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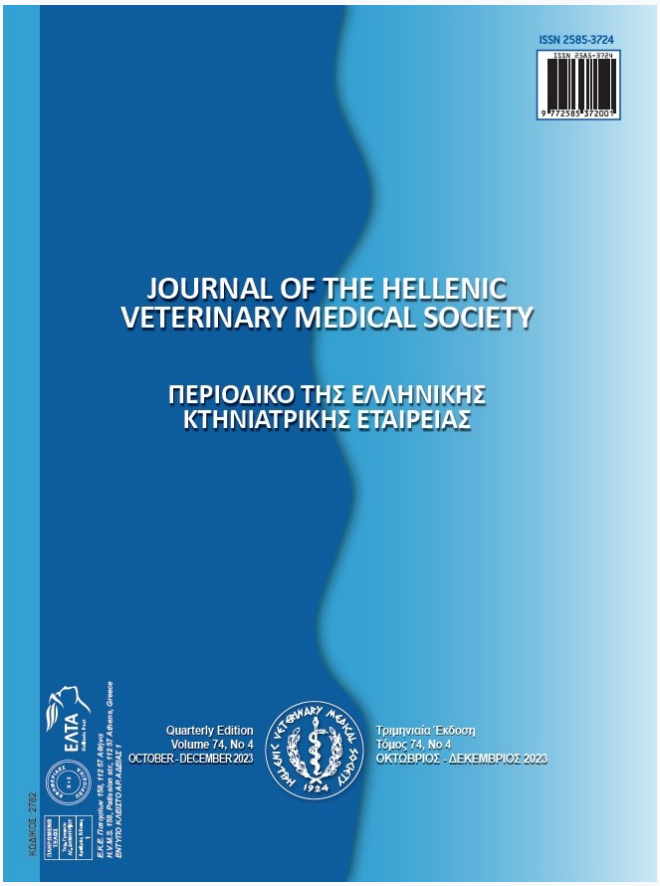

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Research article
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Development and Evaluation of Live Attenuated *Staphylococcus aureus* Vaccine for the Control of Mastitis in Dairy Lactating Buffaloes in Pakistan

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Abstract: This study was designed to assess the evaluation of live attenuated *S. aureus* vaccine (LSAV) in lactating dairy buffaloes. For this purpose, 100 lactating buffaloes one to two months after parturition, were divided into two equal groups i.e., B1 and B2. The prepared LSAV were administered twice at the dose rate of 5 ml/animal subcutaneous (S/C) 30 days apart, while B2 was kept as unvaccinated control (UC) by using a placebo (Normal Saline) at the dose rate 5 ml S/C twice. The animals were monitored for six months' period to check the various parameters. Serum samples were used to measure the geometric mean antibody titers using modified indirect Haemagglutination (IHA) test which were significantly higher in the vaccinated group as compared with the UC group on monthly intervals. Whey geometric mean antibody titer was higher in vaccinated treated animals as compared with UC group during the whole study period. There was significantly higher somatic cell counts in unvaccinated groups as compared with the vaccinated group. The results showed a significant increase of milk fat and protein during the study period in vaccinated group as compared with UC group. The prevalence of mastitis was lower in vaccinated group as compared with UC group. It was concluded that LSAV is associated with reduced risk of mastitis and somatic cell count and it should be an additional tool to prevent mastitis along with other control measures in dairy herds to maximize the profit.

