Probiotic Characteristics of *Ligilactobacillus salivarius* AS22 Isolated from Sheep Dung and Its Application in Corn-Fox Tail Millet Silage

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**Abstract:** The forage crops corn (*Zea mays*) and foxtail millet (*Panicum italics L.*) are widely used as animal feed because of their high nutritive values. The ensiling of corn and foxtail millet is often associated with the growth of undesirable microbes, which cause severe loss of dry matter content during the storage periods. The selection of suitable *Ligilactobacillus* species for corn-fox tail millet silage production can improve the quality. In this study, we aimed to select potent lactic acid bacteria (LAB) from sheep dung and analyses their biological application such as probiotic features, antimicrobial activities and fermentation capability of silage. A total of nine *Lactobacillus* strains were inoculated in MRS medium to evaluate lactic acid concentration. The isolated strain, *Ligilactobacillus salivarius* AS22, produced a higher lactic acid level (40.2 ± 30 µg/mL) with high growth rates (2.2 ± 0.12 OD at 600 nm) compared to other strains. The silage treated with inoculant (*L. salivarius* AS22) decreased the pH value (*p* < 0.05) and enhanced lactic acid production (*p* < 0.05) than the control at ensiled silages. LAB inoculated silage had reduced numbers of fungal colonies on control (*p* < 0.05). In conclusion, the addition of *L. salivarius* AS22 improved the quality of whole corn and foxtail millet silages with significant probiotic potential.

**Keywords:** corn; foxtail millet; lactic acid bacteria; silage; lactic acid; probiotic potential

1. **Introduction**

Silage has rich nutritive content which is used as the feed for ruminants. The *Hordeum vulgare*, *Secale cereal*, *Lolium multiflorum*, *Zea mays* are important forage crops, used for the production of silage for livestock. *S. cereal* has been considered a highly valuable forage crop in silage production. *S. cereal* is an annual plant, overwintering, frost-tolerant, they are able to grow in winter with few daylight hours and low temperatures below 30 ± 2 °C. Generally, *S. cereal* is harvested before the heading is suitable for making silage. After the heading stage, the biomass of cereal enhanced over 30% lignification of cell wall generally enhanced and caused various problems in the digestibility and fermentation process [1]. The application of highly nutritive silage with the lower butyric acid and higher lactic acid content has been considered the basic criteria for high-quality silage production [2].
Lactic acid bacteria are Gram-positive bacteria that produce lactic acid, a key positive indicator by fermentation. The *Lactobacillus, Ligilactobacillus, Lactobacillus, Leuconostoc, Streptococcus, Oenococcus, Enterococcus* and *Lactococcus* have produced a significant level of lactic acid [3]. The genus *Lactobacillus* has more than 100 species and various subspecies. These bacteria are rod-shaped, chains, aero-tolerant, fermentative; grow well under anaerobic conditions which have the potential for carbohydrate fermentation. The genus *Lactobacillus* has been reclassified recently based on core genome phylogeny, clade-specific signature genes; pairwise average amino acid identity, physiological characters and the ecological criteria of the bacteria. Based on this polyphasic approach, the genus *Lactobacillus* has been classified into 25 genera including the emended genus *Lactobacillus* (host-adaptive organism, *Paralactobacillus* and *Lactobacillus delbrueckii* group) and other 23 novel genera such as, *Lapidilactobacillus, Companilactobacillus, Bombilactobacillus, Amylolactobacillus, Holzappelfia, Lacticaseibacillus, Loigolactobacillus, Schleiferilactobacillus, Agrilactobacillus, Lactiplantibacillus, Lignilactobacillus, Liquorilactobacillus, Dellaglioa, Latilactobacillus, Acetilactobacillus, Fructilactobacillus, Limosilactobacillus, Paucilactobacillus, Furfuriilactobacillus, Lentilactobacillus, Secundilactobacillus, Levilactobacillus and Apilactobacillus* [4].

