**PROBIOTIC POTENTIAL BACTERIA OF INDIGENOUS FERMENTED FOOD (GUNDRUK) FROM LOCAL MARKET OF SUNSARI, NEPAL**



A

Dissertation

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(**Environment and Public Health**)

By:

**Sabin K. C.**

T.U. Regd. No. 5-2-0003-0282-2012

Roll No. MB 857/O73

Dharan

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**CERTIFICATE OF APPROVAL**

On the recommendation of Mr. Shiv Nandan Sah the dissertation work of **Mr. Sabin K. C.** entitled **“PROBIOTIC POTENTIAL BACTERIA OF INDIGENOUS FERMENTED FOOD (GUNDRUK) FROM LOCAL MARKET OF SUNSARI, NEPAL”** has been approved for the examination and is submitted to the Tribhuvan University in Partial Fulfillment of the requirements for M. Sc. degree in Microbiology (**Environment and Public Health**).

…………………

**Dr. Kamana Sahani**

Teaching Assistant

Department of Microbiology,

Central Campus of Technology,

Hattisar, Dharan, Sunsari

Nepal

**RECOMMENDATION**

This is to certify that **Mr. Sabin K. C**. has completed this dissertation work entitled**“PROBIOTIC POTENTIAL BACTERIA OF INDIGENOUS FERMENTED FOOD (GUNDRUK) FROM LOCAL MARKET OF SUNSARI, NEPAL”** as a Partial fulfillment of the requirement of M. Sc. degree in Microbiology (**Environment and public Health**) under my supervision. To my knowledge, this work has not been submitted for any others degree/s.

**………………………………….**

**Mr. Shiv Nandan Sah**

***Supervisor,***

Associate Professor

Department of Microbiology,

Central Campus of Technology,

Hattisar, Dharan, Sunsari

Nepal

DATE: ………/……/…….

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**Sabin K.C.**

**Date: ………………………**

**ABSTRACT**

*Gundruk* is a non-salted fermented and acidic vegetable product indigenous to the Nepal. The main objective of this study was to determine the microorganisms found in *Gundruk* sample collected from leaves of *Brassica napus*. A total of 30 samples of *Gundruk* were collected from different places of Sunsari district, Nepal and were analyzed for microbial counts using standard microbial techniques. The colonies grown were identified by conventional methods as described by Bergey’s manual of determinative bacteriology. The antimicrobial susceptibility test and antimicrobial test were also performed by using Mueller Hinton Agar (MHA). No yeast and mold were detected. In order to identify the predominating organisms, a total of 98 strains were isolated. The phenotypic characteristics of these strains were determined by using Gram’s stain, biochemical tests, Carbohydrate fermentation test and capacity to grow on different temperature, pH and different concentration of NaCl. The major representatives of the LAB involved in *Gundruk* were identified as *Lactobacillus plantarum* and *Pediococus pentasaccus*. No pathogenic bacteria were found in gundruk sample collected from different places of Sunsari. No any P-solubilizing bacteria were detected. All isolates were susceptible to most of common antibiotics except Vancomycin. This result suggested that LAB isolated from gundruk have very good potential to be used as probiotics. Antimicrobial activity of gundruk sample was also determined. All gundruk samples were able to inhibit most of the indicator pathogens tested except *Salmonella* spp and *Vibrio cholera*. Attempts were made to produce gundruk using mixed starter culture of LAB previously isolated under lab-respective products. The product prepared under lab condition has scored higher sensory ranking comparable to market products.

Key words: Lactic acid bacteria, *Gundruk,* Probiotics, Antimicrobial Susceptibility Testing

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**ABBREVIATIONS AND SYMBOLS**

CFU Colony forming unit

H₂O₂ Hydrogen peroxide

H₂S Hydrogen sulfide

LAB Lactic acid bacteria

MHA Mueller Hinton Agar

MRS de Man Rogosa and Sharpe

NaCl Sodium chloride

NSLAB Non-starter lactic acid bacteria

P- solubilizing Phosphorus solubilizing

TCBS Thiosulfate- citrate- bile salts- sucrose

µg Microgram

°C Degree Celsius

µl Microlitre

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# CHAPTER I

# INTRODUCTION

### 1.1 Background

Fermented food is usually classified according to the nature of the raw material and nature of fermentation. They may also be classified by the region of origin. *Kinema, Gundruk, Sinki* and *Masyaura* etc are fermented food used by the Nepalese residing both in Nepal and India. It is a fact that microorganisms play an important role during the process of fermentations. It metabolizes a variety of fermented foods into unique and appealing characteristics. Micro flora present on the surface of raw material probably has a major role in the manifestation of important characteristics of the fermented products. As such it is necessary to have a thorough understanding of probable microorganisms, their specific role and their activities in order to make fermentation process more reliable and predictable. Different fermented products have different profile of dominating microbes depending on its raw materials (Caplice and Fitzgerald, 1999).

The production of fermented food is one of the oldest food technologies known to man since the dawn of civilization. In course of time tradition for fermentation of food was established by which handling and storage of certain food raw material in a specific manner resulted in the development of food that had not only the superior quality to those of the original substrate but also had desirable and organoleptically pleasing characteristics. Whatever be the type of food, traditional fermentation in most cases are the methodology and knowledge with the manufacturing of these product is handed down from generation to generation within local communities since time immemorial. Community people in the old days produce relatively small quantities of the fermented food for consumption and distribution in an around the immediate area. The original and primary purpose of fermenting food substrates was to achieve a preservative effect. However, with the development of the many effective alternatives preservation technology which are now commonly available, particularly in the Western world, there is no longer the most pressing requirement and many of these foods are manufactured because their unique flavor, aroma and texture attributes are much appreciated by consumer. However, even in these situations, the conditions generated by the fermentation are essential in ensuring the self life and microbiologically safety of the product. Nevertheless, there are many parts of the world where preservation role is still the essential one and where the fermentation process is performed on a traditional rather than industrial basis. It appeared that during the course of fermentation, the microbial activity leads to a variety of physical, chemical and nutritive changes which arc of complex nature. Some of the fermented products are believed to have a high nutritive and therapeutic property (Singh T. A., Devi K. R. et al. 2014).

Foods such as ripened cheese, pickles, *Sauerkraut*, *Kimchi, Gundruk, Sinki* and fermented sausages are preserved products in which their shelf life is extended considerably over that of raw materials of which they are made of. Now, these fermented foods are the important components of the daily diets of many of the world’s largest population especially in Japan, Indonesia, Thailand, Philippines, Taiwan, Korea, China, Parts of Africa, Pakistan and India. Some of their processes of fermented foods have been based on the acts of mystic, art and science (Vandamme, 1982).

Gundruk is a fermented and acidic vegetable product commonly prepared by the Nepalese of the Himalayan regions of India, Nepal and Bhutan during winter when perishable leafy vegetables are plenty. During preparation of gundruk, fresh leaves of local vegetable known as ‘rayo-saag’ (Brassica rapa L. ssp. campestris (L.) Clapham variety cumifolia Roxb.), leaves of mustard (Brassica juncea (L.) Czern), leaves of radish (Raphanus sativus L.), leaves of cauliflower (Brassica oleracea L. variety botrytis L.) and leaves of cabbages (Brassica oleracea L. variety capitata) are wilted for 1–2 days. Wilted leaves are crushed mildly and pressed into a container or earthenware pot, made air tight and fermented naturally for about 15–22 days. After desirable fermentation, freshly prepared wet gundruk is sun dried for 2–4 days, which can be kept for a year or more at room temperature. Gundruk is eaten as soup and pickles with boiled rice. It is similar to the Korean kimchi and the German sauerkraut. Unlike kimchi and sauerkraut, freshly prepared wet gundruk is normally not preferred. (Tamang J.P.et al, 2005).*Gundruk* and *sinki* are no exception to this and biochemical changes may be present. Further, seasons play a very important part in the variation of biochemical characteristics of the plant materials. Different environmental factors prevailing during different seasons of the year won’t influence directly the activities of the microbes and indirectly affect the physiological parameters. To cite an example, temperature, rainfall and relative humidity play a major role on the occurrence and disappearance of many microbes that brings out the various physiological changes. Relative humidity and temperature cause in the fruit rot (Patil and Pathak, 1993).

Gundruk, a non-salted and fermented leafy vegetable product, has been one of the major appetizers for the Nepalese people since a long time ago. Gundruk making seems to have been evolved as a means of preserving vegetables when they are plentiful during peak harvest season. Gundruk is also a kind of preserved vegetable by fermentation. Gundruk has remained as major popular food items of Nepali people. The quality attributes to Gundruk basically depends upon the typical Gundruk flavour and acidic taste. The following Nepali saying, “More the acidic taste, better is the product” clearly shows the position. The techniques have been limited to the household level and transferred from mother-to-daughter, and quite often the maker is credited or discredited according to the quality of Gundruk. So maintaining a standard quality of gundruk has remained as an unresolved task. (Karki T. et al, 1983).