***Keywords*:** Monovalent mastitis vaccine, Antibody titer, Efficacy, Bovine

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INTRODUCTION

The buffalo is the mainstay of the dairy industry in Pakistan and produces more than 75% of milk to be used all over the country (Sarwer et al., 2002). Mastitis is a global disease of dairy animals (Khan et al., 2020; Saeed et al., 2022) and is associated with severe economic losses (Kassoibati et al., 1998; Ali et al., 2021). This condition deteriorates both quality (Radostitis et al., 2007) and quantity of milk (Raza et al., 2013; Aymen et al., 2022; Dapgh et al., 2022). It can appear in two forms i.e., clinical and sub-clinical (Radostitis et al., 2007). The clinical form causes the change in color and composition of milk along with changes in glandular tissue of udder (Sharif and Muhammad, 2009) whereas in sub-clinical form, there are no visible changes in milk composition but mastitis can be detected by field tests like Surf field mastitis test (Muhammad et al., 1995), California Mastitis test (Kashif et al., 2013) and by many other laboratory tests (Viguier et al., 2009). Subclinical mastitis is 15-40 times more common than the clinical form (Abdeen et al., 2021; Liu et al., 2023; Ullah et al., 2023). In India and Pakistan, its prevalence is 4-48% in buffaloes and 17-93% in cattle (Ali et al., 2021; Allore, 1993).

Mastitis is one of the main diseases affecting the buffaloes in Pakistan (Ahmad, 2001) and effect the productivity very adversely (Firyal et al., 2009). According to a study in Nili Ravi buffalo, mastitis causes 57 days reduction in the lactation period of mastitic affected buffaloes compared to healthy ones and reduces the milk yields up to 438 liters of the total milk production (Cady et al., 1983). Mastitis is caused by several types of bacteria (Allore, 1993 and Hwang et al., 2000). Among these, some are found in the environment and others on the skin of the animal (Sharif and Muhammad, 2009).

As per results of investigations made so far during the last forty years, *Staphylococcus aureus* is the main mastitis related pathogen in buffaloes (Memon et al., 1999 and Ullah et al., 2005; Dad et al., 2022; Ullah et al., 2023) and can cause both sub-clinical and clinical form (Radostitis et al., 2007). Once it enters the udder then it keeps on lodging there, causes damage to milk-producing cells insidiously or overtly (Chakrabarti 2011; Yang et al., 2020). Because of having multifarious defense capabilities, antibiotic becomes ineffective because bacteria lies within the cells and conceals itself in a shell of fibrous tissue in the udder, making the antibiotics inaccessible to that site (Yang et al., 2020).

In Pakistan, there are no mastitis control practices (like dry cow therapy, teat dipping, washing of hands and udder before milking, etc.) in place and the predominance of *S. aureus* (contagious organism) is persistent (Sharif and Muhammad, 2009; Ji et al., 2020). Against this backdrop, the contrivance of vaccination against *S. aureus* seems to be a logical solution to combat this problem. Among these vaccines, a live attenuated vaccine can easily be prepared through a multistep process that requires attenuation, growth, concentration adjusting through spectrophotometric method, and cold chain preservation. So, keeping this fact in view, this study was designed to prepare a live attenuated *S. aureus* vaccine and conduct its trial in lactating buffaloes in association with various evaluation parameters viz serum and milk antibody tires, somatic cell counts (SCC), milk fat, protein and vaccine efficacy.

MATERIALS AND METHODS

Acquisition of *S. aureus*

Milk samples were collected from 100 clinically mastitic quarters of 100 buffaloes from Faisalabad, Punjab, Pakistan, followed by streaking on to blood agar plates containing 5% sheep erythrocytes in the Mastitis Laboratory, University of Agriculture Faisalabad, Pakistan. Morphological and hemolytic patterns were noted. Isolates were identified via Gram staining and biochemical testing including catalase, coagulase, urease and voges proskauer tests (National Mastitis Council, Inc., 1990). Biochemically confirmed *Staphylococcus aureus* were cultured on sheep blood agar plates to observe hemolysis pattern. On the basis of hemolysis pattern, it was revealed that 30 α, 30 β, and 4 α-β *S. aureus* isolates were obtained.

