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RESEARCH ARTICLE

Efficacy Prediction of *Lactobacillus rhamnosus* GG and Platelet-Rich Plasma (PRP) against Sub-Clinical Bubaline Mastitis

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The current study was conducted on Riverine-type buffaloes naturally exposed to Sub-clinical Mastitis (SCM). Probiotic bacteria like *Lactobacillus rhamnosus* GG primarily exert their benefits by competing with harmful bacteria for nutrients and adhesion sites, producing beneficial metabolites, and modulating the immune response. Platelet-Rich Plasma (PRP) comprises various growth factors and cytokines that can modulate the inflammatory response by promoting anti-inflammatory cytokines and inhibiting pro-inflammatory cytokines, thus helping to downregulate excessive inflammation. A total of 96 udder quarters/teats were randomly allotted for three treatment groups, i.e., Probiotic group (n=32), Probiotic plus Platelet-Rich Plasma (PRP) group (n=32), and Antibiotic group (n=32). The probiotic and antibiotic were purchased from the local market. PRP was prepared from the whole blood of the recipient animal. All the treatments were administered intra-mammary to affected animals. Somatic Cell Count (SCC) was significantly ($p<0.05$) decreased in animals that received Probiotic and PRP. The Probiotic alone was nine times more efficient than antibiotic alone, Probiotic plus PRP was 12.43 times more fecund than antibiotic alone and Probiotic plus PRP was 38.1% more prolific than Probiotic alone. Receiver Operating Characteristic (ROC) analysis demonstrated an acceptable predictive value (0.77) for the area under the curve when assessing the effectiveness of Probiotic plus PRP as a substitute for antibiotics in Sub-clinical Bubaline Mastitis. The non-parametric Kruskal-Wallis test revealed a significant difference ($P<0.05$) in the mean rank difference among the three treatment groups. In conclusion, the use of *Lactobacillus rhamnosus* GG and PRP in the management of Sub-clinical Bubaline Mastitis shows promise as a potential therapeutic agent by reducing somatic cell count and inhibiting the growth of mastitis-causing bacteria.

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INTRODUCTION

The water buffalo (*Bubalus bubalis*) plays a crucial role in the global agricultural economy by contributing milk, meat, hides, and draught power. Asia is the region with the largest number of water buffalo worldwide, and many people rely on buffalo per capita of the populace as compared to other livestock species (Young *et al.*, 2019). The buffalo is Pakistan's principal dairy animal (Khan *et al.*, 2007). The estimated number of Asian water buffaloes (*Bubalus bubalis*) in Pakistan during 2022-23 is 45.0 million. The estimated gross milk production of Asian water buffaloes in Pakistan is to be 40,678 tons during 2022-23 (GOP, 2022-23).

Bovine mastitis (BM) is one of the most prevalent ailments affecting dairy cattle, creating issues with welfare and economics in dairy farming worldwide (Kober *et al.*, 2022), which can manifest both clinically and sub-clinically (Anwar *et al.*, 2022). The most common type of mastitis is Sub-clinical Mastitis, an asymptomatic form of intra-mammary inflammation affecting 20–50 percent of cows in a given herd (Forsbäck *et al.*, 2009). Water buffalo mastitis is a significant problem concerning animal health, treatment costs, early culling, and reduced milk production (Catozzi *et al.*, 2019).

In a buffalo's udder, a rise in intramammary infections has been connected to mastitis-causing bacteria and dysbiosis of the commensal intramammary microbiota (Krishnan *et al.*, 2020; Liu *et al*., 2022). Dairy ruminants are frequently given antibiotics for mammary gland infections (Rainard and Foucras, 2018). The increase in antibiotic resistance has prompted researchers to look into ways to prevent or scale back antibiotic-based therapy (Pinheiro *et al*., 2020), mainly in cases of Sub-clinical Mastitis (Catozzi *et al.*, 2019).

Platelet-rich plasma (PRP) is a curative blood component enriched with platelets (PLTs) and growth factors (GFs) with anti-inflammatory, angiogenic, proliferative, and mitogenic effects (Lang *et al.*, 2018). Probiotics provide the host immunological protection by stimulating, regulating, and modulating immune responses (Azad *et al.*, 2018). The probiotic immunomodulatory effect is credited to the discharge of cytokines such as interleukins (ILs), chemokines, transforming growth factor (TGF), tumor necrosis factor (TNFs), and interferons (IFNs) from cells of the immune system (lymphocytes, macrophages, granulocytes, dendritic cells (DCs), mast cells and epithelial cells (Savan and Sakai, 2006), which further control both the adaptive and innate immunity (Foligné *et al.*, 2010).