LABs in the plants are often heterofermentative and low in number which is not sufficient to initiate the fermentation of silage successfully. It increases the ratio of lactic acid into other metabolites such as acetic acid, and ethanol, in general, homo-fermentative bacteria could not be able to convert lactic acid into other products therefore they sustained a high level of the lactic acid in silages, a positive indicator of silages quality [5]. During the growth phase, most of the forage crops are soft targets to several pathogenic fungi, most of the organisms produce mycotoxins. These include *Claviceps, Cladosporium, Alternaria* and *Fusarium* sp. [6], *Penicillium viridicatum* produces ochratoxin A, *Aspergillus parasiticus* and *A. flavus* produce aflatoxin, while *Fusarium* spp. produce fumonisins, zearalenone and trichotheceenes [7]. Aflatoxin B1 is involved in ion deregulation, lipid peroxidation, inhibition of protein synthesis and DNA damage [8]. The lignin is one of the major components of the plant cell wall and is widely considered to be a limiting factor in the digestion of polysaccharides of the cell wall in the rumen of ruminants [9]. The lignin content of the plant cell wall negatively influences forage digestibility. An enzyme, ferulic acid esterase cleaves the ester linkage of the plant lignin and increases the digestibility of silage. The many subspecies of *Lactobacillus* produced ferulate esterase [10]. The application of LAB in silage production has increased the stability and improved the fermentation process [11]. The bacteria such as *Pediococcus, E. faecium* and *L. plantarum* are homofermentative types and are widely used to control silage fermentation by increased production of lactic acid that reduces the pH level [12]. The LAB strains inhibit or reduce the growth of fungi by the production of several organic acids [13].

Many methods have been proposed to preserve the haylage/silage for long time storage [14], particularly, an ensiling method is a process that involves majorly in silage production with enriched nutrients for a long time. It preserves the raw plant materials based on spontaneous lactic acid production under controlled fermentation in anaerobic conditions and has been used for long decades to preserve the various roughages with enriched nutrients [15]. The prime goal of silage preparation is to maintain the original quality of the harvested crop at the maximum limit. For several decades many additives have been recommended to improve the fermentation process through enhancing the lactic acid content of the silage [16]. The addition of LAB to the forage during ensiling is intended to confirm rapid and vigorous fermentation as results in faster accumulation of lactic acid and reduction in pH level and thus inhibiting undesirable microbial growth [15,17]. Many reports claimed that LAB is considered the potent inoculant for silage production due to its advantages because LAB utilizes water-soluble carbohydrates and produces organic acids, which enhances the acidification of silages and is preserved for a long time [18,19].

LAB in foodstuffs enhanced the storage life of fermented products, increases the nutritional quality and beverages by improving antimicrobial properties. These LABs were isolated from various natural sources and foods, including fish waste, cattle faces, poultry
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farms, solid, sugar cane plants, dairy products and fermented foods. Moreover, LAB screened from the mammalian gut is highly relevant to the activity in mammals than LABs that are isolated from other sources [20]. *Ligilactobacillus salivarius* (*Lactobacillus salivarius*) strains can tolerate host antimicrobial defense systems and inhibit various bacterial pathogens demonstrates the adaptation of this bacterial species to the gastrointestinal tract. The probiotic potential of *L. salivarius* has been extensively reviewed [21]. Legumes generally have thick fibrous substances that become least digestible in the case of mature crops. LAB strains are widely used in the silage process to improve the fermentation process of the fibrous stem of the legumes and enhance the feed value of livestock [21]. The utilization of potential LAB with very low-quality roughage is highly effective in combination with other nutrient-rich materials. Molasses is widely used to improve the quality of crop residues and provides various easily digestible water-soluble carbohydrates [22]. LAB derived from animal sources may provide a good approach for silage preparation. LAB in silage not only improve the silage quality but also useful to enhance animal performance [23]. Therefore, we planned to isolate efficient lactic acid bacteria from animal sources and studied their biological potential including probiotic features, antimicrobial and enhancement of corn and foxtail millet silage fermentation.

2. Materials and Methods

2.1. Samples

A sheep dung sample was collected in the morning using a sterile spatula and transported to the laboratory in an ice box for the isolation of LAB. The one gram dung sample was homogenized with 10 mL sterilized distilled water using a homogenizer and filtered by muslin cloth and used for LAB isolation.

2.2. Isolation of LAB Strains

Ten-fold serial dilution was performed with distilled water and the diluted samples (100 µL) were spread onto a Man Rogosa Sharpe (MRS) agar medium (Himedia, Mumbai, India). The plates were incubated at 28 ± 2 °C for 48 h. LAB colonies were picked from the plates based on their morphology and appearance and streaked onto a BCP medium (Bromocresol purple medium) to confirm the identity of LAB. The growth of isolated LAB was monitored in MRS broth at 28 ± 2 °C [2].