LAB in pickles helps to enhance human nutrition by providing vitamins, minerals, and carbohydrates, and produce various aroma components, bacteriocins, and exopolysaccharides. These metabolic products impart some characteristic properties such as taste, texture and longer shelf life to the products (Leroy and de Vuyst 2004). LAB carries out detoxification of toxic compounds and degradation of mycotoxins in specific cases and therefore can reduce the health risk. Lactic acid bacteria are considered as safe additives and Generally Recognized as Safe (GRAS), useful to control the frequent development of pathogens and spoilage microorganisms in food and feed (Namasivayam et al. 2014).

*Lactobacillus plantarum*, *Lactobacillus casei subsp. casei*, *Lactobacillus casei subsp. pseudoplantarum*, *Lactobacillus cellobiosus* and *Pediococcus* are identified from *Gundruk* fermentation. Of the lactics, *Lactobacillus plantarum* and *Pediococcus pentosaceus* are found to be the dominant flora during the natural fermentation of Gundruk. However, *Lactobacillus cellobiosus* is isolated in the earlier part of the fermentation. The sequential pattern of the lactics found in Gundruk fermentation primarily are initiated by heterofermentative rod (*Lactobacillus cellobiosus*) and homofermentative coccus(*Pediococcus pentosaceus*) and later on succeeded by more acid producing homofermentative rod(*L. plantarum*). (Karki T., et al. 1983).

Probiotics are defined as “Living microorganisms which, when administrated in adequate numbers, confer a health benefit to the host” ([FAO/ WHO 2006](https://www.frontiersin.org/articles/10.3389/fmicb.2016.00863/full#B21)). In general, commercially available probiotic bacteria are from *Lactobacillus, Bifidobacterium, Streptococcus* and *Enterococcus genera.* The health benefits of probiotics in treating disorders, including inflammatory bowel disease, irritable bowel syndrome, constipation, antibiotic-associated and acute diarrhea, allergy-related conditions, hypertension, and diabetes, have been well-documented by numerous esteemed scientific reports and systematic reviews (Hill C., et al. 2014). It is desirable for probiotic strains to possess several properties, such as a tolerance to gastrointestinal conditions (gastric, intestinal, and bile acids), attachment to epithelial cells, assimilation of cholesterol in food and the human intestine, bile salt hydrolysis, safety (no virulence genes, absence of hemolytic activity, and sensitive to antibiotics), antimicrobial properties, and survival during the fermentation process and storage (Khan S. U., 2014, Naidu A., et al. 1999). However, it is not essential that potential probiotics possess all of the above characteristics. The industrial characteristic such as tolerance to heat treatment, particularly spray drying, is also preferable. Exopolysaccharide (EPS) production could provide health benefits to consumers as non-digestible fiber or in improving the sensory properties of food. Probiotic strains may also be used to produce fermented functional foods. Functional foods produced using probiotics possess superior health advantages compared with conventional food products. Attempts to screen for new LAB bacteria that possess excellent probiotic characteristics from various food sources are ongoing. (Alkalbani N. S., et al. 2019).

High humidity and temperature also leads to the spoilage of many vegetables and fruits. As the traditional fermentation of *gundruk* and *sinki* is the product of simple fermentation technique involving a very crude method and the fermented products are being consumed by many people, it is worth to assess systematically and scientifically the details of traditional methods of *Gundruk* and *Sinki* preparations, raw materials used, physical-chemical changes in the fermented food and their raw materials, contamination of stored fermented products by microbes and the changes they bring in the biochemical constituents of the fermented food as compared to their fresh raw materials and finally the seasonal variation of microbes associated with the fresh and fermented materials for insight of the fermentation process and products.

Further, the fermented vegetable food has direct impact on human health; quality assessment of fermented food is of utmost importance. It is a fact that in most of the cases, traditionally prepared foods are consumed by the people without getting by the Food Quality Control Department or any other concerned department of the Government. In addition, traditionally prepared foods are given less importance for research and development and occasionally they are misused by the scientific community. Perusal of literature revealed that not much work has been done on the research and development aspect of many traditional fermented foods of the region. It is expected that systematic research of traditionally fermented food in any aspect of biological sciences may lead to an interesting findings as regard the quality improvement of traditional food for betterment of mankind.

## 1.2 OBJECTIVES

#### General objectives

* To determine probiotic potential bacteria of indigenous fermented food (Gundruk) from Local market of Sunsari.

**1.2.2 Specific Objectives**

* To study LAB found in *Gundruk* sample.
* To study pathogenic bacteria in *Gundruk* Sample.
* To ferment leaves of *Brassica napus* prepare *Gundruk* using isolated LAB.
* To perform the antibiotic susceptibility pattern of the LAB.
* To determine the antimicrobial activity of the *Gundruk* sample.

# CHAPTER-II

# LITERATURE REVIEW

## 2. 1 Fermentation

The term "fermentation" comes from latin word *fervere* meaning "to boil". Fermentation is defined as a form of energy-yielding process from an organic substrate, usually carbohydrate, without the involvement of an exogenous oxidizing agent (Bourdichon et al., 2012). Fermentation is being practiced to preserve food safely (Smid and Hugenholtz, 2010). The desirable and edible microorganisms which were overgrown on food substrates become resistant to invasion by spoilage, toxic or food poisoning microorganisms (Steinkraus, 2002). Fermented foods are characterized for their pleasant flavor, aroma, and texture and processing properties (Basappa, 2002).

It is one of the oldest technologies that are practiced by our ancestors which helped them to survive in harsh environmental condition such as drought by improving the shelf-life and food security. Since the dawn of civilization, methods for the fermentation of milks, meats and vegetables have been described, with earliest records dating back to 6000 BC and the civilizations of the Fertile Crescent in the Middle East (Fox, 1993). In ancient time, fermentation is considered to be a natural process and there was no knowledge or appreciation to the role of microorganism. The storage of certain raw material in a specific manner resulted in the development of foods that not only had longer shelf life but were far superior to those of the original substrate, and also had desirable and organoleptically pleasing characteristics. These methodologies and knowledge were transferred from generation to generation within local communities (Caplice and Fitzgerald, 1999).

In recent years, it is widely practiced in the developing countries at a household or village-level technology, but comparatively very few operations are carried out at an industrial level (Holzapfel, 2002). In modern-day life, importance of fermentation is underlined by the wide spectrum of foods marketed, not only for the benefit of preservation and safety, but also for their highly appreciated sensory attributes. Moreover, this process is a low-input enterprise that provides individuals with limited purchasing power, access to safe, inexpensive and nutritious foods (Marshall and Mejia, 2011).

#### 2.1.1 Indigenous Fermented foods

Fermented foods are consumed as a staple diet thus contributing to about one-third of the total world diet (Campbell-Platt, 1994). Fermented products are low-cost, high-value and play an important role in dietary and socio-cultural life of different community. Fermented foods are mostly produced in Europe, North America and Africa. In South America major quantities of beverages and dairy products are fermented while in the Middle East fermented dairy products are important (Campbell-Platt, 1994). In Africa, fermentation of cereals for the production of gruel is well known which is given to infants and young children as a complementary and weaning food (Nout, 2001; Blandino et al., 2003; Tou et al, 2007).

In East and South-east Asia, fish and legumes are the most important fermented foods produced both contributing major protein sources. Also in these regions, cereal products maybe co-fermented with legumes, as in the use of rice or barley with soybeans in misoproduction (Minamiyama and Okada, 2008), wheat used with soybeans in soy sauce production and rice with black gram in *idli* preparation (Prajapati and Nair, 2008).

Production of fermented foods is one of the oldest food processing technologies known to man. Fermentation of milks, meats and vegetables dates back to 6000 B.C. when civilization dawn in the Middle East (Fox, 1993). Solid state fermentation process is found to be most appropriate rural technology for the production of many traditional products as it require little control environment and equipment and technique was adopted for protein enrichment of cassava products (Balagopalan, 1996).

Fermentation is one of the processes which bring about various changes in the food products. It is also considered as the pathway to nutritional security (Anuradham, 1999). Various chemical changes such as the synthesis of fat, enzymes are brought about by the microbes associated in the products (Azdul Azeem et al. 1995). Singh (1987) reported that fermentation decreased the total disaccharide and increase the total monosaccharide on the fermented products. He also reported on the slight increase in total unsaturated fatty acid and decrease in saturated fatty acid. The fermented foods have better organoleptic properties, taste, colour, texture, mouth feel and crispness as reported by Lonsane (1989). They are desirable foods for infants, expectant and nursing mother and invalids due to high fibre content. It is demonstrated that shelf life of fermented food and their increased content of vitamins, proteins and energy makes them more valuable in preventing malnutrition. In the fermented product the high protein content may be due to the production of protein by the microorganisms especially fungi. It has been shown by many workers (Wosten el .al. 1991 and Muller el al. 2000) that protein are found to occur at the tip region of filamentous fungi and thus the high content of protein. These proteins can also pass through the cell wall of the fungi and distributed (Wessels, 1994). Azeem and Neelagund, (1995) reported that surface cultures *Aspergillus sydowii* and *Aspergillus nidulans* synthesized fats from various carbon sources. Wang and Lee (1996) reported that *Aspergillus* sp. utilized starch glucose and yielded maximum alkaline protease.