Numerous studies have shown that the antioxidant properties of *Lactobacillus* aid in defense against pathogen infection (Lebeer *et al.*, 2008; Shen *et al.*, 2011). According to reports, lactic acid bacteria (LAB) found in milk, teat epithelia, and bedding can have probiotic effects (Espeche *et al.*, 2009). Subsequently, the intramammary infusion of probiotics has been suggested as one of the most auspicious options for controlling and preventing bovine mastitis (Sharun *et al.*, 2021). The Generally Regarded As Safe Bacteria (GRAS), like *Lactobacillus rhamnosus* could be used as remedial therapy to repair the imbalance in the microbiota of the mammary gland (Catozzi *et al.*, 2019). Microcine, a bacteriocin with a molecular weight of < 1000 that is impervious to heat and proteases, and seven peptides that have bactericidal action against gram-positive and gram-negative bacteria are formed by LGG (De Keersmaecker *et al.*, 2006; Lu *et al.*, 2009).

To our knowledge, there are no published reports regarding the efficacy and therapeutic effects of *Lactobacillus rhamnosus* GG and PRP for treating sub-clinical mastitis in dairy buffaloes. As a result, we anticipated that *Lactobacillus rhamnosus* GG and PRP would be an effective intra-mammary antibiotic and a viable alternative for treating Sub-clinical Mastitis in dairy buffaloes. Furthermore, *Lactobacillus rhamnosus* GG and PRP would improve the anti-inflammatory character of treated buffalo milk.

MATERIALS AND METHODS

Ethical statement: The present study followed the guidelines issued by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan (Permission Letter No. DR/160 Dated: 05-04-2022).

Experimental animals: A total of sixty (60) lactating buffaloes (96 quarters/teats) were divided into three treatment groups, i.e., Probiotic group, Probiotic plus PRP group, and Antibiotic group; each group consisted of 32 quarters/teats. The experimental trials were performed in seven closely situated private buffalo dairy farms in Lahore, Pakistan. The management and husbandry system of all the studied farms was the same.

Diagnosis of Sub-clinical Bubaline Mastitis: Sub-clinical Bubaline Mastitis was assessed in the milk samples initially using the California Mastitis Test (CMT) Kit (Portland, ME, USA) and then further verified for CMT-positive samples through somatic cell count measurements exceeding 200,000 cells/ml of milk, as well as the presence of bacterial growth on plates containing 5% sheep blood agar and MacConkey agar. The tested positive samples were categorized into three distinct treatment groups, i.e., Probiotic group consisting of 19 animals, Probiotic plus PRP group comprising 21 animals, and Antibiotic group comprising 20 animals. Each group consisted of 32 udder quarters or teats.

Somatic cell count of milk: Newman's Lampert stain was used to dye the somatic cells, and their count was determined using the microscopic count method, as elaborated by Schalm (1971).

Bacteriological culture of milk: Similarly to Guha *et al.* (2012), the milk samples were promptly subjected to culturing. An animal was considered positive for Sub-clinical Mastitis if it tested culturally positive for at least one quarter.

Commercial probiotic Prepro® (*Lactobacillus rhamnosus* GG): A commercial Probiotic sachet with the brand name Prepro® (*Lactobacillus rhamnosus* GG) was purchased from the local market of Lahore, Pakistan. According to the label, each sachet contains $\geq 5 \times 10^9$ cfu of *Lactobacillus rhamnosus* GG.

Isolation of *Lactobacillus rhamnosus* GG: One sachet was diluted in 9 ml of normal saline. Then 100 ul of the diluent was spread on de Man Rogosa and Sharpe (MRS) agar (Bio Chem Scientific, GmbH, Germany) plate and incubated under anaerobic condition in an anaerobic jar for 48 hrs at 37°C. After incubation, the morphology of cells and characteristics of colonies were seen on MRS agar. The distinguished colonies were selected and confirmed through Gram staining (Fig. 1) as Gram-positive and biochemical tests (negative for catalase test). The culture was purified on de Man Rogosa and Sharpe (MRS) agar.

