UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Biomedicina Comparata ed Alimentazione

Corso di laurea magistrale/Second Cycle Degree (MSc)

Biotecnologie perl'alimentazione

Biotechnologies for food science

Isolation and characterization of *Lactobacillus sp.* from raw cow milk of *Bos primigenius indicus* in the Vijayawada Region.

Relatore/Supervisor: Prof.ssa Marina Basaglia

Correlatore/Co-supervisor: Dr. Rajesh Butti

Laureando /Submitted by

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Matricola n./Student no.1237015

ANNO ACCADEMICO/ACADEMIC YEAR 2021/2022

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Contents

List of abbreviations and acronyms 5
ABSTRACT 6
RIASSUNTO 7
1. Introduction 8
  1.1 Probiotic Industry: 8
  1.2 Cow Milk 9
  1.3 Lactic Acid Bacteria 11
  1.4 Lactobacillus and the identification key 12
  1.5 Microbiota and Importance of Gut Probiotics 14
  1.5 Oxidative Stress and Anti-Oxidant ability 20
  1. 6 Methods to determine antioxidant activities 21
Aim of this study 22
F1.FlowChart Methodology Overview 23
2.0 Materials and Methods 24
  2.1 Sample Collection 24
  2.2 Bacterial Isolation: 25
  2.4 Preparation of Stock Culture and Master Plates 27
  2.5 Catalase test 28
  2.6 KOH test 28
  2.7 Acid Tolerance 29
  2.8 Bile tolerance 30
  2.9 Temperature tolerance 30
  2.10 Anti-oxidant assay 31
  2.11 Haemolysis test using blood agar plate. 32
  2.12 Gram Staining 33
  2.13 Endospore staining 34
  2.14 Sugar fermentation based on Bergey's Manual of Systematic Bacteriology 35
3.0 Results 36
3.1 Bacterial isolation 36
3.2 Catalase Test: 37

3.3 KOH Test....37
3.4 Acid Tolerance....37
3.5 Bile Tolerance....39
3.6 Temperature Tolerance....40
3.7 Anti-oxidant assay....41
3.7 Hemolysis test on blood agar plate.....42
3.8 Gram Staining....43
3.9 Identification of strain CO-2 based on sugar fermentation....44
4.Discussion:....45
5. Future Work....47
6.References....48
7.Sitography....56

List of abbreviations and acronyms

- **ATP**: Adenosine triphosphate
- **PBS**: Phosphate Buffered Saline
- **R&D**: Research and Development
- **ROS**: Reactive oxygen species
- **SCFA :** Short Chain Fatty Acids
- **GI:** Gastro intestinal
- **MRS :** De Man, Rogosa and Sharpe agar
- *Lb : Lactobacillus*
- *Sp*.**:** Species
- **OD**: Optical density
- **Nm: Nanometers** meters
- **DPPH**: α, α-diphenyl-β-picrylhydrazyl
- **RSA:** radical scavenging activity
- **UV-Vis spectroscopy** : Ultraviolet-visible spectroscopy

ABSTRACT

Probiotic bacteria are used to confer various beneficial effects to humans as well as animals. In order to achieve a beneficial effect in the gut, probiotic bacteria must be provided in sufficient quantities and alive. To ensure the delivery of sufficient amounts, probiotic bacteria must be evaluated to overcome various stresses during processing, packaging and storage. Stability and viability during the gastric journey also plays a key role in successful colonization. This study was undertaken to isolate, identify and partially characterize strains with high tolerance to various physical and chemical stress and with antioxidant potential. For this purpose, an ideal farm was selected where no supplemental feed and probiotics were used and cows are fed with organic fodder cultivated in the farm itself. In these conditions, the diversity among microorganisms should be higher and thus the chances of isolating a strain with probiotic as well anti-oxidant activity should be higher. Once samples were collected they were serially diluted and transferred to selective culture media using the pour plate method. Isolated colonies with *Lactobacillus* specific characteristics were randomly selected and were made into pure cultures for further investigation.Biochemical tests like Potassium hydroxide string test and Catalase test were performed at first and only negative strains were taken for further evaluation. Firstly tolerance towards acid and bile (gut related stresses) were assayed. Tolerance to temperature was also considered, as this parameter is crucial in the probiotic selection, because of high temperature in the Vijayawada region. As one of the important desired traits is to reduce reactive oxygen species, anti-oxidant activity was measured by the α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity method. Identification of newly isolated strains was performed using culture based methods such as sugar fermentation test and microscopic features according to Bergey's Manual of Determinative Bacteriology. Upon identification the strain with highest anti-oxidant activity is expected to be *Lactobacillus sp.*

RIASSUNTO

I batteri probiotici sono usati per dare vari effetti benefici all'uomo e agli animali. Per ottenere un effetto benefico sull'intestino, i batteri probiotici devono essere somministrati vivi e in una quantità adeguato. Per garantire che un numero sufficiente raggiunga l'intestino, i batteri probiotici devono essere saggiati in modo da valutare la loro stabilità e vitalità dalla somministrazione fino all'intestino e se siano capaci di superare vari stress durante la lavorazione, il confezionamento e lo stoccaggio. Questo studio è stato intrapreso per isolare, identificare e caratterizzare parzialmente ceppi con elevata tolleranza a vari stress fisici e chimici e con potenziale antiossidante. A tal fine è stata selezionata un'azienda agricola ideale dove non sono stati utilizzati probiotici e gli animali vengono alimentati con foraggi biologici coltivati nell'azienda stessa. In queste condizioni la diversità tra i microrganismi dovrebbe essere maggiore e quindi le possibilità di isolare un nuovo ceppo con attività probiotica e antiossidante maggiori. Una volta raccolti, i campioni sono stati diluiti in serie e trasferiti su terreni di coltura selettivi utilizzando la tecnica della diluizione-piastratura. Per le indagini successive, sono state selezionate colonie con caratteristiche specifiche. Dapprima sono stati eseguiti test biochimici come il Potassium hydroxide String test e Catalase test e solo i negativi sono stati ulteriormente saggiati. In primo luogo è stata testata la tolleranza verso l'acido e la bile, stress correlati al transito gastro-intestinale. È stata anche considerata la tolleranza alla temperatura, poiché questo parametro è cruciale nella selezione dei probiotici, a causa dell'elevata temperatura nella regione di Vijayawada. Poiché uno dei tratti importanti desiderati è ridurre le specie reattive dell'ossigeno, l'attività antiossidante è stata misurata tramite l' α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity method. L'identificazione dei nuovi isolati è stata eseguita utilizzando metodi basati sull'utilizzo di specifiche fonti carboniosee e altri caratteri fenotipici secondo la procedura presente ne lBergey's Manual of Determinative Bacteriology. Secondo quanto ottenuto il ceppo con la più alta attività antiossidante sembra appartenere al genere *Lactobacillus*.

1. Introduction

1.1 Probiotic Industry:

In 2020 the global probiotics market was estimated at $34.1 billion, and is expected to grow at an annual rate of 8.6% reaching $73.9 billion by 2030.The growing knowledge of the health and advantages of probiotics, market is predicted to present a major growth opportunity for the industry. This market is predicted to rise as consumer awareness increases, as well as consumer expenditure on healthy food products[1].

The market has experienced a rise in demand for goods that boost immunological health as a result of COVID-19's impact, thus helping the probiotics industry. In emerging nations like India and China, the surge in health-related issues has aided market expansion. To address the demands of the business and remain competitive in the market, companies today have their R&D facilities[1]. In the future, massive R&D efforts are projected to increase the quality of probiotic products. In order to achieve that level of growth, companies have to continuously work towards identifying new and efficient strains which are more beneficial to the consumers as well as to the industry [1].

Probiotic manufacturing industries need to create products to supplement the diet with good microorganisms in adequate amounts to confer health benefits to the consumers. In the development of such products, it is important to identify the microbial strains having greater tolerance to bile and a higher probability of survival in the gut environments. The isolation of microorganisms with such characters is really difficult because of the greater adoption of supplementation by farmers for better yields in animal husbandry. This approach reduces natural diversity and thus the chances of finding “novel” strains. For these reasons, in this work, we have selected an organic farm where the animals were sustained with self-produced feed (Figure 1.1).

[1] https://www.alliedmarketresearch.com/probiotics-market

1.2 Cow Milk

Cow's milk is a nutrient-dense and complex food matrix. Cow milk has a high nutritional content, and contains proteins, lipids, carbohydrates, vitamins, minerals, and essential amino acids (Haug et al.,2007). Milk from the udder cells is sterile and is contaminated by many microbial strains including *Lactobacillus sp.* during routine milking methods due to dirt, manure, grass, silage, and other feeds clinging to the udder, as well as milking equipment and milk storage (Gopal et al.,2015). The milk has a pH very close to neutrality and high water activity and thus sustains the growth of many microorganisms (Gopal et al.,2015).

Bos primigenius indicus

Gir (pronounced Gyr) breed cow has a striking look, with a rounded and domed forehead, long ears, and horns. These cows (Figure 1.1) are commonly bred in Vijayawada region of Andhra Pradesh, India. Usually these cows have a average weight of 368 kg and stand 130 cm tall (Patbandha al.,2020) (Figure 1).

The Classification of *Bos primigenius indicus* is reported in table (Table T1)

Table T1: classification of *Bos primigenius indicus*

Kingdom	Animalia
Phylum	Chordata
Class	Mammalia
Order	Artiodactyla
Family	Bovidae
Genus	*Bos*
Species	*Indicus*

Figure 1.1: ***Bos primigenius indicus*** **at G.Suresh's farm in Rural Vijayawada,India.**

The composition profile (maxmimum and minimum values) of gir cow's milk (Araújo et al.,2018) is given in Table T2 .

Table T2: Composition of the milk of gir cow milk (Araújo et al.,2018).

Parameter	Min	Max
Fat,%	1.79	7.20
Protein,%	2.71	4.24
Lactose,%	4.15	5.03
Total Solids,%	10.61	16.30
Milk production (Kg/Day)	0.50	30.30

Table T3: Milk composition profile of popular Indian cattle indicating Mean maximum Total Solids, Milk fat, Lactose and Protein contents.