##### **2.1.1.1 Fermented vegetables**

Vegetables are good sources of natural antioxidants such as carotenoids, vitamins, flavonoids, phenolic compounds, minerals and dietary fibers (Sun et al., 2009; Kusznierewicz et al., 2010). These vegetables have been recognized as inexpensive and easily accessible sources of food and essential micronutrients (Mwajumwa et al, 1991).

Due to the short shelf life of these crops, fermentation is one of the most effective ways of conserving perishable vegetables. Canned or frozen foods are too expensive or not easily available for the majority of people living in underdeveloped and developing countries, where acid fermentation combined with salting remains one of the most practical methods of preserving and often enhancing the organoleptic and nutritional quality of fresh vegetables (Steinkraus, 1996).

Some traditional fermented vegetables of the world are listed in Table 2.1. Fermented vegetables have a great importance in western countries. Cabbage, olives, cucumbers and peppers account for the largest volume of vegetables and fruits commercially brined and fermented in the western world. German and Scandinavian countries have a tradition in the production of fermented vegetables known as *Sauerkraut. Sauerkraut,* German word for 'sour cabbage’ is made from shredded and salted cabbage by naturally occurring lactic acid bacteria fermentation (Johanningsmeier et al, 2005). It has become popular in the United States and other European countries (Prajapati and Nair, 2008).

In Asia, a variety of fermented vegetable products are available. *Kimchi,* traditional Korean fermented vegetable made from 30 different types of vegetables, such as Chinese cabbage, radish, ponytail radish, young Oriental radish, cucumber and spices such as red pepper, black pepper and cinnamon are frequently used. It is less acid than sauerkraut and is consumed while still carbonated (Kim et al, 2012). *Jeruk* is a homemade fermented pickle indigenous to many races in Malaysia and is prepared from common fruits and vegetables (Merican, 1996). *Pak-gard-dong* is the fermented vegetable product of Thailand prepared from the leaf of mustard (Boon-Long, 1986). Other fermented vegetable product includes *pak-sian-dong* of Thailand, *Sayur asin* of Indonesia, *Suan cai* of China, *Sunki* of Japan, *Futsai* nand *suan-tsai* of Taiwan (Tamang, 2010b).

*Gundruk* is a fermented and acidic vegetable product commonly prepared by the Nepalis of the Himalayan regions of India, Nepal and Bhutan. It is prepared from fresh leaves of a local vegetable called *rayo-sag (Brassica rapa* subspecies *campestris* variety *cuneifolia),* mustard *(Brassica junced),* and cauliflower *(Brassica oleracea* variety *botrytis).* Unlike *kimchi* and sauerkraut, freshly fermented *gundruk* is sun dried for 3-4 days before consumption, and dried *gundruk* is preserved for more than 2 years (Tamang and Tamang, 2010). *Sinki* is a fermented radish tap root product of the Himalayan region of India prepared by pit fermentation (Tamang and Tamang, 2009).

*Khalpi* or *khaipi* is a fermented cucumber *(Cucumis sativus* L.) product, commonly consumed by the Brahmin Nepalese in Sikkim. *Inziangsang* is a traditional fermented leafy vegetable product of Nagaland and Manipur in North East India (Tamang et al., 2005). Similar to *gundruk,* fermented and acidic mixed vegetables based product is prepared by people of Ladakh India. It is made from shredded cabbage, carrot and radish.

**Table 1: List of fermented vegetables prepared and consumed in various parts of the world (Tamang, 2010b).**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Fermented  Food | Substrate | Sensory and  Product Nature | Culinary | Microorganisms | Country |
| ***Anishi*** | Taro leaves | Acidic, wet | Curry | LAB | India |
| ***Bastanga*** | Bamboo shoot | Acidic, soft | Curry | LAB | India |
| ***Ekung*** | Bamboo shoot | Acidic, sour | soft Curry soup | LAB | India |
| ***Goyang*** | Wild vegetable | Acidic, sour, wet | Condiment,soup | LAB | India, Nepal |
| ***Gundruk*** | Leafy vegetable | Acidic, sour, | Dry, Soup pickle | LAB | India, Nepal,  Bhutan |
| ***Inziangsang*** | Mustard leaves | Acidic, sour | dry Curry, soup | LAB | India |
| ***Khalpi*** | Cucumber | Acidic, sour, wet | Pickle | LAB | India, Nepal |
| ***Mesu*** | Bamboo shoot | Acidic, sour, wet | Pickle | LAB | India, Nepal,  Bhutan |
| ***Kinima*** | Soyabean | Acidic, slimy | Curry, soup | LAB | Nepal, Bhutan |
| ***Sinki*** | Radish tap root | Acidic, sour | dry Soup, pickle | LAB | India, Nepal,  Bhutan |

## 2.2 Characteristics of Lactic acid bacteria (LAB):

#### 2.2.1 Introduction of LAB:

The term lactic acid bacteria (LAB) are used for "milk souring" or "lactic acid producing" bacteria. They are Gram-positive, catalase-negative bacteria which grow under microaerophilic to strictly anaerobic conditions, non-spore-forming, mainly non-motile rodsor cocci in shape (Khalid, 2011). They may be mesophilic (optimum temperature D30 °C) or thermophilic (optimum temperature U45 °C). As LAB lack electron transport systems, cytochromes and porphyrins (components of respiratory chains), so they cannot synthesize ATP by creation of a proton gradient and can only obtain ATP by fermentation, usually of sugars (Batt, 2000). The important genera of LAB are *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella, Carno bacterium,Tetragenococcus* and *Bifidobacterium* (Klein et al, 1998). They have less than 55 mol % G +C (guanine plus cytosine) content in their DNA (Stiles and Holzapfel, 1997) and it distanced this group of bacteria from *bifid bacteria* which have greater than 55 mol% G + C in the DNA (Schleifer and Ludwig, 1995).

In the beginning of 1900s, there was pioneering in scientific research leading to the concept of lactic acid bacteria as a group of organisms with potential in food fermentation and human health (Stiles and Holzapfel, 1997). The interactions of LAB in foods resulted in the significant contribution by Pasteur on lactic acid fermentation in 1857. In 1919, Orla-Jensen published a monograph about lactic acid bacteria which had a great impact on the systematic of LAB (Axelsson, 2004). Although taxonomy of lactic acid bacteria has been revised since then, characteristics used by Orla-Jensen are still very important in current classification of LAB. The classical approach to bacterial taxonomy was based on morphological and physiological features. This includes morphology, mode of glucose fermentation, growth at different temperatures, and range of sugar utilization (Khalid, 2011).

Hammes and Vogel (1995) classified *Lactobacilli* on the basis of peptidoglycan type of the cell wall and the fermentation pathway of pentoses and hexoses. However, the taxonomy of LAB has changed considerably with increasing knowledge of genomic structure and phylogenic relationships between *Lactobacillus* species (Klein et al., 1998). Molecular techniques, such as 16S rRNA sequencing have been developed which allows a more consistent and accurate identification of individual strains (Buddhiman et al., 2008). Hencegenetic characterization, such as the mol% G + C content of the DNA, electrophoretic properties of the gene products, DNA: DNA hybridization studies and structures and sequence of ribosomal RNA (rRNA) have become important taxonomic tools (Stiles and Holzapfel,1997).

LAB is widespread in nature and their nutritional requirements are very complex. They predominates the habitat that is rich in carbohydrates, protein breakdown products, vitamins and environments with low oxygen (Tannock, 2004). These are not only involved in food fermentations but they are also closely associated with the human environment. Some LAB strains inhabit the human oral cavity, the intestinal tract, and vagina and may beneficially influence these human ecosystems. This explains why they are considered as “ideal" candidates for application as probiotics (Holzapfel and Schillinger, 2002). Other habitats include soil, water, manure, sewage, and silage. In food products, they are found in dairy products, such as yoghurt and cheese, in fermented vegetables (olives, sauerkraut), in fermented meats (salami) and in sourdough bread (Tannock, 2004).

2.2.2 Lactic acid bacteria as probiotic**:**

*Lactobacilli* have a long history of safe consumption in traditional, fermented dairy products (Ouwehand et al, 2007) and reason for their earliest probiotics (Rettger et al., 1935). They are popular choices because these bacteria are desirable members of the intestinal micro flora, and are thus 'generally regarded as safe' (Tannock, 1997). These microorganisms help to keep the intestinal microbial balance and play role in maintaining health. Fermented products provide an excellent carrier for these probiotic bacteria especially *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium* sp. (Gilliland, 2003) and *Lactobacillus paracasei* (Patrignani et al, 2006).

LAB associated with fermented foods include species of the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997). Most commonly used probiotics are lactic acid bacteria, belonging to the genera *Lactobacillus* and *Bifidobacterium* (Parvez et al, 2006).

Others non-lactic acid bacteria which are most commonly used in probiotic preparations includes *Bacillus, Clostridium, Propionibacterium, Escherichia coli* and yeasts (Foligne et al, 2013). This microorganisms are used as probiotic. *Lactobacillus* and *Lactococcus* isolated from traditional fermented Maasai milk *(kulenato)* in Kenya showed the occurrence of potentially probiotic (Mathara et al, 2004). Eight *L. plantarum* strains from Bulgarian cheeses were characterized and selected for potential probiotic applications as adjunct cultures in cheese (Georgieva et al., 2008).