Preservation of *Lactobacillus rhamnosus* GG culture: The purified cultures of *Lactobacillus rhamnosus* GG were stored in 20% (v/v) Glycerol in de Man Rogosa and Sharpe (MRS) broth at -20°C.

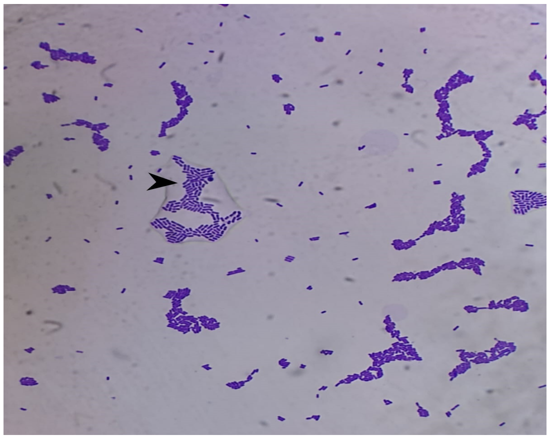

Fig. 1: Arrow showing *Lactobacillus rhamnosus* GG after Gram staining.

Dose preparation of *Lactobacillus rhamnosus* GG: The dose was adjusted to 0.5 McFarland Standard (1.5×10^8 cfu/ml) through a spectrophotometer bearing a 1 cm light path in sterile normal saline at wavelength 625nm and absorbance of 0.08 to 0.1.

Intra-mammary infusion of *Lactobacillus rhamnosus* GG to udder quarters: Five (05) ml of *Lactobacillus rhamnosus* GG (1.5×10^8 cfu/ml) were infused intra-mammary into the teat canal to the animals of the Probiotic group and Probiotic plus PRP group once daily for three consecutive days.

PRP preparation: Whole blood was procured from animals of the Probiotic plus PRP group once daily for three consecutive days from the jugular vein. PRP was prepared using the double-spin open technique per Dashore *et al.* (2021) and Dhurat and Sukesh (2014), with trivial alterations. For the present study, a 30 ml sample of whole blood was harvested that yielded an average of 5 ml PRP. The platelet count was determined to be the typical value of 1×10^9 platelets per milliliter. The PRP was divided into 5 ml aliquots available for immediate use.

Antibiotic (Ceftiofur): Buffaloes suffering from Sub-clinical Mastitis were treated with SPECTRAMAST® LC, a sterile antibiotic suspension administered directly into the udder quarters through the teat canal for three consecutive days at 24-hour intervals, following the regular milking process (Each 10ml of the suspension contains 125mg of Ceftiofur Equivalents as active substance (in the form of hydrochloride salt), 500mg of Oleoyl Polyoxylglyceride, 700mg of Microcrystalline Wax, and a sufficient amount of Cottonseed Oil).

Administration of treatments and milk sample collection: All treatments (Probiotic, Probiotic plus PRP, and Antibiotic) were administered at a dose rate of Probiotic= 5 ml/teat, Probiotic plus PRP= (5ml+5ml)/teat, and Antibiotic= 10 ml/teat were executed intramammary into the affected quarter(s)/teat(s) exceeding somatic cell count 200,000 cells per milliliter, once daily for three days continuously following the regular afternoon milking. Milk samples were collected from all animals in each group on days 0, 1, 3, 7, 14, and 28.

Statistical analysis: The analysis of somatic cell count was performed with a Two-way ANOVA using Minitab® 21.3.1. A binary logistic regression model was utilized to examine the influence of different treatment combinations on the outcome of Sub-clinical Bubaline Mastitis (Recovered or Non-recovered) through Minitab® 21.3.1. Furthermore, the non-parametric Kruskal-Wallis test was employed to analyze the mean rank difference among the three treatments, and Dunn's multiple comparison test was conducted. A P-value below 0.05 (α=0.05) was considered statistically significant.

RESULTS

Somatic cell count of milk: The initial sampling, comprising day 0 to day 3, did not exhibit highly significant differences among the groups concerning somatic cell count. After day 7, the treated groups showed a significant decrease in somatic cell count. Furthermore, the Probiotic and Probiotic plus PRP-treated groups demonstrated a noticeable decline on days 14 and 28 compared to the animals treated with Antibiotic only (Table 1 and Fig. 2).

