V. DISCUSSION

Mastitis remains as one of the most important diseases in dairy cattle despite the progress made in improving general udder health in recent years. It continues to be one of the economically most important diseases of dairy cattle, accounting for 38 per cent of the total direct costs of the common production diseases. Losses have been estimated at $2 billion/year, of which 70 per cent was attributed to reduced milk yield from subclinical mastitis. *Staphylococcus aureus* is one of the most frequently isolated contagious mastitis pathogens that causes either clinical or subclinical or chronic bovine mastitis with high economic losses to the farmers. In cows, intramammary infections (IMI) due to *S. aureus*, which account for 25 to 30 per cent of total IMI, are generally subclinical (Leitner *et al.*, 2000 and Dego and Tareke, 2003). *Staphylococcus aureus* is a common cause of intramammary infections, which frequently become chronic, associated with the ability of the bacteria to produce biofilm and also recurrent infections are often attributable to biofilm growth of bacteria (Cucarella *et al.*, 2004 and Melchior *et al.*, 2006).

Biofilm formation is accompanied by significant genetic and subsequent physiological changes in the bacteria. A group of surface proteins sharing several structural and functional features is emerging as an important element in the biofilm formation process of diverse bacterial species. The first member of this group of proteins was identified in a *S. aureus* mastitis isolate and was named ‘BAP’ for biofilm-associated protein. As common structural features, Bap-related proteins: (i) are present on the bacterial surface; (ii) confer upon bacteria the capacity to form a biofilm; (iii) play a
relevant role in bacterial infectious processes (Lasa and Penades, 2006 and Latasa et al., 2006).

In view of an effective control of mastitis, immunization against mastitis has been a goal of researchers for many years and vaccination against mastitis pathogens is practiced in some dairy farms, especially in western countries. Research on mastitis vaccines has been conducted for at least 35 years. Mastivac I, a newly introduced vaccine designed to protect \textit{S.aureus} mastitis, is being commercially used in Israel since 2004 (Leitner et al., 2008). Many other conventional vaccines are also commercially available against \textit{S.aureus} mastitis. The efficacy of such vaccines in reducing the severity of clinical disease has been demonstrated (Yancey, 1993; Nordhaug et al., 1994a; Tenhagen et al., 2002; Leitner et al., 2003b and Lee et al., 2005) but the vaccines were unable to prevent new intramammary infections. Further, the efficacy of conventional \textit{S.aureus} vaccines has yet to prove their effectiveness with respect to Indian perspective. Recently, \textit{E.coli} BF and FC based vaccines were compared by vaccination trials in rabbits (Kavitha, 2008 and Jyothi, 2009) and in lactating cows (Chandrashekhar, 2009). The study in rabbits indicated the superiority of \textit{E.coli} BF vaccine as the CMT positivity, SCC values and percentage of mammary glands showing lesions on challenging, were significantly less in case of BF vaccinated groups. Further, specific IgG levels in serum and milk and specific IgA levels in milk as detected by ELISA were significantly high in BF vaccinated than FC vaccinated rabbits. The study in lactating cows with \textit{E.coli} biofilm vaccine has indicated better humoral and cell mediated immune response as the serum IgG level was significantly high and percentage of CD4+ and CD8+ cells were increased in BF vaccinated than FC vaccinated and control cattle (Chandrashekhar, 2009).
In order to develop an effective vaccine to control bovine mastitis caused by *S.aureus*, the present study was undertaken to evaluate the *S.aureus* biofilm based vaccine in rabbits.

5.1 **Molecular typing of *S.aureus* isolates for ‘bap’(Biofilm associated protein) gene.**

The ability of *S.aureus* to form biofilm *in vivo* is considered to be a major virulence factor influencing its pathogenesis in mastitis. The implication of biofilm in chronic bacterial infection in many species has triggered an increasing interest in the characterization of genes involved in biofilm formation. The *bap* gene is a newly identified gene that encodes the biofilm-associated protein i.e. ‘BAP’ which is involved in biofilm formation in bovine mastitis causing *S. aureus* (Cucarella *et al.*, 2001, Götz, 2002 and Vautor *et al.*, 2008). The ability of staphylococci to form biofilm affords at least two properties: the adherence of cells to a surface and accumulation to form multilayered cell clusters. A trademark is the production of the slime substance, polysaccharide intercellular adhesion (PIA), an exopolysaccharide composed of beta-1,6-linked N-acetylglucosamines with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defences and antibiotic treatment. Although exopolysaccharides are important and often essential compounds of the biofilm matrix, recent evidences suggest that a biofilm-associated protein in a bovine mastitis causing *S. aureus* isolate plays a leading role during the development of the microbial communities. Later on, other surface proteins homologous to ‘Bap’ and involved in biofilm development have been described in many Gram-positive and Gram-negative bacteria such as *Esp* of *Enterococcus faecalis* and *BapA* of *Salmonella enterica* *ssp.enterica* serotype Enteritidis (Cucarella *et al.*, 2001, Götz, 2002 and Latasa *et al.*, 2008).
Polymerase chain reaction based amplification of *bap* gene not only helps us to identify the potential of *S. aureus* to produce biofilms but also its role in establishment of infection in both clinical and sub clinical mastitis.