Many non-starter LAB (NSLAB) such as *L. paracasei* and *L. plantarum,* which are found in most ripened cheese varieties (Pisano et al., 2008), are used in commercial probiotic products (Zago et al., 2011). Karasu et al. (2010) isolated 12 *L. plantarum* strains from Turkish traditionally fermented vegetables with variable probiotic features. Sung-Mee and Im (2009) reported the potential probiotics properties of LAB isolated from traditional Korean fermentation foods.

2.2.3 Antibiotic resistance OF LAB**:**

One of the required properties by which specific strains can be considered as a potential probiotic bacteria is that they must be safe for human consumption. Such safety includes among other features that they do not harbor acquired and transferable antibiotic resistances (Vizoso-Pinto et al, 2006).

#### 2 .2.4 Antimicrobial properties of LAB:

Antimicrobial activity of bacteria targets the enteric undesirables and pathogens (Klaenhammer and KuUen, 1999). Antimicrobial effects of lactic acid bacteria are due to production of organic acids (lactic acid and acetic acids), hydrogen peroxide, diacetyl and low molecular weight antimicrobial substances bacteriocin (Ouwehand and Vesterlund 2004). *Lactobacillus* produces acetic and lactic acid as a byproduct during the metabolism of substrate. Lactic acid lowers the local pH and thereby inhibits the growth of bacteria sensitive to acidic conditions (Alakomi et al, 2000; De Keersmaecker et al, 2006; Makras et al, 2006). Some *Lactobacillus* strains inhibit the growth of *Salmonella enterica* solely by the production of lactic acid. In a fatal mouse Shiga toxin-producing *E. coli* 0157:H7 infection model, the probiotic *B. breve* produced a high concentration of acetic acid, hence leading to lowering the luminal pH. This pH reduction was associated with increased animal survival (Asahara et al, 2004). The antibacterial effects of *Lactobacilli* may be the result of a combination of lactic acid and other unknown *Lactobacillus* derived bactericidal substances by pH-dependent mechanism (Vanderpool et al. 2008).

The production of hydrogen peroxide (H₂O₂) also explains the inhibitory activity of LAB (Charlier et al., 2009). Secretion of hydrogen peroxide by LAB is considered as an important factor for antimicrobial effect on growth *of E. coli* 0157: H7 (Brashears et al., 1998). Hydrogen peroxide producing *Lactobacilli,* which colonise the urogenital tract, decrease the acquisition of human immune deficiency virus (HIV) infection, gonorrhoea and urinary tract infections (Vallor et al., 2001). Vasiljevic and Shah (2008) found *Lactobacilli* strains inhibited the growth of *S. aureus* by producing hydrogen peroxide at a concentration of 0.18 mmol/l. It was found, hydrogen peroxide has a bacteriostatic effect at these concentrations and bactericidal for concentrations up to 0.6 to 1.0 mmol/l. LAB are known for the production of bacteriocins which enhances their survival in complex ecological systems by preventing the growth of harmful bacteria in the fermentation and preservation of dairy products. Bacteriocins are the peptides with bactericidal activity usually against strains of closely related species (Wohlgemuth et al, 2010). They are classified into low molecular weight bacteriocins (LMWBs) and high molecular weight (class III) peptides. The LMWBs are further sub grouped into three classes: class I, lantibiotics, post-translationally modified peptides harboring unusual amino acids such as lanthionine; class II, heat-stable, non- lantibiotics; and class IV, cyclic antimicrobial peptides (Maqueda et al, 2008; Nishieetal., 2012).

Most of the bacteriocins secreted in the surrounding including lactacin B from *L. acidophilus,* plantaricin from *L. plantarum* and nisin from *Lactococcus lactis* have a narrow antibacterial spectrum (Wohlgemuth et al, 2010), but LAB also produces bacteriocins having broad antibacterial spectrum. Thus, some LAB bacteriocins can inhibit the growth of Gram positive pathogenic and spoilage bacteria as well as yeasts (Cintas et al, 1995; Magnussonand Schnurer, 2001; Atanassova et al, 2003; Ennahar and Deschamps, 2000; Farias et al,1994). Besides, it has been reported that bacteriocins also inhibit the growth of some Gram negative species (Arihara et al, 1996; Cardi, 2002; Stevens et al, 1991).

*Lactobacilli* produce many different bacteriocins of similar activity. *Lactococci* sp.and *Lactobacilli* sp. isolated from traditionally homemade cheeses showed to produce avariety of antimicrobial substances. In this study *Lactococcus lactis* subsp. lactis BGMNl-5was found to produce three narrow spectrum class II heat-stable bacteriocins whereas anotherisolate, *L. lactis* subsp. *lactis* BGSMl-19 produces low molecular mass (7 kDa) bacteriocinSMI9 that showed antimicrobial activity against *Staphylococcus aureus. Micrococcus Jiavus* and partially against *Salmonella paratyphi* (Topisirovic et al, 2006).*Enterococcus durans* LAB 18s, a strain capable of selenium bioaccumulation wereassayed for antimicrobial activity. The antimicrobial activity of culture supernatant and intracellular extract of *E. durans* LAB 18s was tested against different pathogenic microorganisms namely *Listeria monocytogenes, Escherichia coli, Bacillus cereus,* *Staphylococcus aureus, Salmonella typhimurium. Salmonella enteritidis, Pseudomonas aeruginosa, Aeromonas hydrophila and Corynebacterium fimi.* The study showed *E. durans* LAB 18s exhibited a broad inhibitory spectrum, except to *B. cereus, S. aureus* and *S.enteritidis* when the culture supernatant was used, and to *S. typhimurium* when the intracellular extract was tested (Pieniz et al., 2014).

Sucheta and Chhetry (2004) observed significant reduction of total sugar, starch, ascorbic acid, moisture content and pH in *gundruk* and *sinki* compared to their respective mustard leaves and radish where as protein, phenol, phytosterol and fat were enhanced in the fermented food.

Gautam N. and Sharma N. (2015) carried out the experiment on a study on characterization of new bacteriocin produced from a novel strain of Lactobacillus spicheri G2 isolated from Gundruk- a fermented vegetable product of North East India. The bacteriocin producing potential of L. spicheri is being reported for the first time in the present investigation. They concluded that bacteriocin of L. spicheri G2 showed strong antagonism against food spoiling and pathogenic bacteria viz. Listeria monocytogenes, Staphlococcus aureus, Clostridium perfringens, Streptococcus mutans, Lactobacillus plantarum, Leuconostoc mesenteroides and Bacillus cereus.

Karki T. et al (1983) carried out the study on microflora of Nepalese pickles, Gundruk. They investigated the acid producing bacteria and the chemical changes during the natural fermentation of gundruk. They found that the *Lactobacillus plantarum* and *Pediococcus pentasaceus* was dominant flora during the natural fermentation of *Gundruk.*

Tamang J. P. et al (2005) carried out the research on identification of predominant lactic acid bacteria isolated from traditionally fermented vegetable products of the Eastern Himalayas. They analyzed 65 samples of different fermented vegetable products. They found that the population of lactic acid bacteria (LAB) as well as aerobic mesophilic counts was at the level of 10⁻⁷ cfu/g. They did not found molds in any samples and found yeast in few samples of sinki and khalpi. They found that the major representatives of LAB involved in these fermentation were *Lactobacillus brevis, Lactobacillus plantarum, Pediococcus pentasaceus, Pediococcus acidilactici* and *Leuconostoc fallax* determined using phenotypic characteristics followed by genotyping using RAPD-PCR, repetitive element PCR and Species- species PCR technique,

Savitri, Monika et al (2017) carried out the experiment on isolation and characterization of lactic acid bacteria from traditional pickles of Himanchal Pradesh, India. They identified 15 isolates of lactic acid bacteria (LAB). These LAB exhibit antimicrobial activities against food borne pathogenic bacteria i.e. *Bacillus cereus, E. coli, Staphylococus aureus* and *Shigella dysenteriae.* They also concluded that LAB isolated from traditional pickles of Himachal Pradesh has very good potential to be used as probiotics.

Goswami G. et al (2017) carried out the research on identification and functional properties of dominant lactic acid bacteria isolated from Kahudi, a traditional rapeseed fermented food product of Assam, India. They found that LAB was dominant over other microbial flora. They found that *Enterococcus durans, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus casei* as a dominant group of LAB on the basis of phenotypic parameters, biochemical test and 16s rDNA gene sequencing.

Kumar S.R. et al (2012) carried out the research on the traditional Indian fermented food: a rich source of Lactic acid bacteria (LAB). They found that fermented foods such as dahi, gundruk, sinki etc have significant medicinal properties. They also concluded that there is an abundant opportunity available for food microbiologists to explore the Nepalese fermented foods for the isolation of new LAB strains for their potential role in probiotic research.

Tamang J. P. et al (2009) carried out the experiment on the functional properties of lactic acid bacteria (LAB) isolated from ethic fermented vegetables of the Himalayas. They found that all the strains of LAB isolated from fermented vegetables have strong acidification and coagulation activities. They also concluded that LAB show antimicrobial activities against the used indicator strains.