Table 1: The Somatic Cell Count (Mean ± SEM) was measured in every treatment group on various days and assessed by Two-way ANOVA. Cells labeled with distinct superscripts indicate a significant difference at (P<0.05)

Days	Somatic cell count (Mean ± SEM) × 10^4 per ml of milk		
	Probiotic plus PRP	Probiotic	Antibiotic
Day 0	$303±26.8^{a}$	$295±26.2^{ab}$	$276±27.4^{ab}$
Day 1	$230±23.1^{ac}$	$280±27.2^{ab}$	$220±19^{acd}$
Day 3	$214±19.1^{acd}$	$233±17.1^{ac}$	$225±17.7^{ac}$
Day 7	$216±16.4^{acd}$	$207±15.4^{bcd}$	$224±17^{acd}$
Day 14	$89.9±8.68^{ef}$	$132±12^{de}$	$149±16.9^{ce}$
Day 28	$18.2±5^{f}$	$36±9.11^{f}$	$69.4±10.9^{ef}$

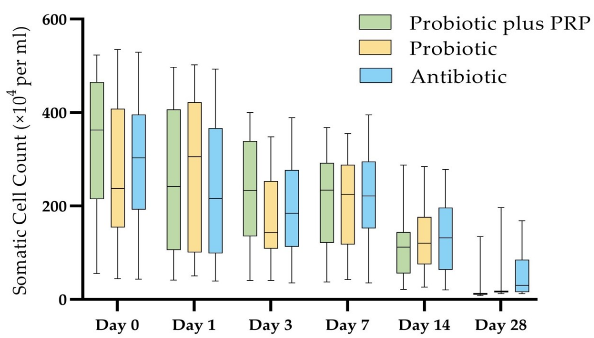

Fig. 2: Comparing the somatic cell count of different groups on various days using a box and whiskers plot.

Probiotic, probiotic plus PRP, and antibiotic treatment: The binary logistic regression results revealed that Probiotic exhibited an effectiveness nine times greater than Antibiotic alone. Conversely, the combined administration of probiotic plus PRP demonstrated an activity of 12.43 times higher than that of antibiotics alone. While the associated treatment of Probiotic plus PRP exhibited 38.1% more dynamism than Probiotic only (Table 2 and Fig. 3).

The mean rank difference among probiotic, probiotic plus PRP, and antibiotic: The mean rank difference between Probiotic and Probiotic plus PRP was insignificant ($p<0.05$). However, there was a significant ($p<0.05$) difference noticed between Probiotic and Antibiotic, as well as between Probiotic plus PRP and Antibiotic (Fig. 4).

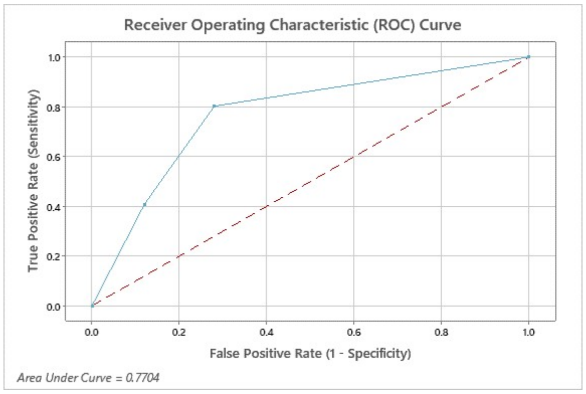

Fig. 3: Receiver Operating Characteristic (ROC) curve for predicting the therapeutic efficacy of Probiotic plus PRP in Sub-clinical Bubaline Mastitis. An area under the curve of (0.77) demonstrates Probiotic plus PRP as an acceptable therapeutic.

Table 2: Odds ratios for categorical predictors

Level A	Level B	Odds Ratio	95% CI
Probiotic	Antibiotic	9.0000	(2.5552, 31.7005)
Probiotic plus PRP	Antibiotic	12.4286	(3.1314, 49.3293)
Probiotic plus PRP	Probiotic	1.3810	(0.2832, 6.7335)

The odds ratio for level A relative to level B.