2.3. Determination of Organic Acid and Enzyme Activities in the Cell-Free Supernatant

The isolated strains were inoculated in MRS broth in a 1000 mL glass bottle containing butyl rubber stoppers with aluminum crimp seals, and then the air was evacuated using a vacuum machine. The bottle was incubated at 28 ± 2 °C for 24 h. The cell-free supernatants were collected by centrifugation at 10,000 × g for 10 min and used for organic acids quantification after filtered by a 0.22 µM syringe filter. High-Performance Liquid Chromatography (HPLC) attached with UV detector was used to determine the lactic acid, acetic acid and succinic acid. The sample (10 µL) was injected and the flow rate was maintained as 0.5 mL/min and the temperature of the column was set as 44 °C. The wavelength was set as 210 nm for the determination of organic acids and the sample was run for 40 min [16]. The protease and cellulose activities were determined in the cell-free supernatant [24].

2.4. Characterization of LAB

Potent LABs were selected based on their growth ability and organic acids production and analyzed their physiochemical properties. 16S rDNA sequencing was performed (SciGenom Labs, Cochin, India). Genomic DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany). Amplicons were sequenced using universal primers 27 F (5′ AGA GTT TGA TCG TGG CTC AG 3′) and 1492 R (3′ GCT TAC CTT GTT ACG ACT T 5′) [25]. The aligned sequence was compared with the other related LAB strains using the NCBI-Blast tool.
2.5. Growth Profile of Isolated Strain

The fresh LAB cultures were inoculated in an MRS broth and incubated at 28 ± 2 °C with mild shaking (150 rpm, Vision shaking incubator, Gyeonggi-do, Korea) for 48 h. The growth profile of LAB was determined at different time intervals by the microplate reader at 600 nm (iMark microplate reader, Bio-Rad, Tokyo, Japan).

2.6. Bile and Acid Tolerance Test of L. salivarius AS22

The active culture (24 h culture) was inoculated in acidic MRS broth (pH was adjusted to 2.0 using hydrochloric acid (1 N)). For bile salt tolerance, the LAB was inoculated in MRS broth containing 0.3% oxgall (SigmaAldrich Missouri, USA) and incubated at 28 ± 2 °C. The culture medium was withdrawn every 0 h, 2 h and 4 h and cultured on MRS agar medium to confirm the survival ability in low pH and toxic bile salt environments. The survival rate was calculated as described previously [13].

2.7. Analysis of Haemolytic Activity

The haemolytic property of the isolated strains was analyzed by Columbia agar containing sheep blood (5%, v/v). Briefly, fresh L. salivarius AS22 (24 h) was streaked on blood agar plates and incubated at 28 ± 2 °C for 48 h and then monitor the α-hemolysis, β-hemolysis, and non-hemolytic activity of isolated strains on the plates [13].

2.8. Antibacterial Activity of L. salivarius AS22

The strain was cultured in MRS broth at 28 ± 2 °C for 24 h and the cell-free supernatant was collected by centrifugation at 10,000 × g for 10 min and then filtered using 0.2 µm syringe filter. Cell-free supernatant (CFS) was used to determine the anti-bacterial study. The intestinal pathogenic bacterial strains (Klebsiella pneumoniae, Psudomonas aeruginosoa, Escherichia coli, Vibrio cholera, Staphylococcus aureus and Enterococcus faecalis) were spread on Muller Hinton Agar plates (1 × 10⁸ CFU/mL) and then made the well on it. A hundred microliters of CFS was loaded into the appropriate well and the plates were left for 48 h in an incubator at 37 °C. Then, zone of inhibition was observed after 24 h [9].

2.9. Antifungal Activity Analysis by Pour Plate Method

Twenty-four hours fresh culture (5 µL) was spotted on the MRS agar medium and incubated at 28 ± 2 °C for 24 h to allow LAB growth. Next 10 mL of sterile Potato dextrose agar (0.8%) was mixed with 50 µL of fungal spore suspension and slowly poured onto the MRS agar medium on the same Petri plates and incubated at 37 °C for 72 h. The conidial growth was monitored every day. The zone inhibition around the well was measured [9].