In the present study, a PCR analysis was carried out using ‘*bap*’ specific primers for 25 bovine mastitis *S. aureus* isolates (SA1 to SA25) which were isolated from clinical and subclinical mastitis and characterized by Rajeev (2006). Out of the 25 isolates, 10 (40 per cent) isolates were ‘*bap*’ positive that showed an amplicon of 971 bp. Among 10 ‘*bap*’ positive isolates, three isolates (SA2, SA3 and SA4) were from clinical mastitis and seven isolates (SA7, SA8, SA10, SA15, SA16, SA17 and SA23) were from sub clinical mastitis cases (Fig.1a & 1b and Table 2). In the previous study conducted by Rajeev (2006) who had subjected these 25 isolates of *S. aureus* for icaA specific PCR and found that 23 (92 per cent) isolates were positive for icaA gene. It was found that all the *bap* positive isolates were also icaA positive in this study (Table 2). These findings are in agreement with Cucarella *et al.* (2004) who analyzed 195 bovine subclinical mastitis *S. aureus* isolates by PCR using icaADBC and *bap* specific primers. Results revealed that 94.36 per cent were icaADBC positive and 25.6 per cent were *bap* positive isolates. They also reported that all the *bap* positive isolates were also ica positive. They also studied the relationship between the ability to produce chronic bovine mastitis and biofilm formation and found that *bap*-positive isolates were significantly more able to colonize and persist in the bovine mammary gland *in vivo* and were less susceptible to antibiotic treatments when forming biofilms *in vitro*. In addition, analysis of the structural *Bap* gene revealed the existence of alternate forms of expression of the Bap protein in *S. aureus* isolates obtained under field conditions throughout the animal's life. The presence of anti-
Bap antibodies in serum samples taken from animals with confirmed *S. aureus* infections indicated the production of Bap during infection. Furthermore, disruption of the *ica* operon in a *bap*-positive strain had no effect on *in vitro* biofilm formation, a finding which strongly suggested that Bap could compensate for the deficiency of the polysaccharide intercellular adhesin / PIA product (a biofilm matrix polysaccharide).

### 5.2 Analysis of proteins of *S. aureus* SA16 and SA2 grown under BF and FC mode

Bacteria do not express the same antigens *in vitro* as *in vivo* — a mechanism known as ‘phase variation’. When bacteria grow *in vivo*, they must cope up with a hostile environment in which certain nutrients are not abundant, and where the host attempts to eliminate them in different ways. The biofilm form of bacteria grown *in vitro* mimic the *in vivo* antigens (Costerton *et al.*, 1999) and it is a fact that only *in vivo* derived antigens can afford protection against homologous and heterologous serotypes (Heddleston and Rebers, 1972). These studies indicated that antigens expressed in biofilm may play a role in the immunoprophylaxis against mastitis caused by *S. aureus*.

#### 5.2.1 Protein expression profile of *S. aureus* SA16 and SA2 grown under BF and FC mode

In the present study, *S. aureus* SA16 and SA2 strains were selected to study the immune response against proteins of these two strains grown under BF and FC mode. The selection of these two isolates was based on presence of *bap* gene which was characterized in the present study and also *icaA* gene which was based on earlier studies carried out by Rajeev (2006). Both *icaA* and *bap* participate in the biofilm formation in *S. aureus* by encoding proteins involved in the synthesis of a biofilm matrix.
polysaccharide and biofilm associated protein respectively. The biofilm associated protein promotes primary attachment to living or inert surfaces and intercellular adhesion which are the two important stages in biofilm formation as reported by Cucarella et al. (2004). \textit{S.aureus} SA16 and SA2 strains were grown in BF mode according to the method standardized by Naveenkumar (2005) using 0.32 per cent TSB with 0.3 per cent bentonite clay as an inert surface for three days, whereas in FC mode, bacteria were grown in three per cent TSB for 16 hrs.

The protein profiles of \textit{S.aureus} SA16 and SA2 grown under BF mode had differed from \textit{S.aureus} SA16 and SA2 grown under FC mode by 56 per cent with unique expression of 26.69, 30.59, 34.02, 40.77, 51.77, 57.22, 65.09, 102.72 and 114.21 kDa and repression of 19.03, 22.61, 25.25, 28.60, 33.30, 38.09, 39.91, 62.45, 79.14 and 149.02 kDa. The unique proteins of 51.77 and 57.22 kDa were detected only in \textit{S. aureus} SA16 BF proteins, but not in \textit{S. aureus} SA2 BF proteins or in FC proteins. The polypeptides of 37.05, 54.39 and 94.91 kDa were expressed in BF and FC of both the strains with more prominent expression of 37.05 kDa protein in BF cells (Fig.2 and Table 3). Similar findings were also observed by Naveenkumar (2005) who reported that the protein profiles of bovine mastitic \textit{S.aureus} BF cells differed from FC by 22 per cent with over expression of 79, 65, 60, 48 and 40 kDa proteins and repression of 85 kDa protein. He also found that the unique proteins of 67, 37, 26 and 20.8 kDa proteins were expressed only in BF cells. The unique expression or over expression of additional proteins or repression of proteins in bacteria grown under BF mode was also reported by Arun, (2002), Veeregowda, (2003), Prakash, (2004) and Lacqua et al. (2006).
Bacteria grown under BF mode are demonstrably and profoundly different from their FC counterparts in antigenic character. The adhesion of bacteria to surface triggers the expression of a number of genes, making the BF cells phenotypically different from the FC of the same species (Costerton et al., 1995). There is a mounting evidence to show that both up and down regulation of number of genes occur in the attaching cells upon initial interaction with the substratum. Combaret et al. (2000) found that 22 per cent of the genes were up regulated and 16 per cent were down regulated in BF forming *Pseudomonas aeruginosa*. Genes encoding for enzymes involved in glycolysis or fermentation (phosphoglycerate mutase, triosephosphate and alcohol dehydrogenase) are up regulated in BF forming *S.aureus* and opined that the up regulation of these genes could be due to oxygen limitation in the developed BF, favouring fermentation (Becker et al., 2001).