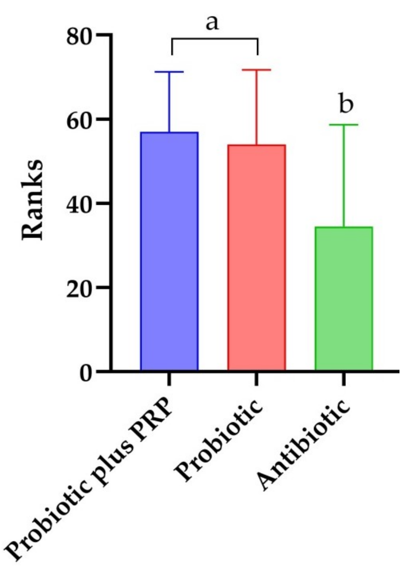

Fig. 4: A comparison of the mean rank difference among probiotic, a combination of probiotic plus PRP, and antibiotic in Sub-clinical Bubaline Mastitis. The rank means labeled with different superscripts denote a significant difference at ($p < 0.05$).

DISCUSSION

Presently, mastitis control approaches in dairy farms primarily revolve around administering antibiotics locally through intramammary infusion or systemically through parenteral administration. Blind antibiotic therapy in lactating cows puts humans at threat of experiencing residual effects through consuming contaminated milk and reduces antibiotic efficacy due to the emergence of mastitis pathogens resistant to antimicrobial treatment (Oliver and Murinda, 2012). The legal limitations on antibiotic usage in numerous developed nations and the persistent rise of antimicrobial-resistant pathogens worldwide underscore the necessity of investigating viable antibiotic alternatives. Various alternative approaches, such as investigating immunomodulatory beneficial microbes, are currently being explored to prevent mastitis in dairy cows. *Lactobacillus* strains are recognized for their diversity in immunoregulatory properties. Lactobacilli are widely recognized as beneficial bacterial species, with multiple strains showing the ability to exert a positive impact against sub-clinical bovine mastitis (Klostermann *et al.*, 2008; Armas *et al.*, 2017).

The microbiota of the mammary gland is unique (Taponen *et al.*, 2019). In the process of antagonism and immune modulation, system dysbiosis is corrected by probiotic contact with the microbiota of the host's system (e.g., udder, intestine, rumen) and controls numerous infectious diseases that are inflammatory (Beecher *et al.*, 2009; Rainard and Foucras, 2018). In polygastric animals like ruminants, administration through the oral route is unlikely to be beneficial because the entero-mammary path is dreadfully functional in these species. This is possibly the rationale behind administering probiotics through the teat canal to the bovine mammary gland (Beecher *et al.*, 2009). Rainard and Foucras (2018) concluded that the chances for intramammary probiotics should be cautiously examined and that the particulars for oral probiotics are not encouraging for ruminants. In mastitis treatment, intra-mammary infusions of a viable culture of Lactic Acid Bacteria (LAB) have been used successfully with similar efficacy as usual antimicrobials (Bouchard *et al.*, 2013). Lactic acid bacteria (LAB) could be a potential antimicrobial alternative. It is a well-known antibacterial producer widely considered safe in the food industry (Athanasiou *et al.*, 2019).

The existence of those bacteria that are not associated with mastitis in the healthy udder strengthens the idea of commensal mammary microbiota, and the microbiota of healthy udder etiological structure can offer an approach to the pathogenesis of intramammary infections (IMI) and opportunities for developing prophylactic or therapeutic products as alternatives to antibiotics (Derakhshani *et al.*, 2018). The prevalence of mastitis-causing bacteria and dysbiosis of the commensal intramammary microbiota in dairy calves has been connected to IMI (Oikonomou *et al.*, 2014). Probiotics are suggested to treat dysbiosis (Rainard and Foucras, 2018). Probiotics have been noted as a substitute for antibiotics owing to their inhibitory possessions and biological action toward pathogenic microorganisms in the host (Ooi *et al.*, 2015; Islam, 2016). Probiotics can hinder the growth of mastitis-causing bacteria by generating antagonistic metabolites like organic acids, H_2O_2, SCFAs, and bacteriocins (Rainard and Foucras, 2018). Organic acids, mainly acetic acid and lactic acid, exhibit potent inhibitory effects on Gram-negative bacteria and are recognized as the principal antimicrobial compounds utilized by probiotics to combat pathogens (Van Zyl *et al.*, 2020). Conversely, bacteriocins, which belong to a category of antimicrobial peptides with a limited spectrum of activity, exert a direct inhibitory impact on pathogenic bacteria (Rainard and Foucras, 2018). The release of cytokines, such as interleukins (ILs), interferons (IFNs), transforming growth factor (TGF), chemokines, and tumor necrosis factors (TNFs) from immune cells (granulocytes, lymphocytes, dendritic cells (DCs), macrophages, epithelial cells, and mast cells is due to the probiotics' immunomodulatory effects (Savan and Sakai, 2006) which further modulate both the adaptive and innate immune system (Foligné *et al.*, 2010). Probiotics can activate B lymphocytes and phagocytes; this process induces the formation of germinal centers (lymphoid follicles) in mucosal lymphoid-like tissue by B lymphocytes, which transform into plasma cells, thus secreting mucosal antibodies known as IgA. As a result, this reinforcement of immune surveillance in mucosal sites outside the intestine contributes to improved tolerance to mastitis in cows (Azad *et al.*, 2018). Pathogens are becoming more resistant to antibiotics; there is growing interest in using the intramammary infusion of lactic acid bacteria (LAB) as a potential antibiotic substitute. This approach aims to treat and prevent bovine mastitis by enhancing the host's immunity (Fukuyama *et al.*, 2020).

