाउडले स्टेफाइलोकोकस औरीयस (*Staphylococcus aureus*), व्यासिलस सिरस (*Bacillus cereus*), इसचेरिचिया कोली (*Escherichia coli*), सिउडोमोनस औरुगीनोसा (*Pseudonmonas aeruginosa*) र क्लेब्सीयला न्युमोनिया (*Klebsiella pneumoniae*) प्रतिनिरोधात्मक प्रतिकृया प्रदर्शन गर्‍यो। सवैभन्दा उच्चत्तम जोन अफ इन्हिविशन BL10 आइसोलेटले *B. cereus* (30mm) लाइ देखायो । ल्याक्टोव्यासिलस प्रजातीबाट अलग गरिएको व्याक्टेरियोसिनले जस्तै कम्पाउडनले भिन्न एमडिआर व्याक्टेरिया प्रति एन्टिमाइक्रोवियल गतिविधिहरु प्रदर्शित गर्‍यो । त्यसैले व्याक्टेरियोसिन जस्तै कम्पाउडबाट बहुप्रतिकात्मक व्याक्टेरियाको लागी नयाँ स्वरुपको एन्टिबायोटिक तयार गर्न सकिन्छ।

मुख्यशब्दहरुः गाइफार्म, माटो, ल्याक्टोव्यासिलस प्रजाती, व्याक्टेरियोसिन

LIST OF ACRONYMS AND ABBREVIATIONS

AST:	Antibiotic Susceptibility test
ATCC:	American Type Culture Collection
$CaCO_3$:	Calcium Carbonate
CFS:	Cell Free Solution
CLSI:	Clinical Laboratory Standards Institute
CO_2:	Carbon Dioxide
DNA:	Deoxy Ribo Nucleic Acid
FDA:	Food and Drug Administration
GRAS:	Generally recognized as Safe
IoST:	Institute of Science and Technology
KDa:	Kilo Dalton
LAB:	Lactic Acid Bacteria
MHA:	Mueller Hinton Agar
Mm:	Millimeter
MRS:	de Mann Rogosa Sharpe
NA:	Nutrient Agar
NaCl:	Sodium Chloride
NaOH:	Sodium Hydroxide
PCR:	Polymerase Chain Reaction
PGPM:	Plant Growth Promoting Microorganisms
$P^{H:}$	Potential of Hydrogen
RAPD-PCR:	Randomly amplified polymorphic DNA- Polymerase Chain Reaction

LIST OF SYMBOLS

%:	Percentage
^{0}C:	Degree Celsius
G:	Grams
Mm:	Millimeter
ml:	Milliliter
ul:	Microliter

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CHAPTER 1

INTRODUCTION

1.1. General Introduction

Among different Lactic Acid Bacteria (LAB), *Lactobacillus* species are rod shape bacteria and able to produce lactic acid by carbohydrates fermentation. Many strains of the genus *Lactobacillus* spp. are capable of colonizing in tract of urogenital as well gastrointestinal and became competitive with pathogen (Antonio, *et al*., 1999; Redondo, *et al*., 1990). In environment, *Lactobacillus* spp. play important role for transformation of bad flavor of substances by decomposition of highly complex substances like macro-molecules and biomolecule (Arena, et al., 2019). So, *Lactobacillus* species are used as soil amendment, plant growth promoting bacteria, disease suppression, bioremediation, composting and organic waste management, soil restoration etc. LAB are also used as probiotics and can be used as a biocontoller (Messaoudi, *et al*, 2013).

Lactobacillus spp. showed antibacterial activity against pathogenic bacteria due to the synthesis of different known molecules like bacteriocin, H_20_2 and lactic acid as well as unknown molecule which is heat-stable and other different molecule which is not lactic acid (Servin, 2004). These molecules have inhibitory activity only after the production of the secondary compounds (metabolites) after 48 hrs of fermentation by LAB (Rouse, *et al*., 2008). Lactic acid bacteria showed antibacterial activity against resistance bacteria (*P. aeruginosa*) which are isolated from clinical samples. So, *Lactobacilli* spp. showed antibacterial activity for the highly resistance bacteria (MDR *P. aeruginosa*) isolated from clinical samples (Jamalifar, *et al*., 2011).

Many clinical problems were caused due to the multi-drug resistance in bacteria. So, resistance bacteria caused many infectious diseases which increased mortality and morbidity as well as increased health cost in many developing countries (Pfaller, *et al*., 1997; Jarvis & Martone, 1992). In recent time, *Lactobacilli* spp. have been reported for prevention as well as treatment for various bacterial infections. So, many strains of *Lactobacillus* spp. are found commercially for oral consumption which are useful for different infections caused by bacteria (Gordon, *et al*., 1957; Carson & Riley, 2003).

1.2 Statement of the problem

Antibiotic resistance in bacteria is the greatest problem in the world which is due the misuse of antibiotics in animals as well as in human treatment for different diseases (Lipsitch, *et al.*, 2000, Roy, 1997, Yoneyama & Katsumata, 2006). So, the different strategies have been used for treatment of different diseases, among which bacteriocins production by LAB is one of them. Bacteriocins are antimicrobial metabolites (peptides) synthesizing by LAB which are capable for controlling the drug- resistant and clinically resistance bacteria. So, there is very importance for production of new antimicrobial producing agents as medicine (Kumar & Schweiser, 2005). Hence, the strains of LAB are able to produce antimicrobial bacteriocin like compound, which may be able to inhibit the pathogens. So, the main aim of study was to extract bacteriocin like compound from *Lactobacillus* spp. isolated from cow farm soil and to study its antimicrobial activity against highly resistance bacteria.

1.3 Objectives

1.3.1 General Objective

- To determine antibacterial assay of *Lactobacillus* spp. isolated from farm soil against MDR bacteria.

1.3.2 Specific Objectives

- To isolate and identify *Lactobacillus* spp. from farm soil.
- To perform antibiotic susceptibility test of MDR bacteria.
- To determine the antimicrobial activity of bacteriocin like compound against MDR bacteria.

1.4 Rationale of the study

In the nature, Lactic acid bacteria (LAB) are widely found in waters, soils, silages, plants, waste products, etc. as well as from humans and animal intestinal tract (Axelsson, 1998). There are several studies in LAB which were isolated from milk, milk products and fermentation products. However, LAB produced secondary metabolites known as bacteriocin having antimicrobial activity against pathogenic bacteria were less studied. To our knowledge in Nepal, little information exists on *Lactobacillus* spp. in soil samples. Only less study was done for isolation of *Lactobacillus* spp. from farm soil and perform antibacterial activity against MDR bacteria. So, this study was aimed for isolation of *Lactobacillus* spp. from farm soil then extraction of bacteriocin like compound was done for the study of antibacterial activity against highly resistance bacteria.

1.5 Limitation of the study

A total of 30 soil samples from Kathmandu valley was collected from the farm side (cow farm only). Only MDR bacteria was studied for antibacterial activity of *Lactobacillus* spp.

CHAPTER 2

LITERATURE REVIEW

Lactic acid bacteria are found in the nature and isolated from waters, soils, silages, plants, waste products. From animals and human intestine, LAB are also isolated (Axelsson, 1998). These bacteria belong to the phylum of Firmicutes. Among LAB, Lactobacillus, Leuconostoc, Carnobacterium, Lactococcus, Enterococcus, Pediococcus, Melissococcus, Streptococcus, Vagococcus, Oenococcus, Tetragenococcus, and Weissella are well known as LAB (Jay, 2000).

Lactic acid bacteria produce different metabolites which are fatty acids with short-chain, organic acids, amines, vitamins, exopolysaccharides and bacteriocins (Arena, *et al*., 2019). Among different metabolites, bacteriocin are toxic to microorganisms. So, Bacteriocin are used for the production of drugs like antibiotic having properties of probiotic. Organic acids which are also known as another metabolite produced by LAB which have antimicrobial activity against fungi. So, these metabolites are used as preservative in some food of fermentation (Gajbhiye, *et al*., 2016).

Among different metabolites produced by LAB, bacteriocins is one of the well study metabolites. These are the metabolites which are highly toxic to microorganisms and used for the production of drug like antibiotic. Actually, ribosomes synthesize the bacteriocins either in proteins or peptides which inhibit the reproduction and growth of a different bacteria (Diep and Nes, 2002).

Bacteriocins are known as protein or peptides that are synthesized by ribosome's and are able to inhibit a variety of bacteria. Bacteriocin are the protein substances which are categorized into two types. Lantibiotics is the first type which either containing or not containing of lanthionine. These lantibiotics are produced by *L. lactis* (polycyclic peptide) which is the causative agent for damaging the cell membrane in bacteria (Gram positive). Helveticin (M and J) is the second type of bacteriocins which are produced by *L. helveticus* and *L. crispatus.* These are types of bacteriocins which are especially used for preservatives of food (Raman, *et al*., 2022).

On the other hand, different fungal infections have caused the severe infection on crops and also affect the livestock feed and quality of seeds. Some strains of *L. plantarum* as well as *Bacillus* spp. were generally used for biological control due to its antifungal activity (Vuyts & Leroy, 2007).

Bacteriocins are synthesized by some bacteria which are capable for controlling both clinically susceptible and resistant types of bacteria. So, there is a continued increasing for the development of new types of antimicrobial agents for production of medicines (Kumar & Schweiser, 2005). Bacteriocins are also studied from the modification to improvement of the properties like physiochemical and effects like pharmacological as well as biosafety. Bacteriocins extracted from same species or from different species were active against different bacteria. So, it is known for narrow spectrum or across genera that is broad spectrum (Mobolaji & Wuraola, 2011).

Variety of bacterial and fungal phytopathogens exist on soil. These will hamper the growth of plant, significant damage in agriculture which gradually decline the productivity and loss the economic value as well as quality of the products in that case, LAB has served as an effective biocontrol agent (Jaffar, *et al*., 2022).

These bacteria are the significant group of probiotic organisms and have protective effects in fermented food preservation, as they are able to produce organic acids in food during their growth. These group species are capable of conversion of carbohydrates to organic acids which will gradually reduce p^H changes, that's the reason to increase the half-life and good quality of such food products. These bacteria are generally known as safe (GRAS) and also considered for one of the important food ingredients (Vijayakumar & Muriana, 2015).

Not only benefited as a food ingredients, these bacteria are able to regulate soil organic matter, biogeochemical cycle, enhancement of plant growth hormone, health of plant and also detoxify the hazardous chemicals. LAB showed the antimicrobial activity against various fungal and bacterial populations of phyllospheric as well as rhizospheric region. Fungal secretion or mycotoxins, heavy deposition of metals affect the natural quality of soil and the organisms of the soil. But, by the use of LAB, the naturality of the soil remain intact and promote the plant growth factors (Raman, *et al*., 2022).

There are many studies which proposed the different mechanism for the inhibitory activity of bacteriocins produced by LAB. Some studies added that bacteriocins showed inhibitory activity to microorganisms by inhibiting protein and nucleic acid synthesis (Kumariya, *et al*., 2019). The lantibiotics are produced from *L. lactis* which are the derivatives of polycyclic group of lanthionine having antibacterial peptides which damaged the cell of many bacteria mostly of Gram-positive (Montville, *et al*., 1998). The second type of bacteriocins are produced by *L. helveticus* and *L. crispatus* which are Helveticin J and Helveticin M. and used for the preservation of food. However, bacteriocin used for controlling the pathogens in crops as commercially showed the resistance in some plants (Rooney, *et al*., 2020)

In the study conducted by Kang (2019) explained that, *Lactobacillus* spp. was used for soil remediation. As, the soil composed of clay, loom or silt. When it was left untreated, soil became harden up and flow of water inside soil became very much harder. So, *Lactobacillus* species in the soil improved the soil friability as well as in land restoration.

Chen, *et al*., (2005) studied in Japan and Taiwan for isolation of 54 LAB from 68 soil samples of rhizospheric area of fruit trees and floor of a horse farm and hen house. In their study, 32 LAB were identified from 54 isolates belonging to five genera:, *Weisssella, Lactococcus, Enterococcus, Leuconostoc* and *Lactobacillus* spp.. From their study, the highest number of LAB were isolated from soil.

Idham, *et al*., (2016) isolated and identified microorganism from cow manure from Indonesia. They reported the decomposers found in cow manure were *Actinomycetes* spp., *Lactobacillus* spp., and *Aspergillus* spp. They isolated these genera on the basis of morphological and physiological characteristics. According to their study, *Lactobacillus* spp. showed circular colony morphology. They observed growth at the temperature of 45^0C and the pH was 3.5. Isolated species able to form acid on sucrose and glucose but not with maltose. Yanagida, *et al*., (2006) conducted the research for the isolation of LAB producing bacteriocin from soil samples in Japan. From their study, 42 LAB were isolated from 55 soil samples This study reported the first production of bacteriocin by *Lactobacillus animalis*. So, this study finding suggested that, the soil is also natural habitat of LAB from which the bacteriocin is isolated as novel antibiotics.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

All the necessary materials, chemicals, equipment's, media reagents required for the study is enlisted in Appendix A.

3.2 Research Methodology

Different cow farms of Kathmandu district (Kritipur area, Thankot area and Budhanilkantha area) were selected for this study. Altogether, 30 soil samples were collected from different local cow farm. All Soil samples were aseptically collected in plastic zip lock bags, and labeled in it with date, time, and sampling sites name. Then, all soil samples were transported to Padma Kanya Campus for the processing in the microbiology laboratory as soon as possible. This study conducted from October 2023 to March 2024.

3.3. Research design

Cross- sectional study was done for this research work. A sampling method was convenience from which 30 different soil samples were from three areas (Kritipur, Thankot and Budhanilkantha) of Kathmandu district for isolation of *Lactobacillus* spp. and extraction of antimicrobial agents (likely bacteriocins) for study of antimicrobial activity against MDR bacteria.

3.4. Experimental procedure

3.4.1. Laboratory Processing

For the isolation of LAB, 1 gm of sample (soil) was mixed properly with 9ml of phosphate buffered saline (1/10 dilution). From this dilution (10^{-1}), serial dilution was done to 10^{-2} to 10^{-6}. For enumeration and isolation of *Lactobacillus* spp., spread plate method was done in MRS agar (De man, Rogosa and Sharpe) containing 1% $CaCO_3$. After solidification of media, all plates were incubated at 37^0C for 48hrs. Then, isolated colonies were again sub-cultured in 1% $CaCO_3$ containing MRS agar for isolation of pure culture of *Lactobacillus* spp. (Kazemipor, *et al*., 2012).

3.4.2. Identification of *Lactobacillus* spp.

Identification of *Lactobacillus* spp. was based on biochemical test result. Gram's test, catalase test and motility test was done for further identification. Fermentation tests (glucose, fructose, galactose, sucrose, arabinose, maltose, lactose and mannitol), NaCl tolerance test and tolerance of acid were also done for further identification of isolates. Then isolation and identification of *Lactobacillus* spp. was followed according to Bergey's manual of determinative bacteriology (Anosike, 2022).

3. 4.3. Antibiotic Susceptibility Tests of test bacteria

Antibiotic susceptibility test for test bacteria was done by modified Kirby- Bauer disc diffusion method. Similarly, antibiotic susceptibility test for isolates *Lactobacillus* spp. were also done (CLSI, 2007). All the test bacteria were collected from Central Department of Microbiology, Tribhuvan University, Kritipur. Firstly, Mueller-Hinton agar was prepared in Petri dishes. Then, a standardized bacterial suspension (*S. aureus*, *B. cereus*, *E. coli*, *P. auroginosa* and *K. pneumonia*) in NB and isolated *Lactobacillus* spp. in MRS broth were created by adjusting the turbidity of the bacterial culture to match the McFarland standard (0.5). Then, the test bacteria and isolated *Lactobacillus* spp. were spread over the MHA plates and different discs of antibiotic were transferred on it. All plates were incubated at 37^0C for 24 hrs. After incubation, all plates were observed and examined for the inhibition zones around each disc. The diameter of each zone of inhibition was measured and compared with standardized zone size according to CLSI (2022).