5.3 Western blotting

Western blotting is an important useful technique extensively used to detect antigenic relatedness and immunogenic components in a crude antigen mixture. This technique was employed to analyse the immunogenicity of BF and FC proteins. In the present study, HIS was raised against *S.aureus* SA16 BF and FC proteins and used to identify the immunogenic proteins and their cross reactivity with heterologous strain of *S.aureus* SA2. Western blot studies using HIS against BF proteins and FC proteins of *S.aureus* showed the evidence of antibody response against proteins of *S.aureus* SA16 and SA2 grown under both planktonic as well as BF mode.
5.3.1 Western blot analysis of *S.aureus* BF and FC proteins probed with *S.aureus* SA16 BF and FC hyper immune serum

On probing with hyper immune sera against *S.aureus* SA16 BF proteins, immunogenic proteins in the region between 29 and 43 kDa with prominence at 30.59, 34.02 and 37.05 kDa were observed in BF proteins of *S. aureus* SA16 and SA2. Further, immunogenic proteins of 94.91 and 114.21 kDa were noticed in BF proteins of *S.aureus* SA16. Whereas, in case of BF proteins of *S.aureus* SA2, diffused immunogenic bands were noticed at this region. An additional 54.39 kDa immunogenic band was observed in BF proteins of *S.aureus* SA16. Probing of FC proteins of *S.aureus* SA16 and SA2 with BF HIS revealed bands in the region 25.25 - 28.60, 54.39 and 62.45 kDa with an additional protein of 38.09 kDa in *S.aureus* SA16 alone (Fig.3a and Table 4).

On probing with hyper immune sera against FC proteins of *S.aureus* SA16, polypeptides of 25.25, 28.60, 54.39 and 62.45 kDa in case of FC proteins of *S.aureus* SA16 and 25.25 and 28.60 kDa in case of FC proteins of *S.aureus* SA2 were observed. Furthermore, with hyper immune sera against FC proteins, a diffused band in the region of 65kDa in case of BF proteins of *S.aureus* SA16 and 29-43 kDa in case of BF proteins of *S.aureus* SA2 were noticed (Fig.3b and Table 5).

In the present study, the important findings of western blot analysis of *S.aureus* proteins was that a total of six immunogenic proteins viz. 30.59, 34.02, 37.05, 54.39, 94.91 and 114.21 kDa were detected in *S.aureus* SA16 BF proteins when probed with *S.aureus* SA16 BF HIS. These proteins were not detected either in BF proteins or FC proteins when probed by FC HIS. This indicates the superiority of BF proteins which are
capable of inducing better antibody response compared to FC proteins. Further, the immunogenic proteins of 30.59, 34.02, 37.05 kDa were observed in *S.aureus* SA2 BF proteins, 25.25, 28.60, 54.39 and 62.45 kDa in FC proteins with an additional protein of 38.09 kDa in *S.aureus* SA16 FC proteins alone when probed with *S.aureus* SA16 BF HIS indicating the cross reactivity of BF proteins. But, such immunogenic cross reactivity was not observed when blot was probed with FC HIS. These findings are in line with observations of Naveenkumar (2005) who analysed bovine mastitic *S.aureus* BF and FC proteins by western blotting. The proteins of BF and FC when probed with BF hyper immune serum showed thirteen immunogenic proteins including over expressed 79, 65, 60, 48 and 40kDa proteins along with the unique proteins of BF cell 67, 37, 26 and 20.8 kDa were found to be immunogenic. Similar observations were also made by Arun (2002) who reported that a maximum of eight and five BF OMPs of *Pasteurella multocida* homologous and heterologous strains respectively were detected by *Pasteurella multocida* A:1 BF hyper immune serum indicating immunogenicity and cross reactivity of BF OMPs.

Antibody response detected against proteins of both the strains with recognition of extra proteins as immunogens confirmed the superiority of BF-based antigen with respect to their cross protection. Additional immunogenic proteins were recognized by *S.aureus* SA16 BF protein HIS in antigen grown under BF mode compared to FC protein HIS probing. Similar findings were also observed in BF of *S. Gallinarum* (Prakash and Krishnappa, 2002), *P. multocida* A: 1 (Arun, 2002), *E. coli* (Veeregowda, 2003) and bovine mastitic *E. coli* (Sumathi (2005) BF cells. Kavitha (2008) studied the western blot analysis of bovine mastitic *E.coli* BF and FC OMPs. She reported that additional
24.4, 28.5 kDa polypeptides in the case of OMPs of both *E.coli* O9 and O147 grown under BF mode and 34.5 kDa polypeptide in the case of *E.coli* O147 grown under BF mode when probed with *E.coli* O9 BF HIS indicating the immunogenicity and cross reactivity of novel proteins expressed when *E.coli* was grown under BF mode.