Biocharacterization of selected isolates

The isolates were further characterized via commercially available kit (API-Staph identification codebook; 1986; API ® AnalyTab Products, Division of Sherwood Medical 200 Express Street Plainview, New York). Selected strains of *S. aureus* characterized via API system were evaluated as vaccinal candidate based on their ability to cause clinical mastitis in natural host. For this purpose, each isolate was used for experimental infection in lactating buffaloes (n= 5) and animals were observed for developed signs of clinical mastitis and isolation of *S. aureus* from milk. Only one isolate (β) developed successful clinical in-

fection in lactating experimental buffaloes. This isolate was given name as AGS-657, washed twice in ice cold PBS and suspended in PBS containing 15% glycerol, then were aliquoted and kept at -80°C until subsequent use. The isolate (AGS-657) was used for preparation of vaccine.

Preparation of live attenuated vaccine

A typical alpha-beta hemolytic selected isolate of *S. aureus* from mastitic buffaloes was repeatedly passage through culture on 5% sheep blood agar (Biolife, Italy) until it lost its hemolytic activity. Then, the live attenuated isolate was grown for 24 hrs in nutrient broth. Organisms were deposited by centrifugation (3000x g; 15 min), washed (x2) with phosphate-buffered saline (PBS) of pH 7.2, and resuspended in sterile PBS (Sigma, USA). The concentration of bacteria was finally adjusted to 10^{10} cells/ml using the spectrophotometric method. Keeping in view per unit volume concentration of antigenic biomass, 5 ml was calculated as appropriate dose (Watson and Lee, 1978; Watson, 1984).

Selection of animals

A total of 100 Nilli-Ravi buffaloes that had calved one to two months earlier were selected. These were maintained at Government farms (LPRI-Bahadurnagar, Okara) in a similar managerial and nutritional regimen. No mastitis control practices were in place. The selected animals were randomly divided into two groups i.e., B1 & B2. Live attenuated *S. aureus* vaccine which proved its worth during the evaluation in pregnant animals was used. Two shots of LSAV were administered at the dose rate of 5 ml S/C 30 days apart, while B2 was kept as unvaccinated control (UC) by using a placebo (Normal Saline) @ 5 ml S/C twice. The animals were monitored for six months' period.

Evaluation Parameters

The following parameters in the pre- and post-vaccinal period at monthly intervals were compared. Serum & milk whey antibody titers against *S. aureus* were measured using standard modified IHA test (Rahman et al., 2004). Modified somatic cell count (SSC) with a little modification to the original method was performed. Briefly, a 10µl of fresh milk was spread over a glass slide having a marked area of 10 mm × 10 mm using a micropipette. The fine milk smear was dried in an oven at a temperature of 35°C for 5 minutes. The slides were dipped in xylene trice to remove fat globules and dried subsequently. The slides were then stained using Newman Lampert's stain (Sigma) for 15 min. The excess stain was removed from the smears with tap water and dried at 35°C. The poorly stained smears were further stained with blue (basis) aliquot of Dip-Quick stain (Jorgensen labs. Inc. Loveland, Colorado, USA) for 15 seconds followed by tap water rinsing and air drying. This enhances the differentiation significantly among cells and the substrate. The somatic cells were counted under a microscope (Olympus, Japan) with 40-fold magnification objective (total magnification 400x) in 50 fields and multiplied by the microscopic factor to get the cells per ml of milk (Feng et al., 2021).

Butterfat concentration in the milk was measured with Gerber's fat test as described by Aggrawala and Sharma (1961). Milk Protein Concentration was determined by the formal titration method as previously described (Feng et al., 2021).

California mastitis test (CMT) was performed to estimate the prevalence of mastitis in both vaccinated and unvaccinated groups according to manufacturer's instructions (Kashif et al., 2013). This test has comparable efficacy with surf field mastitis test as in the earlier studies.

Data analysis

Means for milk antibody titers, whey protein, somatic cell count fat and protein percentages were analyzed using ANOVA with a level of significant $P<0.05$ by using the SAS-2000 software.