Recent research indicates more excellent prospects for recovery when combining probiotics with PRP than antibiotics alone. The combined therapy of probiotic plus PRP was 12.43 times more effective than antibiotic alone, while probiotic itself demonstrated a nine times greater effectiveness than antibiotic alone. Surprisingly, the combined therapy of probiotic plus PRP exhibited 38.1% higher productivity than the probiotic alone. A study demonstrated that *Lactobacillus rhamnosus* could stimulate IL-10 release from macrophages via TLR2, reducing the inflammatory response (Hu *et al.*, 2022). *Lactobacillus rhamnosus* inhibited *S. aureus*-induced keratinocyte cell death on keratinocytes, presumably inhibiting keratinocyte $\alpha 5\beta 1$ integrin (Prince *et al.*, 2012). Taking into consideration that $\alpha 5\beta 1$ integrin is also found in mammary epithelial cells (Taddei *et al.*, 2003), it is believable that Probiotics may prevent *S. aureus* colonization and inhibit *S. aureus* infections in mammary glands by chunking the same integrin.

Platelet-rich plasma (PRP) has been manifested to be helpful in the cure of bovine mastitis with an effect similar to that of antibiotics (Lange-Consiglio Anna *et al.*, 2021). In the current study, a significant reduction in somatic cell count was observed in the groups treated with probiotics and probiotic plus PRP on days 14 and 28, in contrast to the animals treated solely with antibiotics. In a prior investigation, Italian scientists made a significant finding regarding the effectiveness of platelet lysate, an allogeneic hemoderivative related to platelets, in treating cows suffering from acute and chronic clinical mastitis caused by Gram-positive and Gram-negative bacteria. Their study yielded promising results, demonstrating that platelet lysates led to approximately a 67% reduction in somatic cell count for cows with acute clinical mastitis compared to a 52.5% reduction using antibiotic treatment, and a 53% reduction for animals with chronic clinical mastitis, as opposed to 15.4% reduction with antibiotics (Lange-Consiglio A *et al.*, 2014). The findings of Dal *et al.* (2019) study suggest that using intramammary platelet concentrate could be a viable and effective substitute for intramammary antibiotics in treating subclinical mastitis. Contrarily, the study results of Duque-Madrid *et al.* (2021) indicate that subclinical mastitis treated with platelet-rich plasma exhibited a lower rate of bacteriologic cure compared to animals treated with cefquinome sulfate.

Conclusions: In conclusion, *Lactobacillus rhamnosus* GG (*L. rhamnosus* GG) and PRP show great potential as a treatment for Sub-clinical Bubaline Mastitis. This research indicates that *Lactobacillus rhamnosus* GG and PRP can effectively inhibit mastitis-causing pathogens and modulate the immune system, resulting in decreased somatic cell count, reduced bacterial load in milk, and improved udder health. Probiotics and PRP offer a sustainable and cost-effective alternative to traditional antibiotic therapies, promoting animal welfare and ensuring the production of high-quality milk. Further studies are needed to optimize treatment protocols and determine long-term effects, but *Lactobacillus rhamnosus* GG and PRP hold promise for managing Sub-clinical Bubaline Mastitis safely and efficiently.

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Author's contribution: QU, MK, RA, and AAA devised and planned the study. QU conducted the experiments and examined the samples. QU analyzed the data. The manuscript was written by QU and supervised by MK. All authors thoroughly reviewed the manuscript for significant intellectual contributions and ratified the final version for publication.

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