Tamang B., Tamang J. P. (2010) carried out the study on In situ fermentation dynamics during production of gundruk and khalpi, ethnic fermented vegetable products of the Himalayas. They found that significant increase in population of lactic acid bacteria (LAB) during first few days of gundruk and khlapi fermentation respectively. They found that Gundruk fermentation was initiated by *Lactobacillus brevis, Pediococcus pentosaceus* and finally dominated by *Lb. plantarum*. Similarly in khalpi fermentation was initiated by heterofermentative LAB such as *Leuconostoc fallax, Lb. brevis* and *P. pentosaceus* and finally completed by *Lb. plantarum*. They also concluded that gundruk and khalpi prepared by using mixed starter culture of LAB had higher sensory- ranking comparable to market products.

Tamang J.P. et al (2016) carried out the experiment on functional properties of microorganisms in fermented foods. They concluded that the fermented foods have unique functional properties imparting some health benefits to consumers due to presence of functional microorganisms which possess probiotits properties, antimicrobial, antioxidant, peptide production etc.

The number of the indigenous fermented foods and beverages are produced and consumed around the world. Fermented foods are associated with desired and edible microbes which are beneficial for health. Foods on consumption improve or change the intestinal microflora are of particular interest because of increased knowledge of the role the intestinal microflora population plays in health and disease resistance.

Probiotics containing foods, such as fermented milks, yogurts and cheese are called as functional food. Their production continues to grow at an exponential rate in developed countries like Europe, Japan, Australia, and the United States and commercially available in various forms including powders, granules, pastes, liquids, capsules and tablets. Research in the field of probiotics has bloomed in recent years and it has become more essential to promote foods that not only provide adequate nutrition but also may have properties for health promotion and disease prevention. Efforts have to be made to explore the micro flora associated with traditional fermented food and beverages and study their potential probiotic potentials. In addition, proper identification of organisms for specific uses and clearly demonstrating underlying mechanisms with respect to various disease interventions of action is also important. Further research on the controlled human studies, is needed to determine which probiotic strain and in what dosages are associated with the greatest efficacy. These finally selected and fully tested probiotic strains can probably provide alternative options for individuals in whom conventional medical therapies have failed to promote health and perhaps, in the future, it may serve as a first-line choice of therapy for some patients. This targeted translation of science for consumer benefit coupled with technologies will play an important role for paving the path ahead for probiotic use in the country. In future, more fermented foods with health promoting properties will become available on the market, many directed towards consumers with very specific health and metabolism needs.

# CHAPTER III

# MATERIALS AND METHODS

This chapter explores the detailed study of microorganisms from home made *Gundruk*. It also describes the sampling methods, materials required and the methodology employed for the experiment. As this investigation is an experiment based research, the work had been carried in the Microbiological laboratory of Central college of Technology, Hattisar, Dharan. Experimental data was collected and processed very carefully.

## 3.1 Sampling methods:

For potential probiotic bacteria of indigenous food, *Gundruk* sample were collected from different villages of Sunsari district. The period of study was from January to June, 2019.

## 3.2 Transportation of Samples:

The collected *Gundruk* sample were kept in sterile plastic during transportation and analyzed in Microbiology laboratory of CCT, Dharan on the same day.

## 3.3 Isolation of microbes from field sampled (Gundruk):-

Dried *Gundruk* was collected in bulk from villages under Sunsari district where *Gundruk* are made in large scale as an off season use vegetable food. The collected materials were brought to the laboratory in a sterile polythene bag for microbial analysis. Isolation of microorganism was done by isolating the microbes from the sample which were brought to the laboratory. The method adopted for the isolation of microorganism from these fermented products was serial dilution method, i.e. each of *Gundruk* was weighed and washed in separate 100ml conical flask containing sterile distilled water and thoroughly hand shaken for 15 min making 10ˉ² dilution. 1 ml each of the aliquot was taken and serially diluted as above to make 10ˉ³ dilution. 1ml o f 10ˉ³ dilution will be inoculated into Petri plates containing PDA and Plate count agar media for fungi and bacteria respectively. The inoculated Petri plates was well shaken to ensure uniform spread of inoculums on media surface and incubated for 2 days at 30±1°C for bacteria and 5 days at 25±1°C for fungi.

## 3.4 Identification and purification of fungal and bacterial isolates:-

Microbes isolated from the samples were grown in pure culture on PDA slants for fungi and nutrient agar slants for bacteria. Identification was done in pure culture following strictly the keys and relevant manuals (Subramaniam, 1971) for fungi. Bacteria and yeast was identified in the microbiology laboratory of CCT, Dharan.

## 3.5 Isolation and identification of lactic acid bacteria:-

Lactic acid bacterial strain was isolated from *Gundruk* which is a non-salted, fermented acidic vegetable product and is consumed as a soup and pickle by people of Nepal. The leaves of Rape plant were used in its preparation. Isolation was carried out on De Man Ragaosa Sharpe (MRS) agar under anaerobic conditions by standard spread plate method. Anaerobic conditions were maintained under anaerobic gas jars by using gas pack system (Hi-media). The bacterial colonies obtained on MRS agar were purified by streaking twice on MRS agar. The pure cultures were preserved at −20 °C on MRS broth containing 40 % glycerol (v/v) in deep freezer. Screening of isolates was done on the basis of morphological, physiological and biochemical characteristics (Sharpe 1979; Kandler and Weiss 1986). Color, form, margin, elevation and texture of each isolated strain were noted down. Gram staining, catalase test, oxidase test, citrate utilization test, gas production from glucose, casein hydrolysis, H₂S production, nitrate reduction, arginine deamination, growth on different temperature, pH, different percentage of NaCl and sugar fermentation tests were performed with isolated strains.

## 3.6 Isolation and Identification of Pathogenic microorganisms from Gundruk Sample:-

Different selective Medias were used to identify different pathogenic microorganisms from fermented food products called *Gundruk.* Enumeration of pathogenic contaminants from the samples were done in selective media such as *Bacillus cereus* agar base (M833,HiMedia) for *Bacillus cereus*, and Violet Red Bile Glucose agar w/o lactose (M581, HiMedia) for Enterobacteriaceae. *Salmonella* spp. and *Shigella* spp. were isolated by using *Salmonella and Shigella* (SS) agar. Similarly, *E. coli* and *Vibrio* spp*.* were isolated using MacConkey agar and TCBS agar respectively. *Staphylococcus* spp*.* was isolated using Mannitol salt agar and *Streptococcus* spp*.* was isolated using Brain heart infusion agar. *Pseudomonas* spp. was isolated using Malachite green broth.

## 3.7 Antibiotic susceptibility test for lactic acid bacteria isolated from Gundruk sample:-

Antibiotic susceptibility of isolated LAB was assessed using the antibiotic disc diffusion method on MHA (Mueller Hinton Agar) plates. The antibiotic susceptibility test was performed by Kirby- Bauer Sensitivity testing method, according to the guidelines given by the formerly known as National Committee for Clinical Laboratory Standard (NCCLS). Overnight culture (100μL) of each isolate was spread on the MHA agar plate onto which each antibiotic disc was placed separately on the surface of the agar; the plates were incubated at 37°C for 48 hours and observe for the zone of inhibition.

Various types of antibiotics disc used according to the nature of organisms are Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Streptomycin (10 mcg), Ampicillin (10 mcg), Amoxycillin (10 mcg), Ofloxacin(5 mcg), Trimethoprim (5 mcg), Penicillin-G (10 unit) and Vancomycin (10 mcg).

## 3.8 Testing LAB isolates for P-solubilization activity (Pikovskaya, 1948):-

The LAB isolates was spot inoculated on Pikovskaya’s Agar Medium and was incubated for 48 hours and then observed for the Halo zone surrounding the colony.

## 3.9 Antimicrobial activity test by agar well diffusion method:-

Each gundruk samples were prepared. Indicator food pathogens namely, *Bacillus spp, Escherichia coli, Salmonella spp, Vibrio cholerae* and *Staphylococcus aureus* were subcultured in nutrient broth at 37°C for 24 hours. Then, these broth cultures were mixed by vortexing and 100 µl of the culture fluid was diluted to 10 ml of sterilized saline. This suspension was used to evaluate antimicrobial activity of the gundruk samples using agar well diffusion method on Mueller Hinton Agar (MHA).

Antimicrobial activity of isolates against all gundruk samples against all indicator pathogens was determined under aerobic conditions at 37°C. Agar plates were inoculated with diluted 100µl suspension of each indicators microorganism. Wells (5 mm in diameter) were cut in MHA plate and 100µl of gundruk sample was loaded into each well. After incubation at 37°C for 18-24 hours, the diameter (mm) of the inhibition zone around the wells was measured. A negative control consisting sterilized distilled water was used.