3.4.4 Screening for bacteriocin like substances

For screening test, dot plate technique method was used in which all test bacteria were lawn culture in MHA plate then the dot inoculation of isolated *Lactobacillus* spp. was done in 1% $CaCO_3$ containing MRS agar and incubated at 30^oC for 24hrs. Then the screening of bacteriocin like substances producing *Lactobacillus* spp. was done by observing the zone of inhibition in 1% $CaCO_3$ containing MRS agar (Palaniyammal et al., 2019).

3.4.5 Extraction of bacteriocin like compound

For the extraction of bacteriocin, *Lactobacillus* spp. were inoculated in MRS broth at pH 7.0 for 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5day) and 120 hrs (6day) at 37^0C. Then, all culture was centrifuged for 20 mins at 10,000 rpm at 4^0C. After centrifugation, cell free suspension (CFS) was separated then adjusted its pH to 7.0 by 1M NaOH. Then, CFS was found to be precipitated with organic acids presence in the medium which was separated bacteriocin like compound through filtration by using Whatman filter paper no. 1. After filtration, bacteriocin like compound extracted from 10 *Lactobacillus* spp. were collected in potassium phosphate buffer (Yang, *et al*., 1992).

3.4.6 Determination of antimicrobial activity of bacteriocin like compound

For determining the antimicrobial activity, all test bacteria were inoculated in NB for 4hrs at 37^0C and its turbidity was compared with McFarland standard (0.5). Then, all bacterial suspension was swabbed on the Mueller Hinton Agar and wells were made on it. A 70μl of bacteriocin like compound solution along negative control (sterile water) and positive control (Ciprofloxacin) were loaded on the different well in each plate and allowed for 15mins for diffusion then all plates were incubated for 24 hrs to 48hrs at 37^0C. Then, the zone of inhibition was observed and its diameter was measured (Chirom, *et al*., 2016). Antibacterial activity of bacteriocin like compound was done from 24hrs, 48hrs, 72hrs, 96hrs and 120hrs of incubation of *Lactobacillus* spp. in MRS broth at pH 7.0. From this study, the comparative study of antimicrobial activity of bacteriocin like compound extracted from 24hrs, 48hrs, 72hrs, 96hrs and 120hrs of incubation of *Lactobacillus* spp. (Zhennai, 2000).

3.5. Quality control in the laboratory

Quality control was done to obtain for reliable results in microbiology. Quality was also monitored for each laboratory equipment's throughout the study period. Similarly, a high level of aseptic condition was diligently upheld while carrying out all procedure as well as the purity of each plates were carefully preserved to ensure the presence of pure culture.

Standard culture of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) were used for the interpretation of result in antimicrobial activity.

3.6. Data Analysis: All the data was obtained from this study was entered into Microsoft Excel 2016 and analyzed by percentage calculation.

3.7. Ethical Clearance: The ethical consideration was taken from owner of cow farm. After explanation about the purpose of the study with cow farm owner then only soil samples were collected from cow farm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Growth profile of *Lactobacillus* spp.

In this study, *Lactobacillus* spp. were isolated from different soil samples collected from local cow farm. In the study, 30 soil samples were included for isolation of *Lactobacillus* spp from the Kathmandu district. Among total soil samples, 10 (33.3%) isolates were identified as *Lactobacillus* spp. (Figure 1).

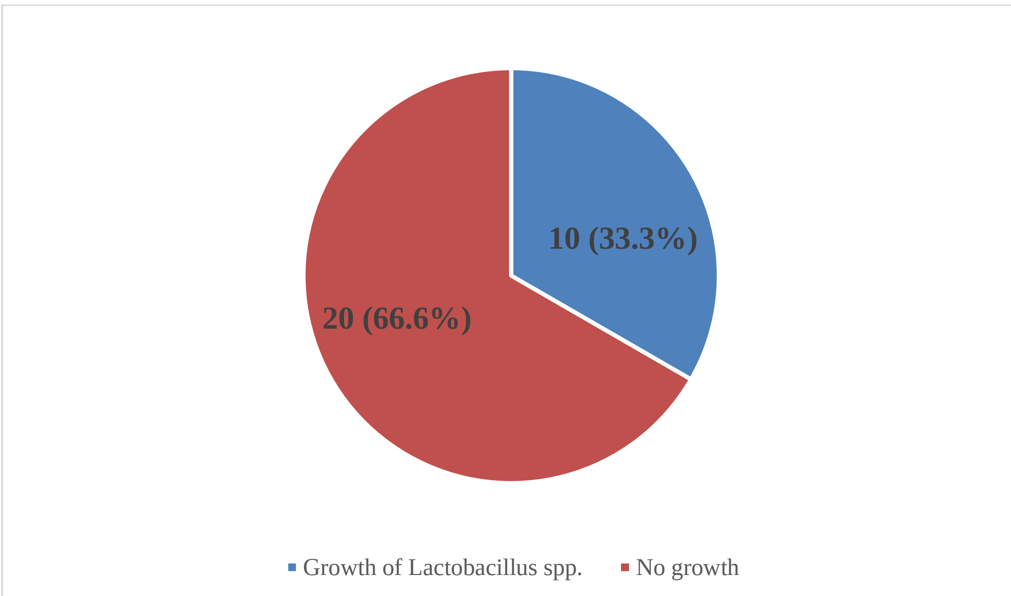


Figure 1: Growth profile of *Lactobacillus* spp.

4.1.2 Distribution of *Lactobacillus* spp. isolates from different location

In this study, cow farm soil samples were taken from the 3 sites which were Kritipur (10 samples), Thankot (10 samples) and Budanilkantha (10 samples). The highest occurrence of *Lactobacillus* spp. was found from Kritipur, 6 (20%) followed by Thankot 2 (6.66%) and Budanilkantha 2 (6.66%). (Table 1).

Table 1: Distribution of *Lactobacillus* spp. from different location

Sites	Location	Number of Samples (N)	Isolated *Lactobacillus* spp. (%)
I	Kritipur	10	6 (20)
II	Thankot	10	2 (6.66)
III	Budanilkantha	10	2 (6.66)

N=Number, %= Percentage

4.1.3. Identification of isolated *Lactobacillus* spp.

4.1.3.1. Identification of isolated *Lactobacillus* spp.

Lactobacillus spp. were isolated and identified from colony morphology and cell morphology then identified from Gram staining, motility, Spore staining, catalase test and oxidase test. The cell morphology of isolates was observed from microscope and found to be rod shaped bacteria (Figure 2).

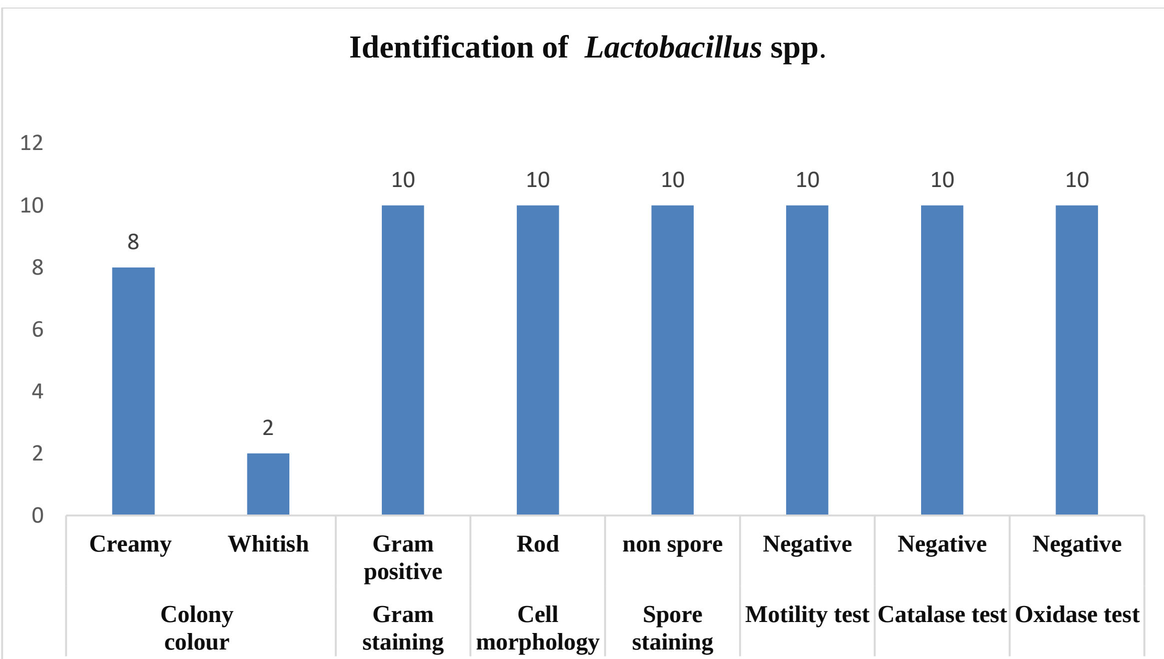


Figure 2: Identification of *Lactobacillus* spp.

4.1.3.2 *Lactobacillus* spp. growth at different temperature

In this study, *Lactobacillus* spp. were able to grow at 10^0c, 15^0c, 35^0c, 37^0c and 45^0c respectively. In different temperature, all ten isolates were able to grow at 35^0c, 37^0c and 45^0c, while no isolates were able to grow at 10^0c and 15^0c respectively (Figure 3).

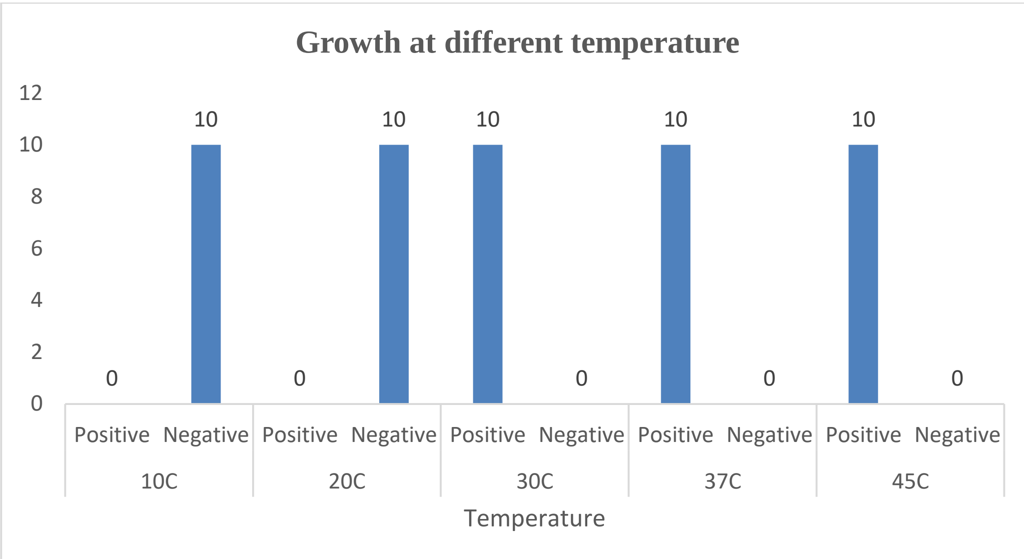


Figure 3: *Lactobacillus* spp. growth at different temperature

4.1.3.3. *Lactobacillus* spp. growth at different (NaCl) Concentration

In this study, 2 *Lactobacillus* spp., 4 *Lactobacillus* spp. and 4 *Lactobacillus* spp were able to grow at 2%, 4% and 6.5% salt concentration respectively (Figure 4).

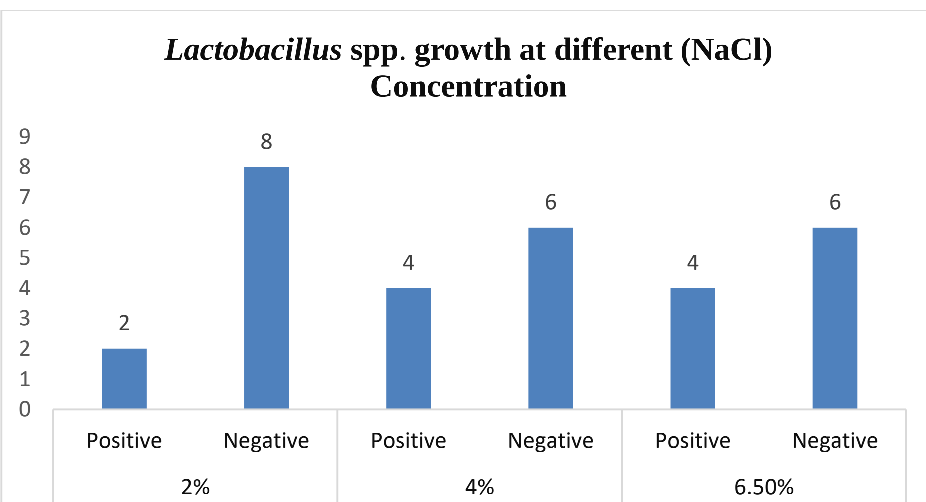


Figure 4: *Lactobacillus* spp. growth at different (NaCl) Concentration

4.1.3.4. *Lactobacillus* spp. growth at different pH

In this study, 2 *Lactobacillus* spp., 8 *Lactobacillus* spp. and 10 *Lactobacillus* spp were able to grow at pH 2, pH 4 and pH 6 respectively (Figure 5).

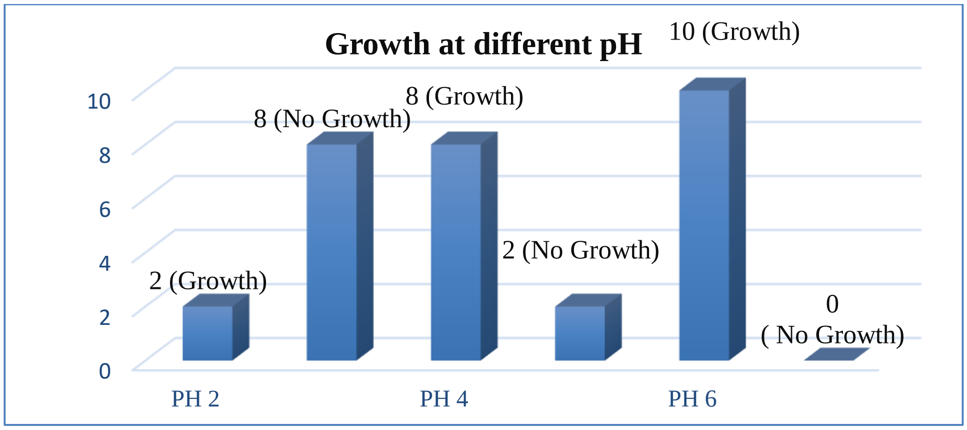


Figure 5: *Lactobacillus* spp. growth at different pH

4.1.3.5. Carbohydrate fermentation test of *Lactobacillus* spp.

In this study, fermentation test for glucose, lactose, fructose, mannitol, sucrose, arabinose, galactose and maltose were done for identification of *Lactobacillus* spp. Different *Lactobacillus* spp. fermented different carbohydrates with gas formation (Table 2).

Table 2: Carbohydrates fermentation test of *Lactobacillus* spp.

***Isolated Lactobacillus* species**	**Carbohydrates Fermentation**							
	Glucose Acid/Gas	**Lactose Acid/Gas**	**Fructose Acid/Gas**	**Mannitol Acid/Gas**	**Sucrose Acid/Gas**	**Arabinose Acid/Gas**	**Galactose Acid/Gas**	**Maltose Acid/Gas**
KL1	+ +	++	++	+-	+-	--	++	++
KL2	+ +	++	++	+-	+-	--	++	++
KL3	++	+ -	++	--	++	+-	+-	+-
KL4	++	+-	+-	--	+-	--	++	+-
KL5	++	+-	+-	--	+-	--	++	+-
KL6	++	+-	+-	--	+-	--	++	+-
TL7	+ +	++	++	+-	+-	--	++	++
TL8	+ +	++	++	+-	+-	--	++	++
BL9	++	+-	+-	--	+-	--	++	+-
BL10	++	+ -	++	--	++	+-	+-	+-

Acid, + = Fermenter, - = Non fermenter, Gas, + = Gas formation, - = No gas formation

4.1.4 Antibiotic susceptibility test of Gram positive bacteria

In this study, Multi Drug Resistance (MDR) Gram positive bacteria were confirmed by Antibiotic susceptibility test for Ampicillin (AMP), Azithromycin (AZM), COT- Co-trimoxazole, Gentamicin (GEN), Ciprofloxacin (CIP), Nitrofurantoin (NIT) and Cefixime (CFM). Among two Gram positive bacteria, *S. aureus* were found to be resistant to GEN, AMP, AZM and susceptible to COT and CIP while *Bacillus cereus* were found to be resistant to AMP, COT, GEN and susceptible to AZM, E and CIP. (Table 3).