These observations proved that the BF cells expressing some unique proteins which are highly immunogenic but are absent in FC and may play an important role in protection. To conclude, BF antigens may be the suitable vaccine candidates against mastitis. The current work, supporting the earlier findings of Naveen Kumar (2005) on immunoblot analysis indicated that the protein expression profile of *S. aureus* isolated from bovine mastitis cases and then grown under BF mode differed from their FC counterparts. It can be concluded that antigen grown under BF mode expressed cross-reactive proteins, which can be subsequently incorporated in the bovine mastitis vaccine to confer cross protection.

### 5.4 Experimental studies on mastitis in rabbits

Rabbits have been considered to be good animal models for mastitis studies as the lactating mammary gland of the rabbit is susceptible to natural infection by staphylococci and the disease could be reproduced in the laboratory by injecting low numbers of organisms into the mammary tissue. It is easier to handle them for intramammary injections or infusions (Adlam *et al.* 1977 and Adlam *et al.* 1980). Bovine and ovine mastitis studies have been carried out in rabbit model by Amorena *et al.* (1991), Reinoso *et al.* (2002) and Kavita (2008).
In the present study, rabbits were used as an experimental model to study bovine mastitis with respect to induction of mastitis as well as protection against challenge infection by biofilm based vaccine. The preliminary study was carried out in the lactating rabbits to optimize the infective dose of *S. aureus* to induce mastitis by bovine mastitis isolates, *S.aureus* SA16 and *S.aureus* SA2 possessing both *bap* and *icaA* genes. In the study, inoculation was done with $10^4$, $10^5$, $10^6$ and $10^7$ cfu/ml of bacterial suspension at the base of the teat. The mammary glands were observed for gross lesions. Milk samples collected from zero day up to 144 hrs were used for the determination of SCC and CMT.

Milk SCC are considered to be an important parameter for assessing mammary health status in lactating animals and milk yield decreases as SCC and incidence of mastitis increase. Thus, SCC in heifer mammary gland secretions were analyzed to measure the degree of inflammation and potential reductions of future milk yield. The SCC of milk samples collected during the study were determined. California mastitis test is an effective cow side proxy for SCC useful to predict IMI in cows (Batra and McAllister, 1984 and Vianni and Filho, 1989). Somatic cell count determination has been widely accepted as a screening test to identify intramammary infections in lactating cows. The higher prevalence of *S.aureus* IMI is associated with higher milk SCC (Schalm and Noorlander 1957, Miljkovic and Milojevic 1962, Sharma and Rajani 1965, Chakraborthy and Hazarika 1977, Okello, 1992, Mohinikumari and Janakiramguptha, 2002, Middleton *et al.*, 2002 and Mdegala *et al.*, 2004).

In the present study, mastitis was induced in rabbits by both *S.aureus* SA16 and *S.aureus* SA2 strains as indicated by gross lesions (Figs.4b, 4c, 4d and 5), increase in
SCC (Figs.8 and 9) and CMT positivity (Figs.10 and 11). The macroscopic lesions were very well appreciated in all the quarters of mammary glands infected with varying concentration of bacterial suspension of both *S. aureus* SA16 and *S. aureus* SA2 strains at 48 hrs post infection (Fig 4c). There was a drastic increase in SCC in the milk samples collected from all the quarters at 48 hours post infection. Also, maximum quarters of mammary glands were found positive for CMT on day two after inoculation with different concentration of bacterial suspension of both *S. aureus* SA16 and *S. aureus* SA2 strains. These findings are in agreement with Reinoso *et al.* (2002) who reported the varying degrees of macroscopic lesions *viz.* swelling, necrosis of the quarters in rabbits inoculated with $10^8$ cfu/ml of virulent and avirulent strains of *S. aureus* isolated from the bovine mastitis cases. Amorena *et al.* (1991) reported that macroscopic lesions such as swelling and induration were observed at 24 and 48 hrs after inoculating with $5 \times 10^5$ cfu/0.5 ml of *S. aureus* bacterial suspension into the mammary gland of lactating rabbits. Craven and Anderson (1982) reported that acute mastitis could be produced by inoculating $10^6$ cfu of bovine mastitis causing *S. aureus* into normal lactating mouse mammary glands. They observed rapid multiplication of bacteria with production of alpha toxin, necrosis and death of the inoculated mice. Reinoso *et al.* (2002) reported increased SCC in milk samples collected from mastitis induced rabbits. The results in the present study clearly show that both the strains were able to induce mastitis in rabbits, which may be used for challenging in vaccinated animals. Based on these indicators of mastitis, $10^4$ cfu / ml of bacterial suspension was found to be an optimum infective dose for challenging the vaccinated animals.
5.5 Immunization and challenge studies in rabbits

In the present study, \textit{S.aureus} grown under BF mode was exploited as potential vaccine candidate against mastitis caused by \textit{S.aureus} in bovines. Rabbit model was used to test the efficacy of this vaccine. Experimental trials in pregnant rabbits using these vaccines showed very promising results necessitating further evaluation in the bovine system under both experimental and field conditions.

