Chapter

Probiotic Supplement Improves the Health Status and Lactation Performance in Dairy Animals

Shakira Ghazanfar, Aayesha Riaz, Muhammad Naeem Tahir, Saad Maqbool, Ghulam Muhammad Ali, Fatima Tariq and Irum Arif

Abstract

Probiotics are essential for the effective growth of beneficial bacteria present in enteric line. They help in the physiological functions of new-born calves that are highly susceptible to a variety of fatal syndromes. The criterion for the selection of strains for the design of probiotic products are based on retaining functional health characteristics. Samples from Nili-Ravi buffaloes were collected, and rumen strains are identified for probiotic product. Microscopic techniques with different biochemical tests and molecular techniques such as BLAST have performed for identification. Following species of *Weisella* has been identified based on genotypic analysis (16S rRNA) under accession number MK336765 (F2) and MK336779 (F4) in the NCBI GenBanK. The strains sharing some of the specific properties evaluated were identified genetically, and their compatibility and exopolysaccharide production were assayed. All of this will be helpful in the production of multi-stain-probiotic product for the nourishment of dairy calves.

Keywords: calves, lactic acid bacteria, probiotic, rumen, product

1. Introduction

The innovative development in the dairy industry is possible only due to scrupulous research, nutrition, genetics, and management strategies and its oriented implementation. The high risk of contagion is due to occasional bouts and improper feed of nutritional contents which become the ultimate cause of debility and economic and resource loss. To avoid the prevalence of such harms on dairy animals' proper nutritional content, management of hygiene adoption is required [1, 2]. For this, a term is defined in the 1960s which is "probiotic," which is a curious mixture of Latin (pro = for, in favor of) and Greek (bios = life). Probiotic which is discovered by Elie Metchnikoff in the early twentieth century is defined as "Live microorganisms which when administrated in an adequate amount to organism body confer a health benefit on the host and alter the gastrointestinal tract flora into the beneficial form" [3]. The nature of probiotics is on the basis of human, animals, and plants [4]. But, here we will focus on the probiotic types of animals because we are dealing with dairy animals.

Microbial infections which become the cause of mortality in dairy animals are animals scouring at early stage and perturbation in microbial GIT and the most enteric infections caused by *Escherichia coli*, *Clostridium perfringens*, *Salmonella*, and some *Streptococcus* and *Staphylococcus* species [5]. The major microbial density is present in the reticulum, rectum, and colon mostly. So, to eradicate the prevalence and outcomes of these infections and to nourish the local microbiota of the gastrointestinal tract. Due to the indiscriminate use of antibiotics, antibiotics resistance has become dominant characteristics in microorganisms [6]. Increase in the dissemination of antibiotic resistance genes is reducing the therapeutic possibilities in infectious disease. So, in order to alleviate the problems associated with the antibiotic use, a number of replacement have been proposed, and one of them is the effectiveness of probiotics [7].

Probiotic microbiota-based feed supplements are used to combat major enteric infections [8]. So, different types of probiotics strains are used for making the GIT congenial for proper health and growth. These probiotics strains are collected from a