Table 3: Antibiotic susceptibility tests of Gram positive bacteria

Gram positive	AMP	AZM	CIP	E	COT	GEN
Staphylococcus aureus	R	R	S	R	S	R
Bacillus cereus	R	S	S	S	R	R

R=Resistance, S=Susceptibility, COT- Co-trimoxazole, AMP-Ampicilin, AZM-Azithromycin, AMX-Amoxicillin, GEN-Gentamicin, CIP-Ciprofloxacin, NIT-Nitrofurantoin and CFM-Cefixime.

4.1.5 Antibiotic susceptibility test of Gram negative bacteria

In this study, Multi Drug Resistance (MDR) Gram negative bacteria were confirmed by Antibiotic susceptibility test for Ampicillin (AMP), Azithromycin (AZM), COT- Co-trimoxazole, Gentamicin (GEN), Ciprofloxacin (CIP), Nitrofurantoin (NIT) and Cefixime (CFM). Among three Gram Negative bacteria, *E. coli* and *P. auroginosa* were resistant to NIT, GEN, AMP and susceptible to CFM, CIP and COT while *K. pneumoniae* were resistant to CIP, AMP, NIT and susceptible to COT and CFM.

Table 4: Antibiotic susceptibility tests of Gram negative bacteria

Gram negative	AMP	CFM	CIP	NIT	COT	GEN
Escherichia coli	R	S	S	R	S	R
Pseudomonas auroginosa	R	S	S	R	S	R
Klebsiella pneumoniae	R	S	R	R	S	R

R=Resistance, S=Susceptibility, COT- Co-trimoxazole, AMP-Ampicilin, AZM-Azithromycin, AMX-Amoxicillin, GEN-Gentamicin, CIP-Ciprofloxacin, NIT-Nitrofurantoin and CFM-Cefixime

4.1.6 Screening of *Lactobacillus* spp. for bacteriocin like substances

For screening of bacteriocin like compound, all ten isolates (*Lactobacillus* spp.) were screened for presence of bacteriocin like compound. All isolates (*Lactobacillus* spp.) showed inhibition zone against all test bacteria except BL9 isolates (*Lactobacillus* spp.) which did not show zone of inhibition against all test bacteria (Table 5).

Table 5: Screening of *Lactobacillus* spp. for bacteriocin like substances

Test bacteria	Zone of inhibition(mm) for screening of *Lactobacillus* spp.									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	+	+	+	-	-	+	+	+	-	-
Staphylococcus aureus	+	+	+	-	-	+	+	+	-	+
Bacillus cereus	+	+	+	+	+	+	+	-	-	+
Gram Negative bacteria										
Escherichia coli 25922	+	+	+	-	-	+	-	+	-	-
Escherichia coli	+	+	+	+	-	+	-	+	-	-
Klebsiella pneumoniae	+	+	+	-	+	+	+	+	-	+
Pseudomonas aeruginosa	+	-	-	-	-	-	-	-	-	-

+= zone of inhibition and - = No zone of inhibition

4.1.7 Antimicrobial activity of bacteriocin like compound extracted from *Lactobacillus* spp.

Antimicrobial activity of bacteriocin like compound extracted from *Lactobacillus* spp. from 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5days) and 120hrs (6days). Antimicrobial activity showed the zone of inhibition against MDR bacteria only after 72hrs (4day), 96hrs (5days) and 120hrs (6days) of extraction of bacteriocin against *S. aureus*, *B.* cereus, *P. aeruginosa* and *E. coli* but not to *K. pneumonia*. In this study, *Lactobacillus* spp. showed zone of inhibition against MDR bacteria (Table 6,7,8 and 9).

Table 6: Antimicrobial activity of bacteriocin like compound extracted from 24hrs (2days)

Test bacteria	Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin)									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-	-	-	-	-
Gram Negative bacteria										
Escherichia coli 25922	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-

Table 7: Antimicrobial activity of bacteriocin like compound extracted from 48hrs (3days)

Test bacteria	Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin)									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-	-	-	-	-
Gram Negative bacteria										
Escherichia coli 25922	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-

Table 8: Antimicrobial activity of bacteriocin like compound extracted from 72hrs (4days)

Test bacteria	Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin)									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	-	-	-	7	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Bacillus cereus	-	-	15	13	18	-	-	-	-	-
Gram Negative bacteria										
Escherichia coli 25922	15	14	22	17	13	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
Escherichia coli	13	16	20	16	17	-	-	-	-	-

Table 9: Antimicrobial activity of bacteriocin like compound extracted from 96 hrs (5 days)

Test bacteria	Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin)									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	10	-	-	-	11	-	-	-	-	-
Staphylococcus aureus	7	-	-	-	-	7	-	-	-	7
Bacillus cereus	20	-	-	-	8	-	11	-	-	30
Gram Negative bacteria										
Escherichia coli 25922	14	-	-	-	11	-	16	-	-	10
Klebsiella pneumoniae	7	-	-	-	8	9	8	-	-	8
Pseudomonas aeruginosa	-	-	-	-	10	-	7	-	-	9
Escherichia coli	18	-	-	-	20	-	19	-	-	10

Table 10: Antimicrobial activity of bacteriocin like compound extracted from 120 hrs (6 days)

Test bacteria	Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin)									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	-	-	-	8	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-	-	-	-	-
Gram Negative bacteria										
Escherichia coli 25922	-	-	-	8	-	-	-	-	-	-
Klebsiella pneumoniae	-	8	-	8	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	8	-	9	-	-		7
Escherichia coli	-	-	-	-	-	-	-	-	-	-

4.1.8 Comparison of antimicrobial activity of bacteriocin like compound extracted from *Lactobacillus* spp. in different time interval

In different time interval, bacteriocin extracted from *Lactobacillus* spp. showed different result. The highest number of zone of inhibition showed in 72hrs (6) followed by 96hrs (4) of extraction of bacteriocin. However, 24hrs and 48hrs did not showed any zone of inhibition for any test bacteria. On the other hand, bacteriocin extracted from 120 hrs also showed the highest zone of inhibition for test bacteria (Figure 6).

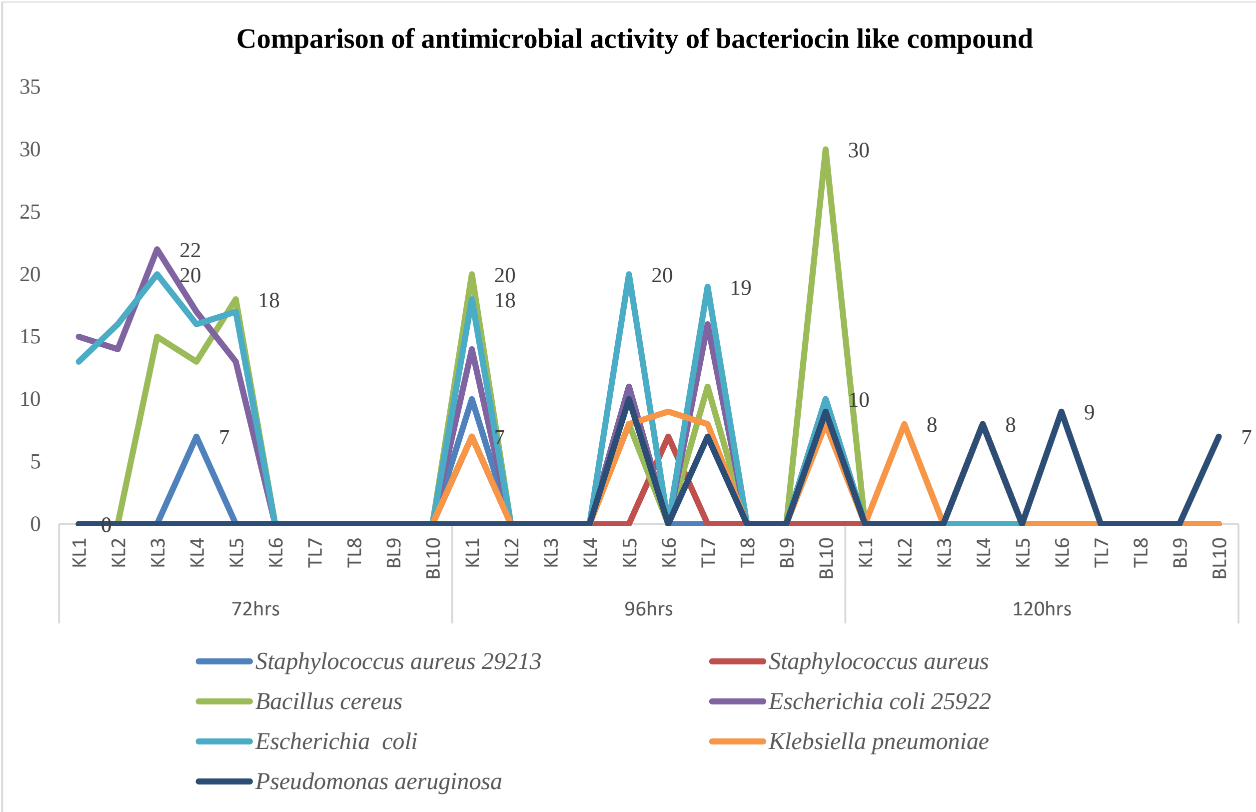
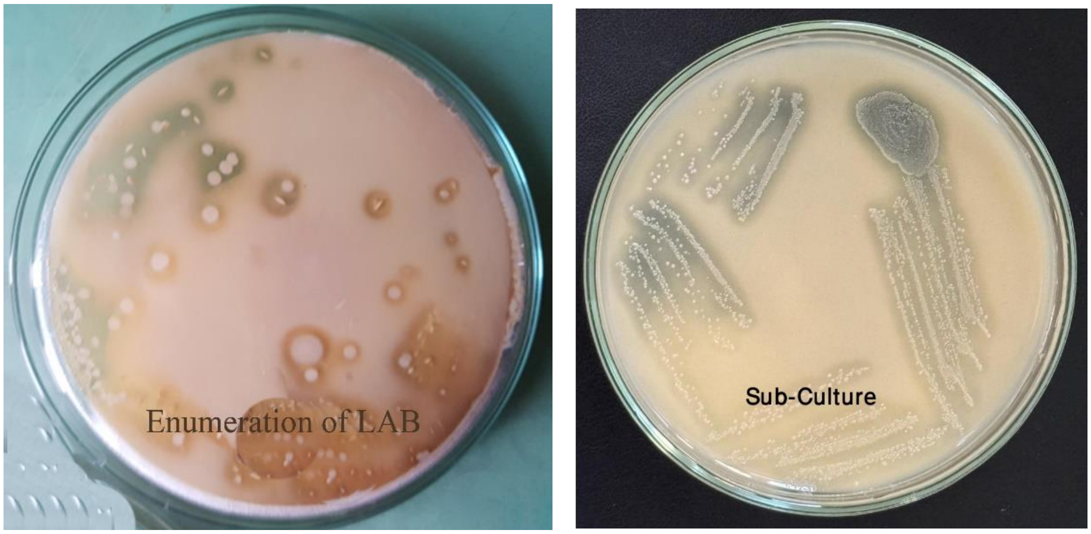


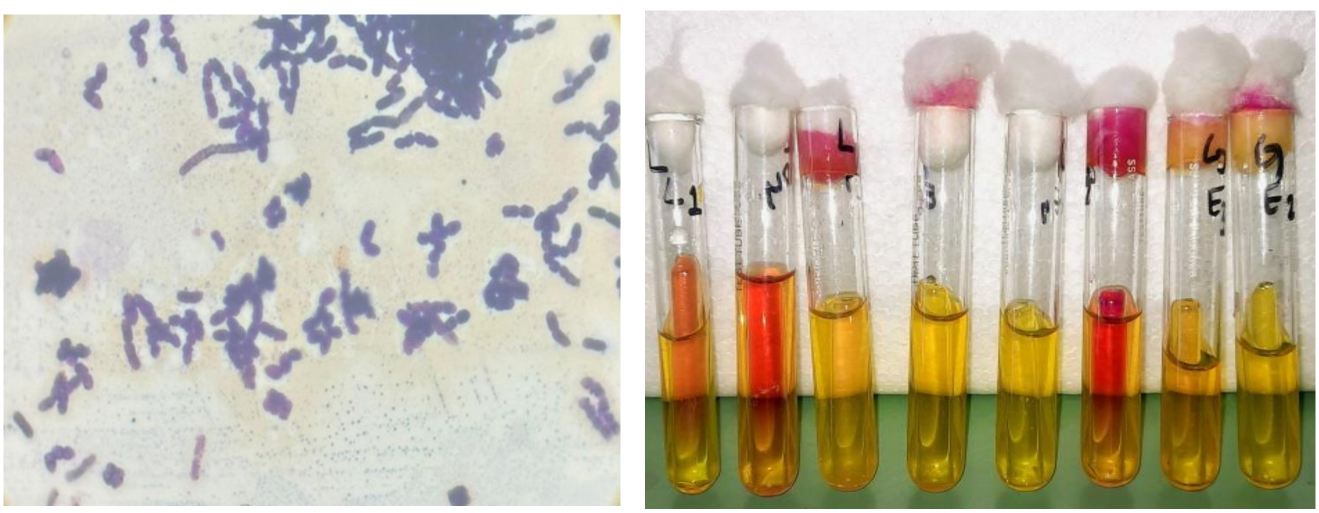
Figure 6: Comparison of zone of inhibition by bacteriocin like compound

LIST OF PHOTOGRAPHS



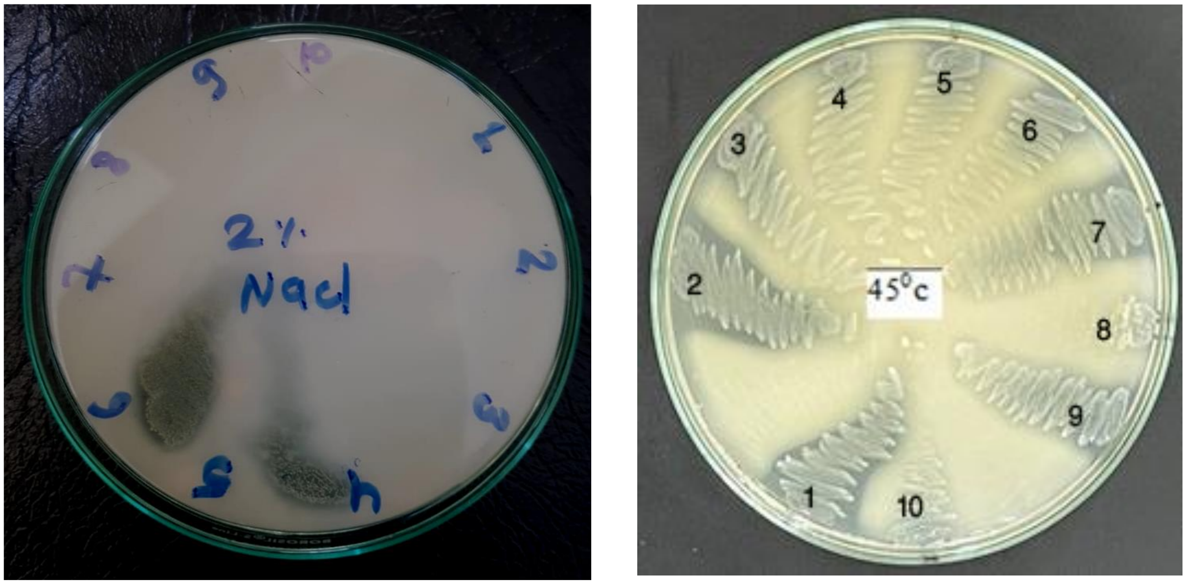
A **B**

Photograph 1: Enumeration of *Lactobacillus* species (A) and subculture of *Lactobacillus* species on de Mann Rogosa Sharpe (MRS) Agar media(B)