Immunization was carried out with \textit{S.aureus} SA16 BF as well as FC vaccine. Comparative evaluation was done by challenging both the groups with \textit{S.aureus} SA16 (homologous strain) and \textit{S.aureus} SA2 (heterologous strain). Rabbits vaccinated with \textit{S.aureus} SA16 BF and FC vaccines were evaluated for gross lesions of mammary glands, SCC, CMT and serum IgG response by ELISA after challenge.

The mean percentage of mammary glands showing lesions, mean SCC and CMT positivity after challenging with homologous strain (\textit{S.aureus} SA16) and with heterologous strain (\textit{S.aureus} SA2) were considered. Milk samples collected at different intervals (days 0, 1, 2, 6, 14 and 21 post challenge) were evaluated for SCC and CMT. Sera samples collected at different intervals (days 0, 15, 22, 29, 30, 31, 35, 43 and day 50) were evaluated for IgG response by indirect ELISA.

5.5.1 Gross lesions

Statistical analysis was carried out using one way ANOVA (Tukey’s multiple comparison test). There was significant difference (P<0.05) between BF vaccinated groups and FC vaccinated groups and also there was a significant difference (P<0.05)
within the BF vaccinated (Group 1 and Group 2) and FC vaccinated groups (Group 3 and Group 4) with respect to mean percentage of mammary glands showing lesions (Fig. 12). The mean percentage of mammary glands showing lesions at 48 hrs after challenging with homologous strain \( S.\text{aureus} \text{SA16} \) and heterologous strain \( S.\text{aureus} \text{SA2} \) was less (14.55 per cent in Group 1 and 24.95 per cent in Group 2) in BF vaccinated rabbits compared to FC vaccinated (50 and 62.5 per cent in Group 3 and 4 respectively) animals indicating BF vaccine had conferred better protection and cross protection against challenge compared to FC vaccine. The results are in accordance with Kavitha (2008) who reported bovine mastitis \( E.\text{coli} \) BF vaccine had conferred higher cross protection upon challenge studies using homologous \( E.\text{coli} \text{O9} \) and heterologous \( E.\text{coli} \text{O147} \) serotypes in rabbits wherein mean percentage of mammary glands showing lesions at 48 hrs after challenging with homologous serotype \( E.\text{coli} \text{O9} \) was 12.5 per cent and 11 per cent with heterologous serotype \( E.\text{coli} \text{O147} \) in BF vaccinated rabbits compared to 41.46 per cent with homologous and 65.62 per cent with heterologous serotype for FC vaccinated group.

In another study by Prakash (2006) observed that progeny from \( E.\text{coli} \) BF vaccinated broiler parents had only 16.66 per cent mortality upon homologous \( E.\text{coli} \text{O78} \) challenge and 16.66, 33.32 and 16.66 per cent mortality upon three heterologous \( E.\text{coli} \text{O2} \) challenges on days 15, 29 and 39 respectively whereas 100 per cent mortality was observed in all the three challenges upon heterologous challenge and 66.64, 50 and 66.64 per cents upon homologous challenge in progeny from unvaccinates. Similar findings were also observed by Shivaraj and Krishnappa (2002) and Veeregowda (2003). These studies supported the hypothesis that BF vaccine is superior to the FC
vaccine against experimentally induced mastitis in rabbits using \textit{S.aureus} isolates from bovine mastitis.

\subsection*{5.5.2 Somatic Cell Count}

In the case of mastitis, an enhanced immune response is not always considered beneficial. One important component of the immune response is the migration of large numbers of white blood cells (in the udder called somatic cells) to the infected gland. The presence of somatic cells in the milk is not considered a positive outcome as somatic cells are evidence of mastitis and reduce the quality of milk. An increase in SCC in milk leads to the release of lipolytic (lipases) and proteolytic (plasmin) enzymes which can degrade the triglycerides of milk fat and casein contents of the milk. This leads to poor quality milk in the mastitis affected animals (Saeman \textit{et al.}, 1988). Unless a vaccine can prevent new infections throughout lactation and dramatically reduce the SCC of affected animals, it may be difficult for a producer to recognize the benefit of using a \textit{S. aureus} vaccine (Ruegg, 2001). However, it is crucial to obtain a recruitment of activated polymorphonuclear leukocytes (PMNL) into the milk of vaccinated animals as early as possible after the entry of \textit{S.aureus} into the mammary gland. This is very much essential for the effective phagocytosis of invading pathogens by the PMNL (Sutra and Poutrel, 1994).

In the present study, statistical analysis was carried out using one way ANOVA (Tukey’s Test) to compare SCC of Group 1 (\textit{S.aureus} SA16 BF vaccinated rabbits challenged with homologous strain \textit{i.e.}, \textit{S.aureus} SA16) and Group 2 (\textit{S.aureus} SA16 BF vaccinated rabbits challenged with heterologous strain \textit{i.e.}, \textit{S.aureus} SA2). Comparisons
were also made between SCC of Group 3 (\textit{S.aureus} SA16 FC vaccinated rabbits challenged with homologous strain) and Group 4 (\textit{S.aureus} SA16 FC vaccinated rabbits challenged with heterologous strain).