## 3.10 Preparation of gundruk using Selected Strains of LAB:-

‘Rayo-saag’ leaves of *Brassica rapa* L. sub-sp. *Campestris* (L.) Clapham variety *cumifolia* Roxb. were purchased from Dharan market. Leaves were washed thoroughly in sterile distilled water and wilted in oven (~30°C) for 6 h. Leaves were crushed, put into sterile warm water (about 90°C) for 5 min and transferred into another sterile glass container. Excess water in the leaves was removed by squeezing and then, about 400 g of crushed leaves of ‘rayo-saag’ were distributed aseptically into each sterile 500 ml capped-bottles, totaling 6 bottles for samplings. Each bottle was inoculated by a mixture of actively grown culture strains of *Lactobacillus plantarum* and *Pediococcus pentosaceus* at the ratio of 10⁷cfu/g, previously isolated from market samples of *Gundruk*. Bottles were tightly capped and incubated at room temperature (25°C) for 6 days. Samplings were done on 3rdand 6thdays for organoleptic test followed by determination of pH and acidity.

# CHAPTER IV

# RESULTS

## 4.1 Sample collection and determination of Microbial load of Gundruk Sample:-

This study was conducted in laboratory of Central Campus of Technology, Dharan, Nepal. In all fermented vegetable products, the population of LAB were in the range of 10⁷to 10⁸ cfu/ g. Yeasts and filamentous moulds were not detected in any of the samples analyzed.

## 4.2 Isolation and Identification of LAB from Gundruk sample:-

In total, 30 samples of gundruk were analyzed for microbial counts (Table 2). All samples were collected from different places of Sunsari districts. Among them, 14 samples (Code-A) were collected from Dharan and were prepared in Glass jar and remaining 16 samples (Code-B) were collected from Prakashpur and were prepared in Earthen jar. Among 98 strains, 46 strains were isolated from A and remaining 52 were isolated from B. All 98 strains isolated from gundruk were considered LAB based on their Gram positive reactions, no motility, absence of spore formation and absence of catalase.

#### Table 2: Number of isolates obtained from different types of Gundruk collected from Locality:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample Code No. | Types of Gundruk | Locality | Isolates | Particulars |
| A (14) | Rape leaves | Dharan, Sunsari | 46 | Glass jar |
| B (16) | Rape leaves | Prakashpur, Sunsari | 52 | Earthen jar |
| Total |  |  | 98 |  |

**Table 3: Selection of Representative strains from *Gundruk* Samples:**

|  |  |
| --- | --- |
| Sample code No. | Representative strains |
| A | A4-G2, A8-G3, A13-G5, A3-G8, A7-G4, A14-G5, A22-G6, A28-G3, A18-G5,A25-G9 |
| B | B2-G11, B4-G4, B17-G6, B9-G1, B15-G9, B12-G5, B26-G8, B13-G7, B23-G8 |

Of the 98 strains of LAB isolated from gundruk samples, 24 strains were homofermentative rods, and 72 were tetrad-forming cocci. All homofermentative rods were presumptively identified as *Lactobacillus plantarum.* Further differentiation of 7 strains from gundruk was performed by using the key proposed by Simpson and Taguchi (1995) based on the ability to grow at different pH, temperature and different concentration of NaCl (Table 4). On the basis of these tests, the 7 strains from gundruk were tentatively identified as *Lactobacillus plantarum.*

**Table 4: General characterization of *Lactobacillus plantarum:***

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| No.  Sample code | 1  A4-G2 | 2  B4-G4 | 3  A7-G4 | 4  A28-G3 | 5  B12-G5 | 6  A18-G5 | 7  B23-G8 |
| Orientation of Cell | R | R | R | R | R | R | R |
| Size ( μm)  Length  Width | 1.6~3  0.7 | 2~4  0.7 | 2  0.7 | 2.5  0.7 | 1.3~3  0.7 | 2~4  0.7 | 2  0.7 |
| Motility | - | - | - | - | - | - | - |
| Spore forming | - | - | - | - | - | - | - |
| Gram staining | + | + | + | + | + | + | + |
| Catalase reaction | - | - | - | - | - | - | - |
| Nitrate reduction | - | - | - | - | - | - | - |
| Gelatin liquefaction | - | - | - | - | - | - | - |
| Gas from Glucose | - | - | - | - | - | - | - |
| Growth temperature  15°C  30°C  45°C | +  +  - | +  +  - | +  +  - | +  +  - | +  +  - | +  +  - | +  +  - |
| pH  3.5  6.5  8.5  10.5 | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - |
| Nacl  1.5%  2.5%  5%  6.5%  8.5%  10% | +++  ++  -  -  -  - | +++  ++  -  -  -  - | +++  +++  +  -  -  - | +++  ++  +  -  -  - | +++  +++  +  -  -  - | +++  ++  -  -  -  - | ++  ++  -  -  -  - |
| Metabolism | Homo | Homo | Homo | Homo | Homo | Homo | Homo |
| Arginine deamination | - | - | + | - | - | + | - |
| Carbohydrate Fermentation Test:  Sucrose  Lactose  Maltose  Mannitol  Dextrose  Starch | ++  ++  ++  ++  ++  - | ++  ++  ++  ++  ++  - | ++  +  ++  +  ++  - | ++  ++  ++  +  +  - | ++  ++  ++  +  ++  - | ++  ++  +  +  ++  - | ++  ++  ++  ++  +  - |

-;Negative, +;Positive, R=rod, (For NaCl; +++= maximum growth, ++= moderate growth, += minimum growth, -= no growth), (For Carbohydrate fermentation test; ++= very strong, += strong, -= negative)

All cocci forming tetrads were presumptively identified as *Pediococci* (Table 5). Further differentiation of 13 strains from gundruk was performed by using the key proposed by Simpson and Taguchi (1995) based on the ability to grow at pH 8.5, pH 6.5, at 45⁰C and in the presence of 8.5% NaCl (Table 5). On the basis of these tests, the 12 strains from gundruk were tentatively identified as *Pediococcus pentosaceus.*

**Table 5: General characterization of *Pediococcus pentosaceus*:**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| No.  Sample code | 1  A8-G3 | 2  B2-G11 | 3  B9-G1 | 4  A13-G5 | 5  A3-G8 | 6  B26-G8 | 7  A22-G6 | 8  A14-G5 | 9  B15-G9 | 10  B17-G6 | 11  A25-G9 | 12  B13-G7 |
| Orientation of Cell | T | T | T | T | T | T | T | T | T | T | T | T |
| Size (μm) | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| Motility | - | - | - | - | - | - | - | - | - | - | - | - |
| Spore forming | - | - | - | - | - | - | - | - | - | - | - | - |
| Gram staining | + | + | + | + | + | + | + | + | + | + | + | + |
| Catalase test | - | - | - | - | - | - | - | - | - | - | - | - |
| Nitrate reduction | - | - | - | - | - | - | - | - | - | - | - | - |
| Gelatin liquefaction | - | - | - | - | - | - | - | - | - | - | - | - |
| Gas from Glucose | - | - | - | - | - | - | - | - | - | - | - | - |
| Growth temperature  15°C  30°C  45°C  60°C | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - |
| pH  3.5  6.5  8.5  10.5 | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - | +  +  +  - |
| Nacl  1.5%  2.5%  5%  6.5%  8.5%  10% | +++  +++  ++  +  +  - | +++  ++  ++  +  +  - | +++  +++  ++  +  +  - | +++  +++  ++  +  +  - | +++  ++  ++  +  +  - | +++  +++  ++  +  +  - | +++  ++  ++  +  +  - | +++  +++  ++  +  +  - | +++  +++  ++  +  +  - | +++  +++  ++  +  +  - | +++  ++  ++  +  +  - | +++  +++  ++  +  +  - |
| Arginine deamination | + | + | + | + | + | + | + | + | + | + | + | + |
| Carbohydrate Fermentation Test:  Sucrose  Lactose  Maltose  Mannitol  Dextrose  Starch | ++  ++  ++  -  ++  - | ++  ++  ++  -  ++  - | +  -  ++  +  ++  - | ++  ++  ++  -  +  - | ++  ++  ++  -  ++  - | ++  +  +  -  ++  - | -  ++  ++  +  +  - | ++  -  +  -  ++  - | +  ++  ++  -  ++  - | ++  -  ++  +  ++  - | -  ++  +  -  +  - | +  ++  ++  +  +  - |

-;Negative, +;Positive, T= tetrads, (For Nacl; +++= maximum growth, ++= moderate growth, += minimum growth, -= no growth), (For Carbohydrate fermentation test; ++= very strong, += strong, -= negative)

#### 4.2.1 Identification of Pathogenic microorganisms from Gundruk Sample:-

No pathogenic microorganisms were found in any culture done in selective media such as *Bacillus cereus* agar base (M833, HiMedia) for *Bacillus cereus*, and Violet Red Bile Glucose agar w/o lactose (M581, HiMedia) for *Enterobacteriaceae*, *Salmonella and Shigella* (SS) agar for *Salmonella* spp. and *Shigella* spp., MacConkey agar for *E. coli,* TCBS agar for *Vibrio* spp, Mannitol salt agar for *Staphylococcus* spp, Brain heart infusion agar for *Streptococcus* spp and Malachite green broth for *Pseudomonas* spp.