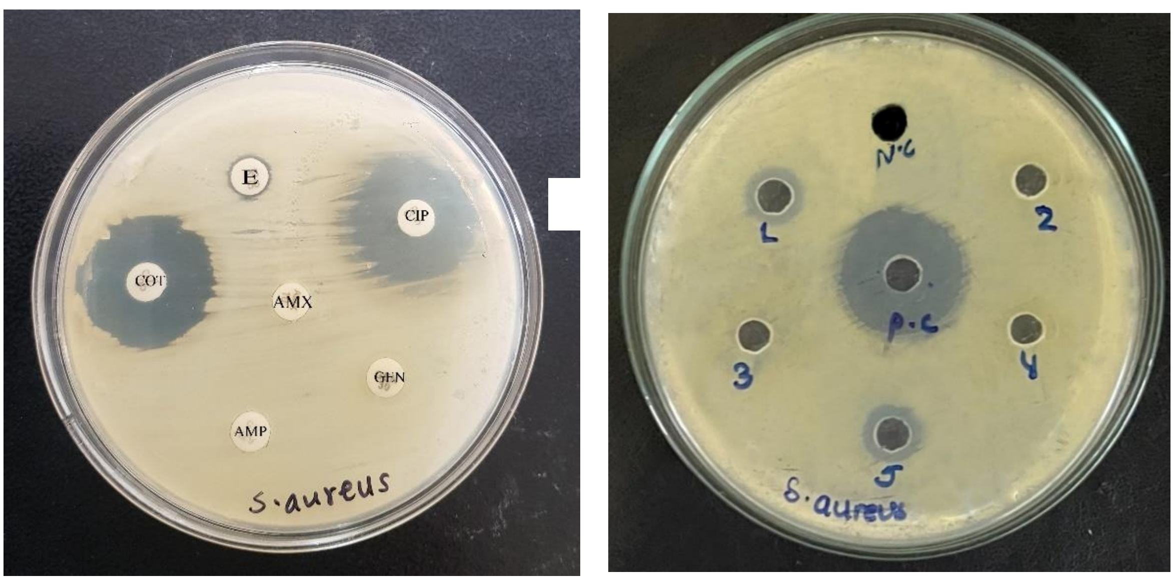
A **B**

Photograph 2: Identification of *Lactobacillus* spp. by Gram staining (A) and carbohydrate fermentation test (glucose, maltose, fructose, galactose, sucrose, arabinose, lactose and mannitol)



A **B**

Photograph 3: ***Lactobacillus*** **species growth at 2% NaCl (A) and 45^{0}c (B)**



A (AST) **B (72hrs)**

Photograph 4: Antibiotic susceptibility test of test bacteria (A) and Antimicrobial activity of bacteriocin like compound extracted from ***Lactobacillus*** **species at 72hrs (B)**

AZM-Azithromycin, CIP-Ciplofloxacin, GEN-Gentamicin, AMP-Ampicillin, COT-Co-trimoxazole, CAC= Ceftazidime and E-Erythromycin, P.C =Positive control, N.C=Negative control and 1,2,3,4 &5=Isolates

4.2 DISCUSSION

In this study, 30 different soil samples were collected from various local farm area of Kathmandu district of 3 sites including Kritipur (10 samples), Thankot (10 samples) and Budanilkantha (10 samples). The highest occurrence of *Lactobacillus* spp. were found from Kritipur followed by Thankot and Budanilkantha which were 6 (20%), 2 (6.66%) and 2 (6.66%) respectively. Among total samples, 10 strains (33.3%) were identified as *Lactobacillus* spp. by various morphological, biochemical and physiological test.

Similar study was done by Yanagida, *et al*., (2005) for isolation of new types of bacteriocin producing LAB from soil in which 55 soil samples were taken and 42 were found as acid producing bacterial strains. On the other hand, Ekundayo (2014) obtained 21 LAB obtained from rhizosphere soil, gastrointestinal tract, gills of fish, pond sediments as well as yellow maize gruel. According to Ekundayo (2014), LAB can be detected in soil which may be considered as a source of LAB as well as its ability to grow at different temperature, salt concentration and pH level.

For identification of *Lactobacillus* spp. different laboratories techniques were performed including isolation, identification by microscopic examination, culturing and biochemical test. The morphological characteristics of all the isolates basically showed same type of feature of *Lactobacillus* spp. Similarly, *Lactobacillus* spp. were also found to be Gram positive rod shape, nonmotile, nonspore forming, catalase negative and oxidase negative bacteria. According to George, *et al*., (2018), *Lactobacillus* spp. were also identified by catalase negative, non-motile and Gram positive bacteria.

For further identification, *Lactobacillus* spp. growth at different temperate, different pH and different concentration of NaCl were studied. In different temperature, all ten isolates were able to grow at 35^0c, 37^0c and 45^0c, while no isolates were able to grow at 10^0c and 15^0c respectively. Similarly, Tsvetkovn & Shishkova (1982) studied the various strains of *Lactobacillus* species for their ability to sustained in freezing temperature. According to their study, the viability as well as activity of LAB cell increase in cooling. They concluded that, *S. thermophiles*, *Streptococci* spp. and *S. lactis* were found to be resistant than rods shape *L. casei*.

Lactobacillus species displayed the wide range of growth at different salt concentration value. *Lactobacillus* species were able to grow at low salt concentration to medium salt concentration that is 2% to 4%. While, some of the strains were able to grow at high salt concentration that is in 6.5%. In this study, 2 *Lactobacillus* spp., 4 *Lactobacillus* spp. and 4 *Lactobacillus* spp. were able to grow at 2%, 4% and 6.5% salt concentration respectively. Similar type of test was performed for identification of LAB isolated from raw milk of cow (Wassie and Wassie, 2016).

LAB are also known for bacteria having high resistance against lower pH and are generally cocci or rod shaped (Mokoena, 2017). In this study, 2 *Lactobacillus* spp., 8 *Lactobacillus* spp. and 10 *Lactobacillus* spp. were able to grow at pH 2, pH 4 and pH 6 respectively. Similar study done by Arya, *et al*., (2020) for study of LAB able to grow in different pH. The different genera of LAB able to grow in lower temperature are *Lactobacillus brevis*, *Lactobacillus plantarum, Pediococcus pentasaceus, Pediococcus acidilactic,* and *Leuconostoc* (Tamang, *et al.*, 2005).

Carbohydrate fermentation test was done for all isolates were tested using eight different sugars. In this study, fermentation test of *Lactobacillus* spp. for different carbohydrates like glucose, lactose, fructose, mannitol, sucrose, arabinose, galactose and maltose were done. Different *Lactobacillus* spp. were able to ferment different carbohydrates with gas formation or without gas formation. Certain test of sugar fermentation was performed by Gunkova, *et al*., (2021) on lactic acid starter culture. Similar study done by Arya, *et al*., (2020) for LAB identification from colostrum of human. Lindquist (1998) reported that medium to support the proper growth of LAB in different growth conditions and different carbohydrates.

In this study, test bacteria were confirmed as Multi Drug Resistance (MDR) bacteria by Antibiotic susceptibility test for Ampicillin (AMP), Azithromycin (AZM), COT- Co-trimoxazole, Gentamicin (GEN), Ciprofloxacin (CIP). *S. aureus* were resistant to COT, AMP, AZM and susceptible to COT and CIP. Similarly, *E. coli* and *P. auroginosa* were resistant to NIT, GEN, AMP and susceptible to CFM, CIP and COT. Furthermore, *K. pneumoniae* were resistant to CIP, AMP, NIT and susceptible to COT and CFM. After conforming the test bacteria were MDR bacteria then only antimicrobial activity of isolated

Lactobacillus spp. was done. Many studies done the antimicrobial activity of *Lactobacillus* spp. against ATCC bacteria, one of the study was done by Arya, *et al*., (2020).

In this study, Antibiotic susceptibility test of all *Lactobacillus* spp. were also done and found to be resistance to Ampicillin (AMP) and Ceptazidime (CAC) while found to sensitive to Co-trimoxazole (COT), Gentamicin (GEN) and Ciprofloxacin (CIP). Similarly, Arya, *et al*., (2020) also reported LAB were resistance against different antibiotics. However, many studies were not reporting antibiotic resistance in *Lactobacillus* spp. and in LAB.

Many studies have already done for the isolation, characterization as well as production of antimicrobial substance Bacteriocin. The use of these antimicrobial compounds usually applicable for the preservation of food, increase shelf life of product, as well as to inhibit the harmful pathogenic bacteria. So, the bacteriocin were the very essential substance produced by Lactic acid bacteria (Adenike, *et al.,* 2007). A naturally occurring organic acids called lactic acid was created when LAB fermented carbohydrates. This contributed the acidic environment which eventually prevented the growth of other harmful bacteria and microbes. As this lowered the pH and prevent the spoilage as well as considered as the preservative substance. LAB were responsible for the production of proteinaceous substance bacteriocin which act as antimicrobial agent and also carried huge potential to use as alternatives to artificial preservatives in many foods after extensively used in research (Mobolaji & Wuraola, 2011). So, bacteriocins are also considered as antibacterial substances (proteins) which are produced from different LAB (Gaur, *et al*., 2004).

For screening test of bacteriocin in all *Lactobacillus* spp., dot inoculation of isolated *Lactobacillus* spp. was done and incubated at 30^{o}C for 24hrs. Among 10 isolates, 9 *Lactobacillus* spp. showed inhibition zone against all test bacteria. Similarly, Palaniyammal, et al., (2019) also studied the screening of bacteriocin producing *Lactobacillus* spp. However, many studies were not doing this screening test before antimicrobial activity study of bacteriocin extracted from *Lactobacillus* spp.

In this study, bacteriocin like compound were extracted from *Lactobacillus* spp. were from 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5days), 120hrs (6days) and 144 hrs (7days). However, antimicrobial activity was showed against MDR bacteria only after 72hrs (4day), 96hrs (5days) and 120hrs (6days) of extraction of bacteriocin against MDR bacteria. From 96hrs (5days) of extraction of antimicrobial of bacteriocin like compound was able to inhibit all test bacteria which were *S. aureus, B. cereus*., *E. coli and P. aeruginosa* and *K. pneumoniae*. The highest zone of inhibition was showed by BL10 isolate against *B. cereus* (30mm) followed by KL1 isolate against *E. coli* (21mm) and KL5 isolate against *B. cereus* (20mm). In this study, 5 extracted antimicrobial substance bacteriocin were able to show inhibition zone against all MDR bacteria.

Nguyen, *et al*. (2020) isolated and identified LAB from vegetable growing soils. Different inhibitory action of these isolates were studied against various pathogens such as *E. coli, Staphylococcus, Bacillus spizizenii, Salmonella Typhi.* But, the isolates were not able to inhibit *E. coli* and *Staphylococcus* but able to show inhibitory action against *Bacillus spizizenii* and *Salmonella Typhi.* Several studies were performed to study the antimicrobial properties against various pathogens that includes *E. coli, Shigella* species, *Salmonella* species and so on. Gilliland & Speck, (1977) reported *Lactobacilli* spp. showed antibacterial activity against Gram- positive bacteria (*C. perfringens* and *S. aureus*) than Gram- negative bacteria (*S.* Typhi, *E coli,* and *S. Paratyphi*).

On the study done by Elayaraja, *et al*. (2014) bacteriocin were produced, purified and characterized for the inhibition of wide range of pathogens also created greater interest for food safety and as a preservative. Lactic acid bacteria displayed a wide range of antimicrobial activities. Thus, bacteriocin produced by various LAB have huge potential as preservative (Bromberg, *et al.*, 2004). Such study had become a great importance in today's world which has the greater potential to lead the nation by its potentiality as a natural preservatives and also have ability to inhibit growth of pathogenic microorganisms in food and feed industries Liasi, *et al.* (2009).

CHAPTER 5

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Among 30 farm soil samples, 10 *Lactobacillus* species were identified. The highest occurrence of *Lactobacillus* species was found in the sample collected from Kritipur (20%). From this study it was concluded that *Lactobacillus* species were isolated from local cow farm can inhibit the growth of MDR bacteria included *S. aureus*, *Bacillus* spp., *P. aeruginosa*, *E. coli* and *K. pneumonia*. This is due to the antimicrobial substance bacteriocin like compound extracted from of *Lactobacillus* spp. which inhibited MDR bacteria which was highly resistant.

5.2 Novelty/ Association to National priority and needs

This study showed that *Lactobacillus* spp. isolated from soil of cow farm showed antimicrobial activity. So, this is the novelty of the antibacterial activity of *Lactobacillus* species against MDR bacteria. So, there is need for study of *Lactobacillus* spp. in large number in national level for production of antibiotics.

5.3 Future prospect of the research proposal

Further research can be done on antibiotics production by *Lactobacillus* spp. in future.

5.4. Recommendations for further work

From this study, *Lactobacillus* spp. were able to isolate from soil of different farm of Kathmandu district. So, further study can be done for isolation of different potential species from farm soil to reduce the development of antibiotic resistance. Antimicrobial activity of bacteriocin like compound extracted from *Lactobacillus* spp. showed the antimicrobial activity against different MDR bacteria. So, next generation antibiotics can be prepared for targeting the multi drug resistant bacteria.

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APPENDIX A

List of materials: Chemicals, Reagent, Equipment’s and Media

Chemicals and Reagents

1. Crystal violet
2. Gram's iodine
3. 95% Ethanol
4. Safranin
5. 3% Hydrogen Peroxide
6. Oxidase strip
7. Ammonium Sulphate
8. Potassium Phosphate Buffer
9. Distilled water
10. Phenol red indicator

Equipment

1. Autoclave
2. Hot air oven
3. Incubator
4. Microscope
5. weighing machine
6. Refrigerator
7. Micropipette
8. Micropipette tips
9. Centrifuge machine
10. Centrifuge tubes

Glass Wares

1. Conical flask
2. Beaker
3. Petri plates
4. Test tubes
5. Glass slides
6. Measuring Cylinder
7. Volumetric Flask
8. Cover slip
9. Glass rod
10. Micropipette

Miscellaneous

1. Inoculating wire
2. Inoculating loop
3. Forceps
4. Cotton Swab
5. Dropper
6. Cotton
7. Lysol
8. Alcohol
9. Bunsen Burner
10. Marker

Microbiological Media

1. Nutrient Broth
2. Nutrient Agar
3. MRS broth
4. MRS Agar
5. Mueller-Hinton Agar
6. Simmon's Citrate Agar

APPENDIX B

I. Composition and preparation of different cultural media

1. Mueller Hinton Agar

Ingredients	gm/liter
Beef infusion	300 gm
Casamino acid	17.5 gm
Starch	1.5 gm
Agar	17 gm
Distilled water	1000ml
Final P^H	7.2±0.2

The agar was accurately weighed and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs. pressure) for 15 min. The media was then poured into sterile petriplates.

2. Nutrient Agar

Ingredients	gm/liter
Peptic digest of animal tissue	5 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Sodium chloride	5 gm
Agar	15 gm
Distilled water	1000 ml

The agar was accurately weighed and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs. pressure) for 15 min. The media was then poured into sterile petriplates.

3. Nutrient Broth

Ingredients	gm/liter
Peptic digest of animal tissue	5 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Sodium chloride	5 gm
Distilled water	1000 ml

The media was accurately weighted and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was poured in sterile test tubes and sterilized by autoclaving at 121°C (15 lbs. pressure) for 15 min.

4. MRS Broth (Himedia)

Component	Amount(g/l)
Dextrose	20.00
Protease peptone	10.0
Yeast extract	5.000
Sodium acetate	5.00
2-Phenyl ethanol	3.00
Ammonium citrate	2.00
Dipotassium hydrogen phosphate	2.00
Magnesium sulphate	0.100
Maganese sulphate	0.050
Bromocresol green	0.040
Cycloheximide	0.004
Final pH (25^0C)	4.3+-0.2
Distilled water	1000 ml

The agar 47.2 gm of agar was accurately weighed and was suspended into 1000 ml of distilled water and dissolve completely. Then the broth was dispensed and sterilized by autoclaving at 121°C (15 lbs. pressure) for 15 min.