Analysis showed that the difference in the SCC of milk collected on day ‘0’ \textit{i.e.}, before challenge was ‘non-significant’ in all the groups. The values were varied from $5 \times 10^5$ cells/ml to $8 \times 10^5$ cells/ml (Fig.13). Meanwhile, on day one \textit{i.e.}, 24 hrs post challenge, there was sudden increase in SCC in all the four groups ($10 \times 10^5$ in Group 1, $27 \times 10^5$ in Group 2, $15 \times 10^5$ in Group 3 and $37 \times 10^5$ cells/ml in Group 4) indicated the recruitment of activated PMNL into milk of vaccinated animals. Similar observations were made by Kavitha (2008) who also reported a sudden increase in SCC in milk samples from rabbits vaccinated with \textit{E.coli} BF vaccine and challenged with homologous and heterologous serotypes. These findings were in accordance with Sutra and Poutrel (1994) who reported that it is very much essential to obtain a recruitment of activated PMNL into the milk of vaccinated animals as early as possible after the entry of \textit{S.aureus} into the mammary gland to prevent the establishment of the infection. They also opined that the migration of large number of PMNL into the mammary gland must coincide with the presence of opsonising antibodies in milk for effective phagocytic killing of the invading pathogens. In a study conducted by Jyothi (2009) who analysed the milk samples collected from the same rabbits for \textit{S.aureus} specific IgG response and \textit{E.coli} specific IgG response from rabbits vaccinated with \textit{E.coli} BF vaccine and reported that a significant increase in the specific IgG antibodies to \textit{E.coli} and \textit{S.aureus} BF vaccine in milk samples collected from four groups of rabbits supported this hypothesis. When SCC were compared between different groups, a highly significant difference ($P < 0.001$) was
observed within the BF vaccinated (Group 1 and 2) and FC vaccinated groups (Group 3 and 4). But, there was no significant difference (P>0.05) between BF vaccinated-homologous challenged and FC vaccinated-heterologous challenged groups. On day two post challenge, peak SCC response was obtained with a highly significant difference (P<0.001) within the BF and FC vaccinated group. But, the BF and FC vaccinated groups challenged with homologous strain did not differ significantly (P>0.05).

Milk samples collected on days 6, 14 and 21 post challenge have also yielded similar results (Fig.13). It was observed that there was a reduction in the SCC in BF and FC vaccinated groups after 48 hrs of challenge. Similar observations were made by Pankey et al. (1985), Leitner et al. (2003a) and Shakoor et al. (2006). Pankey et al. (1985) reported that the somatic cell counts were significantly lower for cows vaccinated with protein A and a commercial staphylococcal bacterin (Somatostaph®) and challenged with S.aureus. Leitner et al. (2003a) also found that the SCC were very low in cows vaccinated with S. aureus vaccine composed of three field isolates of bovine mastitis and challenged with a highly virulent S. aureus strain. Shakoor et al. (2006) observed that S.aureus vaccines (live attenuated, simple bacterin, dextran sulphate adjuvanted and oil adjuvanted) reduced the somatic cell count significantly as compared to control group and concluded that S.aureus mastitis vaccines were helpful in improving the quality and quantity of milk in buffaloes. However, in Group 1 i.e., S.aureus BF vaccinated rabbits challenged with homologous strain, the mean SCC were reduced to below 10 × 10^5 cells per ml compared to other groups indicating the superiority of BF vaccine over FC vaccine in reducing the SCC in milk thereby protecting the mammary gland against challenge.
5.5.3 California Mastitis Test

California mastitis test is an effective cow side proxy for determination of SCC in milk and useful to predict IMI in cows. It has been widely implemented as a screening test to identify intramammary infections in lactating cows (Okello, 1992, Mohinikumari and Janakiramguptha, 2002 and Mdegala et al., 2004). In the present study, statistical analysis was carried out using one way ANOVA (Tukey’s Test) to compare percentage of CMT positive mammary glands of Group 1 and 2 at 48 hrs post challenge. Comparisons were also made between Group 3 and 4. Analysis indicated that there was a significant difference (P < 0.05) between BF vaccinated and FC vaccinated groups. But, there was no significant difference (P > 0.05) within the BF vaccinated groups with respect to mean percentage of CMT positive mammary glands (Fig.14) at 48 hrs post challenge. Mean percentage of CMT positive mammary glands was 9.37 and 12.5 per cent in Group 1 and 2 (BF vaccinated) respectively as against 46.87 and 56.25 per cent in Group 3 and 4 (FC vaccinated) respectively. Further, on day 6, it had reduced to 4 per cent in both the BF vaccinated groups and 29.16 and 25 per cent in FC vaccinated – homologous and heterologous challenged groups respectively. But in case of BF vaccinated groups, there was a drastic reduction in mean percentage of CMT positivity to zero at 14 days after challenge as against 20 per cent in FC vaccinated groups. These findings were in agreement with Kavitha (2008) who also analysed the mean percentage of CMT positive mammary glands in rabbits vaccinated with bovine mastitis causing *E.coli* BF vaccine and observed a significant difference (P < 0.05) between BF vaccinated and FC vaccinated group and no significant difference (P > 0.05) within the BF vaccinated group with respect to mean percentage of CMT positive mammary glands indicating cross protection conferred by BF vaccine unlike FC vaccine. This analysis
supported the hypothesis that BF vaccine is superior to FC vaccine with respect to protection and cross protection against \textit{S.aureus} challenge infection.