Breed	Total Solids(%)	Fat(%)	Lactose(%)	Protein(%)	Reference
Gir	14.98	5.38	5.29	3.85	(Patbandha et al.,2020)
Vechur	12.69[c] ±0.71	3.61[c] ±0.27	4.99[a] ±0.04	3.33[a] ±0.03	(Abraham et al.,2015)
Sahiwal	12.72[c] ±0.41	4.36[b] ±0.29	4.62[d] ±0.07	3.04[c] ±0.04	(Abraham et al.,2015
Kankrej	14.33[a] ±0.16	5.54[a] ±0.12	4.89[b] ±0.28	3.19[b] ±0.27	(Abraham et al.,2015

According to data obtained across studies (Patbandha et al.,2020; Abraham et al.,2015) comparing the milk composition profile of other common Indian cows breeds like Vechur, Sahiwal, Kankrej, the Gir cow's milk has greater total solid, lactose and protein content. Fat content is higher than the Sahiwal, Vechur breeds (Table T3).

1.3 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming, non-respiring but aerotolerant, which produce lactic acid as one of the key fermentation products by utilizing carbohydrates during fermentation (Ayivi et al.,2020). These bacteria produce lactic acid as an end product of carbohydrate catabolism and also synthesize organic substances including short-chain fatty acids, amines, bacteriocins, vitamins, and exopolysaccharides that contribute to the flavor, texture, and aroma resulting in unique organoleptic characteristics of the final fermented food (Wang et al.,2021).

Lactic acid bacteria have been classified into different genera/species based on their acid production characteristics by fermenting sugars and their growth at specific temperatures. LAB can be classified as homofermentative or heterofermentative organisms based on their ability to ferment carbohydrates (Mokoena et al.,2017). The homofermentative lactic acid bacteria yield two molecules of lactates from one glucose molecule whereas heterofermentative lactic acid bacteria generate lactate, ethanol, and carbon dioxide from one molecule of glucose. Homofermentative LAB include *Lactococcus* sp. that are used in dairy starter culture applications where the rapid development of lactic acid and reduced pH

are desirable. Other homofermentative LAB include yogurt strains consisting of rods (*Lactobacillus delbruckii* subspecies *bulgaricus*, *Lb. acidophilus*) and cocci (*Streptococcus salivarius* subsp. *thermophilus*) and thermophilic strains (e.g., *Lb. helveticus*). Other homofermentative cocci that might be found in milk and dairy products, include other *Streptococcus* spp., *Enterococcus*, *Pediococcus* and *Aerococcus* (Mokoena et al.,2017).

Heterofermentative LAB ferment glucose with lactic acid, ethanol/acetic acid and carbon dioxide (CO_2) as by-products. Testing for heterofermentative fermentation generally involves the detection of gas (e.g., CO_2). With the exception of certain fermented milk products, heterofermentative LAB are rarely used as dairy starter cultures, although they are not uncommon in milk and dairy products. If allowed to grow to significant numbers, they can cause defects related to their acid and CO_2 production, such as slits in hard cheeses or bloated packaging in other dairy products. Heterofermentative LAB include *Leuconostoc* spp. (Gram-positive cocci) and Gram-positive rods such as *Lactobacillus brevis*, *Lb. fermentum*, and *Lb. reuteri*. Other *Lactobacillus* species are considered "facultatively" heterofermentative, meaning they will produce CO_2 and other by-products only under certain conditions or from specific substrates. These strains would include *Lb. plantarum*, *Lb. casei* and *Lb. curvatus* [2] .

1.4 *Lactobacillus* and the identification key

The genus *Lactobacillus* has recently been reclassified by scientists into 25 genera. This reclassification was necessitated to the extent of how diverse the original genus was, (Zheng et al., 2020; Beijerinck ,1901). The new genera are *Lactobacillus*, *Paralactobacillus* and the 23 novel genera. The twenty three (23) novel genera includes: *Amylolactobacillus, Acetilactobacillus, Agrilactobacillus, Apilactobacillus, Bombilactobacillus, Companilactobacillus, Dellaglioa, Fructilactobacillus, Furfurilactobacillus, Holzapfelia, Lacticaseibacillus, Lactiplantibacillus, Lapidilactobacillus, Latilactobacillus, Lentilactobacillus, Levilactobacillus, Ligilactobacillus, Limosilactobacillus, Liquorilactobacillus, Loigolactobacilus, Paucilactobacillus, Schleiferilactobacillus,* and *Secundilactobacillus* (Zheng et al.,2020).

[2] https://foodsafety.foodscience.cornell.edu/sites/foodsafety.foodscience.cornell.edu/files/shared/FACT-homo-hetero%2008.doc

For the sake of clarity in this thesis the old classification, following the Bergey's Manual of Determinative Bacteriology has been pursued.

Heterofermentative strains along with glucose ferment other sugars and may produce gas. The Bergey's Manual of Determinative Bacteriology may be used as an identification tool. After performing microscopic analysis, Gram status is determined, then the appropriate identification flow chart was selected from the Bergey's Manual of Determinative Bacteriology (Figure 1.2): *Lactobacillus sp.* have thick peptidoglycan in their cell wall thus they appear Gram positive rods (Chapot et al., 2014). Only Gram positive rods are then tested for spores formation. Usually *Lactobacillus* sp doesn't form spores (Mokoena et al., 2017) . *Lactobacillus* is described as Gram-positive rod, non-spore-forming, acid-fast negative and catalase-negative bacterium. Thus only non spore forming Gram positive, acid fast-negative, and catalase-negative rods, are considered for sugar fermentation test. Firstly glucose fermentation is checked, and the production of Acid and Gas evaluated. If both are positive, the isolated strain can be *Lactobacillus fermenti*. If only acid is produced and no gas production observed, further fermentation test with mannitol need to be carried out. Upon positive fermentation of mannitol, the isolate can be classified as *Lactobacillus casei*. If negative for fermentation of mannitol, the isolate can be *Latobacillus delbruecki* (Figure 1.2).

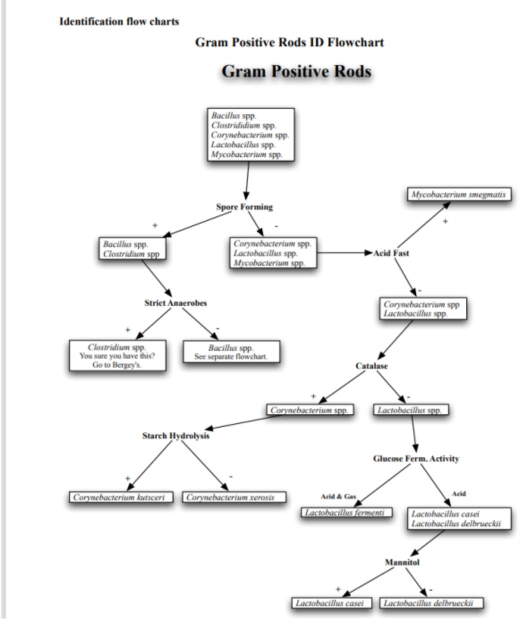


Figure1.2: Identification key of Lactic Acid Bacteria following the Bergey's Manual of Determinative Bacteriology.

Lactobacillus

Lactobacillus is a LAB genus that contains a large number of GRAS (Generally Recognized As Safe) species, and several strains are among the most common bacteria in food microbiology and human nutrition due to their usage as probiotics (Salvetti et al., 2012). Members of this group have the same peptidoglycan types, are facultatively heterofermentative, and have DNA G+C contents of 45–47 mol% (Huang et al., 2018). The current taxonomic hierarchy for *Lactobacillus* sp. is given in the Table T4.

Table T4: classification of *Lactobacillus* genus

Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	*Lactobacillus*

1.5 Microbiota and Importance of Gut Probiotics

Microbiota is usually defined as the assemblage of living microorganisms present in a defined environment (Marchesi al., 2015). As phages, viruses, plasmids, prions, viroids, and free DNA are usually not considered as living microorganisms (Dupre al., 2009), they should not belong to the microbiota. The term microbiome, as it was originally postulated by Whipps and coworkers (Whipps et al., 1988) , includes not only the community of the microorganisms, but also their “theatre of activity”. The latter involves the whole spectrum of molecules produced by the microorganisms, including their structural elements (nucleic acids, proteins, lipids, polysaccharides), metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules produced by coexisting hosts and structured by the surrounding environmental conditions (Berg et al.,2020). Therefore, all mobile genetic elements, such as phages, viruses, and “relic” and extracellular DNA, should be included in the term microbiome, but are not a part of microbiota (Figure 1.3)