2.10. Antioxidant Properties

Cell-free supernatant was collected after 48 h by centrifugation at 4 °C, in 4000 × g for 10 min and filtered by whatman no 1 filter paper and further filtered by 0.2 µm syringe filter and then it was lyophilized below −40 °C in less than 50 m Torr pressure. Cell-free supernatant (CFS) at various concentrations (20, 40, 60, 80, 100, 125 and 150 µg/mL) was used to determine the antioxidant study. The sample was mixed with DPPH solution prepared in ethanol at 0.05 mM and incubated for 30 min at 37 °C. Positive control (vitamin C) and negative control (DPPH solution) were also used for the study. Then, scavenging activity of the sample was measured at 517 nm. The scavenging activity (%) of the CFS was calculated using the following formula:

\[
\text{DPPH free radical}(\%) = \frac{\text{Absorbance of the sample} - \text{Absorbance of the blank}}{\text{Absorbance of the control}} \times 100
\]

2.11. Bacterial Cultivation and Enumeration

LABs were inoculated in MRS broth and incubated at 30 ± 2 °C for 24 h. Then, the sample was serially diluted with sterile distilled water. A hundred microliter of the sample was plated onto an MRS agar medium and incubated at 30 ± 2 °C for 48 h. The growth of
LAB colonies was numerated manually. For silage production LAB was harvested after 24 h by centrifugation at $4000 \times g$ for 10 min and diluted in sterilized distilled water and used for silage production [26].

2.12. Silage Preparation

Corn and foxtail millet forages in the early bloom stage was harvested from the farm and wilted under field condition. After reaching expected moisture (70%), the forages were cut into small segments (1.5 cm in length). Silage was formulated using a large-scale system. It consists of approximately 20 kg forage of corn and foxtail millet was chopped and packed in the silage sample bags (50 cm $\times$ 80 cm size). The experiment was divided into two groups each consist of three replicates. Group I Non-Inoculated and Group II sample was inoculated with AS22 ($1 \times 10^5$). The air was evacuated from experimental bags by a vacuum sealer and stored in laboratory condition for 70 days [27].

2.13. Microbial Population and Lactic Acid Analysis

The silage sample (10 g) was mixed with double distilled water (90 mL) and kept in a rotary shaker at 150 rpm for 1 h. Then, ten-fold serial dilution of sample was performed in sterile distilled water. A hundred microliter of diluted samples was poured onto an MRS agar for LAB, onto a potato dextrose agar (Himedia, India) for fungi; onto an Actinomycetes Isolation Agar (Himedia, Mumbai, India) for actinomycetes. The lactic acid content of the silage was assayed by the HPLC method [28].

2.14. Statistical Analysis

Three different experiments were performed. Statistical analysis was carried out using SAS software and the results were expressed as Mean $\pm$ SD. Duncan’s multiple range analysis methods were applied to evaluate the important variation between experimental groups. The “$p$-value” < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Isolation and Characterization of Ligilactobacillus sp. from the Sheep Dung

We isolated nine lactic acid bacteria (LAB) based on their color, morphology and growth. Selected LAB showed Gram-positive, non-motile, rod-shaped, absence of endospore formation and catalase-negative. The isolated strains were named AS1, AS2, AS3, AS4, AS22, AS30, AS47, AS58 and AS90. The amount of lactic acid, acetic acid and succinic acid in culture media were estimated and the results were presented in Table 1. Among the strains, AS3, AS22 and AS90 showed maximum amount of lactic acid in the cell-free extract (40.2 $\pm$ 2.2 µg/mL, 83.2 $\pm$ 1.8 µg/mL and 76 $\pm$ 4.3, respectively) compared to other strains.