RESULTS

In buffaloes vaccinated with LSAV serum antibody titers (GMT) against the vaccinal *S. aureus* at day 0, 30, 60, 90, 120, 150, and 180 postpartum were 6.24, 128, 291, 261, 142, and 58, respectively. For unvaccinated control (B2), antibody titers (GMT) were 5.3 at day 0, which remained almost the same until the termination of the trial. There was a highly significant difference ($P<0.01$) between two groups as shown in Figure 1.

In vaccinated animals, whey IHA antibody titers on days 0, 30, 60, 90, 120, 150, and 180 post-vaccination were 2, 4.3, 4, 3.5, 2.8, 2.3, and 2.3, respectively. In unvaccinated group, IHA antibody titer was 2.7, 3.2, 2.5, 2.3, 1, 2, and 2.1 on days 0, 30, 60, 90, 120, 150, and 180, respectively. The whey antibody titer was significantly higher in vaccinated animals as compared with non-vaccinated (Figure 2).

In buffaloes vaccinated with LSAV, somatic cell count (SCC) was highest at day 0 (12.72 ± 4.15 x10^5/ml of milk) followed by a decline to 4.58 ± 2.43 x 10^5, 3.03 ± 1.82 x 10^5, 2.46 ± 2.03 x 10^5, and 2.36 ± 1.17 x 10^5/ml at day 30, 60, 90, and 120, respectively. With the advancement of lactation, the mean SCC steady increased. In group B2 (control) mean somatic cell count was significantly higher ($P< 0.05$) as compared with the vaccinated group (Figure 3).

In group B1, the average milk fat percentage was 6.10 ± 0.58 at day 0 which increased slightly at day 30 and day 60 post-vaccination. After registering a slight

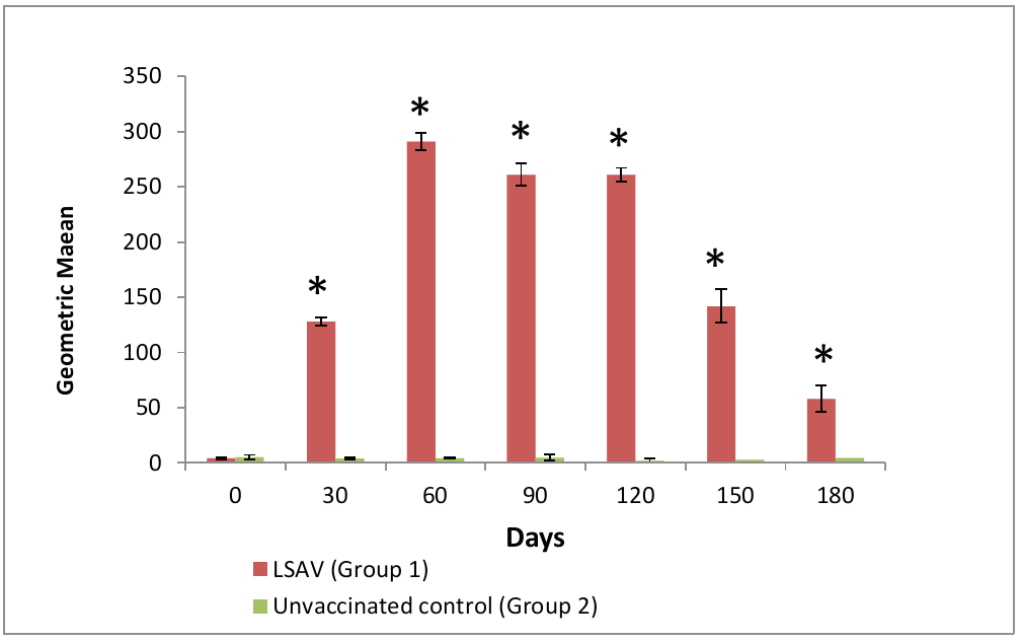

Fig. 1: Comparative geometric mean antibody titers (mean±SD) in lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. Aureus* vaccine and unvaccinated control group. * indicate the significance difference between vaccinated and control group