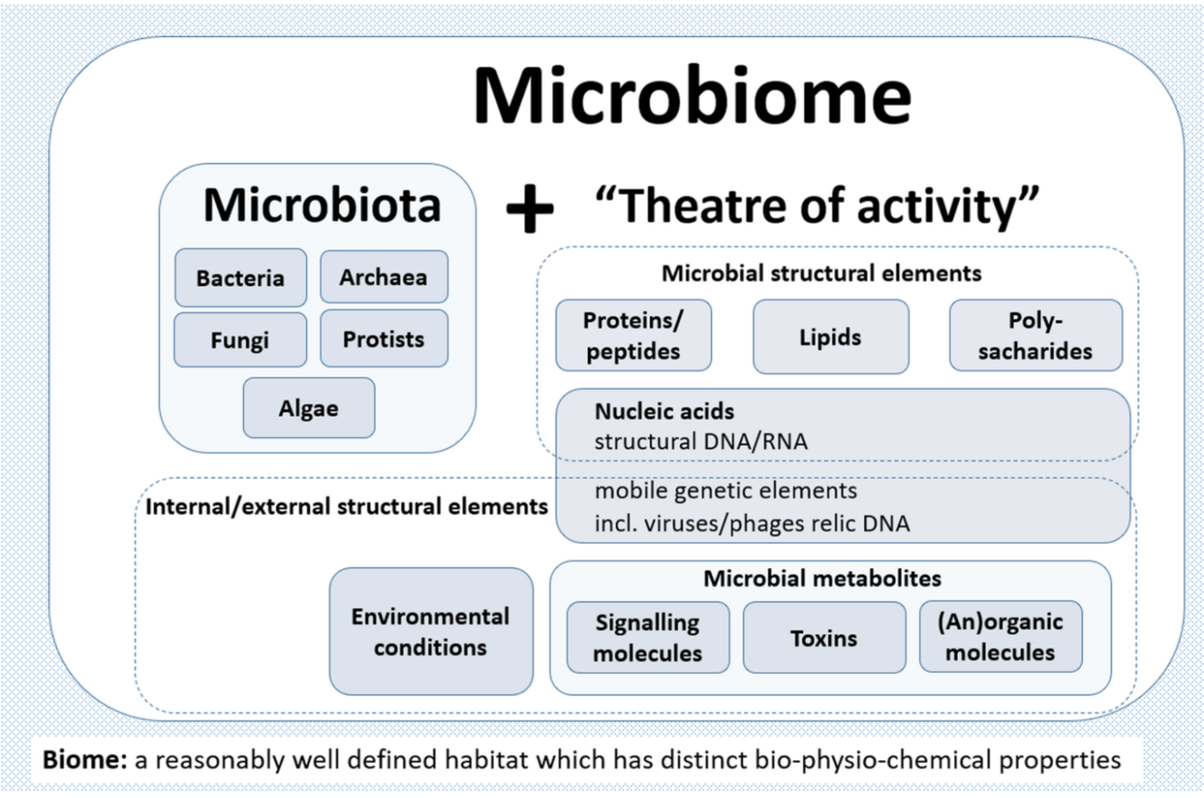


Figure 1.3:Microbiome definition re-visited: old concepts and new challenges (from Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C. C., Charles, T., ... & Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. Microbiome, 8(1), 1-22).

Microbes interact with one another, with diverse consequences for microbial fitness, population dynamics, and functional capacities within the microbiome. These interactions can be between microorganisms of the same species or between different species, genera, families, and domains of life. The interactive patterns within these webs may be positive (mutualism, synergism, or commensalism), negative (amensalism, including predation, parasitism, antagonism, or competition), or neutral—where there are no (or no observed) effect on the functional capacities or fitness of interacting species (Berg et al., 2020). The stability of a complex microbial ecosystem depends on trophic interactions for the same substrate at different concentration levels. It is important to highlight that microbial social adaptations in nature are so far understudied. Secondary metabolites play an essential role in mediating complex interspecies interactions ensuring survival in competitive environments. Unfortunately, the communication and the interaction within the microbiome remain understudied (Berg et al., 2020).

The colonization of hosts by microbiota is also not uniform. It is well known that in plants, leaves harbor a different microbiota compared to the root, and the root itself is heterogeneously colonized by microbes, with different microbiota along the length of the rhizosphere and at the root surface vs. the root interior (Estendorfer et al., 2017). Similarly to plants, the human body is not homogeneously colonized by microbes: each body compartment contains its own microbiota (Proctor et al., 2017) , and even microbiota from one body site may differ depending on the area of sampling (e.g. the skin microbiota), dietary habits, food preferences, climate, age, gender etc (Perez et al., 2016).

Nowadays microbiota is of special interest because of various new discoveries linking its role to health and disease prevention, although there are various gaps in understanding of mechanisms and modes of action (Zec et al., 2020). Microbiota is as unique as a signature, very specific to the particular individual. Moreover gut microbiota seems to play a crucial role in nutrient uptake, bioavailability and synthesis of various macro and micro molecules.microbiota composition of patients compared with that of healthy individuals has been related with certain disease conditions (Zec et al., 2020). Usually, diverse gut microbiota is considered for good nutritional and healthy outcome (Lee et al., 2009).

The composition of gut microbiota is broadly dependent of the host's food habits, food composition. Fluctuations among microbial population in the gut are also related to the age . As an example Bacteroidetes bacteria are higher in youth but decline significantly as age progresses and Firmicutes phylum bacteria are lower in younger populations but tend to become higher with time progression (Zec et al., 2020). Even though gut microbiota consists of all sorts of microorganisms including yeasts, mostly bacteria are of great interest to researchers as well as to consumers in providing health benefits and aiding in disease prevention (Julio et al., 2019).

Having a healthy microbiome confers various health benefits to the host including digestion of food, immunomodulation, competition and prevention of pathogenic microorganisms colonization (Pickard et al., 2017). Examples of common gut bacteria in adults are *Lactobacillus, Bifidobacterium, Clostridium, Escherichia, Bacteroides, Streptococcus etc.* (Zec et al., 2020; Reale et al., 2015)

Probiotic bacteria are non-pathogenic living micro-organisms that, when consumed in adequate amounts, provide health benefits to the host (Kechagia et al. 2013). Prebiotics are compounds that support, induce, and enhance the growth of probiotic microorganisms (eg. inulin) (Markowiak et al., 2017) . The Gut is colonized by both probiotic as well as the pathogenic microorganisms but the ratio of these strains' colonization purely depends upon the food habits of the individual (Markowiak et al., 2017). Higher concentrations of good (probiotic) microorganism in Gastrointestinal tract helps in reducing the growth of pathogenic microorganisms thus aiding in disease prevention (Markowiak et al., 2017).

Probiotic microrganisms can be supplemented to diet thus increasing their number in the gut, although they must be present for a long time in adequate amounts inside the gut to confer health benefit. Probiotics are consumed to maintain good gut microbiome in gastrointestinal tract (Kobyliak et al., 2015). Various factors like pH, availability of fermentable fibres etc. are affecting the survival and successful colonization.(Parada et al., 2019)

The use of live microorganisms for beneficial purposes as probiotics such as those in fermented milk dates back to ancient times. Scientists such as Hippocrates and others considered fermented milk to be not only a food product but also medicine, and sour milk was prescribed for curing disorders of the stomach and intestines. In 1908, a Russian bacteriologist, Eli Metchnikoff (Pasteur Institute, France) was the first to put forth a scientific explanation of the benefits of lactic acid bacteria in fermented milk. Metchnikoff attributed the good health and longevity of Bulgarians to their high consumption of a fermented milk called "yahourth" (Ayivi et al., 2020) .

In 1899, Henry Tissier (Pasteur Institute, France) isolated bifidobacteria from the stools of breast-fed infants and discovered that these bacteria were a predominant component of the human intestinal microflora. Tissier, thus, proposed the administration of bifidobacteria to infants diagnosed with diarrhea, "believing" that bifidobacteria would displace bacteria responsible for gastric upsets while re-establishing themselves as the dominant intestinal microorganisms. Recently El-Soud, et al. (2015) found that the supplementation of milk formula with *Bifidobacterium lactis* in children diagnosed with acute diarrhea, decreased the frequency, sickness duration, and ospitalization period of diagnosed children than the conventional treatment approach (El-Soud, et al., 2015).

Probiotic lactic acid bacteria can be isolated from different sources such as fermented foods, animals, and from humans as well. A probiotic strain must also be safe and not pose any threat to the host (Ayivi et al., 2020). The most commonly used probiotics generally come from the genera *Lactobacillus* and *Bifidobacterium*. Other bacteria that could be considered and are similar include *Streptococcus thermophilus*, non-pathogenic strains of *E. coli, Enterococcus, Bacillus*, and yeasts, such as *Saccharomyces boulardii* (Ayivi et al., 2020) . The genera *Streptococcus* and *Enterococcus* are considered members of the lactic acid bacteria group, although, these bacteria contain several strains ,associated with severe infections, such as *Streptococcus pyogenes, Streptococcus pneumoniae,* and vancomycin-resistant *Enterococcus faecium*, there are other strains that form part of the commensal human microbiome of the mouth, skin, and intestine, such as *Enterococcus faecium* PC4.1 (Sfaxi et al., 2011).

Lactic Acid Bacteria are the more extensively studied probiotics and have confirmed efficacy in preventing complications such as colorectal cancer and carcinogenesis (Zhong et al., 2014). There are reports of LAB (*Lactobacillus casei* BL23) used to treat cancers induced by mutagens like azoxymethane in various animal models (Jacouton et al., 2017). Although there are still strong research gaps and further correlations between microbiota and some diseases have to be drawn, it can be concluded that the gut microbiome’s composition plays a very important role in human nutrition and well-being (Zec et al.,2020).

In fact in s,ome animal models, many probiotic strains from the *Bifidobacterium* and *Lactobacillus* genera were reported and the strains seem to exhibit anti-carcinogenic, anti-mutagenic, and anti-tumorigenic properties (Śliżewska et al., 2020) .It is obviously critical that probiotics conform to international standards and specific norms for usage have to be defined.

According to the Joint FAO/WHO (2002) guidelines on probiotics, the use of probiotic microorganisms to confer health benefits on the host must indicate the dosage regimens and duration of use as recommended by the manufacturer of each individual strain or product based on scientific evidence, and as approved in the country of sale.

Probiotics confer an array of health benefits including the following (Ayivi et al., 2020) : (1) maintain the healthy microbiota balance of the intestine by competing and excluding

harmful pathogens adhering to the gastrointestinal gut (2). stimulate and enhance the immune response by increasing the release of serum antibodies. Probiotics also help to prevent or decrease the duration of intestinal diseases such as Inflammatory bowel disease, diarrhea, and constipation by colonizing and modulating the gut microflora, synthesizing antimicrobial compounds, and enhancing the immune response and secretion of mucus (Ezendam et al., 2006).

Moreover, probiotics are important in the prevention of metabolic disorders such as obesity, diabetes, and cardiovascular diseases by enhancing gut microbiota, restoring the antioxidant systems, decreasing insulin resistance and inflammation, and inhibiting the growth of *Candida* and *Helicobacter pylori*; thus they prevent intestinal infection by competing and adhering to the mucosal surface and enhancing immune responses (Markowiak et al., 2017; Parvez et al., 2006; Nagpal et al., 2012). They also help to avoid the growth of cancer by the detoxification of chemical carcinogens, by enhancing the antioxidant system, modulating the immune response, and generating metabolites like the SCFAs (short chain fatty acids) and butyrate which increases cancer cell death (Śliżewska et al.,2020; Kedare et al., 2011).