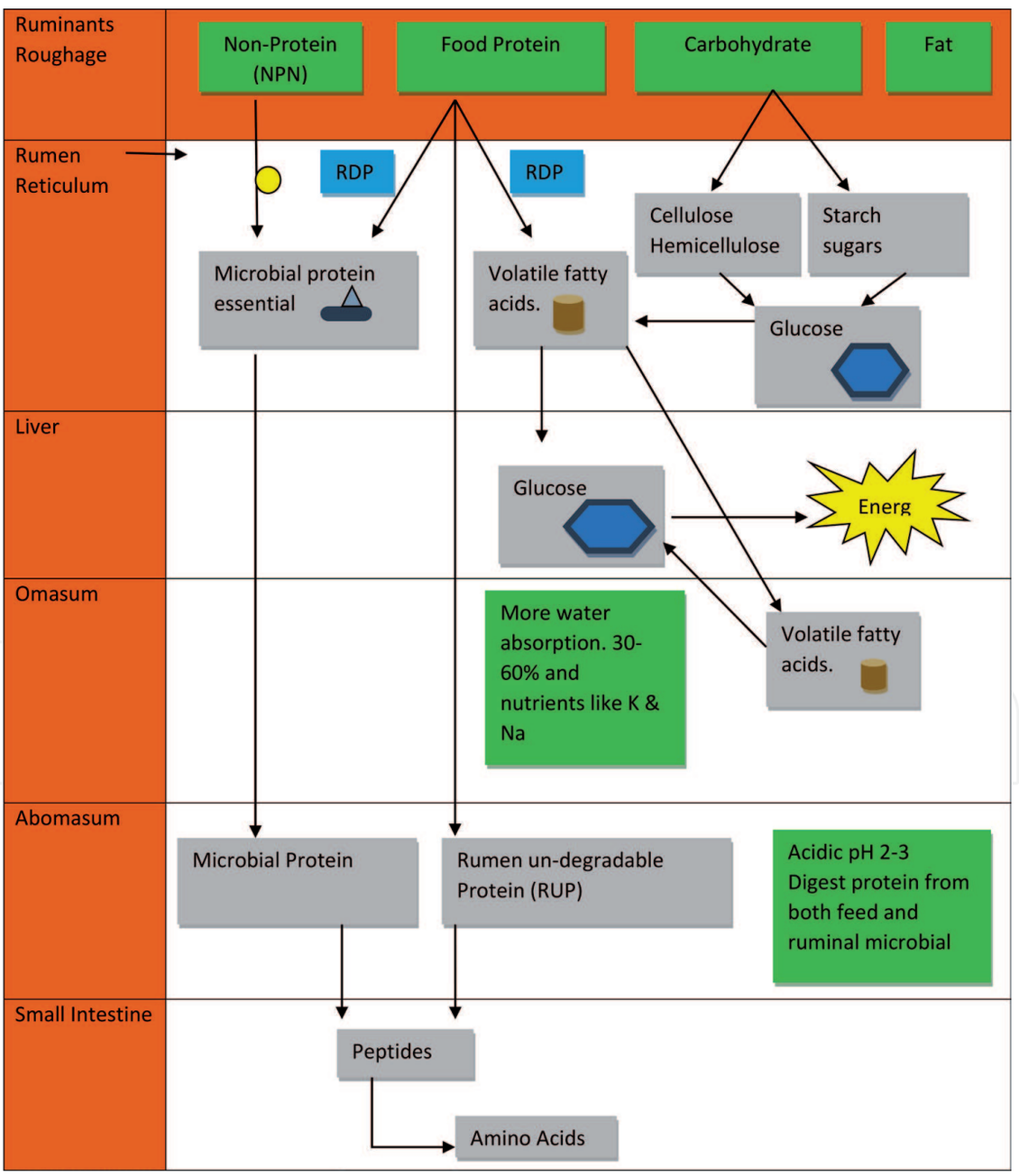

Figure 1.
Impact of the Probiotics on the GIT of dairy animal: The microbial flora degrades the feed and improve feed intake and ultimately improve milk production.

different source of host such as feces, milk, and directly from GIT. Probiotic bacteria produce protein segments or polypeptide bacteriocins which reduce the growth of harmful bacteria [9]. Probiotics help to prevent and control gastrointestinal pathogens and improve the performance and production of animals through various biochemical mechanisms. Closely related strains may differ in their mode of action [10]. Increased nutrient digestion in the diet may be due to the speed-up of enzyme activity in the intestine due to probiotics [11]. *Lactobacillus* probiotics altered the digestive enzyme activity in the GIT of dairy animals and enhanced the growth rate [12]. However, there is no change in proteolytic and lipolytic activity of the animal's digestive enzyme activity. This improvement in amylase activity is associated with a 4.6% increase in body weight gain and 5% improvement in feed use efficiency [13]. Probiotics increased the height of intestinal villi and villus height crypt ratio in dairy animals, thus increasing the surface area for nutrient absorption [14, 15].

The rumen has complex integrated microbial ecology which degrades the ingested polysaccharide and proteins resulting in short-chain fatty acids which are further used by a host as energy and protein source [16]. The probiotic concept was raised around 1900 which is hypothesized by Elin Metchnikoff, and later he was convinced that yoghurt contained the organisms which are necessary for protecting the intestine from the damaging effects of other harmful bacteria [17]. The first clinical trials were performed in the 1930s. In the 1950s, a probiotic product was licenced by the United States Department of Agriculture as a drug for the treatment of scouring (*Escherichia coli* infection) among pigs [18]. In 1994, the World Health Organization deemed probiotics to be the next most important immune defence system when the commonly prescribed antibiotics are rendered useless by antibiotic resistance, altering the natural mechanism of the body [19] (**Figure 1**).

2. Microbial composition of the GIT of dairy animals

The most common organism used in the vital preparation of probiotics in the lactic acid bacteria (LAB) is highly effective because it is also the natural flora of organism GIT system and it is regarded as safe in the words of US FDA [20]. Microorganisms other than LABs which are currently used in probiotic preparation are *Bacillus* sp. and yeasts (*Saccharomyces cerevisiae* and *S. boulardii*). Different species of *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* are used for probiotic preparation with fructooligosaccharides (FOS). The probiotic products are in the form of spray, pastes, tablets, powder, and capsules [21].