Table 1. Analysis of organic acid in the cell-free extract of the supernatant.

| Strain Number | Lactic Acid (µg/mL) | Acetic Acid (µg/mL) | Succinic Acid (µg/mL) |
|---------------|---------------------|---------------------|-----------------------|
| AS1           | 22.4 $\pm$ 3.2      | 2.3 $\pm$ 0.32      | 1.20 $\pm$ 0.04       |
| AS2           | 32.1 $\pm$ 1.9      | 5.7 $\pm$ 0.87      | 1.08 $\pm$ 0.32       |
| AS3           | 40.2 $\pm$ 2.2      | ND                  | ND                    |
| AS4           | 30.3 $\pm$ 5.2      | 4.4 $\pm$ 1.2       | 0.52 $\pm$ 0.05       |
| AS22          | 83.2 $\pm$1.8       | ND                  | ND                    |
| AS30          | 29.2 $\pm$ 2.1      | 5.2 $\pm$ 0.82      | 0.209 $\pm$ 0.109     |
| AS47          | 5.2 $\pm$ 1.1       | 4.1 $\pm$ 0.32      | 1.98 $\pm$ 0.28       |
| AS58          | 28.1 $\pm$ 2.3      | 8.2 $\pm$ 0.49      | 0.272 $\pm$ 0.41      |
| AS90          | 76.0 $\pm$ 4.3      | ND                  | ND                    |

ND: not detected. The LAB isolates were inoculated in MRS medium and incubated for 48 h. Experiment was performed in triplicate analysis.

3.2. Growth Kinetics of LAB

The three isolates (AS3, AS22 and AS90) were further cultured in MRS broth and the growth kinetics was analyzed. The strains (AS3, AS22 and AS90) exhibited maximum
growth (2.09 ± 0.05, 2.24 ± 0.12 and 2.03 ± 0.38 OD at 600 nm and 30 h) (Figure 1). Based on growth performance, the strain AS22 was selected. The isolated strain had significant growth rate at wide ranges of pH (4.0 to 8.0), grow well between 20 and 35 °C and optimum growth was achieved at 5.5% sodium chloride concentration. The morphological, biochemical and growth parameters of strain AS22 were described in Table 2.

![Figure 1](image-url). Growth kinetics of LAB in MRS broth medium at 28 ± 2 °C. The bacterial isolates (AS3, AS22 and AS90) were inoculated in 100 mL Erlenmeyer flask containing 50 mL culture medium and incubated for 48 h.

Table 2. Morphological, physiological and biochemical parameters of strain AS22 isolated from the sheep dung.

| Characters                | Result  |
|---------------------------|---------|
| Gram staining             | Positive|
| Shape                     | Rod     |
| Gas production            | Negative|
| Catalase                  | Negative|
| Growth in NaCl            | ++      |
| 2% NaCl                   | ++      |
| 4% NaCl                   | ++      |
| 6% NaCl                   | ++      |
| Growth at temperature     |         |
| 15 °C                     | +       |
| 20 °C                     | ++      |
| 25 °C                     | ++      |
| 30 °C                     | ++      |
| 35 °C                     | +       |
| Enzyme activity           |         |
| Protease                  | ++      |
| Cellulase                 | +       |
| Fibrolytic activity       | ++      |
| Carbohydrate fermentation |         |
| Sorbitol                  | +       |
| Maltose                   | +       |
| Esculin                   | +       |
| Salicin                   | +       |
| Glucose                   | +       |

Growth was analyzed at 600 nm using a UV-visible spectrophotometer (>1.0 OD at 600 nm ** referred as ++; <1.0 OD at 600 nm referred as +) and enzyme activity was assayed by spectrophotometer method (<50 U/mL * referred as +; >100 U/mL ** referred as ++). Carbohydrate fermentation test: strong fermentation referred as ++; week fermentation referred as +.
3.3. Probiotic Properties of LAB

LAB strains were subjected to analyze the acid and bile acid tolerance at a very low pH range. The isolated strain displayed the ability to survive in low acidic conditions for 2 h and 4 h. The survival ability (%) at low pH and bile salt concentrations were described in Figure 2a,b. Among three isolates, the isolate AS22 showed 89 ± 1% survival rates in acidic conditions and showed 88 ± 1% survival rates at 0.3% bile salt concentration. Fermented food consists of various LAB and protected the host organism by the production of various vitamins, stimulate the metabolism and ingestion in the intestine [28]. Acid tolerance is the main criteria to be considered as an important feature for the selection of a potent probiotic strain. The findings represented that AS22 effectively survived in the extreme pH (low pH) condition. In the case of *Lactobacilli*, the ability to survive in lower pH in the medium is depends on the intracellular enzyme activity. Glucose plays a potent role in enhancing the intracellular pH of the strain [29], thereby increasing the survival rate of LAB at a very low pH value. The acid tolerance potential of bacteria was mainly based on the outer membrane properties in stress conditions. The isolate AS22 showed high acid tolerance potential at low pH which was significantly concurrent with Vega et al., results, that stated as *Lactobacillus salivarius* TUCO-L2 had significant resistance to low pH and high bile salts concentrations [30].