Furthermore, probiotics assist in the maintenance and alleviation of lactose intolerance by providing the β-galactosidase (lactase) enzyme which breaks down lactose into simple sugars (Ayivi et al.,2020).Gut Bacteria like *Lactobacillus* sp.can produce vitamins like K, B_{12}, Folate, Biotin, Thiamine (Rossi et al., 2011). Under healthy and normal conditions these "good bacteria" outcompete the pathogenic bacteria thus deprived of essential nutrients and therefore probiotics reduce the risk of disease (Zec et al., 2020). Beneficial gut microorganisms can hydrolyse high molecular weight polysaccharides which will be then converted into simpler units of glucose, easily metabolized by the host for generating energy. From the carbohydrates, microrganisms can also produce organic acids and nutrients for other good microorganisms. Specifically, dietary fibre which is indigestible by humans is the food for some gut bacteria that ferment them into weak organic acids reducing the pH and determining unfavourable conditions for pathogenic bacteria (Zhang et al., 2010). *Lactobacillus* sp.require iron to be able to thrive in the gut; when these good bacteria prosper, they produce SCFAs which reduce the pH and are used by other bacteria.

This pH reduction contribute to a more iron uptake (Rusu et al., 1993; den Besten et al.,2013).

The prebiotic fibers (such as inulin), are compounds that support, induce and enhance growth of probiotic microorganisms in the gastrointestinal tract . Unfortunately, studies demonstrated that the health benefit to the body obtained by supplementing probiotics, prebiotics and nutrients had a short effect and once the supplementation stopped, the microbiota returned to its original state (except in infants of certain age group) (Zoetendal et al.,1998); therefore a continuous supplementation of microorganisms is necessary. This fact gives a great opportunity to the industries.

Generally diverse microbiome is an indication of good health (Valdes et al., 2018). The diversity in microbiota can be affected by many factors such as a diet rich in fats and proteins that encourage the growth of specific microbes thus unbalancing the microbiota. Consequently a well-adjusted diet with enough dietary fibres, proteins, good fats, vitamins, and minerals is the key not only to good health but also to a more diverse microbiome that aids in disease prevention and stronger immunity (Conlon et al., 2014).

1.5 Oxidative Stress and Anti-Oxidant ability

Cows suffer from various udder diseases, like mastitis, due to oxygen free radicals(ROS) (Turk et al., 2017). This stress may cause cell and DNA damages which may lead to cancers (Scott et al., 2014). Moreover these molecules also determine inflammation which can cause alterations in the taste of milk. Despite the fact that animal models and human research are more suitable, chemical techniques such as hydroxyl, superoxide, and DPPH radical scavenging activities are usually used to determine the antioxidant activity of *Lactobacillus* sp.strains for verifying anti-oxidant activity (Mishra et al., 2015).

Studies indicate that some LAB strains have anti-oxidant and anti-inflammatory properties (Kuda et al., 2014; Mu et al, 2018; Tien et al.,2006). For this reason, the industry is developing probiotic products with potential anti-oxidant characteristics, with the aim to reduce cellular damage and provide benefit to host. As Lactobacilli *have* long history of consumption and are generally regarded as safe (GRAS) (Salvetti et al., 2017), they would be ideal microorganisms for identification of strains with anti-oxidative properties.

1. 6 Methods to determine antioxidant activities

DPPH Method

The reactive oxygen species (ROS) are extremely reactive oxidation products with high electron acceptability produced during natural mechanisms in the body. ROS can harm the body's inherent cells or organs. In light of this, antioxidants are necessary to prevent ROS damage to body cells (Kedare et al., 2011). Some bacterial strains also synthetize metabolites that protect them from ROS damage, e.g. some *Lactobacillus* sp., release beneficial molecules having anti-oxidant properties into the milk which can be analysed using DPPH assay (Kedare et al., 2011).

The α, α-diphenyl-β-picrylhydrazyl (DPPH) assay is a test aimed to determine the antioxidant properties of a sample. It can be performed by mixing the potential anti-oxidant sample with the DPPH solution and measuring the absorbance after a defined time (Lin et al., 2018). In Figure 1.4, principle of DPPH anti-oxidant assay isreported. if the sample contains anti-oxidant capability when added to the deep purple-colored DPPH solution, diphenylpicryl hydrazine is formed which is a pale yellow-colored product. This change in colour is measured using a spectrophotometer at 517 nm; the fluctuation in optical density is related to the rate of scavenging activity of that particular sample that can be calculated.

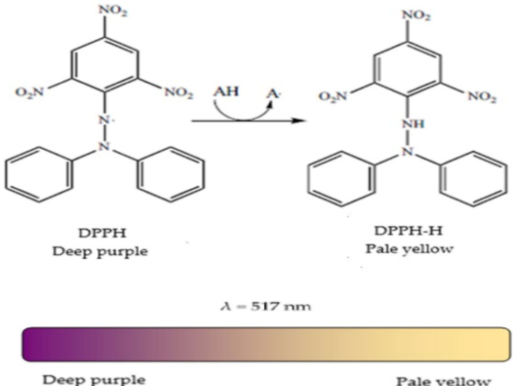


Figure1.4: DPPH scavenging mechanism by an anti-oxidant(AH).

Aim of this study

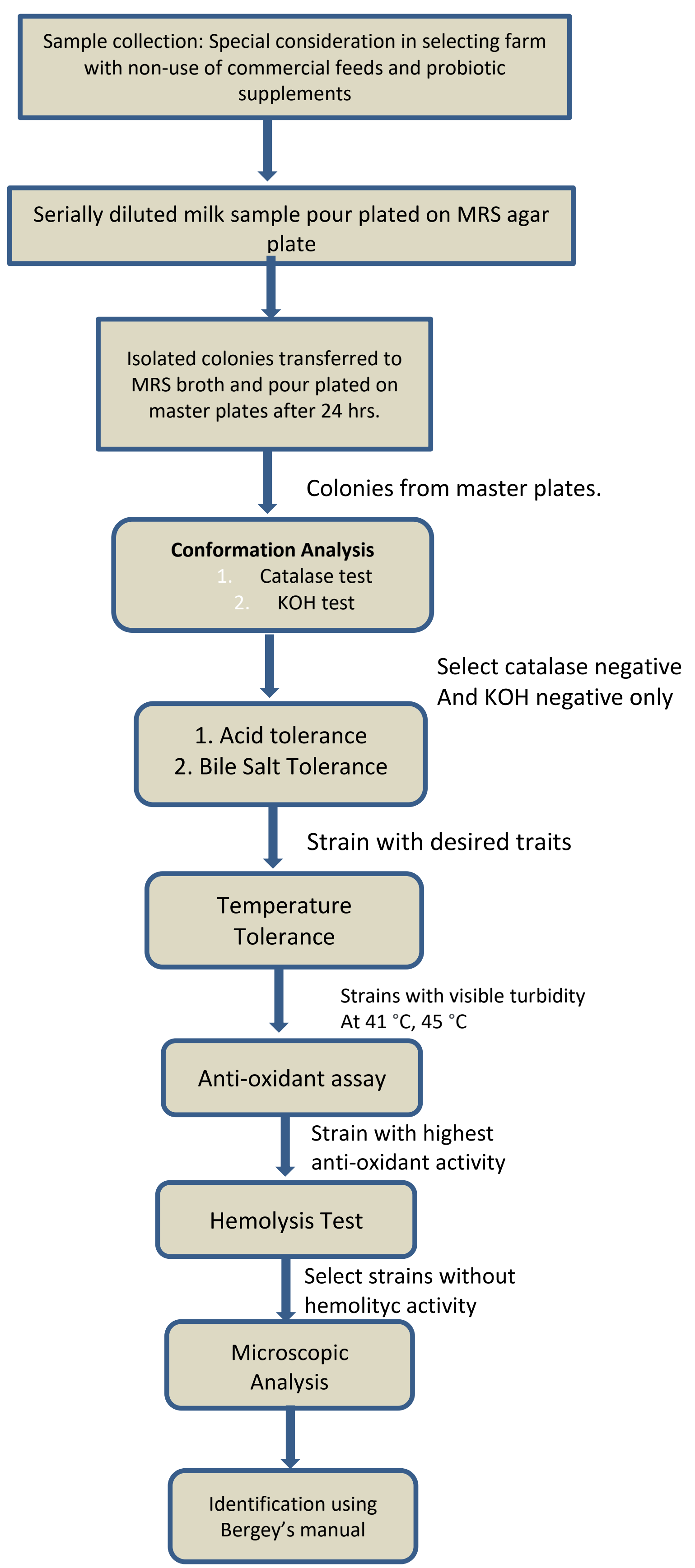
This study is aimed to isolate and characterize strains with probiotic and anti-oxidant capabilities which could be utilized into the formulation development and commercial production of a probiodic product from Gir cow's milk in Vijayawada region ,India.

Thus the specific objectives of this thesis were:

1. To isolate and characterize bacterial strains showing tolerance to acid, bile and temperature, isolated from cow milk.
2. To test anti oxidant activity of the selected strains exhibiting desired traits.
3. To carry out preliminary identification of newly isolated strains showing the highest anti-oxidant activity, using the Bergey's Manual of Determinative Bacteriology. The methodology is summarized into the F1.Flowchart Methodology Overview.

Isolation of strains was obtained by the cultivation on selective medium (MRS). The pure cultures were selected based on colony characteristics and confirmation analysis. The isolated strains were tested for important traits that are desired in an ideal probiotic strain such as tolerance to acid,bile and temperature. Strains exhibiting probiotic traits were tested for anti-oxidant activity and the strain exhibiting highest anti oxidant was checked for hemolysis on blood agar plate and then identified using the Bergey's Manual of Determinative Bacteriology.