5. MRS Agar (Himedia)

Component	**Amount(g/l)**
Peptone	10.00
HM peptone B#	8.00
Yeast extract	5.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.20
Maganese sulphate	0.05
Dipotassium hydrogen phosphate	2.00
Dextrose	20.00
Polysorbate 80 (Tween 80)	1.00
Agar	12.00
Final pH (25^0C)	5.7+-0.2
Distilled water	1000 ml

The media (65.13gm) was accurately weighted and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was poured in sterile test tubes and sterilized by autoclaving at 121°C (15 lbs. pressure) for 15 min.

6. Sugar Fermentation Broth

Component	Amount(g/l)
Trypticase	10.0
Sodium chloride	5.0
Sugar (Glucose, Fructose, Galactose, Mannitol, Lactose, Maltose, Sucrose and Arabinose)	10.0
Phenol red indicator	0.018 g
Distilled and deionized water	1,000 ml

APPENDIX C

Reagents Composition

Crystal Violet (200ml)

- In 40ml 95% ethyl alcohol, 4 gm crystal violet was dissolved
- In 160ml distilled water,1.6gm ammonium oxalate was dissolved
- The two solutions were mixed and stored in a reagent bottle at room temperature.

Gram's Iodine(150ml)

- In 150ml distilled water, 0.5g iodine and 1gm potassium iodide was added
- The solution was mixed and kept overnight and was transferred to reagent bottle and stored at room temperature.

Safranin(50ml)

- In 5ml 95% ethanol, 1.25g safranin was dissolved.
- Distilled water was added to the solution to make final volume of 50ml.
- Then, the solution was stored at room temperature.

Oxidase Reagent(50ml)

- In 50ml distilled water, 1% tetra-methyl-p-phenylenediamine dihydrochloride was added
- It was stored in reagent bottle and was covered with aluminum foil to prevent the exposure of light at 4^0C.

Catalase Reagent

- 3.5% Hydrogen peroxide was used.

0.5 McFarland Standards

- 0.6ml of 1% w/v solution of barium chloride solution was taken
- It was then mixed up with 99.4ml of 1% v/v solution of sulphuric acid
- The mixture was shaked well
- This can be stored in a well-sealed container at 20-28^{0}C for up to 6 months
- Small amount of the solution was taken during the testing of turbidity of the inoculums.

Ethyl alcohol (95%)

- 95ml of ethyl alcohol was added to 5ml of distilled water.

Antibiotic Susceptibility Test (AST)

- Mueller-Hinton Agar**,** Antibiotic disc, 0.5 McFarland Standard, Test MDR bacterial suspension.

APPENDIX D

Bacterial identification

S.No	Bacteria	Test Performed	Result
1	*S. aureus*	Gram Staining	Gram +ve cocci,nonmotile
		Catalase test	Catalase +ve
		Oxidase test	Oxidase –ve
		O/F test	Fermentative
		Coagulase test	Coagulase +ve
2	*B. cereus*	Gram Staining	Gram +ve rod, motile
		Catalase test	Catalase +ve
		Oxidase test	Oxidase -ve
		O/F test	Fermentative
3	*E. coli*	Gram Staining	Gram –ve rod
		Catalase test	Catalase +ve
		Oxidase test	Oxidase –ve
		O/F test	Fermentative
		Indole test	Indole +ve, motile
		MR test	MR +ve
		VP test	VP –ve
		Citrate Utilization test	Citrate –ve
		TSI test	A/A, H_2S^- Gas^{+ve}
		Urease test	Urease -ve
4	*P. aeruginosa*	Gram Staining	Gram –ve, rod
		Catalase test	Catalase +ve
		Oxidase test	Oxidase +ve
		O/F test	Oxidative
		Indole test	Indole –ve, motile
		MR test	MR –ve
		VP test	VP –ve
		Citrate Utilization test	Citrate +ve
		TSI test	Alk/Alk , H_2S^-, Gas^{-ve}
		Urease test	Urease –ve

5.	*Klebsiella pneumoniae*	Gram Staining	Gram –ve rod
		Catalase test	Catalase +ve
		Oxidase test	Oxidase –ve
		O/F test	Fermentative
		Indole test	Indole –ve
		MR test	MR -ve
		VP test	VP +ve
		Citrate Utilization test	Citrate +ve
		TSI test	A/A,, Gas^{+ve}
		Urease test	Urease +ve

Antibiotic susceptibility test (AST) of test bacteria (MDR)

1. *S. aureus*

Antibiotic Disc	Symbol	Disc content (mcg)	Interpretative chart			Zone of inhibition (mm)	Result
			Sensitive (mm) or more	Intermediate (mm)	Resistant (mm) or less		
Ampicillin	AMP	10	29	-	28	22	Resistant
Azithromycin	AZM	15	18	14-17	13	-	Resistant
Ciprofloxacin	CIP	5	21	16-20	15	32	Sensitive
Erythromycin	E	15	23	14-22	13	-	Resistant
Co-Trimoxazole	COT	25	16	11-15	10	28	Sensitive
Getamicin	GEN	10	15	13-14	12	8	Resistant

2. *Bacillus cereus*

Antibiotic Disc	Symbol	Disc content (mcg)	Interpretative chart			Zone of inhibition (mm)	Result
			Sensitive (mm) or more	Intermediate (mm)	Resistant (mm) or less		
Ampicillin	AMP	10	29	-	28	-	Resistant
Azithromycin	AZM	15	18	14-17	13	26	Sensitive
Ciprofloxacin	CIP	5	21	16-20	15	35	Sensitive
Erythro	E	15	23	14-22	28	20	Resistant
Co-Trimoxazole	COT	25	16	11-15	10	-	Sensitive
Getamicin	GEN	10	15	13-14	15	25	Sensitive

3. *E. coli*

Antibiotic Disc	Symbol	Disc content (mcg)	Interpretative chart			Zone of inhibition (mm)	Result
			Sensitive (mm) or more	Intermediate (mm)	Resistant (mm) or less		
Ampicillin	AMP	10	29	-	28	-	Resistant
Cefixime	CFM	5	19	16-18	15	-	Resistant
Ciprofloxacin	CIP	5	21	16-20	15	36	Sensitive
Nitrofurantoin	NIT	300	17	15-16	14	19	Sensitive
Co-Trimoxazole	COT	25	16	11-15	10	30	Sensitive
Gentamicin	GEN	10	15	13-14	15	20	Sensitive

4. ***Pseudomonas auroginosa***

Antibiotic Disc	Symbol	Disc content (mcg)	Interpretative chart			Zone of inhibition (mm)	Result
			Sensitive (mm) or more	Intermediate (mm)	Resistant (mm) or less		
Ampicillin	AMP	10	29	-	28	-	Resistant
Cefixime	CFM	10	19	16-18	15	-	Resistant
Ciprofloxacin	CIP	5	21	16-20	15	38	Sensitive
Nitrofurantoin	NIT	300	17	15-16	14	-	Resistant
Co-Trimoxazole	COT	25	16	11-15	10	-	Resistant
Gentamicin	GEN	5	15	13-14	15	15	Sensitive

5. ***Klebsiella pneumoniae***

Antibiotic Disc	Symbol	Disc content (mcg)	Interpretative chart			Zone of inhibition (mm)	Result
			Sensitive (mm) or more	Intermediate (mm)	Resistant (mm) or less		
Ampicillin	AMP	10	29	-	28	-	Resistant
Cefixime	CFM	10	18	16-18	15	-	Resistant
Ciprofloxacin	CIP	5	21	16-20	15	27	Sensitive
Nitrofurantoin	NIT	300	17	15-16	14	14	Sensitive
Co-Trimoxazole	COT	25	16	11-15	10	23	Sensitive
Gentamicin	GEN	5	21	13-14	15	16	Sensitive

6. Antibiotic susceptibility test of isolated *Lactobacillus* spp.

In this study, Antibiotic susceptibility test of all *Lactobacillus* spp. were done and found to be resistance to Ampicillin (AMP) and Ceptazidime (CAC) while found to sensitive to Co-trimoxazole (COT), Gentamicin (GEN) and Ciprofloxacin (CIP). (Table 4).

Antibiotic susceptibility tests of *Lactobacillus* species

***Lactobacillus* species**	**AMP**	**CAC**	**CIP**	**COT**	**GEN**
KL1	-	-	30	40	15
KL2	-	-	35	36	12
KL3	-	-	37	38	15
KL4	-	-	33	26	20
KL5	-	-	32	32	11
KL6	-	-	34	28	22
TL7	-	-	35	44	21
TL8	-	-	38	28	20
BL9	-	-	35	30	20
BL10	-	-	33	30	16

Res.-Resistance, AMP-Ampicilin, CAC= Ceftazidime, CIP-Ciprofloxacin, COT- Co-trimoxazole and GEN-Gentamicin

7. Antimicrobial activity of bacteriocin like compound extracted from 144 hrs (7days)

Test bacteria	Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin)									
	KL1	**KL2**	**KL3**	**KL4**	**KL5**	**KL6**	**TL7**	**TL8**	**BL9**	**BL10**
Gram positive bacteria										
Staphylococcus aureus 29213	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-	-	-	-	-
Gram Negative bacteria										
Escherichia coli 25922	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-

Total budget

S.No.	Details	Amounts
1.	Chemicals, reagents and laboratory instruments, Culture media and others accessories	45,876/-
3.	Transportation and sample collection	5,188/-
4.	Typing, printing and hard bindings of reports	5, 000/-
5.	Stationaries	4,666/-
6.	Plagiarism	2,000/-
7.	Refreshment Expenses	5,007/-
6.	Report writing	3,000/-
	Total	70,737/-

ANTIMICROBIAL ASSAY OF LACTOBACILLUS SPECIES AG...