![Figure 2](image-url)

**Figure 2.** (a) Survival rate of LAB isolated at low pH (2.0) after 2 and 4 h incubation. The strains were cultured in MRS medium at 28 ± 2 °C and survival rate was expressed as percentage; (b). Survival rate of LAB isolates at 0.3% bile salt concentration after 2 and 4 h incubation. The strains were cultured in MRS medium at 28 ± 2 °C and survival rate was expressed as percentage.
Among the isolated bacterial strains, AS22 showed higher resistance properties in bile salt condition than the other isolates. The isolated strain was survived at 0.3% bile salt concentrations and the results were described in Figure 2b. Tolerance of LAB at low pH and at high concentration of bile salt is the prerequisite for the screening of probiotic organisms. The stress-tolerant probiotic organisms secrete bacteriocin and various organic acids [16].

The isolate AS22 showed maximum survivability in an acidic environment and bile salt (>80%). In the present study, AS22 significantly survived in the low pH and bile salts environment, our data were strongly concurrent with Sanhueza et al. and Messaoudi et al., they have reported that the \textit{L. salivarius} SMXD5 [31], \textit{L. salivarius} UCO-979C-1 [32] had the significant survival rate at low pH and bile salts environment. Bile salts play a significant role in the non-specific and specific defense system in the gastrointestinal tract. In general probiotics must tolerate and survive in numerous environmental factors including bile salt related stress, low pH and nutrient limitations [33].

3.4. Hemolytic Property of Probiotics

Hemolytic properties of the isolated strains were analyzed using blood agar plates. No haemolytic property was noted on blood agar plates. Evaluation of the haemolytic property of any GRAS status of bacteria is a prerequisite for food applications. The present finding was in good agreement with the previous finding. In a study, Oh and Jung [34] analyzed the hemolytic activity of various \textit{Lactobacillus} species isolated from alcoholic beverages. Previously reported data evidenced that the probiotic LAB did not show hemolytic property [35–37]. Similarly, our strain did not exhibit any negative impact on blood agar plates. This suggested that isolated strain does not exhibit any hemolytic activity which is essential criteria for the selection of potent probiotics.

3.5. Antibacterial Activity

The AS22 showed noticeable antibacterial activity against the indicator bacteria \((1 \times 10^8 \text{ CFU/mL})\) such as \textit{K. pneumoniae}, \textit{P. aeruginosa}, \textit{E. coli}, \textit{V. cholera}, \textit{S. aureus} and \textit{E. faecalis}. The antibacterial activity is may be due to the synthesis of various organic acids, proteins and peptides, etc. The present finding showed that the isolated LAB strain exhibited the various ranges of the zone of inhibition between 15 and 25 mm against different indicators. The inhibitory effect was maximum (32 mm zone of inhibition) for \textit{S. aureus} (Figure 3) followed by \textit{V. cholera} (30 mm) and \textit{K. pneumoniae} (27 mm), \textit{E. coli} (22 mm), \textit{E. faecalis} (21 mm) and \textit{P. aeruginosa} (16 mm). The present finding showed better antagonistic activity compared to Ligilactobacillus salivarius (Lactobacillus salivarius) and Lactobacillus plantarum characterized previously [38]. Similarly, several researchers have reported that the Lactobacillus salivarius showed good antibacterial activity against various pathogens such as \textit{Enterococcus hirae} staphylococcus, \textit{E. coli}, \textit{Listeria innocua}, \textit{L. monocytogenes}, \textit{Salmoella enteritidis}, \textit{Enterobacter cloacae}, \textit{Klebsiella spp.}, \textit{S. epidermidis}, \textit{ Proteus vulgaris}, and \textit{Salmonella thyphimurium} [39–43]. In addition, Vega et al. reported that the \textit{L. salivarius} TUCO-L2 possessed strong probiotic potential with antibacterial activities against both Gram-positive and negative pathogens [30].