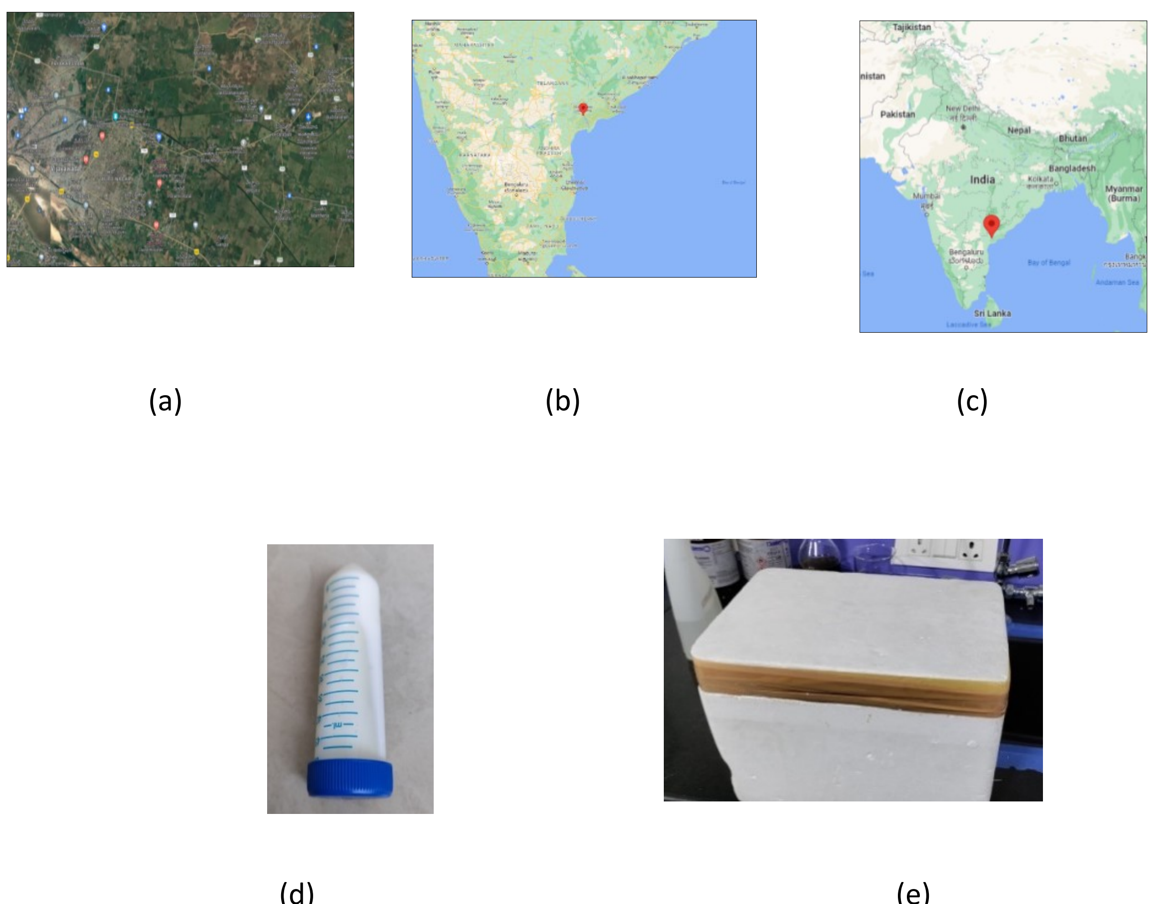
F1.FlowChart Methodology Overview



2.0 Materials and Methods

2.1 Sample Collection

As the aim of this thesis was to isolate novel strains showing anti-oxidant benefit to the host, multiple farms in and around Vijayawada at a 30KM radius were contacted and an ideal farm was identified (Location in Figure 2.1(a),(b)). The farmer, Mr G.Suresh runs an organic farming awareness centre and herds cows for the manure and milk. Upon visit to the farm and discussion with the farmer, it resulted that no commercial probiotic supplement was administered to the cows and the animals were fed with the fodder from his farm. This wasan ideal farm as the possibility to isolate already commercialized strains was less. An healthy Gir cow, free from any disease at the time of sample collection, was then selected. The udder was properly washed and cleaned outside with 70% ethanol and milking was done in stripping method. The milk sampling was carried out in three consecutive days from the same cow. The samples were collected aseptically into sterile Eppendorf tube(Figure 2.1(e)) and were put into an icepack (Figure 2.1 (d)) to reduce contamination and proliferation risk and were transported to the lab at PVS Laboratories Limited the same day.PVS Laboratories Limited situated in Mangalagiri, Andhra Pradesh, India (Figure 2.1(c)) is a company specialized in manufacturing of feeds and probiotic formulations for veterinary use.



Figures 2.1: Location of sample collection (a, b), location of PVS Labratories (c) and collected milk sample (d, e).

2.2 Bacterial Isolation:

Most samples collected from a natural source include a large number of microorganisms thus they must be serially diluted in order to be quantitated properly. This process involves step by step dilutions of a solution. The extent of dilution is defined by the estimated concentration of cells in a sample. Since it is not possible to collect colonies from an extremely dense Petri plate, it is mandatory to dilute the samples as indicated in the Figure 2.2 (Wassie et al., 2016).

Figure 2.2: Procedure for serial dilution and pour plating technique.

Pour plating was done by transferring 1 mL of serially diluted milk samples into Petri plate and adding 20-25 ml of sterile and molten MRS Agar (Figure 2.3) at 40-45°C. The medium was stirred to mix well and then the plates anaerobically incubated.

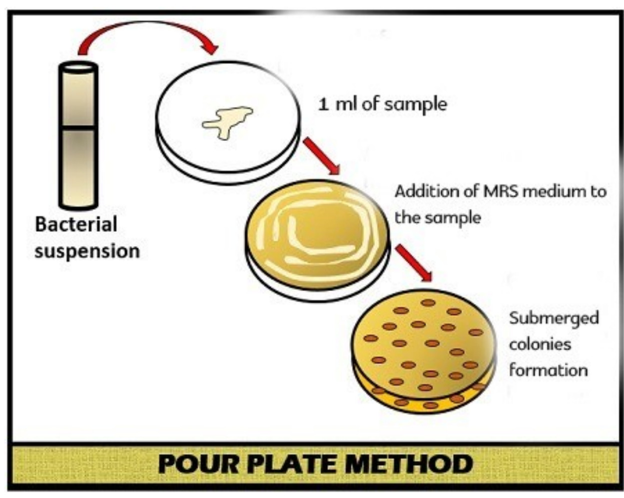


Figure 2.3: Procedure for pour plating technique.

MRS (de Man, Rogosa and Sharpe broth), is a selective medium for *Lactobacillus* sp. and was used for isolation process. MRS medium (HI-Media, Mumbai) compositions is reported in the Table M1.

Table M1: Composition of MRS medium (HI-Media, Mumbai)

Ingredients	g/L
Proteose peptone	10.00
HM Peptone B#	10.00
Yeast extract	5.00
Dextrose(Glucose)	20.00
Polysorbate 80 (Tween 80)	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium hydrogen phosphate	2.00
Final pH (at 25°C)	6.5±0.2

Equivalent to Beef Extract

To Prepare MRS agar, Agar was added as 12 g/L

The medium was sterilized in autoclave at 121 °C for 15 minutes. After sterilization, the culture medium was brought to around 40-45 °C before utilization.

In short, the collected milk samples were placed in the ice pack and were brought to lab the same day. 1 ml of milk sample was serially diluted using distilled water up to 10^{-6} under aseptic conditions..

Each of 1ml of 10^{-4} and 10^{-5} diluted milk samples were placed in sterile Petri plates using micropipettes. Approximately 20-25 mL of previously prepared and sterilized MRS agar were added to these plates, gently mixed and the plates incubated anaerobically at 37°C for 48 hours (Wassie et al., 2016). All analyses were conducted in triplicates.

2.4 Preparation of Stock Culture and Master Plates

After 48 hours of incubation, the plates containing the serially diluted milk sample were observed for colonies growing inside the agar. The selected colonies were aseptically transferred to sterile MRS broth tubes using a sterile inoculation needle and allowed for anaerobic incubation at 37 °C for 24 hours. After incubation, the a drop of the broth was observed under the microscope using 40X and 100X magnifications for microbial cell appearance and purity. Then tubes were taken for further assessment and storage in the

refrigerator. 1mL was collected from each tube aseptically and was pour-plated into MRS agar plates and incubated for 24 hours at 37 °C anaerobically. These plates were maintained in triplicate. Only plates with uniform colonies and without contamination were used for the next processes of identification and characterization. These plates were labeled with a unique sample code and were considered to be master plates.

2.5 Catalase test

As the desired strain was of *Lactobacillus* sp., this is expected not to have the enzyme catalase which won't breakdown hydrogen peroxide to water and gaseous oxygen. Catalase test was performed to screen for any positives placing a loop full of inoculum in a drop of 3% H_2O_2 on a clean glass slide. Strains that did not produce gas bubbles were taken for further evaluation (Shuhadha et al., 2017).

2.6 KOH test

KOH string test is based on the differential resistance of Gram positive and Gram negative bacteria towards 3% KOH (Figure 2.4). In the presence of potassium hydroxide, Gram negative cell walls are dissolved releasing cellular contents creating stringy or visicid-like texture on slide.On the other hand Gram positive bacteria are not affected by KOH, because they have thicker peptidoglycan layer in the cell wall. A drop of 3% KOH was added on a glass slide, then a bacterial colony was picked up and mixed in KOH solution(Powers, 1995) . As most of LAB are Gram positive, 3% KOH should not cause cell lysis and viscid like/stringy structures should not be observed. Negative strains that did not produce viscid like structures were taken for further evaluation. (Shuhadha et al., 2017)

Figure 2.4 : Negative and Positive KOH result on a test slide.(Ref: https://www.uwyo.edu/molb2210_lab/images/catalase.jpg)

2.7 Acid Tolerance

In order to prosper in the gut and provide probiotic benefit, the microorganisms must be able to pass through the acid in stomach. Thus acid tolerance test was designed to check for ability to survive in an acid environment. The isolates were tested following the protocol of (Shehata et al., 2016) with modifications. In short, 25 mL of 24 hour old MRS broth cultures of each isolate were centrifuged (REMI India centrifuge) at 4300rpm for 10 minutes. The pellet was washed with PBS (pH=7.0) for 3 times. Cell density was adjusted to 10^8 CFU/ml using MacFarland 0.5 standard (PBS was used to set the cell density) using a densitometer. 1ml of bacterial suspension was added to 5 ml of sterile MRS broth with pH adjusted at 2.0 with HCl and then incubated for 3h at 37 ° C. After 3h the broth was collected and serial dilutions were made (up to 10^{-4}). 100 µL of the serially diluted suspensions were pour plated into MRS agar plates and incubated at 37°C for 24h to assess survival (Shehata et al.,2016). *Lactobacillus acidophilus* IHC20047 (a well-known tolerant) and PBS were used as positive and negative controls respectively. The appearance of colonies after incubation period on the solid medium was considered an indication of acid tolerance and only strains exhibiting this trait were taken for further assessment.