5.5.4 Enzyme Linked Immuno Sorbent Assay

Seromonitoring of animals for the presence of antibodies against mastitis causing pathogens has an immense value as a measure of level of protection. Further, periodical monitoring of \textit{S.aureus} antibodies has a distinct advantage of providing an insight into the immune status against mastitis. Enzyme-linked immunosorbent assay is one of the sensitive methods for the detection of \textit{S.aureus} antibodies in the serum of vaccinated as well as mastitis affected animals (Matsushita \textit{et al.}, 1990; Grove and Jones, 1992; Herbeline \textit{et al.}, 1997 and Leitner \textit{et al.}, 2000).

5.5.4.1 Optimization of \textit{S.aureus} BF antigen

An Indirect ELISA was standardized by optimizing \textit{S.aureus} BF antigen dilution at 1.25 µg/ml against a constant 1:100 dilution of positive serum control (C+) and negative serum control (C−) by using anti-rabbit IgG-HRP conjugate at a constant 1:25,000 dilution. The end point titration of antigen showed a sudden drop in the OD values at 1:80 dilution of antigen (Fig.15 and Table 6). At this concentration of antigen, the OD value of strong positive at 490 nm was 0.824. Hence, 1.25 µg/ml (1:80) dilution of \textit{S.aureus} BF protein antigen was used as optimum working dilution of antigen for monitoring \textit{S.aureus} BF and FC vaccinal antibodies in sera samples collected on days 0, 15, 22, 29, 30, 31, 35, 43 and 50 from different groups of rabbits. Kavitha (2008) used higher concentration of antigen \textit{i.e.}, 5 µg/ml of \textit{E.coli} BF OMP antigen and a constant serum dilution of 1:100 as optimum working dilution for monitoring \textit{E.coli} BF vaccinal
IgG antibodies in sera collected from the *E. coli* BF vaccinated rabbits. Herbeline *et al*. (1997) used 4 μg/ml of purified *S. aureus* α - toxin (alpha toxin) and test sera samples diluted at 1:2000 and 1: 4,000 were used as optimum dilutions for indirect ELISA to measure antibodies against *S. aureus* α - toxin in cows vaccinated with staphylococcal α - toxin. Gilbert *et al*. (1994) used 1 μg/ml of purified capsular polysaccharide type 5 of *S. aureus* to measure the specific antibodies in lactating cows immunized with purified capsular polysaccharide type 5 of *S. aureus*. The lower concentration of antigen used in the present study was probably an indication of better quality of antigen employed in the test and which was also economical.

### 5.5.4.2 Monitoring of pre and post challenged specific IgG antibodies in *S. aureus* BF, FC vaccinated and control rabbits

Sera samples collected on days ‘0’ (corresponded to 12th day of gestation/pre immunization) 15, 22, 29, 30, 31, 35, 43 and 50 from *S. aureus* BF, FC vaccinated challenged with homologous and heterologous strains and control rabbits were subjected to indirect IgG ELISA. In the present study, it was observed that there was a gradual increase in the PP values of sera samples collected from different/ four groups (viz., 1, 2, 3 and 4 and 5) at days 15 and 22 of the experiment compared to control *i.e.*, OD values were increased after immunization and after each booster and remained elevated till the end of the experiment at 50 day (Fig.16 and Table 7). These findings are supported by Guidry *et al*. (1991) who reported that serum agglutination and ELISA titers of cows immunized with two different strains of mastitis causing *S. aureus* were increased after immunization and after each booster and maintained till the end of the experiment at 112 day.
At the same time, no significant difference among Group 1 and 2 and also Group 3 and 4 was noticed in the PP values of sera samples collected at the same periods. Meanwhile, there was a significant increase in the PP values of sera samples collected at day 29 of the experiment *i.e.*, a week after 2nd booster dose given to the animals indicating the boosting of humoral response in immunized animals. However, there was a sudden drop in the PP values on day ‘30’ of the experiment *i.e.*, on the first day of post challenge and then increased gradually after 2nd day till 21st day post challenge. The sudden drop in the level of IgG antibodies in the sera samples collected on day one after challenge could be due to the selective passive transfer of IgG from blood circulation into the mammary gland and subsequent involvement of specific IgG in phagocytic killing of bacteria at the site of infection. Immunoglobulin G is a very good opsonin and a primary component of immune system responsible for promoting phagocytosis by PMNL as the polymorphonuclear neutrophil phagocytosis is the most effective defense against *S.aureus* intramammary infection (Leitner *et al.*, 2000). In the present study, the sudden drop in the level of specific IgG antibodies in the sera samples was coincided with the significant increase in the SCC in milk of vaccinated rabbits after 24 hrs of challenge with *S.aureus* (Fig.13 and 16). These findings were in agreement with Gilbert *et al.* (1994) who opined that the concurrent presence of specific antibodies and early recruitment of PMNL in the milk is required for efficient phagocytosis of *S.aureus* during intramammary infections. Sutra and Poutrel (1994) also opined that the migration of large number of PMNL into the mammary gland must coincide with the presence of opsonising antibodies in milk for effective phagocytic killing of the invading pathogens. Similar observations were also made by Miller *et al.* (1988); Watson (1989) and Leitner *et al.* (2000) who reported the enhancement of neutrophil phagocytosis by specific antibodies
of IgG isotype is responsible for protection of ruminant mammary gland against challenge exposure after immunization with staphylococcal vaccines. Subsequently, a significant increase in the antibody levels after 48 hrs post challenge may be because of intra mammary antigenic stimulation which served as intra mammary booster to increase antibody response and remained elevated till the end of the experiment up to 50 days.