3.6. Antifungal Activity of Cell-Free Extract of \textit{L. salivarius} AS22

Antifungal activity of \textit{L. salivarius} AS22 was analyzed against \textit{R. microsporus}, \textit{F. oxysporum}, \textit{P. chrysogenum}, \textit{A. fumigatus}, \textit{A. niger} and \textit{A. flavus}. The cell-free extract of \textit{L. salivarius} AS22 exhibited potent antifungal activity against \textit{R. microsporus} (41 ± 3.0 mm) and \textit{P. chrysogenum} (35 ± 3.2 mm) (Figure 4). The lowest antifungal activity was noted for the \textit{A. niger} (13 mm) compared to other fungi. The moderate inhibitory activity showed against \textit{A. flavus} (32), \textit{A. fumigatus} (27 mm), and \textit{F. oxysporum} (28 mm). In general, cell-free metabolites produced by LABs possess strong antifungal activities against various fungi. The Lactobacillus plantarum KCC-24 and Pediococcus pentosaceus KCC-3 have produced various organic acids that inhibited the growth of \textit{F. oxysporum}, \textit{B. elliptica}, \textit{P. roqueforti}, \textit{P. chrysogenum} and \textit{A. fumigatus} [44,45]. The antifungal activity of LAB depended on
the growth, environmental conditions and microbial compositions [46]. The isolate AS22 produced a significant amount of lactic acid which acts as a strong antifungal agent. Overall data confirmed that the AS22 inhibits growth of various fungi at different levels. The fungal species belonging to Fusarium, Penicillium and Aspergillus affect the quality of cereals, fodders, vegetables, fruits and animal feed. Lactobacillus species have a great deal of potential to increase the shelf-life of peanuts, animal feed, and cheese and cereal products [16]. The present study recommended that the use of AS22 as an additive could prevent the above-mentioned fungi-based loss of cereals, fodders, vegetables, fruits and animal feed quality, as well as extend their shelf-life by a significant amount of time.

![Diagram](image1)

**Figure 3.** Antagonistic activities of *L. salivarius* AS22 isolated from sheep dung. The cell-free extract was tested against bacterial pathogens and the result was expressed as zone of inhibition (mm).

![Diagram](image2)

**Figure 4.** Antifungal activities of *L. salivarius* AS22 against fungi. The cell-free extract was tested against fungi pathogens and the result was expressed as zone of inhibition (mm).
3.7. Antioxidant Activity of *L. salivarius* AS22

Figure 5 showed the In vitro antioxidant acidity of cell-free supernatant (CFS) in different concentrations. DPPH has been used widely as a free radical to determine antioxidant activity. Oxidized DPPH was reduced by the addition of CFS. The scavenging activity on hydrogen radicals is one of the key indexes of the reduction process. DPPH scavenging activity CFS of *L. salivarius* AS22 was closely related to the concentration of CFS used. Higher antioxidant activity was showed with an increased concentration of CFS. The antioxidant activity of AS22 was increased in a concentration dependent manner. The maximum antioxidant activity was noted at the concentration of 125 µg/mL CFS. Further increases in the concentration of CFS reduced the antioxidant activity. Various LABs include *L. salivarius* [47,48], *P. pentosaceus*, *L. plantarm* and *L. brevis* showed a good antioxidant activity [49–51]. Our result was significantly concurrent with the above-mentioned statements.

![Figure 5. DPPH scavenging properties of *L. salivarius* AS22 at various concentrations. The cell-free extract was assayed at various concentrations and the scavenging effect was determined.](image)