3. Selection of probiotics to improve milk yield

The following abilities should be manifested by bacteria used as lactic acid bacteria:

- It should exert a beneficial effect on the host's life and metabolic activities.
- It should withstand into a foodstuff at high cell counts and remain viable throughout the shelf life of the probiotic-containing product.
- It should withstand through the GIT tract and help in colonization of beneficial bacteria.
- It should adhere to the intestinal epithelium cell lining.

Common bacterial type of probiotics			Yeast probiotics
***Lactobacillus* sp.**	***Bifidobacterium* sp.**	***Enterococcus***	***Saccharomyces***
L. acidophilus	*B. bifidum*	*E. faecalis*	*S. cerevisiae*
L. casei	*B. infantis*	*E. faecium*	*S. boulardii*
L. bulgaricus	*B. longum*		
L. fermentum	*B. animalis*		
L. lactis	*B. thermophilum*		
L. plantarum			
L. brevis			

Table 1.
Most Common species of LAB'S in animal probiotic preparation.

- It should stabilize the intestinal microflora and be associated with the health benefits.
- It should contain viable cells at the time of consumption.
- It should reduce symptoms of lactose intolerance.
- It should enhance the functionality of the immune system and enhance the bioavailability of nutrients.

These strains are used for the preparation of probiotics with or without FOS (**Table 1**).

4. Physiology of dairy animal's digestive system

The primary roles of the gastrointestinal epithelium (GE) are to shield the host from the mixture of pathogenic microorganisms, toxins, and chemicals in the lumen and to prevent unregulated movement of these compounds into the lymphatic or portal circulation [22]. The GE continuously endeavors to enhance nutrient absorption. Careful consideration of gut health—promoting the action of a particular nutrient or feeding strategy—is important. Food goes down to the reticulorumen from the esophagus, and this is like a fermentation chamber which converts plant carbohydrate to volatile fatty acids, lactate, hydrogen, and methane which are used by the ruminant host. In ruminants, process starts with the peptic digestion in the abomasum [23]. The digestive system of the rumen is composed of the first reticulum then rumen, then omasum, and finally abomasum. The rumen is a complex biological system which is like a fermentative vat where nutrients are consumed by different organisms. Energy from forages are acquired by ruminants through fermentation process which is done by microorganisms by different enzymatic activities [24]. Different factors including pH, temperature, osmotic pressure, buffering capacity, and redox potential affect the activity and growth of rumen microorganisms [25]. Different environmental and physiological conditions determined these prime factors. The normal temperature of the rumen is in the range of 39–39.5°C, but as fermentation occurs after food intake, the rumen generates heat which increases temperature up to the limit of 41°C. pH is affected by short-chain fatty acid production, feed intake level, as well as exchange and absorption of ions like phosphate and bicarbonate [25] (**Figure 2**).