#### 4.2.2 Antibiotic susceptibility test for Lactobacillus plantarum:

In this study, 9 antibiotics, Ampicillin, Chloramphenicol, Penicillin G, Streptomycin, Tetracycline, Vancomycin, Trimethoprim, Ofloxacin and Amoxycillin were used to test the susceptibility of the isolates to these antibiotics. All the test isolates were susceptible to most of the common antibiotics (e.g. ampicillin, tetracycline, chloramphenical, penicillin G) as well as other antibiotics, but most of them were resistant to Vancomycin.

**Table 6: Antibiotic susceptibility test for *Lactobacillus plantarum:***

|  |  |  |
| --- | --- | --- |
| Antibiotic used | *Lactobacillus plantarum* (N=7)  Sensitive Resistant  n n | |
| 1. Chloramphenicol (30 mcg) 2. Ciprofloxacin (5 mcg) 3. Streptomycin (10 mcg) 4. Ampicillin (10 mcg) 5. Amoxycillin (10 mcg) 6. Ofloxacin (5 mcg) 7. Trimethoprim (5 mcg) 8. Penicillin-G (10 unit) 9. Vancomycin (10 mcg) | 7 (100%)  7 (100%)  7 (100%)  7 (100%)  7 (100%)  7 (100%)  7 (100%)  7 (100%)  3 (42.86%) | -  -  -  -  -  -  -  -  4 (57.14%) |

#### 4.2.3 Antibiotic susceptibility test for Pediococcus pentosaceus

In this study, 9 antibiotics, Ampicillin, Chloramphenicol, Penicillin G, Streptomycin, Tetracycline, Vancomycin, Trimethoprim, Ofloxacin and Amoxycillin were used to test the susceptibility of the isolates to these antibiotics. All the test isolates were susceptible to most of the common antibiotics (e.g. ampicillin, tetracycline, chloramphenical, penicillin G) as well as other antibiotics, but most of them were resistant to Vancomycin.

**Table 7: Antibiotic susceptibility test for *Pediococcus pentosaceus:***

|  |  |  |
| --- | --- | --- |
| Antibiotic used | *Pediococcus pentosaceus* (N=12)  Sensitive Resistant  n n | |
| 1. Chloramphenicol (30 mcg) 2. Ciprofloxacin (5 mcg) 3. Streptomycin (10 mcg) 4. Ampicillin (10 mcg) 5. Amoxycillin (10 mcg) 6. Ofloxacin (5 mcg) 7. Trimethoprim (5 mcg) 8. Penicillin-G (10 unit) 9. Vancomycin (10 mcg) | 12 (100%)  12 (100%)  12 (100%)  12 (100%)  12 (100%)  12 (100%)  12 (100%)  12 (100%)  8 (66.66%) | -  -  -  -  -  -  -  -  4 (33.34%) |

## 4.3 Testing LAB isolates for P-solubilization activity (Pikovskaya, 1948)

No Halo zone surrounding the colony was observed using Pikovskaya’s Agar Medium.

## 4.4 Testing of Antimicrobial activity test by agar well diffusion method:

In this study, 30 gundruk samples were used to test antimicrobial activity. Antimicrobial activity by well diffusion assay showed that gundruk samples are able to inhibit the most of the indicator pathogens tested, shown in Table 8.

**Table 8: Antimicrobial activity test of *Gundruk* sample:**

|  |  |  |
| --- | --- | --- |
| No. of pathogens | No. of *Gundruk* samples capable of inhibiting(N=30) | Inhibition Zone (mm) |
| 1. *Staphylococcus aureus* | 20 (66.66%) | 6-14 |
| 1. *Escherichia coli* | 18 (60%) | 5-16 |
| 1. *Salmonella* spp | - | - |
| 1. *Bacillus* spp | 25 (83.33%) | 8-18 |
| 1. *Vibrio cholerae* | - | - |

## 4.5Testing of Gundruk prepared by using Selected Strains of LAB:

The final product is not always consistent in natural fermentation; the use of a mixed lactic starter culture could provide fermentations that are more consistent and products of higher quality. Besides, use of mixed starter culture complements different technological properties to attain better products. In this study, *Lb. plantarum (*A4-G2) and *P. pentosaceus (*B15-G9) were selected as a starter culture for production of *gundruk*, as described in materials and methods. *Gundruk* prepared by the starter culture was evaluated organoleptically, shown in Table 9.

**Table 9: Sensory evaluation of Gundruk prepared from leaves of ‘rayo-saag’ using a mixed pure culture strains:**

|  |  |  |
| --- | --- | --- |
| Fermentation periods (day) | Attribute  Aroma Taste Texture Colour General acceptability | pH |
| Fermentation at 25⁰C  3  6 | 3.0±0.4 2.9±0.5 3.5±0.4 3.8±0.5 2.8±0.5  3.9±0.4 3.5±0.5 3.4±0.5 3.6±0.5 3.5±0.5 | 4.6±0.20  4.4±0.20 |

Data represents the mean score ±SD (n=7)

Market Gundruk was used as control (Score 3); Score1, bad; Score 5, good.

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# CHAPTER V

# DISCUSSION

Although *Gundruk* is commonly prepared from mustard leaves, *Gundruk* can be prepared from many other plants including some wild plants. Manandhar, (1998) listed nine domestic plants and also four wild plants used in the preparation of *Gundruk* in Terai and hill regions of Nepal. Plants belonging to *Brassicaceae, Raphanus* and *Araceae* are found cultivated by the majority of Nepali Community as winter crop for oil and vegetable production. During their peak season of growth these leafy vegetables are in surplus and, their green leaves lasting only for a month or so are used as vegetables. During the remaining season of the year, scarcity of vegetables is often encountered and to tide over the scarcity, villages have resorted to production of *Gundruk.* Different names are given to *gundruk* prepared from different plant leaves *Gundruk* made of *Brassica juncea* var. *rugosa* and *Brassica juncea* var. *cureifolia* Roxb. Commonly called *Rayo* (in Nepali) is called ‘*Rayo Gundruk’* or ‘*Rayo ko Gundruk ’.*

*Gundruk* made up of *Brassica compestries* i.e. *sarson* in called ‘*Sarson Gundruk or Tori Gundruk*,*Kalo-tori Gundruk, phool-gobi Gundruk, Bandagobi Gundruk. Shalgam pata Gundruk, Mula pata Gundruk* and *Kachchhu Gundruk* are made up of *B. napus, B.nigra. B. oleraceae* var *botrytis, B. oleraceae* var. *capitata, B. rapa, Raphanussativus* and *Colocasica esculenta* respectively. These plants are available in plenty during the seasons. Both *Gundruk* and *Sinki* are fermented by traditional method using available surrounding resources. Fermentation of vegetable food by the people all over the world is not recent. It has been continuing process through generations since time immemorial, (Caplice andFitzgerald, 1999).

Fermented vegetables are used in different parts of the world using different traditional technique and few of which such as *Sauerkraut, Kimchi, Tempeh, Oncom, Soy sauce, Idli, Ogi* and *Gari; e*tc are very common in Europe and USA, Korea, Indonesia, Orient (Japan, china and Phillipines), India and Africa respectively and prepared at the commercial level (Caplice andFitzgerald, 1999; Oliver and Nunez, 1999; Battcock and Ali, 1998; Adams and Moss, 1996 and Jay, 1986).

*Gundruk* and *sinki* preparation is confined to the village level only for domestic use. Only few families are associated with large scale production and are sold in the weekly village market at the rate of about Rs200 per kg or so. The reason that *Gundruk* and *Sinki* preparation are confined to traditional technology using crude method and hence quality production of *Gundruk* has to go a long way ahead. Unless the traditional method of *Gundruk* preparation is improved, quality production of *Gundruk* and *Sinki* will be a far cry. Moreover, *Gundruk* and *Sinki* are consumed by the Nepalese only and is not very much popular, at least at the consumption level in other communities at the moment. However, some indigenous community, who are inhabited nearby Nepali villages are slowly adopting the technology and *Gundruk* and *Sinki* are being prepared by them too. Of course, at the initial level *Gundruk* and *Sinki* are made by them using traditional Nepali method and it is expected that this traditional technique will improve and *Gundruk* and *Sinki* will be a popular vegetable item *among* other communities too. Not only the processing of the raw materials for the preparation of *Gundruk* and *Sinki* need to be improved, fermented types both underground and over ground need drastic improvement.

Till date, fermenter used for preparation of *Gundruk* and *Sinki* are very crude and there is no specially designed over ground fermenter. In contrast, fermenter used for the preparation of other fermented vegetable foods such as *Sauerkraust, Kimchi, Tempeh, Oncom, Gari* use improved type of fermenter. To mention few wooden trays lined with perforated plastic sheets, large perforated stainless steel plate, deep fermentation tank, wooden clay or glass tank, polythene bags are relatively improved type (Dubey, 1998; Oliver and Nune;z, 1999; Kim *et al.* 2004 and Smith *et al..* 1983).