3.8. Lactic Acid Content and Microbial Profiles of Silages

This isolate showed significant antimicrobial, antioxidant with sufficient probiotic characteristics, next, we investigated the efficacy of this isolate on acidification of silage and lactic acid production corn and foxtail millet forages. In the current study, we used corn and foxtail millet forages at the ratio of 1:1 for silage production by ensiling process (Table 3). However, limited populations of LAB have been found in natural plants, it is not sufficient to induce expected fermentation of silages results favoring undesirable microbial growth. Hence, the addition of LAB is essential for silage production. In the current study, we used *L. salivarius* as an inoculant for corn and foxtail millet silage production by the ensiled method. The lower number of LAB was noted in the control silage (5.3 ± 0.29 Log CFU/g). In contrast, the higher LAB population was noted in silage inoculated with AS22 (6.8 ± 0.14 Log CFU/g), indicating that the addition of AS22 competes for the epiphytic communities present in the plants. In addition, fungi and actinomycetes population were recorded in AS22 inoculated silage compared to control silage; it suggested that the AS22 had the ability to inhibit the spores of fungi (1.2 ± 0.36 vs. 0.9 ± 0.29) and actinomycetes (0.72 ± 0.28 vs. 0.43 ± 0.19 Log CFU/g) present in the natural plants compared to non-inoculated silages. Modification of the microbial population during this phase in successfully fermented silages indicated the disappearance of entero-bacteria. These changes are closely associated with lowering pH and higher lactic acid production in LAB inoculated silages [52]. Similarly, in the current study, we noted lower pH level
(5.29 ± 0.03 vs. 3.84 ± 0.07 Log CFU/g) and higher lactic acid production (22.3 ± 3.9 vs. 38.7 ± 1.4 µg/g) in silage inoculated with AS22 compared to the control. Many researches have confirmed the efficacy of *L. salivarius* on the fermentation of silages produced from various plants. Most of the studies suggested the use of *L. salivarius* as an additive during silage production to enhance silage quality via increasing essential organic acids, particularly lactic acid which lowers pH and inhibits unwanted microbial growth [53,54]. Previously published data significantly correlated with our present experimental results, here we found the addition of *L. salivarius* strongly reduced pH and increased lactic acid content than non-inoculated silages, and this condition could preserve the silage for a long time. In general, an increase in acidification is closely associated with the preservation of silages and it reduces proteolytic enzyme-mediated degradation and controls the growth of the enterobacteria and clostridia [55]. The pH is generally used to monitor the silage quality and the pH range between 3.8 and 4.2 is considered a benchmark for well-preserved silage [56]. Lactic acid is a key acid present higher level in the fermented silages and it reduces the pH of the silages during fermentation, approximately 10–20 times higher than the other acids [56]. Similarly, more LAB population, higher lactic acid and lower pH range were noted in silage inoculated with AS22 compared to control. These are the major factors to enhance fermentation quality and prevent undesirable microbial growth such as enterobacteria, fungi and actinomycetes.

### Table 3. Microbial population and lactic acid content of experimental silages.

| Parameters                  | Control         | AS22 Treated   | p-Value     |
|-----------------------------|-----------------|----------------|-------------|
| LAB (Log CFU/g)             | 5.3 ± 0.29      | 6.80 ± 0.14    | 0.0005 **   |
| Fungi (Log CFU/g)           | 1.2 ± 0.36      | 0.90 ± 0.29    | 0.0156 *    |
| Actinomycetes (Log CFU/g)   | 0.72 ± 0.28     | 0.43 ± 0.19    | 0.0000 **** |
| pH                          | 5.29 ± 0.03     | 3.84 ± 0.07    | 0.00262 *** |
| Lactic acid (µg/g)          | 22.3 ± 3.9      | 38.7 ± 1.4     | 0.0002 ***  |

Total LAB, fungi, and actinomycetes populations were determined and the result was expressed as Log CFU/g. Lactic acid concentration was assayed and expressed as µg/g. (** statistically significant at <0.05 (*), at <0.005 (**), <0.0005 (***), and <0.0001 (****) level, respectively).

### 4. Conclusions

The isolated strain *L. salivarius* AS22 had significant probiotic potentials as evident; AS22 showed low pH and high bile salt tolerant features without hemolytic activity and possessed intense antagonistic activity against various intestinal bacteria and phyto fungi with good antioxidant activity. AS22 produced a significant level of lactic acid in MRS broth (an essential acid). *L. salivarius* AS22 exert potential effects on silage fermentation by increasing lactic acid concentration which enhanced acidification of silage and this condition inhibited undesirable microbial growth at ensiled condition. Overall data suggest that the AS22 showed significant probiotics characteristics with antagonistic activities with great potential to improve the silage quality with enriched nutrients and can be preserved for long time storage. It is an in vitro laboratory-scale level experiment. However, in-depth investigation is essential to confirm the efficacy of the isolated LAB on silage production and preservation of different legumes and grass plants with an animal performance study.

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