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ANTIMICROBIAL ASSAY OF LACTOBACILLUS SPECIES AGAINST MULTIGRUG RESISTANCE BACTERIA Submitted to Institute of Science and Technology Dean's office, Tribhuvan University Submitted by Rama Khadka Institute of Science and Technology Padma Kanya Multiple Campus Bagbazar, Kathmandu, Nepal i 2024 DECLARATION This project work entitled "Antimicrobial Assay of Lactobacillus Species against Multidrug Resistance Bacteria" is being submitted to the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for carried out mini research project work. This work is original and has not been submitted for any other research work. .... Ms. Rama Khadka Institute of Science and Technology (IoST), Padma Kanya Multiple Campus Tribhuvan University (T.U.), Kathmandu, Nepal ii March, 2024 RECOMMENDATION This is to recommend that Ms. Rama Khadka, has carried out project work entitled "Antimicrobial Assay of Lactobacillus species against Multidrug Resistance Bacteria", for the mini research project work under my supervision of Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal. She has fulfilled the entire requirement laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the submission of the mini research project work. .... Assoc. Prof. Dr. Shyam Prakash Dumre Mentor Central Department Microbiology Tribhuvan University Kritipur, Kathmandu, Nepal March, 2024 iii LETTER OF APPROVAL On the recommendation of Assoc. Prof. Dr. Shyam Prakash Dumre, this mini research project work is submitted by Ms. Rama Khadka, carried out project work entitled "Antimicrobial Assay of Lactobacillus species against Multidrug Resistance Bacteria" is forwarded for the approval to the Evaluation Committee, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal. .... Assistant Dean Institute of Science and Technology Tribhuvan University Kathmandu, Nepal .... Evaluator Institute of Science and Technology Tribhuvan University Kathmandu, Nepal iv ACKNOWLEDGEMENT I would like to express my heartfelt gratitude towards Institute of Science Technology, Dean's office, Tribhuvan University for providing this project. I would like to express my deepest gratitude to my Mentor Associate Professor Dr. Shyam Prakash Dumre, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal for his invaluable guidance and support throughout the research process. Her expertise, insightful feedback, and unwavering encouragement have been instrumental shaping this work. I would also like to acknowledge our respected Campus chief, Prof. Dr. Jaya Laxmi Pradhan, coordinator Mrs. Bindra Devi Shakya, and former Assistant Campus Chief, Mr. Achut Ram Pradhananga of Padma Kanya Multiple Campus for providing the platform to conduct this project work. I would like to extend my sincere thanks to all the lecturers and staffs of Padma Kanya Multiple Campus for their generous support allowed me to conduct the necessary experiments and gather crucial data. Lastly, I am immensely grateful to my students Anusuya Sigdel and Anita Thapa Magar for their continuously help throughout this journey. Special thanks to owner of cow farm for providing me soil samples for this research work. Without the incredible support of these individuals, this research would not have been possible. .... Rama Khadka Institute of Science and Technology Tribhuvan University Kathmandu, Nepal v ABSTRACT Lactic acid bacteria are the group of bacteria which are commonly found in soil and in the guts of animals and they are favorable for both human and animal health. In this study, Lactobacillus species were isolated and identify from soil of a cow farm and perform its antimicrobial activity against MDR bacteria from October 2024 to March 2024 in the microbiology laboratory of Padma Kanya Multiple Campus, Bagbazar, Nepal. A total of 30 soil samples were collected from 3 locations of farm of Kathmandu valley for enumeration and isolation of Lactobacillus species on selective media (MRS). Pure cultures were obtained through sub culturing on MRS media and further morphological as well as biochemical test was be carried to identify the isolated strains. For the confirmation of Lactobacillus species, Gram staining, motility test and catalase test were done. For the further analysis, fermentation test, growth on different temperature, different salt concentration and different pH were also done. Antibiotic susceptibility test (AST) of Lactobacillus species and test bacteria was done by a modified Kirby- Bauer method. The screening test for bacteriocin was done by dot plate technique method. Then, the extraction of bacteriocin from isolated Lactobacillus species was done by precipitation method and antimicrobial assay was done against MDR test bacteria. From this study, 10 Lactobacillus species were identified. In this study, bacteriocin were extracted from Lactobacillus spp. were from 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5days) and 120hrs (6days). However, antimicrobial activity was showed against MDR bacteria only after 72hrs (4day), 96hrs (5days) and 120hrs (6days). From 96hrs (5days) of extraction of antimicrobial substance bacteriocin was able to inhibit all test bacteria which were S. aureus, B. cereus., E. coli and P. aeruginosa and K. pneumoniae. The highest zone of inhibition was showed by BL10 isolate against B. cereus (30mm) followed by KL1 isolate against E. coli (21mm) and KL5 isolate against B. cereus (20mm). From this study, antimicrobial activity of bacteriocin extracted from Lactobacillus spp. showed the antimicrobial activity against all MDR bacteria. So, next generation antibiotics can be prepared for targeting the multi drug resistant bacteria. Keywords: Cow farms, soil, Lactobacillus species, AST, Bacteriocin. vi vii zf]wzf/ NoflS6s Pl;8 JofS6]l/of (Lactic acid bacteria), JofS6]l/of s} Ps k|hftL xf] . of] ljz]ifu/L df6f] / hgjf/x?sf] kfrggnLdf kfOG5 / of] k|hftLsf] JofS6]l/ofn] dfgj / kz'sf] :jf:Yodf cg's'n k|efj kf/]sf] x'G5 . o; cWoogdf ufOkfng If]qx?df /x]sf] df6f]sf] gd'gfnfO{ lnP/ NofS6f]Jofl;n; k|hftLnfO{ (Lactobacillus species) klxrfg ul/Psf] 5 . o;kl5 ;f] gd'gfx?nfO{ ;g\ @)@$;fnsf] dfr{ b]vL cS6f]j/;Dd kBsGof ax'd'vL SofDk;, af3jhf/df /x]sf] dfOs|fafof]nf]hL k|of]uzfnfdf PG6LdfOs|f]ljon kl/If0fx? u/\of}+ . sf7df8f}+ pkTosfsf ljleGg # j6f If]qx?df /x]sf] kmfd{x?af6 s'n #) j6f gd"gfx? ;+sng / cnu u/\of}+ . o;kl5 tL gd'gfx?df lglZrt ldl8ofsf] (MRS) k|of]u u/L NofS6f]Jofl;n;sf] u0fgf ul/of] . NofS6f]Jofl;n; (Lactobacillus spp.) cnu ug{ Pdcf/P; ldl8of ;fy} ;f] k|hftLsf] afx\o :j?k klxrfg ug{ cGo afof]s]ldsn kl/If0fx? ul/of] . NofS6f]Jofl;n;sf] k'li6s/0f ug{ u|fd :6]lg+u, ultlzntf kl/If0f / Sof6fn]h kl/If0fx? ul/of] . yk ljZn]if0fsf] nfuL kmd{]G6]zg kl/If0f / o;sf] a[l4 dfkgsf] nfuL ljleGg tfkdfqf tyf ;fN6 3gTj kl/If0fx? klg ul/of] . o;kl5 NofS6f]Jofl;n; k|hftLaf6 k|]l;lk6]zg lj lw4f/f JofS6]l/of]l;gnfO{ cnu ul/of] . o; cWoogaf6 !) NofS6f]Jofl;n; k|hftL klxrfg ul/of] . cnu ul/Psf] JofS6]l/of]l;gn] :6]kmfOnf]sf]s; cf}/Lo; (Staphylococcus aureus), Osf]nL (E. coli) / l;p8f]df]g; cf}?uLgf];f (Pseudonmonas aeruginosa) k|lt lg/f]wfTds k|lts[of k|bz{g u/\of] t/ Sn]A;Lonf Go'df]lgof (Klebsiella pneumoniae) k|lt eg] k|lts[of lbPg . NofS6f]Jofl;n; k|hftLaf6 cnu ul/Psf] JofS6]l/of]l;gn] leGg Pdl8cf/ JofS6]l/of k|lt PlG6dfOs|f]ljon ultljlwx? k|blz{t u/\of] . To;}n] ax' k|ltsfTds JofS6]l/ofsf] nfuL gof :j?ksf] PlG6afof]l6s tof/ ug{ ;lsG5 . viii d'VozAbx?M ufOkmfd{, df6f], NofS6f]Jofl;n; k|hftL, JofS6]l/of]l;g ix LIST OF ACRONYMS AND ABBREVIATIONS AST: ATCC: CaCO3: CFS: CLSI: CO2: DNA: FDA: GRAS: IoST: KDa: LAB: MHA: Mm: MRS: NA: NaCl: NaOH: PCR: PGPM: PH: RAPD-PCR: Antibiotic Susceptibility test American Type Culture Collection Calcium Carbonate Cell Free Solution Clinical Laboratory Standards Institute Carbon Dioxide Deoxy Ribo Nucleic Acid Food and Drug Administration Generally recognized as Safe Institute of Science and Technology Kilo Dalton Lactic Acid Bacteria Mueller Hinton Agar Millimeter de Mann Rogosa Sharpe Nutrient Agar Sodium Chloride Sodium Hydroxide Polymerase Chain Reaction Plant Growth Promoting Microorganisms Potential of Hydrogen Randomly amplified polymorphic DNA- Polymerase Chain Reaction x LIST OF SYMBOLS %: Percentage 0C: Degree Celsius G: Grams Mm: Millimeter ml: Milliliter ul: Microliter xi LIST OF TABLES Table 1: Table 2: Table 3: Table 4: Table 5: Table 6: Table 7: Table 8: Table 9: Distribution of Lactobacillus spp. from different location Fermentation of different carbohydrates by Lactobacillus spp. Antibiotic susceptibility test of test bacteria Antibiotic susceptibility tests of Lactobacillus spp. Screening of Lactobacillus spp. Antimicrobial activity of bacteriocin extracted from 48hrs Antimicrobial activity of bacteriocin extracted from 72hrs Antimicrobial activity of bacteriocin extracted from 96 hrs Antimicrobial activity of bacteriocin extracted from 120 hrs xii Figure 1: Figure 2: Figure 3: Figure 4: Figure 5: Figure 6: LIST OF FIGURES Growth profile of Lactobacillus spp. Identification of Lactobacillus spp. Lactobacillus spp. growth at different temperature Lactobacillus spp. growth at different (NaCl) Concentration Lactobacillus spp. growth at different pH Comparison of zone of inhibition by bacteriocin xiii LIST OF PHOTOGRAPHS Photograph 1: Enumeration of Lactobacillus species and subculture on de Mann Rogosa Sharpe (MRS) Agar media Photograph 2: Identification of Lactobacillus spp. by carbohydrate fermentation (glucose, maltose, fructose, galactose, sucrose, arabinose, lactose and mannitol) and growth at 450c Photograph 3: Antibiotic susceptibility of test bacteria and isolated Lactobacillus spp. Photograph 4: Antimicrobial activity bacteriocin extracted from Lactobacillus species at 24hrs and 72hrs xiv LIST OF APPENDIX Appendix A: List of materials Appendix B: Composition and preparation of different culture media Appendix C: List of chemicals Appendix D: Protocol xv TABLE OF CONTENTS CHAPTER 1 INTRODUCTION 1.1. General Introduction Among different Lactic Acid Bacteria (LAB), Lactobacillus species are rod shape bacteria and able to produce lactic acid by carbohydrates fermentation. Many strains of the genus Lactobacillus spp. are capable of colonizing in tract of urogenital as well gastrointestinal and became competitive with pathogen (Antonio, et al., 1999; Redondo, et al., 1990). In environment, Lactobacillus spp. play important role for transformation of bad flavor of substances by decomposition of highly complex substances like macro-molecules and biomolecule (Arena, et al., 2019). So, Lactobacillus species are used as soil amendment, plant growth promoting bacteria, disease suppression, bioremediation, composting and organic waste management, soil restoration etc. LAB are also used as probiotics and can be used as a biocontoller (Messaoudi, et al, 2013). Lactobacillus spp. showed antibacterial activity against pathogenic bacteria due to the synthesis of different known molecules like bacteriocin, H2O2 and lactic acid as well as unknown molecule which is heat-stable and other different molecule which is not lactic acid (Servin, 2004). These molecules have inhibitory activity only after the production of the secondary compounds (metabolites) after 48 hrs of fermentation by LAB (Rouse, et al., 2008). Lactic acid bacteria showed antibacterial activity against resistance bacteria (P. aeruginosa) which are isolated from clinical samples. So, Lactobacilli spp. showed antibacterial activity for the highly resistance bacteria (MDR P. aeruginosa) isolated from clinical samples (Jamalifar, et al., 2011). Many clinical problems were caused due to the multi-drug resistance in bacteria. So, resistance bacteria caused many infectious diseases which increased mortality and morbidity as well as increased health cost in many developing countries (Pfaller, et al., 1997; Jarvis & Martone, 1992). In recent time, Lactobacilli spp. have been reported for prevention as well as treatment for various bacterial infections. So, many strains of Lactobacillus spp. are found commercially for oral consumption which are useful for different infections caused by bacteria (Gordon, et al., 1957; Carson & Riley, 2003). 1.2 Statement of the problem Antibiotic resistance in bacteria is the greatest problem in the world which is due the misuse of antibiotics in animals as well as in human treatment for different diseases (Lipsitch, et al., 2000, Roy, 1997, Yoneyama & Katsumata, 2006). So, the different strategies have been used for treatment of different diseases, among which bacteriocins production by LAB is one of them. Bacteriocins are antimicrobial metabolites (peptides) synthesizing by LAB which are capable for controlling the drug- resistant and clinically resistance bacteria. So, there is very importance for production of new antimicrobial producing agents as medicine (Kumar & Schweiser, 2005). Hence, the strains of LAB are able to produce antimicrobial bacteriocin like compound, which may be able to inhibit the pathogens. So, the main aim of study was to extract bacteriocins from Lactobacillus spp. isolated from cow farm soil and to study its antimicrobial activity against highly resistance bacteria. 1.3 Objectives 1.3.1 General Objective ? To determine antibacterial of Lactobacillus spp. isolated from farm soil against MDR bacteria. 1.3.2 Specific Objectives ? To isolate and identify Lactobacillus spp. from farm soil. ? To perform antibiotic susceptibility test for MDR bacteria and isolates. ? To determine the antimicrobial activity of antimicrobial substances (Bacteriocin) against MDR bacteria. 1.4 Rationale of the study In the nature, Lactic acid bacteria (LAB) are widely found in waters, soils, silages, plants, waste products, etc. as well as from humans and animal intestinal tract (Axelsson, 1998). There are several studies in LAB which were isolation from milk, milk products and fermentation products. However, LAB produced secondary metabolites known as bacteriocin having antimicrobial activity against pathogenic bacteria were less studied. To our knowledge in Nepal, little information exists on Lactobacillus spp. in soil samples. Only less study was done for isolation of Lactobacillus spp. from farm soil and perform antibacterial activity against MDR bacteria. So, this study was aimed for isolation of Lactobacillus spp. from farm soil then extraction of bacteriocin was done for the study of antibacterial activity against highly resistance bacteria. 1.5 Limitation of the study A total of 30 soil samples from Kathmandu valley was collected from the farm side (cow farm). Only MDR bacteria was studied for antibacterial activity of Lactobacillus spp. CHAPTER 2 LITERATURE REVIEW Lactic acid bacteria are found in the nature and isolated from waters, soils, silages, plants, waste products. From animals and human intestine, LAB are also isolated (Axelsson, 1998). These bacteria belong to the phylum of Firmicutes. Among LAB, Lactobacillus, Leuconostoc, Carnobacterium, Lactococcus, Enterococcus, Pediococcus, Melissococcus, Streptococcus, Vagococcus, Oenococcus, Tetragenococcus, and Weissella are well known LAB (Jay, 2000). Lactic acid bacteria produce different metabolites which are fatty acids with short-chain, organic acids, amines, vitamins, exopolysaccharides and bacteriocins (Arena, et al., 2019). Among different metabolites, bacteriocin are toxic to microorganisms. So, Bacteriocin are used for the production of drugs like antibiotic having properties of probiotic. Organic acids which are also known as another metabolite produced by LAB which have antimicrobial activity against fungi. So, these metabolites are used as preservative in some food of fermentation (Gajbhiye, et al., 2016). Among different metabolites produced by LAB, bacteriocins is one of the well study metabolites. These are the metabolites which are highly toxic to microorganisms and used for the production of drug like antibiotic. Actually, ribosomes synthesize the bacteriocins either in proteins or peptides which inhibit the reproduction and growth of a different bacteria (Diep and Nes, 2002). Bacteriocins are known as protein or peptides that are synthesized by ribosome's and are able to inhibit a variety of bacteria. Bacteriocin are the protein substances which are categoried into two types. Lantibiotics is the first type which either containing or not containing of lanthionine. These lantibiotics are produced by L. lactis (polycyclic peptide) which is the causative agent for damaging the cell membrane in bacteria (Gram positive). Helveticin (M and J) is the second type of bacteriocins which are produced by L. helveticus and L. crispatus. These are types of bacteriocins which are especially used for preservatives of food (Raman, et al.,2022). On the other hand, different fungal infections have caused the severe infection on crops and also affect the livestock feed and quality of seeds. Some strains of L. plantarum 4 as well as Bacillus spp. were generally used for biological control due to its antifungal activity (Vuyts & Leroy, 2007). Bacteriocins are synthesized by some bacteria which are capable for controlling both clinically susceptible and resistant types of bacteria. So, there is a continued increasing for the development of new types of antimicrobial agents for production of medicines (Kumar & Schweiser, 2005). Bacteriocins are also studied from the modification to improvement of the properties like physiochemical and effects like pharmacological as well as biosafety. Bacteriocins extracted from same species or from different species were active against different bacteria. So, it is known for narrow spectrum or across genera that is broad spectrum (Mobolaji & Wuraola, 2011). Variety of bacterial and fungal phytopathogens exist on soil. These will hamper the growth of plant, significant damage in agriculture which gradually decline the productivity and loss the economic value as well as quality of the products in that case, LAB has served as an effective biocontrol agent (Jaffar, et al., 2022). These bacteria are the significant group of probiotic organisms and have protective effects in fermented food preservation, as they are able to produce organic acids in food during their growth. These group species are capable of conversion of carbohydrates to organic acids which will gradually reduce pH changes, that's the reason to increase the half-life and good quality of such food products. These bacteria are generally known as safe (GRAS) and also considered for one of the important food ingredients (Vijayakumar & Muriana, 2015). Not only benefited as a food ingredients, these bacteria are able to regulate soil organic matter, biogeochemical cycle, enhancement of plant growth hormone, health of plant and also detoxify the hazardous chemicals. LAB showed the antimicrobial activity against various fungal and bacterial populations of phyllospheric as well as rhizospheric region. Fungal secretion or mycotoxins, heavy deposition of metals affect the natural quality of soil and the organisms of the soil. But, by the use of LAB, the naturality of the soil remain intact and promote the plant growth factors (Raman, et al., 2022). There are many studies which proposed the different mechanism for the inhibitory activity of bacteriocins produced by LAB. Some studies added that bacteriocins showed inhibitory 5 activity to microorganisms by inhibiting protein and nucleic acid synthesis (Kumariya, et al., 2019). The lantibiotics are produced from L. lactis which are the derivatives of polycyclic group of lanthionine having antibacterial peptides which damaged the cell of many bacteria mostly of Gram-positive (Montville, et al., 1998). The second type of bacteriocins are produced by L. helveticus and L. crispatus which are Helveticin J and Helveticin M. and used for the preservation of food. However, bacteriocin used for controlling the pathogens in crops as commercially showed the resistance in some plants (Rooney, et al., 2020) In the study conducted by Kang (2019) explained that, Lactobacillus spp. was used for soil remediation. As, the soil composed of clay, loom or silt. When it was left untreated, soil became harden up and flow of water inside soil became very much harder so, according to him, Lactobacillus species in the soil improved the soil friability as well as in land restoration. Chen, et al., (2005) studied in Japan and Taiwan for isolation of 54 LAB from 68 soil samples of rhizospheric area of fruit trees and floor of a horse farm and henhouse. In their study, 32 LAB were identified from 54 isolates belonging to five genera:, Weisssella, Lactococcus, Enterococcus, Leuconostoc and Lactobacillus spp.. From their study, the highest number of LAB were isolated from soil. Idham, et al., (2016) isolated and identified microorganism from cow manure from Indonesia. They reported the decomposers found in cow manure were Actinomycetes spp., Lactobacillus spp., and Aspergillus spp. They isolated these genera on the basis of morphological and physiological characteristics. According to their study, Lactobacillus spp. showed circular colony morphology. They observed growth at the temperature of 450C and the pH was 3.5. Isolated species able to form acid on sucrose and glucose but not with maltose. Yanagida, et al., (2006) conducted the research for the isolation of LAB producing bacteriocin from soil samples in Japan. From their study, 42 LAB were isolated from 55 soil samples This study reported the first production of bacteriocin by Lactobacillus animalis. So, this study finding suggested that, the soil is also natural habitat of LAB from which the bacteriocin is isolated as novel antibiotics. CHAPTER 3 MATERIALS AND METHODS 3.1 Materials All the necessary materials, chemicals, equipment's, media reagents required for the study is enlisted in Appendix A. 3.2 Research Methodology Different cow farms of Kathmandu district (Kritipur area, Thankot area and Budhanilkantha area) were selected for this study. Altogether, 30 soil samples were collected from different local cow farm. All Soil samples were aseptically collected in plastic zip lock bags, and labeled in it with date, time, and sampling sites name. Then, all soil samples were transported to Padma Kanya Campus for the processing in the microbiology laboratory as soon as possible. This study conducted from October 2023 to March 2024. 3.3. Research design Cross- sectional study was done for the project work. A sampling method was convenience from which 30 different soil samples were from three areas (Kritipur, Thankot and Budhanilkantha) of Kathmandu district for isolation of of Lactobacillus spp. and extraction of antimicrobial agents (bacteriocins) for study of antimicrobial activity against MDR bacteria. 3.4. Experimental procedure 3.4.1. Laboratory Processing For the isolation of LAB, 1 gm of sample (soil) was mixed properly with 9ml of phosphate buffered saline (1/10 dilution). From this dilution (10-1), serial dilution was done to10-2 to 10-6. For enumeration and isolation of Lactobacillus spp., spread plate method was done in MRS agar (De man, Rogosa and Sharpe) containing 1% CaCO3. After solidification of media, all plates were incubated at 370C for 48hrs. Then, isolated colonies were again sub- cultured in 1% CaCO3 containing MRS agar for isolation of pure culture of Lactobacillus spp. (Kazemipor, et al., 2012). 7 3.4.2. Identification Lactobacillus spp. Identification of Lactobacillus spp. was based on biochemical test result. Gram's test, catalase test and motility test was done for further identification. Fermentation tests (glucose, fructose, galactose, sucrose, arabinose, maltose, lactose and mannitol), NaCl tolerance test and tolerance of acid were also done for further identification of isolates. Then isolation and identification of Lactobacillus spp. was followed according to Bergey's manual of determinative bacteriology (Anosike, 2022). 3. 4.3. Antibiotic Susceptibility Tests of test bacteria Antibiotic susceptibility test for test bacteria and Lactobacillus spp. was done by modified Kirby- Bauer disc diffusion method. Firstly, Mueller-Hinton agar was prepared in Petri dishes. Then, a standardized bacterial suspension (S. aureus, B. cereus, E. coli, P. auroginosa and K. pneumonia) in NB and isolated Lactobacillus spp. in MRS broth were created by adjusting the turbidity of the bacterial culture to match the McFarland standard (0.5). Then, the test bacteria and isolated Lactobacillus spp. were spread over the MHA plates and different discs of antibiotic were transferred on it. All plates were incubated at 370C for 24 hrs. After incubation, all plates were observed and examined for the inhibition zones around each disc. The diameter of each zone of inhibition was measured and compared with standardized zone size according to CLSI (2016). 3.4.4 Screening for bacteriocin For screening test, dot plate technique method was used in which all test bacteria were lawn culture in MHA plate then the dot inoculation of isolated Lactobacillus spp. was done and incubated at 30oC for 24hrs. Then the screening of bacteriocin producing Lactobacillus spp. was done by measurement of zone of inhibition (Palaniyammal et al., 2019). 3.4.5 Extraction of bacteriocin For the extraction of bacteriocin, Lactobacillus spp. were inoculated in MRS broth at pH 7.0 for 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5day) and 120 hrs (6day) at 370C. Then, all culture was centrifuged for 20 mins at 10,000 rpm at 40C. After centrifugation, cell free suspension (CFS) was separated then adjusted its pH to 7.0 by 1M NaOH. Then, CFS was 8 found to be precipitated with organic acids presence in the medium which was separated from bacteriocin through filtration by using Whatman filter paper no.1. After filtration, bacteriocin extracted from 10 Lactobacillus spp. were collected in potassium phosphate buffer (Yang, et al., 1992). 