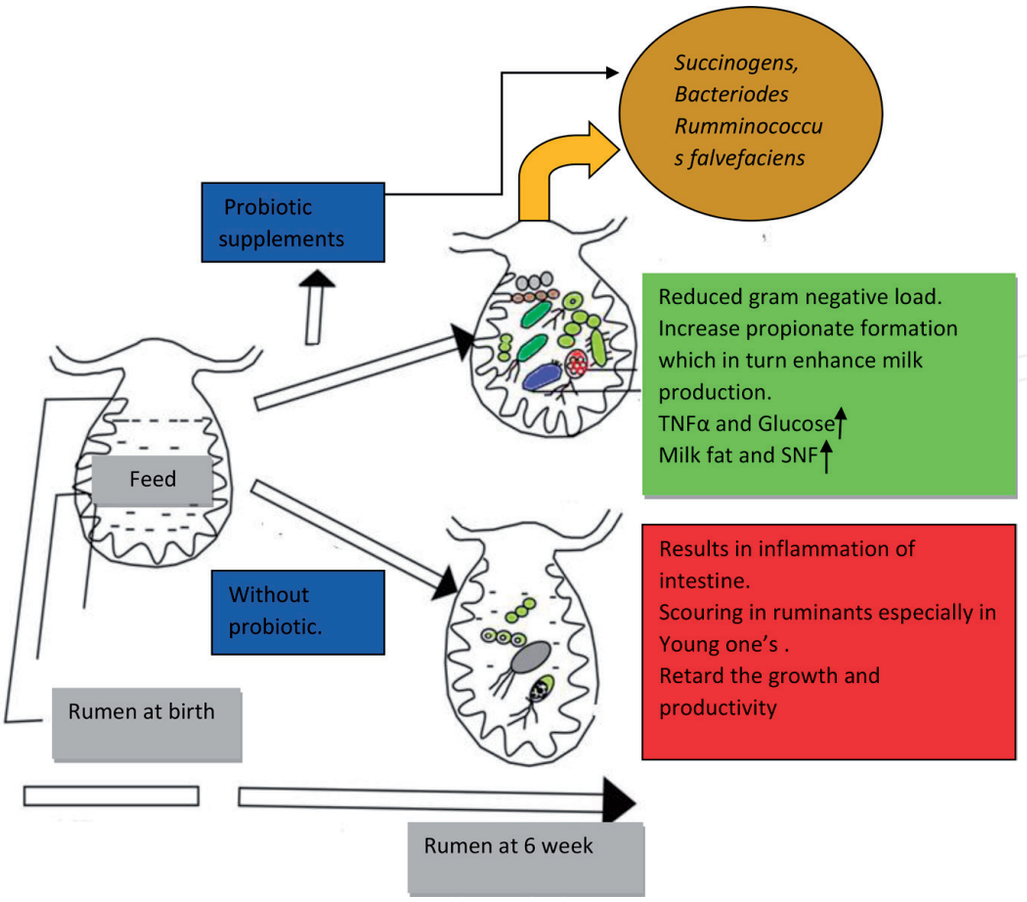

Figure 2.
Effect of probiotic on the development of the microbial flora in newborn calves.

5. Rumen microbiology

Different bacteria are present in the rumen, and they are more in ratio than other microbes which include *Megasphaera elsdenii*, *Lactobacillus ruminis*, *Streptococcus bovis*, *Fibrobacter succinogenes*, *Prevotella*, *Bacteroidaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae*, *Succinivibrionaceae*, and *Veillonellaceae* [26]. There are a total of five groups of bacteria: 1, free living in liquid phase; 2, loosely attached with feed; 3, firmly attached with feed; 4, attached with rumen epithelial lining; and 5, attached with protozoa/fungi.

6. Culture-based method to develop the indigenous probiotic feed to improve milk yield in dairy animals

We have finalized the simple protocols that will guide researchers in identifying the most ideal probiotics for animal use to improve milk yield. There are two methods which have been utilized till now for the identification and characterization of the microbial flora, i.e. culture-dependent method and culture-independent method. Milk products own the major economic importance all over the globe especially in countries where agriculture and livestock cover the major area of industry. The milk we consume is derived from cattle, buffaloes, goats, sheep, and camels that come under categories of ruminants. And 99% of this milk is produced from ruminant [1].

LABs as feed supplements can help in improving the milk quality and quantity in lactating dairy buffaloes [27]. The literature showed that the species-specific probiotic can improve the host performance in a better way than the nonspecific. In our lab, we have isolated and molecularly characterized the bacterial strains that are basically animal origin probiotics. We used the culture-dependent method to isolate the animal probiotic-bacteria strains.

7. Experimental proof

7.1 Experiment no. 1: isolation, identification, and characterization of LAB from the gut of dairy lactating buffalo

Three healthy lactating *Nili-Ravi* buffaloes raised at NARC, Islamabad, Pakistan, were randomly selected for sampling. A sample was taken fresh from deep rumen with the hand using aseptic techniques. Samples were transported to the laboratory under controlled conditions for further processing. For pure isolates, 1 g of feces sample was diluted in PBS (phosphate buffer saline). Commercially available MRS (De Man, Rogosa, and Sharpe) agar media plates were inoculated with diluted fecal samples and incubated at 37°C for 24 h. Initial screening was done by using the basic microbiological methods. For that purpose, colony morphology was examined, and gram staining was done. For complete morphology scanning electron microscopy was performed. Common biochemical tests like catalase and oxidase were done. For molecular identification of the isolated strains, we used the PCR. The pure cultures were subjected to a polymerase chain reaction for amplification of DNA. Amplified products were sequenced and identified at the species level, and a phylogenetic tree was constructed. A total of 30 bacterial strains was isolated from buffalo gut. These were mostly gram-positive and catalase-negative bacterial strains. We noted that very important bacterial strains were isolated from buffalo gut (**Table 2**).

Gram staining showed that isolated strains were either gram-positive rod or gram-positive cocci, as the strains retained primary stain (crystal violet) that is one of the major characteristics of LAB (**Figure 3**). The strains appeared as a single cell or in the form of short chains or small clusters under a microscope. The colony on MRS agar was round, irregular with a smooth shiny surface, cream in color, and with entire or convex margins. If we talk about elevation and opacity, most of gram-positive colonies were raised and opaque (**Figure 4**).