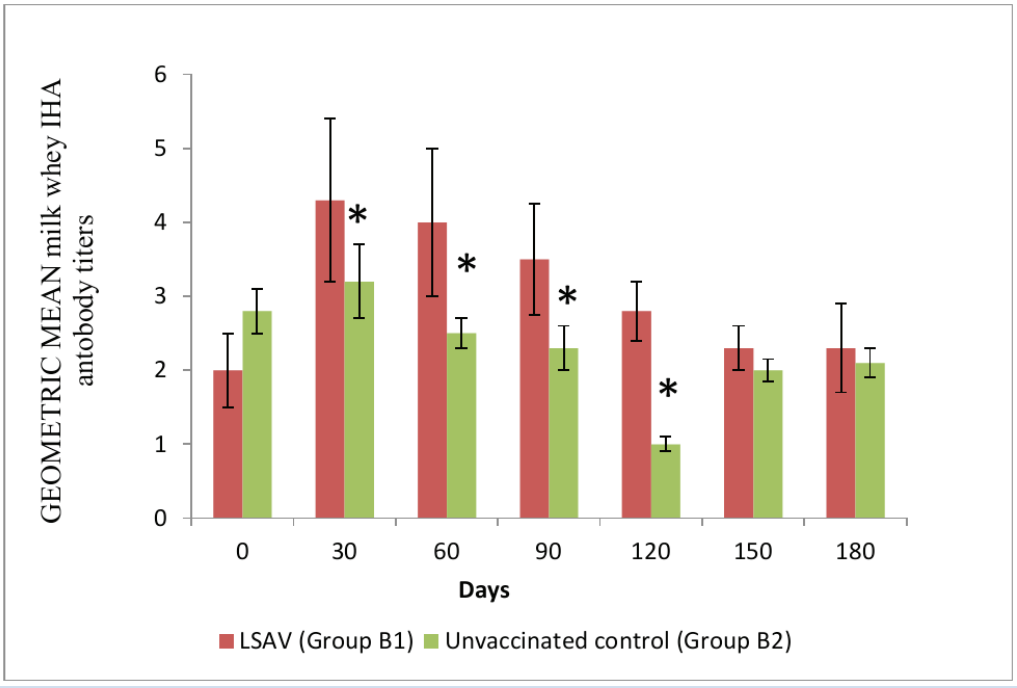

Fig.2: Comparative geometric mean milk whey IHA antibody titers (mean±SD) in lactating buffaloes vaccinated twice during post-partum period with live attenuated **S**. *Aureus* vaccine and unvaccinated control group. * indicate the significance difference between vaccinated and control group

decrease at day 90, a progressive increment in the milk fat contents was recorded until the end of the trial. In animals kept as unvaccinated control, the mean milk fat percentage was 6.00 ± 0.38, gradually decreased until day 120, then remained the same at day 150 followed by a rise at day 180. The fat contents were significantly higher ($P<0.05$) in the vaccinated group than in the unvaccinated control (Figure 4).

In a group B1, protein concentration (percent) was 3.39 ± 0.24 at the start of the field trial (day 0) which increased to 3.43 ± 0.19 at day 30 post-vaccination. It