3.4.6 Determination of antimicrobial activity of bacteriocin For determining the antimicrobial activity, all test bacteria were inoculated in NB for 4hrs at 370C and its turbidity was compared with McFarland standard (0.5). Then, all bacterial suspension was swabbed on the Mueller Hinton Agar and wells were made on it. A 70μl of bacteriocin solution along negative control (sterile water) and positive control (Ciprofloxacin) were loaded on the different well in each plate and allowed for 15mins for diffusion then all plates were incubated for 24 hrs to 48hrs at 370C. Then, the zone of inhibition was observed and its diameter was measured (Chirom, et al., 2016). Antibacterial activity of bacteriocin was done from 24hrs, 48hrs, 72hrs, 96hrs and 120hrs of incubation of Lactobacillus spp. in MRS broth at pH 7.0. From this study, the comparative study of antimicrobial activity of bacteriocin extracted from 24hrs, 48hrs, 72hrs, 96hrs and 120hrs of incubation of Lactobacillus spp. (Zhennai, 2000). 3.5. Quality control in the laboratory Quality control was done to obtain for reliable results in microbiology. Quality was also monitored for each laboratory equipment's throughout the study period. Similarly, a high level of aseptic condition was diligently upheld while carrying out all procedure as well as the purity of each plates were carefully preserved to ensure the presence of pure culture. Standard culture of E. coli (ATCC 25922) and S. aureus (ATCC 25923) were used for the interpretation of result in antimicrobial activity. 3.6. Data Analysis All the data was obtained from this study was entered into Microsoft Excel 2016 and analyzed by percentage calculation. 3.7. Ethical Clearance: The ethical consideration was taken from owner of cow farm. After explanation about the purpose of the study with cow farm owner then only soil samples were collected from cow farm. 9 CHAPTER 4 RESULTS AND DISCUSSION 4.1 RESULTS 4.1.1 Growth profile of Lactobacillus spp. In this study, Lactobacillus spp. were isolated from different soil samples collected from local cow farm. In the study, 30 soil samples were included for isolation of Lactobacillus spp from the Kathmandu district. Among total soil samples, 10 (33.3%) isolates were identified as Lactobacillus spp. (Figure 1). 10 (33.3%) 20 (66.6%) Growth of Lactobacillus spp. No growth Figure 1: Growth profile of Lactobacillus spp. 4.1.2 Distribution of Lactobacillus spp. isolates from different location In this study, cow farm soil samples were taken from the 3 sites which were Kritipur (10 samples), Thankot (10 samples) and Budanilkantha (10 samples). The highest occurrence of Lactobacillus spp. was found from Kritipur, 6 (20%) followed by Thankot 2 (6.66%) and Budanilkantha 2 (6.66%). (Table 1). Table 1: Distribution of Lactobacillus spp. from different location Sites Location Number of Samples Isolated Lactobacillus spp. (%) I Kritipur 10 II Thankot 10 III Budanilkantha 10 6 (20) 2 (6.66) 2 (6.66) N=Number, %= Percentage 4.1.3. Identification of isolated Lactobacillus spp. 4.1.3.1. Identification of isolated Lactobacillus spp. Lactobacillus spp. were isolated and identified from colony morphology and cell morphology then identified from Gram staining, Motility, Spore staining, catalase test and oxidase test. The cell morphology of isolates was observed from microscope and found to be rod shaped bacteria (Figure 2). Identification of Lactobacillus spp. 12 10 8 8 10 10 10 10 10 10 4 2 2 0 Creamy Colony colour Whitish Gram positive Gram staining morphology staining Rod Cell non spore Negative Negative Negative Spore Motility test Catalase test Oxidase test 6 Figure 2: Identification of Lactobacillus spp. 4.1.3.2 Lactobacillus spp. growth at different temperature In this study, Lactobacillus spp. were able to grow at 100c, 150c, 350c, 370c and 450c respectively. In different temperature, all ten isolates were able to grow at 350c, 370c and 450c, while no isolates were able to grow at 100c and 150c respectively (Figure 3). Lactobacillus spp. growth at different temperature 12 10 10 10 10 10 10 10 8 6 4 2 0 0 Positive Negative 10C 0 0 0 Positive Negative Positive Negative Positive Negative Positive 20C 30C 37C 0 Negative Positive 45C 0 Negative Figure 3: Lactobacillus spp. growth at different temperature 4.1.3.3. Lactobacillus spp. growth at different (NaCl) Concentration In this study, 2 Lactobacillus spp., 4 Lactobacillus spp. and 4 Lactobacillus spp were able to grow at 2%, 4% and 6.5% salt concentration respectively (Figure 4). Lactobacillus spp. growth at different (NaCl) Concentration 9 8 8 7 6 6 6 5 4 4 4 3 2 2 1 0 Positive Negative Positive Negative Positive Negative 2% 4% 6.50% Figure 4: Lactobacillus spp. growth at different (NaCl) Concentration 12 4.1.3.3. Lactobacillus spp. growth at different pH In this study, 2 Lactobacillus spp., 8 Lactobacillus spp. and 10 Lactobacillus spp were able to grow at 2 pH, 4 pH and 6 pH respectively (Figure 5). Growth at different pH10 (Growth) 10 8 (Growth) 8 (No Growth) 8 6 4 2 (No Growth) 2 (Growth) 2 0 (No Growth) 0 PH 2 PH 4 PH 6 Figure 5: Lactobacillus spp. growth at different pH 4.1.3.5. Carbohydrate Fermentation test of Lactobacillus spp. In this study, fermentation test of Lactobacillus spp. for glucose, lactose, fructose, mannitol, sucrose, arabinose, galactose and maltose were done. Different Lactobacillus spp. were able to ferment different carbohydrates with gas formation or no gas formation (Table 2). Table 2: Fermentation of different carbohydrates by Lactobacillus spp. Isolated Carbohydrates Fermentation Lactobacillus Glucose Lactose Fructose Mannitol Sucrose Arabinose Galactose Maltose species Acid/Gas Acid/Gas Acid/Gas Acid/Gas Acid/Gas Acid/Gas Acid/Gas Acid/Gas KL1 + + ++ ++ +- +- -- KL2 + + ++ ++ +- +- -- KL3 ++ + - ++ -- ++ +- KL4 ++ +- +- -- +- -- KL5 ++ +- +- -- +- -- KL6 ++ +- +- -- +- -- TL7 + + ++ ++ +- +- -- TL8 + + ++ ++ +- +- -- BL9 ++ +- +- -- +- -- BL10 ++ + - ++ -- ++ +- ++ ++ ++ ++ +- +- ++ +- ++ +- ++ +- ++ ++ ++ ++ ++ +- +- +- Acid, + = Fermenter, - = Non fermenter, Gas, + = Gas formation, - = No gas formation 4.1.4 Antibiotic susceptibility test In this study, Multi Drug Resistance (MDR) bacteria were found confirmed by Antibiotic susceptibility test for Ampicillin (AMP), Azithromycin (AZM), COT- Co-trimoxazole, Gentamicin (GEN), Ciprofloxacin (CIP), Nitrofurantoin (NIT) and Cefixime (CFM). In Gram positive bacteria, S. aureus were found to be resistant to GEN, AMP, AZM and susceptible to COT and CIP while Bacillus cereus were found to be resistant to AMP, COT, GEN and susceptible to AZM, E and CIP. Similarly, E. coli and P. auroginosa were resistant to NIT, GEN, AMP and susceptible to CFM, CIP and COT while K. pneumoniae were resistant to CIP, AMP, NIT and susceptible to COT and CFM. (Table 3). Table 3: Antibiotic susceptibility tests of test bacteria Gram positive AMP AZM CIP E COT GEN Staphylococcus Res. Res. Sen. Res. Sen. Res. aureus Bacillus cereus Res. Sen. Sen. Sen. Res. Res. Gram negative AMP CFM CIP NIT COT GEN Escherichia Res. Sen. Sen. Res. Sen. Res. coli Pseudomonas Res. Sen. Sen. Res. Sen. Res. auroginosa Klebsiella Res. Sen. Res. Res. Sen. Res. pneumoniae Res.=Resistance, Sen.=Susceptibility, COT- Co-trimoxazole, AMP-Ampicilin, AZM- Azithromycin, AMX-Amoxicillin, GEN-Gentamicin, CIP-Ciprofloxacin, NIT-Nitrofurantoin and CFM-Cefixime. 4.1.5 Antibiotic susceptibility test of isolated Lactobacillus spp. In this study, Antibiotic susceptibility test of all Lactobacillus spp. were done and found to be resistance to Ampicillin (AMP) and Ceptazidime (CAC) while found to sensitive to Co-trimoxazole (COT), Gentamicin (GEN) and Ciprofloxacin (CIP). (Table 4). Table 4: Antibiotic susceptibility tests of Lactobacillus species Gram positive AMP CAC CIP COT GEN KL1 Res. Res. 30 40 15 KL2 Res. Res. 35 36 12 KL3 Res. Res. 37 38 15 KL4 Res. Res. 33 26 20 KL5 Res. Res. 32 32 11 KL6 Res. Res. 34 28 22 TL7 Res. Res. 35 44 21 TL8 Res. Res. 38 28 20 BL9 Res. Res. 35 30 20 BL10 Res. Res. 33 30 16 Res.=Resistance, AMP-Ampicilin, CAC= Ceftazidime, CIP-Ciprofloxacin, COT- Co- trimoxazole and GEN-Gentamicin 4.1.6 Screening of Lactobacillus spp. for bacteriocin For screening of bacteriocin, all Lactobacillus spp. showed inhibition zone against all test bacteria except BL9 Lactobacillus spp. isolates which did not show zone of inhibition against all test bacteria (Table 5). Table 5: Screening of Lactobacillus spp. Test bacteria Zone of inhibition(mm) for screening of Lactobacillus spp. Klebsiella pneumoniae Pseudomonas aeruginosa + + + - + - - - + - + - + - + - - - + - Escherichia coli + + + + - + - + - - += zone of inhibition and - = No zone of inhibition Gram positive bacteria KL1 KL2 KL3 KL4 KL5 KL6 TL7 TL8 BL9 BL10 Staphylococcus aureus Bacillus cereus + + + + + + - + - + + + + + + + - - - + + Gram Negative bacteria 4.1.7 Antimicrobial activity of bacteriocin extracted from Lactobacillus spp. Antimicrobial activity of bacteriocin extracted from Lactobacillus spp. from 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5days) and 120hrs (6days). Antimicrobial activity was showed the zone of inhibition against MDR bacteria only after 72hrs (4day), 96hrs (5days) and 120hrs (6days) of extraction of bacteriocin against S. aureus, B. cereus, P. aeruginosa and E. coli but not to K. pneumonia. In this study, Lactobacillus spp. showed zone of inhibition against MDR bacteria (Table 6,7,8 and 9). Table 6: Antimicrobial activity of bacteriocin extracted from 48hrs Test bacteria Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin) Gram positive bacteria Staphylococcus aureus Bacillus cereus Gram Negative bacteria Klebsiella pneumoniae Pseudomonas aeruginosa Escherichia coli KL1 KL2 KL3 KL4 KL5 KL6 TL7 TL8 BL9 BL10 - Table 7: Antimicrobial activity of bacteriocin extracted from 72hrs Test bacteria Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin) Gram positive bacteria Staphylococcus aureus Bacillus cereus Gram Negative bacteria Klebsiella pneumoniae Pseudomonas aeruginosa Escherichia coli KL1 KL2 KL3 KL4 KL5 KL6 TL7 TL8 BL9 BL10 - - - 7 - - - - - - - - 15 13 18 - - - - - - - - - - - - - - 15 14 22 17 - - - - - - - - - - - 13 8 - - - - Table 8: Antimicrobial activity of bacteriocin extracted from 96 hrs Test bacteria Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin) Gram positive bacteria Staphylococcus aureus Bacillus cereus Gram Negative bacteria Klebsiella pneumoniae Pseudomonas aeruginosa Escherichia coli KL1 KL2 KL3 KL4 KL5 KL6 TL7 TL8 BL9 BL10 10 - - - 11 - - - - 7 20 - - - 8 - 11 - - 30 7 - - - 8 9 8 - - 8 - - - - 10 - 7 - - 9 18 - - - 21 - 19 - - 10 Table 9: Antimicrobial activity of bacteriocin extracted from 120 hrs Test bacteria Zone of inhibition(mm) showed by antimicrobial substances (bacteriocin) Gram positive bacteria Staphylococcus aureus Bacillus cereus Gram Negative bacteria Klebsiella pneumoniae Pseudomonas aeruginosa Escherichia coli KL1 KL2 KL3 KL4 KL5 KL6 TL7 TL8 BL9 BL10 - 8 - 8 - - - - - - - - - 8 - 9 - - 7 - - - - - - - - - - 4.1.8 Comparison of antimicrobial activity of bacteriocin extracted from Lactobacillus spp. in different time interval In different time interval, bacteriocin extracted from Lactobacillus spp. showed different result from 24hrs to 120hrs. The highest number of zone of inhibition showed in 72hrs (6) followed by 96hrs (4) of extraction of bacteriocin. However, 24hrs and 48hrs did not showed any zone of inhibition for any test bacteria. On the other hand, bacteriocin extracted from 120 hrs also showed the highest zone of inhibition for test bacteria (Figure 6). 35 30 25 20 15 10 5 0 Comparision of zone of inhibition by bacteriocin KL1 KL2 KL3 KL4 KL5 KL6 TL7 TL8 BL9 BL10 24hrs (2day) 48hrs (3day) 72hrs (4day), 96hrs (5days) 120hrs (6days) Figure 6: Comparison of zone of inhibition by bacteriocin LIST OF PHOTOGRAPHS A B Photograph 1: Enumeration of Lactobacillus species (A) and subculture (B) on de Mann Rogosa Sharpe (MRS) Agar media A B Photograph 2: Identification of Lactobacillus spp. by carbohydrate fermentation (A) (glucose, maltose, fructose, galactose, sucrose, arabinose, lactose and mannitol) and growth at 450c (B) A B Photograph 3: Antibiotic susceptibility of test bacteria (A) and isolated Lactobacillus spp. (B) AZM-Azithromycin, CIP-Ciplofloxacin, GEN-Gentamicin, AMP-Ampicillin, COT-Co-trimoxazole, CAC= Ceftazidime and E-Erythromycin A (24hrs) B (72hrs) Photograph 4: Antimicrobial activity bacteriocin extracted from Lactobacillus species at 24hrs (A) and 72hrs (B) 4.2 DISCUSSION In this study, 30 different soil samples were collected from various local farm area of Kathmandu district of 3 sites including Kritipur (10 samples), Thankot (10 samples) and Budanilkantha (10 samples). The highest occurrence of Lactobacillus spp. were found from Kritipur followed by Thankot and Budanilkantha which were 6 (20%), 2 (6.66%) and 2 (6.66%) respectively. Among total samples, 10 strains (33.3%) were identified as Lactobacillus spp. by various morphological, biochemical and physiological test. Similar study was done by Yanagida, et al., (2005) for isolation of new types of bacteriocin producing LAB from soil in which 55 soil samples were taken and 42 were found as acid producing bacterial strains. On the other hand, Ekundayo (2014) obtained 21 LAB obtained from rhizosphere soil, gastrointestinal tract, gills of fish, pond sediments as well as yellow maize gruel. According to Ekundayo (2014), LAB can be detected in soil which may be considered as a source of LAB as well as its ability to grow at different temperature, salt concentration and pH level. For identification of Lactobacillus spp. different laboratories techniques were performed including isolation, identification by microscopic examination, culturing and biochemical test. The morphological characteristics of all the isolates basically showed same type of feature of Lactobacillus spp. Similarly, Lactobacillus spp. were also found to be Gram positive rod shape, nonmotile, nonspore forming, catalase negative and oxidase negative bacteria. According to George, et al., (2018), Lactobacillus spp. were also identified by catalase negative, non-motile and Gram positive bacteria. For further identification, Lactobacillus spp. growth at different temperate, different pH and different concentration of NaCl were studied. In different temperature, all ten isolates were able to grow at 350c, 370c and 450c, while no isolates were able to grow at 100c and 150c respectively. Similarly, Tsvetkovn & Shishkova (1982) studied the various strains of Lactobacillus species for their ability to sustained in freezing temperature. According to their study, the viability as well as activity of LAB cell increase in cooling. They concluded that, S. thermophiles, Streptococci spp. and S. lactis were found to be resistant than rods shape L. casei. Lactobacillus species displayed the wide range of growth at different salt concentration value. Lactobacillus species were able to grow at low salt concentration to medium salt concentration that is 2% to 4%. While, some of the strains were able to grow at high salt concentration that is in 6.5%. In this study, 2 Lactobacillus spp., 4 Lactobacillus spp. and 4 Lactobacillus spp. were able to grow at 2%, 4% and 6.5% salt concentration respectively. Similar type of test was performed for identification of LAB isolated from raw milk of cow (Wassie and Wassie, 2016). LAB are also known for bacteria having high resistance against lower pH and are generally cocci or rod shaped (Mokoena, 2017). In this study, 2 Lactobacillus spp., 8 Lactobacillus spp. and 10 Lactobacillus spp were able to grow at 2 pH, 4 pH and 6 pH respectively. Similar study done by Arya, et al., (2020) for study of LAB able to grow in different pH. The different genera of LAB able to grow in lower temperature are Lactobacillus brevis, Lactobacillus plantarum, Pediococcus pentasaceus, Pediococcus acidilactic, and Leuconostoc (Tamang, et al., 2005). Carbohydrate fermentation test was done for all isolates were tested using eight different sugars. In this study, fermentation test of Lactobacillus spp. for different carbohydrates like glucose, lactose, fructose, mannitol, sucrose, arabinose, galactose and maltose were done. Different Lactobacillus spp. were able to ferment different carbohydrates with gas formation or without gas formation. Certain test of sugar fermentation was performed by Gunkova, et al., (2021) on lactic acid starter culture. Similar study done by Arya, et al., (2020) for LAB identification from colostrum of human. Lindquist (1998) reported that medium to support the proper growth of LAB in different growth conditions and different carbohydrates. In this study, test bacteria were confirmed as Multi Drug Resistance (MDR) bacteria by Antibiotic susceptibility test for Ampicillin (AMP), Azithromycin (AZM), COT- Co- trimoxazole, Gentamicin (GEN), Ciprofloxacin (CIP). S. aureus were resistant to COT, AMP, AZM and susceptible to COT and CIP. Similarly, E. coli and P. auroginosa were resistant to NIT, GEN, AMP and susceptible to CFM, CIP and COT. Furthermore, K. pneumoniae were resistant to CIP, AMP, NIT and susceptible to COT and CFM. After conforming the test bacteria were MDR bacteria then only antimicrobial activity of isolated Lactobacillus spp. was done. Many studies done the antimicrobial activity of Lactobacillus spp. against ATCC bacteria, one of the study was done by Arya, et al., (2020). In this study, Antibiotic susceptibility test of all Lactobacillus spp. were also done and found to be resistance to Ampicillin (AMP) and Ceptazidime (CAC) while found to sensitive to Co-trimoxazole (COT), Gentamicin (GEN) and Ciprofloxacin (CIP). Similarly, Arya, et al., (2020) also reported LAB were resistance against different antibiotics. However, many studies were not reporting antibiotic resistance in Lactobacillus spp. and in LAB. Many studies have already done for the isolation, characterization as well as production of antimicrobial substance Bacteriocin. The use of these antimicrobial compounds usually applicable for the preservation of food, increase shelf life of product, as well as to inhibit the harmful pathogenic bacteria. So, the bacteriocin were the very essential substance produced by Lactic acid bacteria (Adenike, et al., 2007). A naturally occurring organic acids called lactic acid was created when LAB fermented carbohydrates. This contributed the acidic environment which eventually prevented the growth of other harmful bacteria and microbes. As this lowered the pH and prevent the spoilage as well as considered as the preservative substance. LAB were responsible for the production of proteinaceous substance bacteriocin which act as antimicrobial agent and also carried huge potential to use as alternatives to artificial preservatives in many foods after extensively used in research (Mobolaji & Wuraola, 2011). So, bacteriocins are also considered as antibacterial substances (proteins) which are produced from different LAB (Gaur, et al., 2004). For screening test of bacteriocin in all Lactobacillus spp., dot inoculation of isolated Lactobacillus spp. was done and incubated at 30oC for 24hrs. Among 10 isolates, 9 Lactobacillus spp. showed inhibition zone against all test bacteria. Similarly, Palaniyammal, et al., (2019) also studied the screening of bacteriocin producing Lactobacillus spp. However, many studies were not doing this screening test before antimicrobial activity study of bacteriocin extracted from Lactobacillus spp. In this study, bacteriocin were extracted from Lactobacillus spp. were from 24hrs (2day), 48hrs (3day), 72hrs (4day), 96hrs (5days) and 120hrs (6days). However, antimicrobial activity was showed against MDR bacteria only after 72hrs (4day), 96hrs (5days) and 120hrs (6days) of extraction of bacteriocin against MDR bacteria. From 96hrs (5days) of extraction of antimicrobial substance bacteriocin was able to inhibit all test bacteria which were S. aureus, B. cereus., E. coli and P. aeruginosa and K. pneumoniae. The highest zone of inhibition was showed by BL10 isolate against B. cereus (30mm) followed by KL1 isolate against E. coli (21mm) and KL5 isolate against B. cereus (20mm). In this study, 5 extracted antimicrobial substance bacteriocin were able to show inhibition zone against all MDR bacteria. Nguyen, et al. (2020) isolated and identified LAB from vegetable growing soils. Different inhibitory action of these isolates were studied against various pathogens such as E. coli, Staphylococcus, Bacillus spizizenii, Salmonella Typhi. But, the isolates were not able to inhibit E. coli and Staphylococcus but able to show inhibitory action against Bacillus spizizenii and Salmonella Typhi. Several studies were performed to study the antimicrobial properties against various pathogens that includes E. coli, Shigella species, Salmonella species and so on. Gilliland & Speck, (1977) reported Lactobacilli spp. showed antibacterial activity against Gram- positive bacteria (C. perfringens and S. aureus) than Gram- negative bacteria (S. Typhi, E coli, and S. Paratyphi). On the study done by Elayaraja, et al. (2014) bacteriocin were produced, purified and characterized for the inhibition of wide range of pathogens also created greater interest for food safety and as a preservative. Lactic acid bacteria displayed a wide range of antimicrobial activities. Thus, bacteriocin produced by various LAB have huge potential as preservative (Bromberg, et al., 2004). Such study had become a great importance in today's world which has the greater potential to lead the nation by its potentiality as a natural preservatives and also have ability to inhibit growth of pathogenic microorganisms in food and feed industries Liasi, et al. (2009). CHAPTER 5 5. CONCLUSION AND RECOMMENDATION 5.1 Conclusion Among 30 farm soil samples, 10 Lactobacillus species were identified. The highest occurrence of Lactobacillus species was found in the sample collected from Kritipur (20%). From this study it was concluded that Lactobacillus species were isolated from local cow farm can inhibit the growth of MDR bacteria included S. aureus, Bacillus spp., P. aeruginosa, E. coli and K. pneumonia. This is due to the antimicrobial substance bacteriocin extracted from of Lactobacillus spp which inhibited MDR bacteria which was highly resistant. 5.2 Novelty/ Association to National priority and needs This study showed that Lactobacillus spp. isolated from soil of cow farm showed antimicrobial activity. So, this is the novelty of the antibacterial activity of Lactobacillus species against MDR bacteria. So, there is need for study of Lactobacillus spp.in large number in national level for production of antibiotics. 5.3 Future prospect of the research proposal Further research can be done on antibiotics production by Lactobacillus spp. in future. 5.4. Recommendations for further work From this study, Lactobacillus spp. were able to isolate from soil of different farm of Kathmandu district. So, further study can be done for isolation of different potential species from farm soil to reduce the development of antibiotic resistance. Antimicrobial activity of bacteriocin extracted from Lactobacillus spp. showed the antimicrobial activity against different MDR bacteria. So, next generation antibiotics can be prepared for targeting the multi drug resistant bacteria. REFERENCES Adebayo,F., Afolabio, O., & Akintokun, A. 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bacteria", structure and properties. Academic Dissertation. Department of Food Technology, University of Helsinki: 31.ISBN: 951-45-9146-1. APPENDIX A List of materials: Chemicals, Reagent, Equipment's and Media Chemicals and Reagents 1. Crystal violet 2. Gram's iodine 3. 95% Ethanol 4. Safranin 5. 3% Hydrogen Peroxide 6. Oxidase strip 7. Ammonium Sulphate 8. Potassium Phosphate Buffer 9. Distilled water 10. Phenol red indicator Equipment 1. Autoclave 2. Hot air oven 3. Incubator 4. Microscope 5. weighing machine 6. Refrigerator 7. Micropipette 8. Micropipette tips 9. Centrifuge machine 10. Centrifuge tubes Glass Wares 1. Conical flask 2. Beaker 3. Petri plates 4. Test tubes 5. Glass slides 6. Measuring Cylinder 7. Volumetric Flask 8. Cover slip 9. Glass rod 10. Micropipette I Miscellaneous 1. Inoculating wire 2. Inoculating loop 3. Forceps 4. Cotton Swab 5. Dropper 6. Cotton 7. Lysol 8. Alcohol 9. Bunsen Burner 10. Marker Microbiological Media 1. Nutrient Broth 2. Nutrient Agar 3. MRS broth 4. MRS Agar 5. Mueller-Hinton Agar 6. Simmon's Citrate Agar II APPENDIX B I. Composition and preparation of different cultural media 1. Mueller Hinton Agar Ingredients gm/liter Beef infusion 300 gm Casamino acid 17.5 gm Starch 1.5 gm Agar 17 gm Distilled water 1000ml Final PH 7.2?0.2 The agar was accurately weighed and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121?C (15 lbs. pressure) for 15 min. The media was then poured into sterile petriplates. 2. Nutrient Agar Ingredients Peptic digest of animal tissue Beef extract Yeast extract Sodium chloride Agar Distilled water gm/liter 5 gm 1.5 gm 1.5 gm 5 gm 15 gm 1000 ml The agar was accurately weighed and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121?C (15 lbs. pressure) for 15 min. The media was then poured into sterile petriplates. III 3. Nutrient Broth Ingredients Peptic digest of animal tissue Beef extract Yeast extract Sodium chloride Distilled water gm/liter 5 gm 1.5 gm 1.5 gm 5 gm 1000 ml The media was accurately weighted and was suspended into 1000 ml of distilled water and boiled to dissolve completely. Then the medium was poured in sterile test tubes and sterilized by autoclaving at 121?C (15 lbs. pressure) for 15 min. MRS Broth (Himedia) Component Protease peptone Dextrose Sodium acetate Ammonium citrate Yeast extract Dipotassium phosphate Beef extract Amount(g/l) 10.0 20.0 5.0 2.0 5.0 2.0 10.0 IV Mueller-Hinton Agar Component Beef, infusion Casein acid hydrolysate Starch Agar Amount(g/l 300.0 17.5 1.50 17.0