The isolated strains were further subjected to biochemical characterization. We performed a catalase test. I took an isolated colony using a sterilized toothpick and mixed with a drop of hydrogen peroxide and noted the bubble formation. Many strains resulted in negative result and few were positive. I noted that the negative results were of the strains that retained crystal violet stain during gram staining, i.e. those were gram-positive rods or cocci. Selected strains were identified on a molecular level by blasting the amplified DNA using the BLAST tool at the National Centre for Biotechnology Information (NCBI) website. And the strains F2 and F4 were identified as different species of *Weisella*, on the basis of genotypic analysis. These 16S rRNA sequences were submitted to the NCBI GenBank under the accession numbers MK336765 and MK336779 assigned to strains F2 and F4, respectively (**Table 3**).

7.2 Phylogenetic analysis

Phylogenetic trees of the strains were constructed to see the closely related species of the strains (**Figure 5**). We found the following results.

Selected bacterial strains	Colony characteristics								Biochemical characteristics
	Gram staining	Shape	Form	Surface	Color	Margin	Elevation	Opacity	Catalase
F1	+ve	Rod	Round	Smooth/shiny	Cream white	Entire	Raised	Opaque	−ve
F2	+ve	Rod	Round	Smooth	Cream	Entire	Raised	Transparent	−ve
F3	+ve	Cocci	Circular	Smooth/shiny	Cream white	Convex	Slightly raised	Opaque	−ve
F4	+ve	Curved Rod	Round	Shiny	White	Entire	Raised	Moist	−ve
F5	+ve	Cocci	Circular	Smooth	Pinkish white	Entire	Raised	Opaque	−ve
F6	+ve	Cocci	Round	Smooth/shiny	Cream white	Entire	Raised	Opaque	−ve
F7	+ve	Rod	Round	Smooth	White	Entire	Convex	Translucent	−ve
F8	−ve	Rod	Round	Smooth	Cream	Convex	Raised	Opaque	+ve
F9	−ve	Rod	Circular	Shiny	Cream white	Entire	Slightly raised	Opaque	+ve
F10	+ve	Cocci	Round	Smooth/shiny	cream	Entire	raised	Opaque	−ve

Table 2.
Morphological, biochemical identification of bacterial isolates on MRS agar.

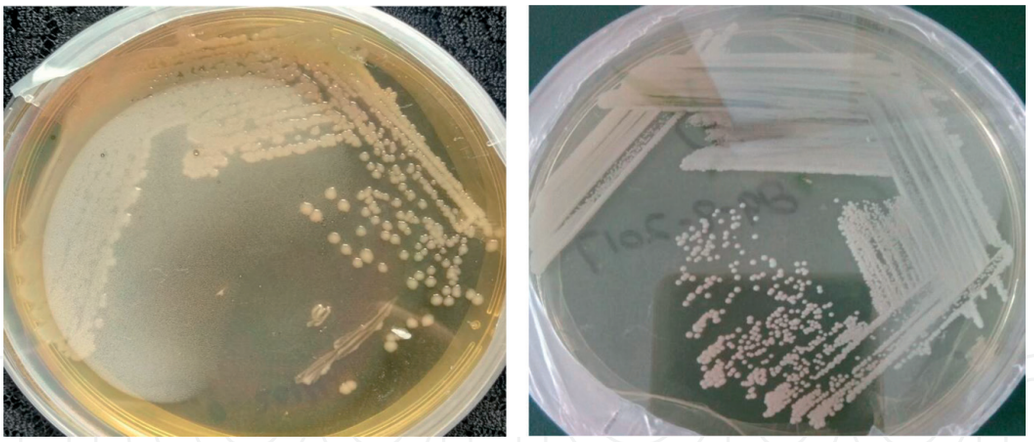

Figure 3.
Colony morphology of strain F2 and F4 isolated from animal gut.