2.8 Bile tolerance

Tolerance to bile salts is one important trait required to microorganisms that determines how successfully a strain can colonize the gut. For this reason, isolates showing tolerance to acid were tested for bile tolerance (Shehata et al., 2016). Firstly, 25 mL of 24-hour old MRS broth cultures of each isolate were centrifuged at 3400 rpm for 10 minutes. Supernatant was discarded and the pellet washed with PBS (pH=7.0) for 3 times and then re-suspended into PBS (pH=7.0). Cell density was adjusted to 10^8 CFU/ml using MacFarland 0.5 standard (PBS was used to set the cell density) using a densitometer. 1ml was added to 5 ml of sterile MRS broth which with 0.3% bile and were incubated for 3h at 37 ° C. After 3h the broth was collected and serial dilutions made (up to 10^{-5}). 100 µL was pour plated into MRS agar plate. *Lactobacillus acidophilus* IHC20047 (known tolerant to bile) and PBS were used as positive and negative controls. Appearance of colonies after incubation period on the solid medium were considred as the indication of bile tolerance and only positive strains were taken for further assessment.

2.9 Temperature tolerance

Vijayawada region is hot and humid weather, during summers, maximum temperatures range from 40- 45°C. Thus a strain used in an industrial mass production should exhibit tolerance and ability to grow at higher temperatures. 25 mL of24-hour-old MRS broth cultures of each isolate were centrifuged at 3400 rpm for 10 minutes. Supernatants were discarded, the pellets washed with PBS (pH=7.0) 3 times, and then re-suspended into PBS (pH=7.0). Cell density was adjusted to 10^8 CFU/ml using MacFarland 0.5 standard (PBS was used to set the cell density) using a densitometer.

To assess tolerance to high temperature 100 µL of the samples were inoculated into fresh centrifuge tubes containing 30 ml MRS broth and incubated at 41°C and 45°C for 24 hours in water bath. A tube with MRS and PBS was kept as negative control and incubated along with samples. Results were interpreted based on turbidity of the broth after 24 hours at

41°C, 45°C (Shehata et al., 2016). After the incubation the turbidity of samples was compared visually with that of the Control sample by comparing the clarity of the lines on the Wickerham card. Samples that were able to grow at the desired temperatures showed turbidity and reduced the clarity of lines on Wickerham card in comparision with the control. Strains exhibiting clear and distinct turbidity at both the test temperatures were considered for further assessment. Slow growers and strains lacking tolerance to test temperatures were not taken into consideration because the possible difficulties for the application in an industrial production.

2.10 Anti-oxidant assay

Antioxidant ability is an important trait that is extremely desired in a probiotic microorganism because of the benefit it could provide to the host. Reactive oxygen species (ROS) are extremely reactive oxidation compounds formed as a result of natural processes in body. ROS can harm the organs or the body's inherent cells .Thus antioxidants are necessary to stop these ROS and protect from the effects of oxidation. Previous research (Hyemin et al.,2020) demonstrated the antioxidant properties of *Lactobacilli* .Thus an anti-oxidant assay (DPPH assay) was performed according to (Lin et al., 2018) to select the strain with highest antioxidant properties.

10 mL of 24 hour freshly prepared bacterial culture was centrifuged at 10000 RPM for 10 minutes and supernatant was discarded. Pellet was washed twice and resuspended with 0.85% sterile saline. Cell density was adjusted to 10^8 CFU/ml using MacFarland 0.5 standard (0.85% saline was used to set the cell density) using a densitometer. A solution of 4mM of DPPH (α,α-diphenyl-β-picrylhydrazyl) in methanol was prepared and wrapped in aluminium foil. 2mL of the cell suspension sample were added to 1mL of previously prepared DPPH and kept in dark place for 30 min. Suspension was centrifuged at 6000 rpm for 10 minutes and the supernatant was collected for OD measurement. OD values were measured at 517 nm using spectrophotometer(Figure 2.5). Methanol was used as the blank and DPPH along with methanol was used as the control. Scavenging rate % was calculated using the equation below as descibed in (Lin et al.,2018).

- Scavenging rate (%) = [1−(Absorbance of sample−Absorbance of blank)/Absorbance of control] ×100 (in%).

The strain with highest antioxidant activity was taken for further tests.

Figure 2.5: Lab India Spectrophotometer.

2.11 Haemolysis test using blood agar plate.

The ability of bacterial strains to cause hemolysis when grown on blood agar is used to characterize and classify certain microorganisms. Microbial haemolytic activity is an important parameter related to the possible patogenicity of newly isolated strains and thus should be assessed. This parameter is usually estimated by growing the bacteria in an agar containing red blood cells and evaluating the hydrolysis haloes after growth. β-hemolysis is associated with complete lysis of red cells surrounding the colony. Example of β-hemolytic bacteria are *Streptococcus pyogenes,* or Group A β -hemolytic Strep (GAS). Weakly β -hemolytic species are *Streptococcus agalactiae, Clostridium perfringens* or *Listeria monocytogenes.*

α-hemolysis is a partial or "green" hemolysis associated with reduction of red cell hemoglobin. α-hemolysis is caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobin to green methemoglobin. Examples of α-hemolytic bacteria are *Streptococcus pneumoniae* and a group of oral streptococci (*Streptococcus viridans*)

In γ -hemolytic (Non-haemolytic) Streptococci colonies show neither typical α- nor β haemolysis. There may be, however, slight discoloration in the medium. The streptococci included in this group are usually not pathogenic (Figure 2.6)

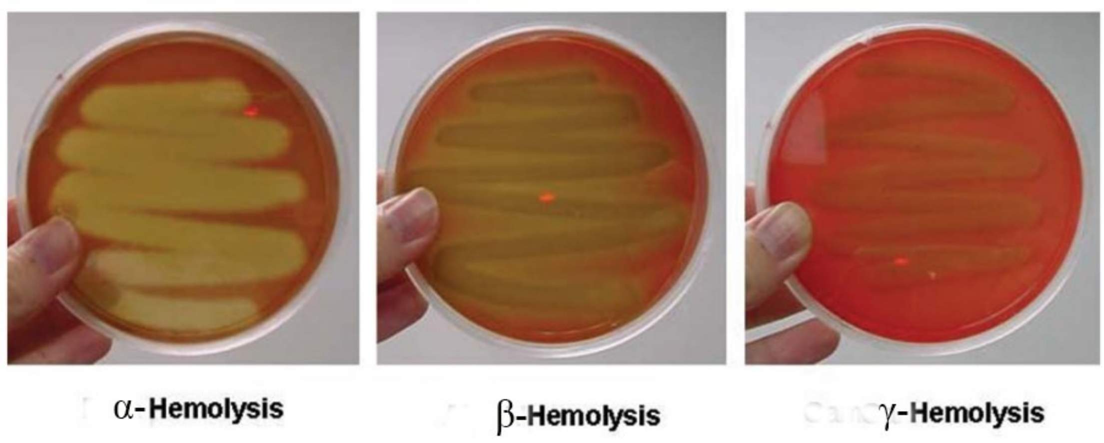


Figure 2.6. Different kind of hemolysis on Colombia red blood agar

To assess haemolytic activity, Columbia Blood agar base (Hi-Media, Mumbai) was prepared following manufacturer's instruction. 5% v/v sterile defibrinated sheep blood was added after sterilization in autoclave. Then the 30-35 mL were poured into sterile petri plates and allowed to solidify. Then the strains exhibiting highest anti-oxidant activity were streaked into previously prepared blood agar plates alog with positive control (*Staphylococcus aureus*) and negative control (*Lactobacillus acidophilus)* for comparision with the sample. Petri plates were then incubated at 37°C for 24 hours and checked for hydrolysis haloes. Only non-haemolytic strains were taken into consideration for further microscopic evaluation and identification.

2.12 Gram Staining

To determine Gram status, a colony from master plate of strain with highest anti-oxidant activity was picked. Gram staining was done according to protocol (Shuhadha et al., 2017) and flow process as in Figure 2.6. A drop of Crystal violet was added o a heat-fixed smear of a bacterial culture and kept for 1 minute. The slide was then rinsed with water until ran

clear. The slide was then flooded with the Iodine and again was rinsed with water. Then few drops of decolourizer (95 % Ethanol) was added and was again rinsed with water. Few drops of counter stain Safranin was added added for 30 seconds and was again rinsed with water.Once the slide is dry the sample is observed under the microscope. Gram positive were then selected for identification, based on sugar fermentation.

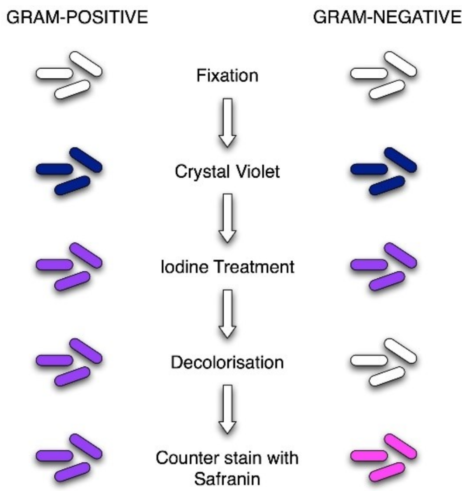


Figure 2.7: Flow chart for the Gram staining procedure

2.13 Endospore staining

The endospore stain method was performed following the protocol of Shuhadha et al. 2017 with a modification, safranin was used instead of diluted carbol fuschsin as counter strain. Malachite green (primary stain) was applied to bacterial smear on microscopic slide which was then heat fixed. The heat fixed slide was , rinsed with water until ran clear. The slide was then flooded with the counter stain (safranin) for 30 seconds and rinsed with water. The endospore, if present would appear green. As the desired strains are non spore forming, only the non-spore forming were taken for further evaluation.