Sugar Fermentation Broth Component Amount(g/l) Trypticase 10.0 Sodium chloride 5.0

Sugar (Glucose, Fructose, Lactose, Maltose, Sucrose) 5.0 Phenol red indicator Few drops V APPENDIX C Reagents Composition Crystal Violet(200ml) ? In 40ml 95% ethyl alcohol, 4 gm crystal violet was dissolved ? In 160ml distilled water,1.6gm ammonium oxalate was dissolved ? The two solutions were mixed and stored in a reagent bottle at room temperature. Gram's Iodine(150ml) ? In 150ml distilled water, 0.5g iodine and 1gm potassium iodide was added ? The solution was mixed and kept

overnight and was **transferred to reagent bottle and stored at room temperature**

. Safranin(50ml) ? In 5ml 95% ethanol, 1.25g

safranin was dissolved. ? Distilled water was added to the solution to make final volume of 50ml. ? Then, **the solution was stored**

at room temperature. Oxidase Reagent(50ml) ? In 50ml

distilled water, 1% tetra-methyl-p-phenylenediamine dihydrochloride was added ? It was **stored in reagent bottle** and was **covered with aluminum foil to prevent** the **exposure** of **light** at 40C. **Catalase Reagent**

? 3.5% Hydrogen peroxide was used. 0.5 McFarland Standards ? 0.6ml of 1% w/v solution of barium chloride solution was taken ? It was then mixed up with 99.4ml of 1% v/v solution of sulphuric acid VI ? The mixture was shaked well ? This can be stored in a well sealed container at 20-280C for up to 6 months ? Small amount of the solution was taken during the testing of turbidity of the inoculums.

Ethyl alcohol (95 %) ? 95ml **of ethyl alcohol was added to** 5ml **of distilled water**

. Antibiotic Susceptibility Test (AST) ? Mueller-Hinton Agar, Antibiotic disc, 0.5 McFarland Standard, Test MDR bacterial suspension. Antibiotic Susceptibility Test (AST) of test bacteria (MDR) Gram positive AMP AZM CIP E COT GEN Staphylococcus R R S R S R aureus Bacillus spp. R S S S R R Gram negative AMP CFM CIP NIT COT GEN Escherichia coli R S S R S R Pseudomonas R S S R S R auroginosa Klebsiella R S R R S R pneumoniae VII VIII 1 2 3 6 10 11 13 14 15 16 17 18 19 20 21 22 23 24 25 23 24 25 26 27 28 32