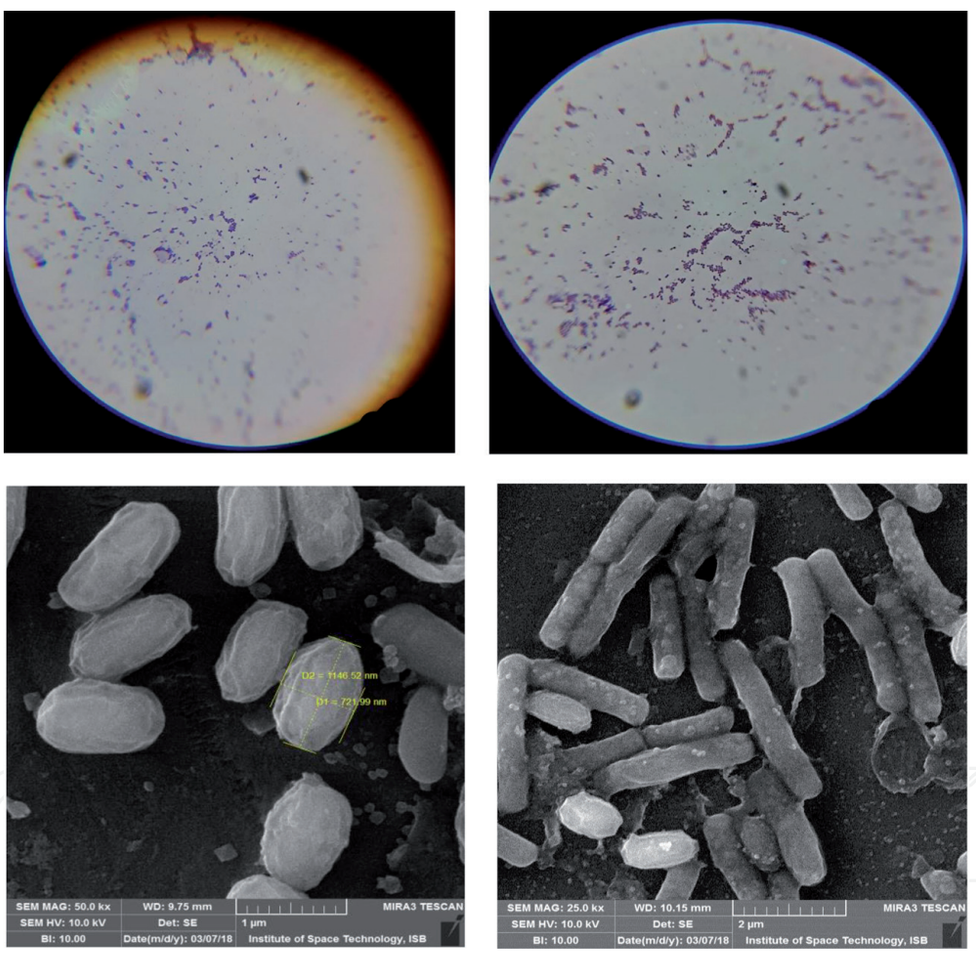

Figure 4.
Gram staining and electron microscopy of strain F2 and F4 isolated from animal gut.

7.2.1 Weisella species

The strain similarity was found using NCBI BLAST; *Weisella* MK336780 (NMCC-M14) has similarity with *Weisella* JX1880721 (AB13), and *Weisella* MK336765 (NMCC-M11) has high similarity with *Weisella* JX1880721 (AB13).

7.2.2 Staphylococcus species

The strain similarity was found using NCBI BLAST; *Staphylococcus* MK355570 (NMCC-path-2) has high similarity with *Staphylococcus aureus* strain DSTNMRM17,

Strain ID	Strain name/ genus	Accession number	Closely related valid published species	Similarity % of 16S rRNA gene sequencing
F2	*Weisella*	MK336765	*Weisella confusa* strain AB13	97%
F4	*Weisella*	MK336779	*Weisella confusa* strain AB13	96.5%
F5	*Staphylococcus*	MK355570	*Staphylococcus aureus* strain DSTNMRM17	98%
F6	*Staphylococcus*	MK355562	*Staphylococcus aureus* strain YT-3	99%

Table 3.
Phylogenetic tree of the F2 and F4 isolated from dairy animals on 16S rRNA gene sequence.