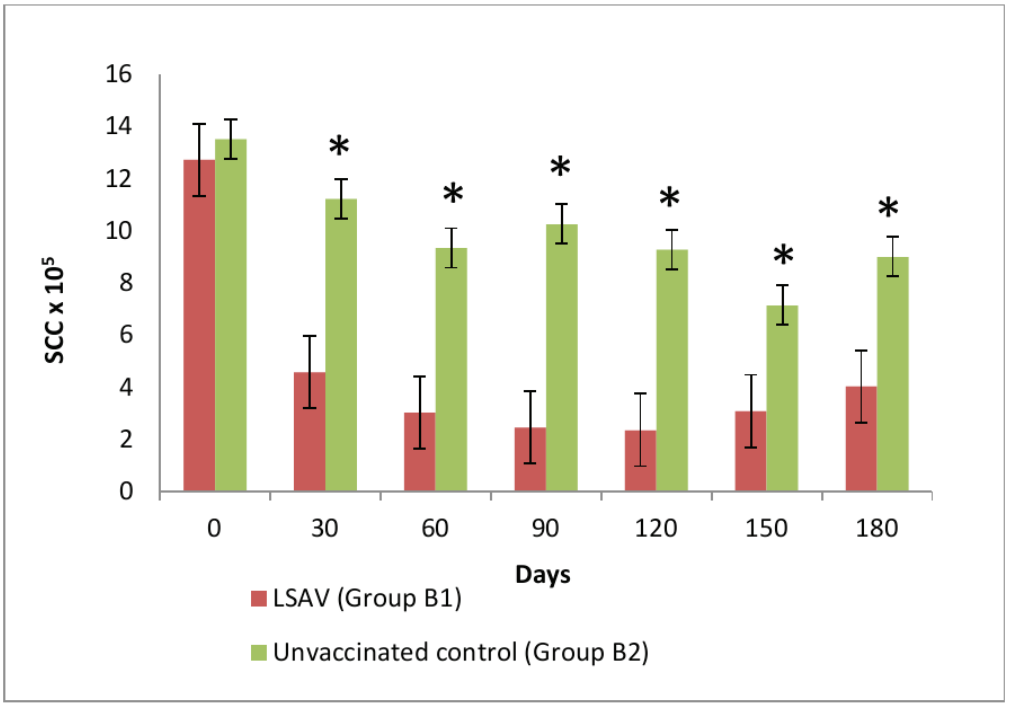

Fig.3: Comparative milk somatic cell count (mean±SD); 10^5/ml of milk) in lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. Aureus* vaccine and unvaccinated control group. * indicate the significance difference between vaccinated and control group

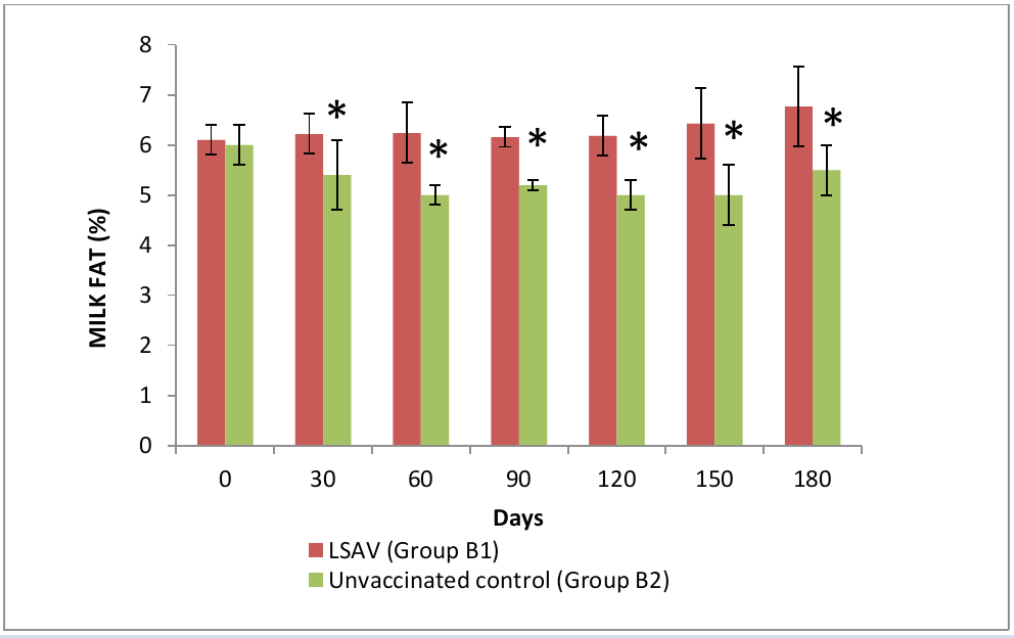

Fig.4: Comparative milk fat percentage (mean±SD) in lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. Aureus* vaccine and unvaccinated control group. * indicate the significance difference between vaccinated and control group