2.14 Sugar fermentation based on Bergey's Manual of Systematic Bacteriology

Bergey's Manual of Systematic Bacteriology is the main resource for determining the identity of prokaryotic organisms, emphasizing bacterial species, using every characterizing phenotypic aspect. The manual does not classify bacteria according to evolutionary relatedness but provides identification (determining) schemes, based on such phenotypic criteria as cell wall composition, morphology, differential staining, oxygen requirement, and biochemical testing as the ability of bacteria to selectively ferment certain substrates. In this work glucose and mannitol were used as sugars to investigate the ability of selected strains to ferment and to produce acid. Durham tubes were inserted into each test tube to determine gas generation in addition to sugar fermentation. The utilised medium was MRS with 1 % sugar along with bromocresol purple (0.12g/L) as a pH indicator. 25 ml of 24 hour culture were centrifuged at 10000 RPM for 10 minutes and supernatant was discarded. Pellet was washed twice in sterile 0.85% saline. Cell density was adjusted to 10^8 CFU/ml using MacFarland 0.5 standard (0.85% saline was used to set the cell density) using densitometer. 50µL of the sample were inoculated into tubes containing test sugar and were incubated for 24 hours at 37°C. Change in colour and gas development in the tube was noted after the incubation period.

3.0 Results

3.1 Bacterial isolation

As described in Materials and Methods, MRS agar was used as selective media for the isolation of strains having probiotic properties from Gir cow's milk (figure 3.1).

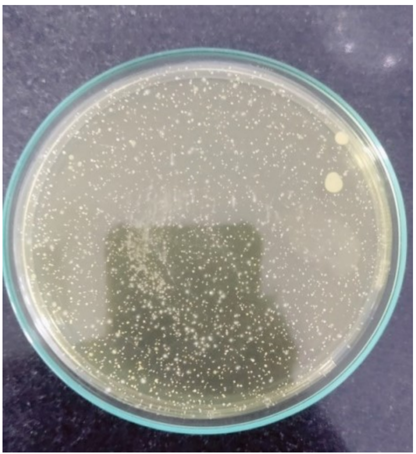


Figure 3.1: MRS agar plate inoculated with milk sample by the pour plate method.

After 48 hours at 37°C under anaerobic conditions 5 submerged colonies exhibiting *Lactobacillus* characteristics were randomly chosen for this study among the 86 Isolated colonies extracted from the sample by the company.

The 5 colonies were aseptically transferred into sterile centrifuge tubes (Figure 3.2) containing MRS broth labeled as CO-1, CO-2, CO-3, CO-4, CO-5, sealed air tight and incubated anaerobically for 24h at 37°C. After incubation, the cultured broth was observed under microscope for microbial cell appearance and purity.

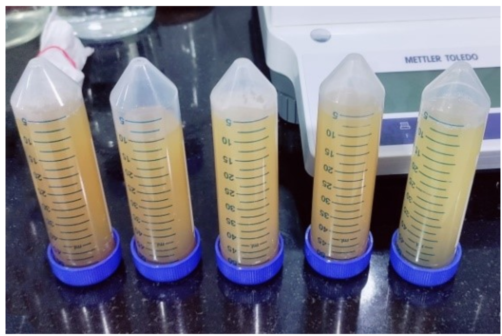


Figure 3.2 : 24 hour old pure isolated strains in MRS broth.

3.2 Catalase Test:

All isolated strains were catalase negative as shown in Figure 3.3. There was no gas bubbles formed and thus all isolates were selected for the next steps.

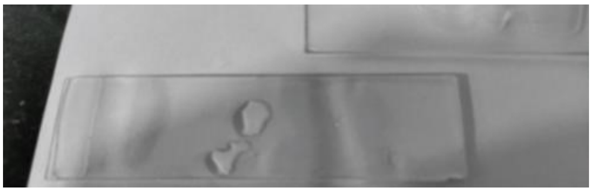


Figure 3.3 : Negative catalase tests.

3.3 KOH Test

The KOH test revealed that all 5 isolates were negative (figure 3.4) as they did not produce a viscousity/string like structure and thus were taken forward for further investigation.

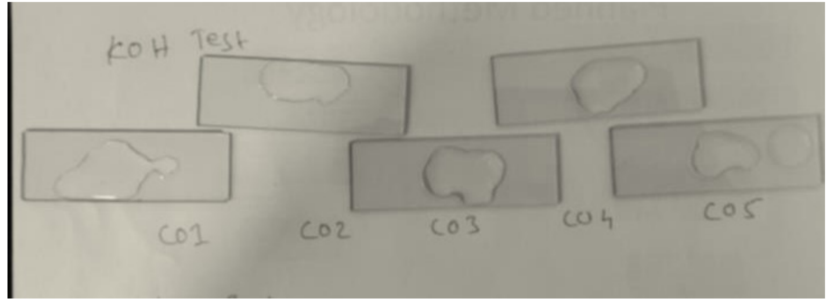


Figure 3.4: KOH test negative results

3.4 Acid Tolerance

After cells were incubated in acidic MRS broth for 3 hours they were transferred to MRS agar plate to assess survival. Only strains with tolerance to acid were able to survive and develop colonies (Table R1). After 24 hour incubation out of 5 isolates, colonies appeared on the solid medium for 3 strains while 2 were completely inhibited by acid and did't show any growth (Table R1). The three strains (CO-1.CO-2,CO-4) were thus considered for further analysis.

Table R1: Acid tolerance of selected strains after 24 hour incubation.

Strain number	Sample Name	**Appearance of Colonies after 24 hours**
1	**CO-1**	Yes
2	**CO-2**	Yes
3	**CO-3**	No
4	**CO-4**	Yes
5	**CO-5**	No

3.5 Bile Tolerance

Only the 3 strains tolerating acidity were tested for bile tolerance. After incubation in MRS broth containing 0.3% Bile for 3 hours they were transferred to MRS plate (Figure 3.5) to assess survival and occurrence or absence of colonies after incubation. The appearance of colonies after 24 hours of incubation was considered as positive result while the absence of colonies as negative result (Table R2). After 24 hour incubation out of three strains, colonies appeared on the solid medium for two (CO-2,CO-4) strains, while CO-1 was completely inhibited by Bile and did't show any growth. A negative control (MRS and PBS without sample) and *Lactobacillus acidophilus* IHC20047 a know strain tolerant to bile as positive control were kept.

Table R2 : Bile tolerance test of the strains CO-1, CO-2 and CO-4.

Strain number	Sample Name	Appearance of Colonies after 24 hours
1	CO-1	No
2	CO-2	Yes
4	CO-4	Yes

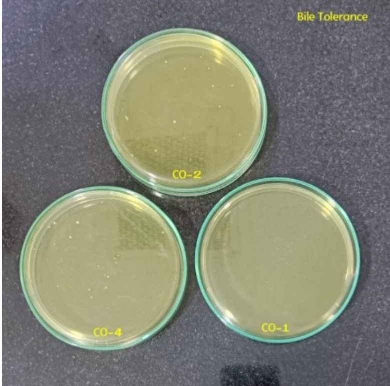


Figure 3.5: Colonies toleranting bile after incubation of bacteria in MRS agar + bile.

3.6 Temperature Tolerance

The temperature tolerance test was performed in MRS broth according to (Mulaw et al. 2019, with modifications). The tested temperatures were 41 °C and 45 °C. After 24 hours of incubation in water bath (Figure 3.6) at the desired temperature turbidity was evaluated. Only CO-2 and CO-4 isolates were considered for this analysis, because the other three failed in previous tests.

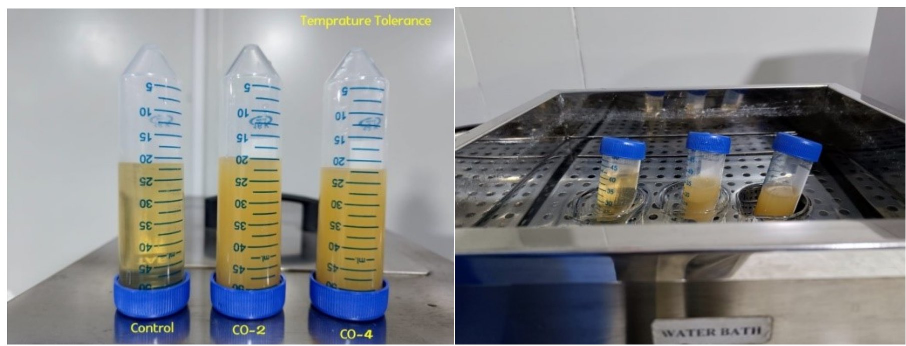


Figure 3.6: Growth of strains CO-2 and CO-4 at 45°C after 24 hours of incubation.

After incubation, the turbidity of the test suspension was compared with that of the control sample by evaluating the clarity of the lines on the a Wickerham card. Results are reported in Table R3.

Table R3 : Turbidity status for temperature tolerance test.

Sample Name	Growth at 41°C	Growth at 45°C
CO-2	Turbidity Observed	Turbidity Observed
CO-4	Turbidity Observed	Turbidity Observed

Both the samples were able to grow as demonstrated by the increased turbidity of the broth (Figure 3.6) at both the tested temperatures, thus were taken forward for further assessment.

3.7 Anti-oxidant assay

DPPH method was followed to screen the bacterial strains CO-2 and CO-4 for anti-oxidant activity as described in materials and methods. Among the two isolates CO-2 showed higher rate of scavenging activity when compared with CO-4 (Table R5) . Thus CO-2 was taken for further analysis.

Table R5 : Table indicating Absorbance and Rate of Scavenging Activity (RSA) of the strains CO-2 and CO-4.

Sample	Absorbance	Blank	Control	RSA %
CO-2	0.118	0	0.285	58.59
CO-4	0.192	0	0.285	32.63

The RSA % results are represented also in the Bar graph (Figure 3.7) for easy reference.