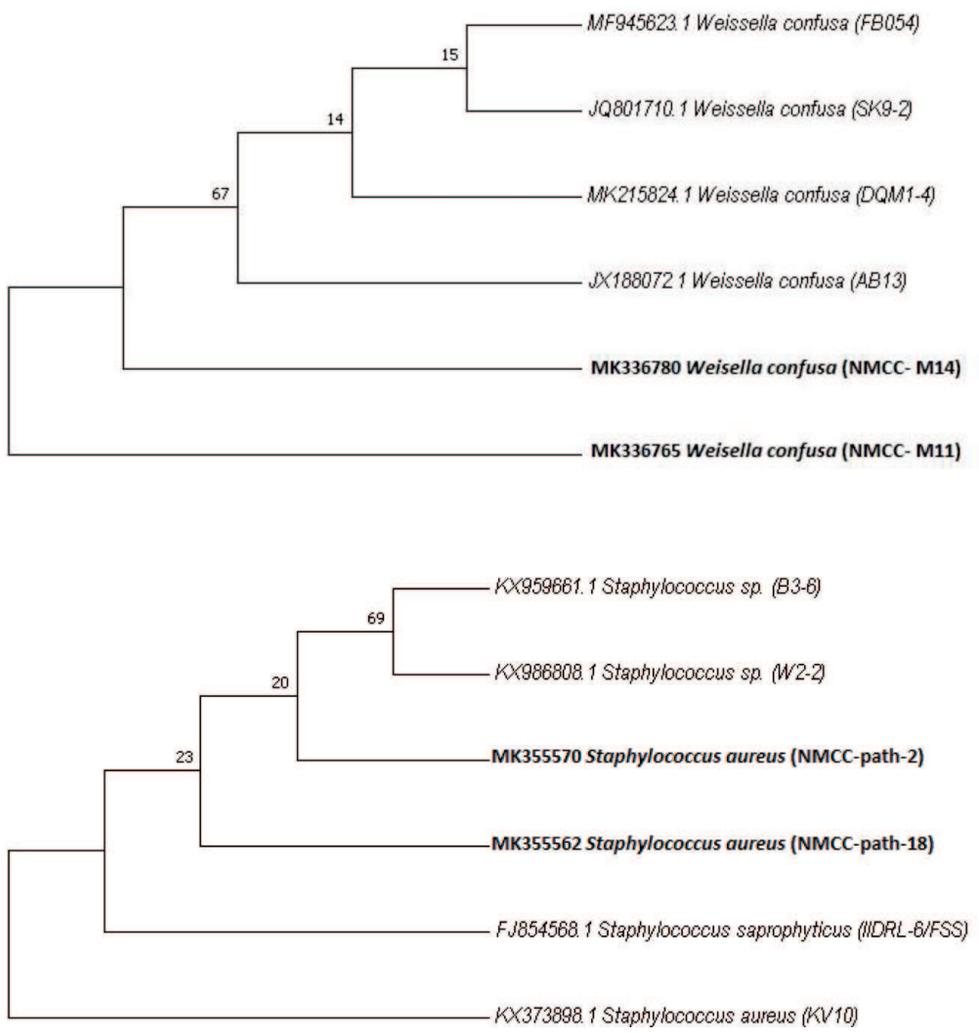

Figure 5.
Phylogenetic tree of Weisella confusa, and S. aureus isolated from animal gut.

and *Staphylococcus* MK355562 (NMCC-path-18) has high similarity with *Staphylococcus aureus* strain YT-3.

7.3 Experiment no. 2: determination of probiotic potential

Selected strains were subjected to further testing to determine the probiotic potential. Different tests like bile tolerance activity, cholesterol assimilation test, antimicrobial activity, and antibiotic susceptibility test. Bile tolerance activity was

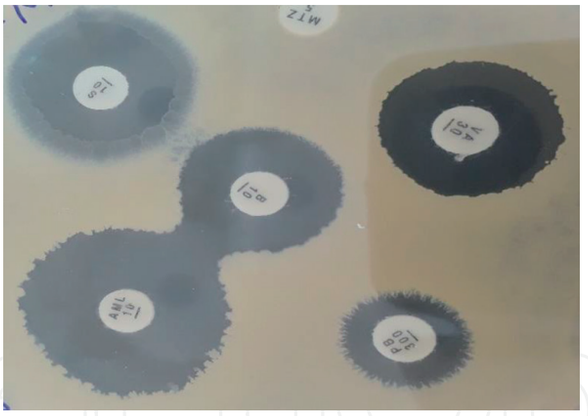

Figure 6.
Antibiotic susceptibility testing of strain F2isolated from animal gut.