*Gundruk* and *sinki* are non-salted and fermented leafy vegetable of different plant leaves which are processed either by wilting or boiling the leaves. In other fermented vegetable foods prepared in developed countries like Korea, Japan and China salts are basically added to quicken the process of fermentation whereas *Gundruk* and *Sinki* are non-salted fermented product as a result fermentation process takes longer time. Although *Gundruk* and *Sinki* are non-salted vegetable food, it constitutes a very important vegetable food items among the Nepalese people, for the reason of simple preparation procedure. Secondly the recipe preparation from *Gundruk* is very quick and makes the food tasty and palatable due to its pleasant aroma, flavour and sour taste. Shredding, i.e. breaking of hard tissues of leaves and radish are solely done using *‘Dhiki ’ Musli* and *Okhli'* because of the fact that they are readily available wooden equipments . Shredding of leaves and radish for the preparation of *Gundruk* and *Sinki* is necessary for removing excess moisture of the raw materials. Soil tunnel fermenters were burnt before lining its wall possibly for removing moisture from immediate surrounding and for activating the microorganism of the soil tunnel fermenter. Lining the soil tunnel fermenter by different lining materials including leaves, bamboo mats and paddy straw may be for the checking easy passage of moisture from the wall of the fermenter.

Fermentation of *Gundruk* and *Sinki* usually takes a minimum of 15 days to maximum 23 days. Variation in the duration of fermentation period of *Gundruk* and *Sinki* may be attributed to different factors (i) one of which is the longer time taken for fermentation which could be due to non addition of salt because salt act as an excellent tissue softening agent by forming a fermenting brine as in other salt fermented vegetables such as *Sauerkraut, Kimchi,* pickled *Gherkins.* (Oliverand Nunez, 1999). (ii) *Gundruk* and *Sinki* undergoes natural fermentation which is affected by other factors like soil types, depth of soil tunnel, types of lining material used for lining the soil tunnel fermenter etc. (in case of soil tunnel fermenter), types of overground fermenter and environmental factors etc.

Indigenous knowledge of ethnic people, living in the Nepal, Eastern Himalayan regions of India and Bhutan, on production of fermented vegetable products is worth documentation. *Gundruk* and *Sinki* are important fermented vegetable foods in local diet of the Nepal which is prepared at individual household. The microbial load of *Gundruk* sample revealed that LAB comprising lactobacilli and pediococci were the predominant microorganisms present in viable numbers above 10⁷cfu/g. Out of 98 strains isolated from 30 *Gundruk* samples collected from different places of Sunsari district, 24 were identified as *Lactobacillus plantarum* and 72 were identified as *Pediococcus pentasaceus* which were identified on the basis of morphology, physiological and biological characteristics followed by casein hydrolysis, H₂S production, nitrate reduction, arginine deamination, growth on different temperature, pH, different percentage of NaCl and sugar fermentation tests. The identity of the LAB seems to correspond with that of LAB typically reported for fermented vegetable products (Lee, 1994; Steinkraus, 1996, Karki T. et al 1983 and Tamang J.P. et al, 2005).

The isolated, identified and preserved microorganisms from lesser-known fermented vegetable products may contribute significant information on unknown microbial gene pool as genetic resources of the different regions.

One of the required properties by which specific strains can be considered as a potential probiotic bacteria is that they must be safe for human consumption. Such safety includes among other features that they do not harbor acquired and transferable antibiotic resistances (Vizoso-Pinto et al, 2006). In this study, 9 antibiotics, Ampicillin, Chloramphenicol, Penicillin G, Streptomycin, Ciprofloxacin, Amoxycillin Ofloxacin, Trimethoprim and Vancomycin were used to test the susceptibility of the isolates to these antibiotics. All the test isolates were susceptible to most of the common antibiotics (e.g. ampicillin, tetracycline, chloramphenical, penicillin G) as well as other antibiotics, but were resistant to Vancomycin. The resistance to vancomycin observed in this study was in accordance with other reported study (Monika et al. 2017 and Zhou et al. 2005). An important drawback of antibiotic resistance is that transfer of antibiotic resistance gene is possible. Because of antibiotic resistance genes are generally carried on plasmids, they can be transferred to other bacteria by means of conjugation. This resistance is due to the presence of D-Ala-D-Lactate in their peptidoglycan rather than D-Ala-D-Ala dipeptide which is target of antibiotic (Danielsen and Wind 2003). There is a risk associated with the ability of these resistant strains to transmit the resistance gene to pathogenic bacteria ( Mathur and Singh 2005). This may result to highly antibiotic resistance enteropathogenic bacteria. All LABS was inoculated into Pikovskaya’s Agar Media and incubated for 48 hours for P-solubilization activity. No any halo zone surrounding the colony was observed.

Antimicrobial activity by well diffusion assay showed that gundruk samples are able to inhibit the most of the indicator pathogens tested. *Bacillus* spp was inhibited the most by the gundruk sample (87.33%) and showed the highest diameter of inhibition (8-18 mm) followed by *Staphylococcus aureus* and *Escherichia coli*. No any inhibition zones were observed in plate containing *Salmonella* spp and *Vibrio cholerae.* This report was similar to previous study done by Rahman S.A.et al (2017). They also reported that *Bacillus* spp as the most inhibited pathogens. This happed probably due to the presence of other antimicrobial compounds such as hydrogen peroxide, diacetyl, acetoin and bacteriocins.

The final product is not always consistent in natural fermentation; the use of a mixed lactic starter culture could provide fermentations that are more consistent and products of higher quality. Besides, use of mixed starter culture complements different technological properties to attain better products. On the basis of superior technological properties of LAB strains such as acidification ability, antimicrobial activities, non-production of biogenic amines, ability to degrade anti nutritive factors, and even high degree of hydrophobicity, *Lb. plantarum (*A4-G2) and *P. pentosaceus (*B15-G9) were selected as a starter culture for production of *Gundruk*, as described in materials and methods. *Gundruk* prepared by the starter culture was evaluated organoleptically, shown in Table 6. With respect to general acceptability, 6 day-old *Gundruk* fermented at 20°C had the highest score with better aroma, acidic taste typical of *Gundruk* and thus acceptable to consumers (Table 5). Significant (*p<0.05*) difference in aroma and taste, typical to *Gundruk*, was found on 6 day-old *Gundruk*. This result is similar to the report of Tamang J.P. et al (2010).

The fermented vegetable food has direct impact on human health; quality assessment of fermented food is of utmost importance. It is a fact that in most of the cases, traditionally prepared foods are consumed by the people without getting by the Food Quality Control Department or any other concerned department of the Government. In addition, traditionally prepared foods are given less importance for research and development and occasionally they are misused by the scientific community. Perusal of literature revealed that not much work has been done on the research and development aspect of many traditional fermented foods of the region. It is expected that systematic research of traditionally fermented food in any aspect of biological sciences may lead to an interesting findings as regard the quality improvement of traditional food for betterment of mankind.

The main purpose of the study was to find out the microbial count of *Gundruk*. One of the main rationales of the study is to determine the presence of any pathogenic microorganisms and to test antimicrobial activities and perform antibiotic test from *Gundruk* samples. Beside this, this study also estimates the quality of *Gundruk* prepared in laboratory using selected strains of LAB by organoleptic test. Thus, this study helps to play the important role in quality determination and improvement of *Gundruk* for betterment of man kinds.

# CHAPTER VI

# CONCLUSION AND RECOMMENDATIONS

## 6.1 CONCLUSION

*Gundruk,* a non salted and fermented leafy vegetable product, has been one of the major appetizers for the Nepalese people since a long time ago. From the result of this study, it was concluded that the predominating organisms of *Gundruk* were *Lactobacillus plantarum* and *Pediococcus pentasaceus.* No any pathogenic bacteria, yeast and molds were detected in gundruk samples which indicate that gundruk is good for human consumptions. No any P-solubilizing bacteria were detected. All isolates were susceptible to most of common antibiotics except Vancomycin. This result suggested that LAB isolated from gundruk have very good potential to be used as probiotics. Antimicrobial activity of gundruk sample was also determined. All gundruk samples were able to inhibit most of the indicator pathogens tested except *Salmonella spp* and *Vibrio cholera*. Attempts were made to produce gundruk using mixed starter culture of LAB previously isolated under lab-respective products. The product prepared under lab condition has scored higher sensory ranking comparable to market products.

In the Nepal, most of the ethnic fermented foods are prepared by spontaneous fermentation, except production of ethnic alcoholic beverages by using mixed starter cultures. Use of starter cultures may appear appropriate in *Gundruk* production at household level since it is cost-effective and may contribute to effective control and safeguarding of the fermentation process. Interesting there was no inter-antimicrobial activities among the selected pure cultures. *Gundruk* prepared by using mixture of pure starter cultures had thus advantages over the conventional method, which resulted in a lesser fermentation period that may eliminate non-lactic contaminants, may ensure the hygienic conditions, maintaining consistency with better quality and flavour.

## 6.2 RECOMMENDATION

Based on the result of this study, the following recommendations are made:-

1. This study stresses the need for the continuous screening and surveillance for antibiotic resistance.
2. Further study on the volatile flavor compounds-matrix interactions, flavor release mechanisms, synergistic effect of flavor compounds and correlating these compounds to sensory attributes of *Gundruk* could be done.
3. Molecular researches can also be conducted within this topic.

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# APPENDICES