kept on increasing until day 90 and declined slightly at day 120 as well as on day 150. A slight increase in milk protein concentration was noted at day 180 post-vaccination. In group B2 a constant decline in milk protein concentration was recorded from day 0 to day 180 post-vaccination (Figure 5).

The prevalence of mastitis was lower in group B1 on different days as compared with group B2 on different days as mentioned in Figure 6. This showed the marvelous effect of live attenuated *S. aureus* vaccine in control of mastitis.

DISCUSSION

Several studies have been conducted emphasizing

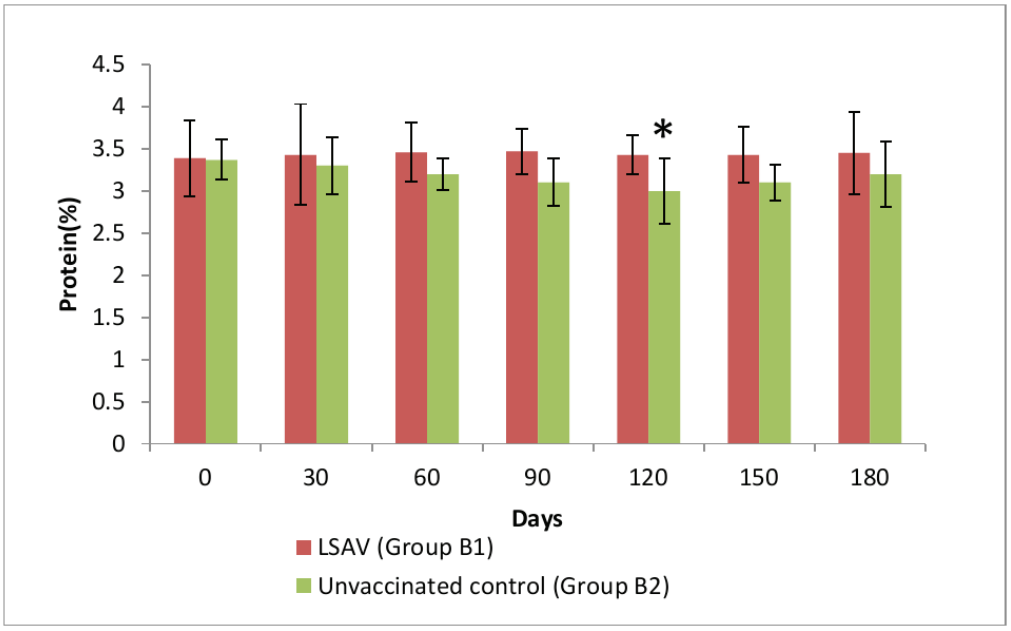

Fig. 5. Comparative milk protein concentration (mean±SD) in lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. Aureus* vaccine and unvaccinated control group. * indicate the significance difference between vaccinated and control group