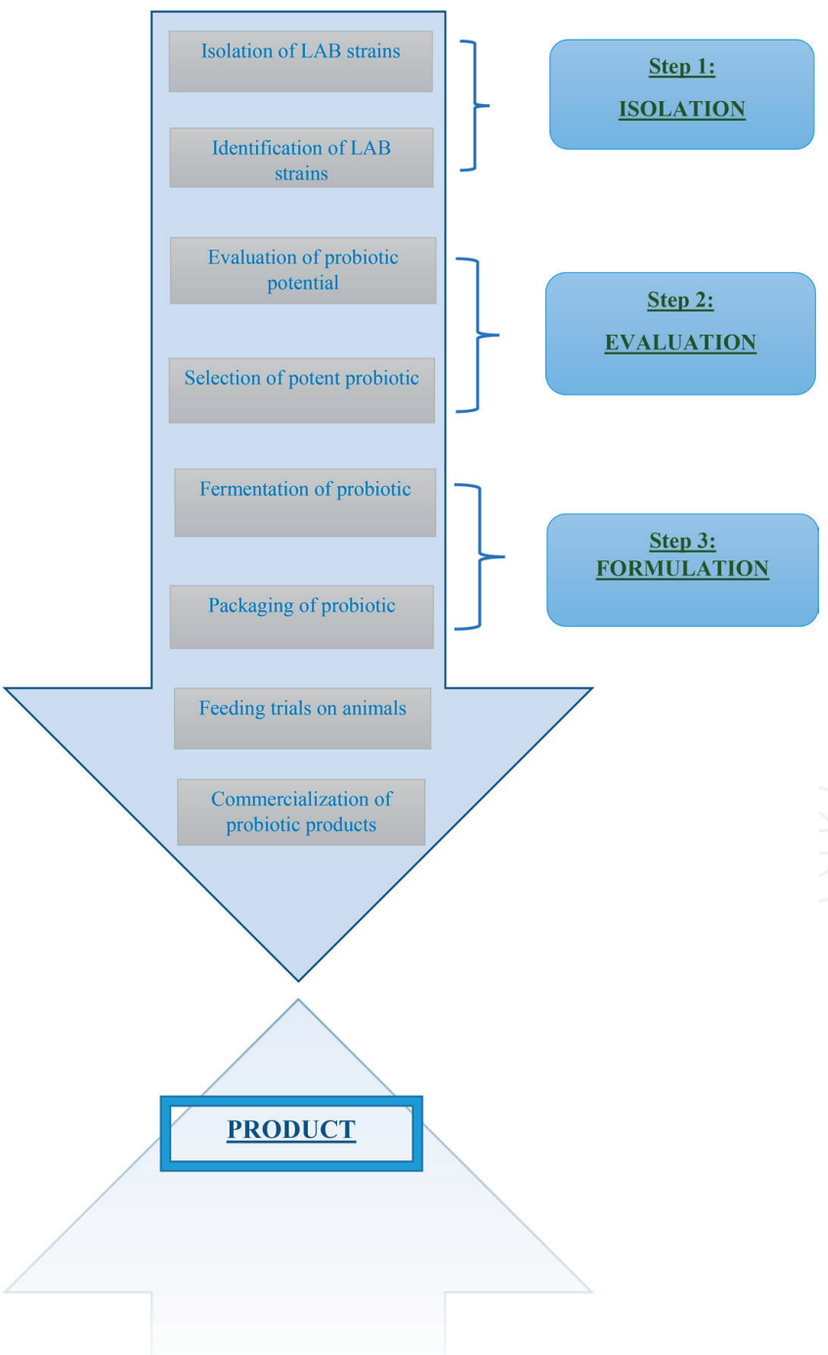

Figure 7.
Steps involved in the preparation of animal probiotic product development.

performed by inoculating the two selected strains on sterilized TSB (tryptic soy broth) in Erlenmeyer flasks incubated at 37°C in shaking incubator, 150 rpm for 24–48 h. Stock solutions of bile salts and lysozyme were added after incubation. The pH of the solution was adjusted to 3. Control was kept aside. After intervals of 30 min, samples were inoculated on TSA (tryptic soy agar) after serial dilution and incubated for 24–48 h at 37°C. After incubation, CFU was determined, and the tolerance rate was analyzed (**Figure 6**).

The livestock sector is mostly based on traditional lines which lead to unbalanced nutrition resulting in poor growth and productive performance in dairy animals. Nowadays, increasing the performance of dairy animals through the use of probiotics has become a useful and economical method to overcome the effects of malnutrition. The use of probiotic yeast enhances nutrient utilization, which may lead to improved performance and increased immunity in dairy heifers. Literature reveals that suitability and profitability of the probiotic yeast depend on many factors including animal breed, age, and probiotic strains. From this line of research, we look forward and develop a new probiotic yeast strain for our local breed, which provides a positive effect on milk yield and fat contents in lactating dairy cattle and moreover is cost-effective. At the same time, the dietary supplementation of probiotic yeast could also have an enhancing effect on the microbial balance of the GIT that leads to improved growth, health, and production performance in a dairy animal (**Figure 7**).

In the situation of a high feed cost, probiotic gives a useful nutritional strategy which allows increasing diet digestibility and consequently enhances the performance parameters of dairy animals in a cost-effective manner. Future research is needed to see the impact of the yeast cells in the GIT of the dairy animals. Future research will also need to address the behaviour of the yeast cells in the digestive environment. We look forward to the development of the new probiotic strains, which will hopefully mean that the rumen microbiologist in Pakistan instead of following the nutritious in an exploratory mood as has been the role for so long, will instead lead advances in ruminant nutrition in a year to come.

8. Recommendations

The recommendations are outlined as follows:

- For the preparation of the probiotic the sampling, the source should be indigenous/local-based.
- Internationally validated molecular methods should be used to identify the microbial strains.
- The probiotic, as well as genetic properties of the probiotic strains, should be studied. Good manufacturing practices must be applied with quality assurance, and shelf life conditions must be established, and labelling must be made clear to include minimum dosage and verifiable health claims.

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