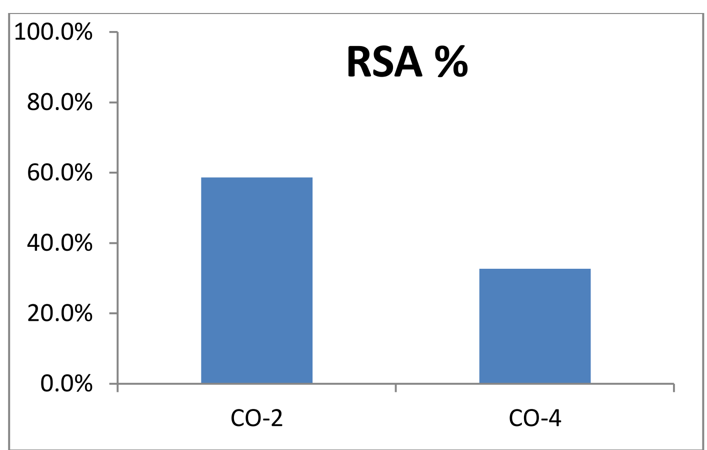


Figure 3.7: Rate of Scavenging activity of of the strains CO-2 and CO-4

The strain CO-2 has the highest DPPH scavenging activity and thus was evaluated for other characters.

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3.7 Hemolysis test on blood agar plate.

The strain CO-2 was streaked on Columbia blood agar and plate were kept in incubator 37 °C for 24 hours; the surroundings of colonies were checked for blood lysis haloes. No lysis was observed (figure 3.8).

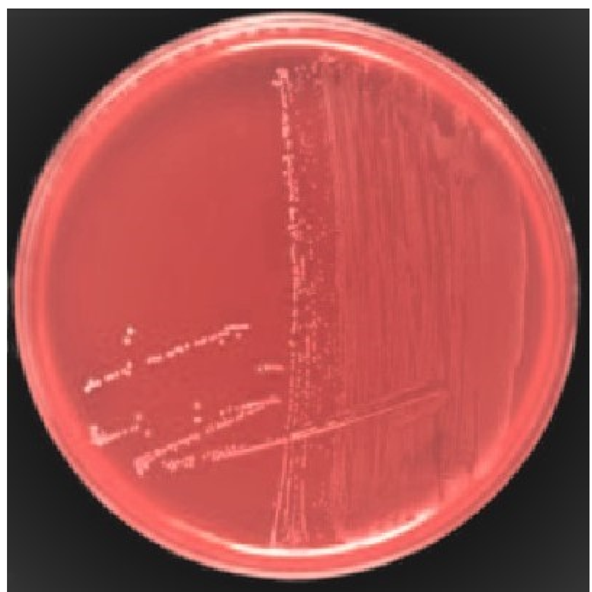

Figure 3.8 Growth of strain CO-2 on Columbia blood agar plate.

Staphylococcus aureus was streaked on the Columbia blood agar plates as a positive control and *Lactobacillus acidophilus* as negative control along with sample CO-2 (Figure 3.9).

After 24 hour incubation at 37 °C a clear yellow zone was observed around the the positive control while the non haemolytic ability (Figure 3.9) of the sample CO-2 was confirmed in comparison. Thus CO-2 was carried for further analysis.

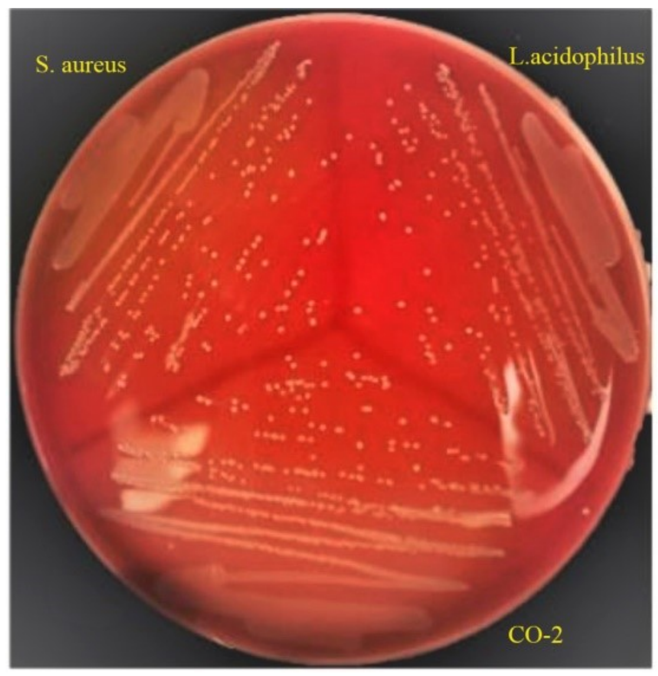

Figure 3.9 : Growth of CO-2 in comparison with positive control *(S. aureus*) and negative control (*L. acidophilus).*

3.8 Gram Staining

Gram staining was performed to determine the Gram status and the morphological characteristics of CO-2. The isolate CO-2 appears in violet color after Gram staining hence the strain was confirmed as Gram positive and with a rod shape (Figure 3.10).

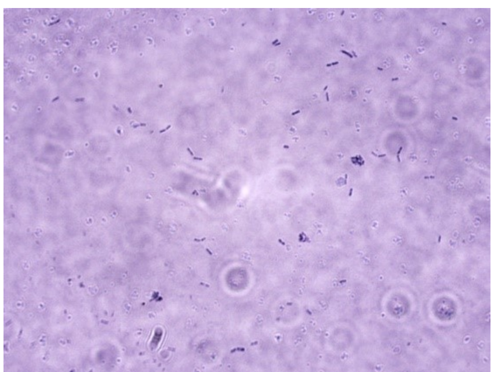

Figure 3.10 : CO-2 strain at the light microscope under 100X magnification after Gram staining procedure.

3.9 Identification of strain CO-2 based on sugar fermentation

Upon microscopic analysis for Gram staining we found CO-2 to be non-spore forming, Gram positive rods (Figure 3.10). Thus sugar fermentation analyses with glucose and mannitol were carried out. All observations were made after 48 hours. For each of the sugars, a negative control (without the sugar) was utilised to compare the colour change due to the acid production. The results are reported in the Table R6.

Table R6 : Phenotipic tests conducted with strain CO-2.

Phenotipic analyses	Result
Spore Forming	Non-Spore Forming
Acid Fast	Negative
Glucose	Fermentation Positive, Gas Production Negative
Mannitol	Fermentation Positive, Gas Production Positive
Possible identification	*Lactobacillus casei*

4.Discussion:

This study was aimed to isolate and characterize a strain of LAB showing probiotic and anti-oxidant capabilities which could be used in a formulation development and in a commercial production. These experiments were carried out at *R&D department of* PVS Laboratories Limited, Mangalagiri, Andhra Pradesh, India. This work is a part of a wider study conducted by a research team, working for the Company with the aim of isolating a new probiotic strain. The experiments were designed and carried out in the interest of the company to identify a suitable probiotic strain with good anti-oxidant properties, as the first step for the developing of a commercial product and/or for the inclusion in a formulation. Probiotic bacteria may reduce oxidative damage, and increase activity of crucial anti oxidative enzymes. Thus identifying and adding them to specific formulations can be very beneficial to the consumer. To increase a chance of identifying novel strains, a farm where no commercial starters and feed supplements were used, was identified. Mr.G.Suresh's farm in rural Vijayawada was visited and with the consent of farmer, a healthy non pregnant cow was selected for the collection of sample. Sampling and isolation was performed as a team effort.

Colonies were firsty selected and were assigned labels of convenience as (CO-1, CO-2, CO-3, CO-4,CO-5) and carried out the experimentation based on the F1. Methodology flow chart. All isolates were grown on specific selective medium (MRS) and were tested first for some probiotic potential to reduce complexity and increase efficiency. Only strains that possess tolerance to all tested stress as well as exhibit certain level of anti-oxidant activity were preliminary identified. At the end of experiments the CO-2 strain showed promising potential probiotic features such as tolerance to temperature, acid and bile and antioxidant characters. Although the species and phylogenetic relationship could not be accurately determined, based on some phenotypic characteristics, the isolate CO-2 could be classified as *Lactobacillus casei. Lactobacillus casei* is a gram positive, non-spore forming, rod shaped bacteria which is used as a probiotic and according to (Reale et al, 2015) *Lactobacillus casei* strains were able to grow at temperature higher than 45°C and are tolerant to 1.5% bile salts, pH 2.0 which coincides with the results obtained in this study. Further molecular

approaches such as 16S sequencing, RAPD and ERIC-PCR could be utilized to provide confirmation as well as more accurate identification of the isolated strain. Moreover additional studies will be carried out before the introduction into the market of such microrganisms: to check tolerance towards phenol and salt (NaCl) to confirm probiotic characteristics and explore uses in biopreservation and food industry. Antibiotic susceptibility test will be also performed to identify resistance and susceptibility of the isolated strain towards antibiotics . Antagonistic activity test will be also taken into consideration to assess the strain's inhibitory effect against pathogenic bacteria which is also one of the desired trait in a probiotic microorganism. *In-vivo* colonization pattern of the isolated strain will also be studied using model organisms for effective formulation development and to determine the benefits of probiotic supplementation essential for acquiring approval from the Regulatory agencies as well as gain credibility in the market.

5. Future Work

The aim of this work is to understand and asses various physiochemical properties of the probiotic strain, gain hands on experience in strain identification and also generates useful information for formulator in developing stable probiotic formula for bulk manufacturing. As part of future study, further identication and characterization using molecular approaches like 16S sequencing, RAPD and ERIC-PCR will be performed. .After sucessful identification, a suitable formulation will be studied as the strain needs to be viable for longer duration. Along with this, compatibility with other probiotic organism will be also studied as most of the probiotic supplement products contain more than one strain . Then suitable fermentation media which can give maximum growth in lesser time should be optimized. Studies on model animals can be carried out in order to know about the colonization pattern, based on which further formulation improvement will be pursued.

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7.Sitography

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