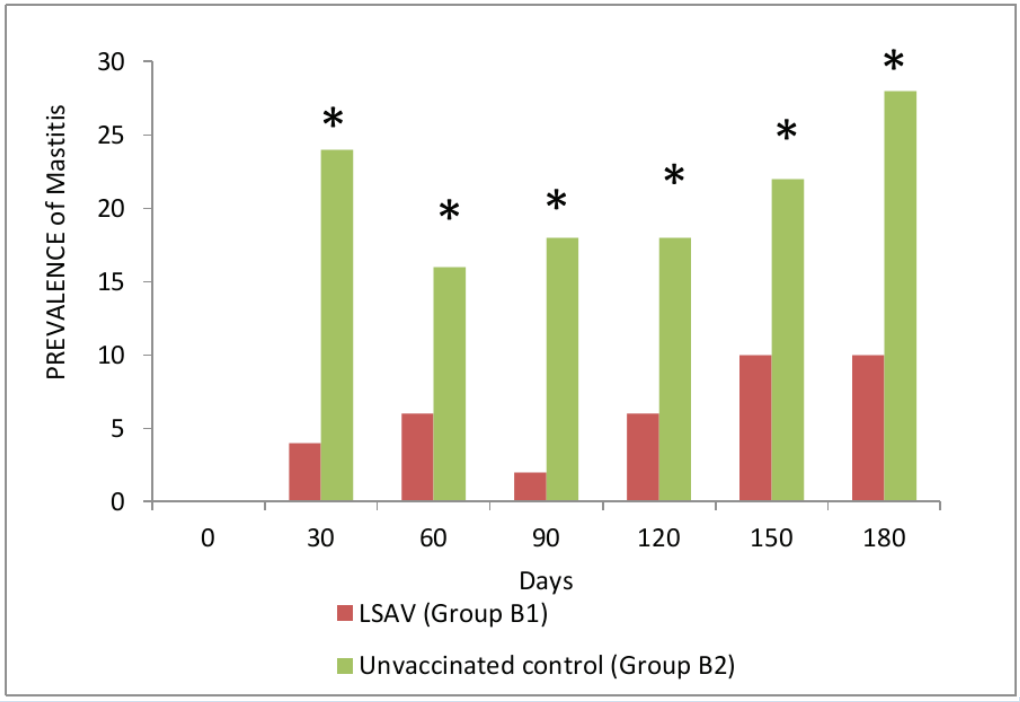

Fig.6: Prevalence of mastitis through CMT in lactating buffalo vaccinated twice during postpartum period with live attenuated *S. Aureus* vaccine and unvaccinated control group. * indicate the significance difference between vaccinated and control group

the evaluation of serum and whey antibody titers to assess the efficacy of mastitis vaccines (Kirlin & Watson, 1987; Nordhaug et al., 1994a-b). It is also pertinent to mention that LSAV stimulates the IgG_2 which is cytophilic to neutrophils coming into the udder within 6 hrs in response to mastitis. Contrary to it, oil-based vaccines initiate the formation of IgG_1 which is not required to subjugate the infection (Kirlin and Watson, 1987). In this study, we observed that vaccine neither exert any side effect nor become virulent with no possibility of leading to disease occurrence. Hence, all the animals administered with vaccine remained clinically healthy during the trial suggesting that this vaccine can be used safely in the field conditions.

Somatic cell count is an index of the udder health for the detection of sub-clinical mastitis in dairy animals (Dohoo and Meek, 1982). In the current study, a significant increase in SCC in unvaccinated group as compared to vaccinated group. This is in line with the findings of Yoshida et al*.,* (1984) and Raza et al., (2013) who conducted the trials of different *S. aureus* vaccines (adjuvanted) in buffaloes and cows and concluded in a drastic reduction in SCC after vaccination for 5-6 months.

The increased fat percentage may be ascribed to the impact of LASV negating the chances of mastitis. On the other hand, decreased fat percentage may be due to the presence of infection that influence negatively the fat formation process. Our findings are consonant with the results of Ahmad and Muhammad (2008), and Raza et al., (2013) who also observed a similar impact on milk fat of buffalo and cattle by using polyvalent mastitis vaccines. The LSAV caused an increase in milk protein. On the other hand, a significant decrease in the un-vaccinated control group may be due to the degradation of milk protein by *S. aureus* infection.

CONCLUSION

It was concluded that LSAV increased antibody titer was efficient in reducing the incidence of mastitis, somatic cell count and increased fat and protein concentration. Thus, it should be adopted as an additional tool to prevent mastitis along with other control measures in dairy buffaloes to maximize the farm profit.

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