5.5.4.3 Comparison of homologous v/s heterologous challenged groups

The statistical analysis was carried out to find out differences between homologous and heterologous challenged rabbits within BF and FC vaccinated groups using two tailed unpaired ‘t’ test. The differences among PP values of sera samples collected from four groups (viz., 1, 2, 3 and 4) at days 0, 15, 22, 29, 30, 31, 35, 43 and 50 (Fig.16 and Table 7). It was observed that the difference in the level of specific IgG antibodies in the sera collected from BF vaccinated group challenged with homologous strain v/s BF vaccinated group challenged with heterologous strain, on days 0, 15, 22, 29, 30, 31, 35, 43 and 50 was ‘non-significant’ (P>0.05). This confirms the ability of BF vaccine to confer cross protection against heterologous strain. Further, when FC vaccinated group challenged with homologous and heterologous strain were compared on days 43 and 50, the difference was ‘significant’ (P<0.05), indicating inability of FC vaccine to confer cross protection against heterologous strain. Thus, the hypothesis of cross protection conferred by BF vaccines is supported by this analysis. These findings are in agreement with Veeregowda (2003) who reported that experimental vaccination trials with *E.coli* BF vaccine against colibacillosis conferred 100 per cent cross protection compared to 58 per cent with FC vaccine against IM. challenge infection in poultry. Field trials conducted with BF vaccine against colibacillosis indicated 100 per cent cross
protection in poultry. Using a live attenuated strain of *S. aureus* (W 79) as a vaccine, Watson and Colditz, (1985) were able to provide moderate protection in ewes and heifers from challenge with homologous (W 79) and heterologous *S. aureus* strains.

5.5.4.4 Comparison of biofilm v/s free cell vaccinated groups

Statistical analysis was also performed to compare BF vaccinated and FC vaccinated groups by two tailed ‘t’ test, based on PP values of sera samples collected at days 0, 15, 22, 29, 30, 31, 35, 43 and 50. The highly significant (P<0.01) difference was seen in the PP values of sera collected from BF vaccinated and FC vaccinated groups on days 22, 29, 30, 31 and 35. The highly significant (P<0.001) difference was obtained in the PP values of sera collected from BF vaccinated and FC vaccinated groups on days 43 and 50 (Fig.16 and Table 7). The significantly high level of antibodies in BF vaccinated groups than FC vaccinated groups emphasized the supremacy of BF vaccine.

Further, in post challenge period, although there was a sudden drop in detectable antibody levels immediately after challenge during first two days (48 hrs), followed by a sudden significant rise in the antibody levels, especially in the BF vaccinated group (Fig.16). The sudden drop in the level of IgG antibodies within 48 hours of challenge could be due to the influx of IgG antibodies from blood into the mammary gland to opsonize the invading *S. aureus* organisms for effective phagocytic killing of bacteria at the site of infection. However, subsequent highly significant increase in the antibody level from 31st day onwards, till the day 50 of experiment induced by BF vaccine is probably due to the enhanced uptake and longer retention of the BF antigens compared to that of FC vaccine. These findings are in agreement with Azad *et al.*., (1999) who reported a progressive improvement in serum antibody titer and protective response with
time following *Aeromonas hydrophila* biofilm oral vaccination in common carps. They also opined that the possibility of BF antigens being available continuously for the immune system with minimally altered immunogenic epitopes could have contributed the observed higher antibody titre and subsequent protection. The glycocalyx of BF, is a polymer of neutral hexoses (Costerton *et al.*, 1981) which encapsulates and possibly protects the bacterial surface antigens from any destruction or alteration of immunogenic epitopes. The progressive increase in IgG response till day 50 of experiment may also be due to the peripheral antigenic stimulation resulting in the seeding of a proportion of lymphoid cells into mammary tissues, due to challenge which would have served as intramammary booster. This hypothesis is supported by the findings of Guidry *et al.* (1991) who obtained an elevated serum anticapsular antibodies till the end of the experiment at 112 days in cows immunized against *S.aureus* mastitis wherein the cows were immunized in the area of the supramammary lymph node and intramuscularly and were boosted on days 14, 42, and 70. On elevation of serum IgG titers for intra mammarily immunized cows, present results support the suggestions of Saif *et al.* (1984), that an intramammary booster following systemic immunization with viral antigens could elicit an enhanced systemic antibody response in addition to local immunity. Lymphoid cells stimulated locally in the gland following an intra mammary sensitization may traffic to local lymphatic tissues, bolstering systemic antibody responses. Thus, the relationship between IgG titers in serum and intra mammary sensitization can possibly be explained by the selective transfer of IgG across secretary cells (Saif *et al.*, 1984).

The mechanism of action by which immunization with BF vaccine provides protection appears to be related to the enhanced uptake, longer retention and slow release
of BF antigens (Azad et al., 1999) and subsequent production of antibodies to novel immunogenic proteins expressed by *S.aureus* when grown under BF mode. Further, the cross reactivity of antisera from rabbits immunized with BF proteins with heterologous strain was due to antibodies directed against such novel proteins expressed and shared by both homologous as well as heterologous strains of *S.aureus*. Enhanced immunoglobulin level of sera may lead to increased opsonization of homologous as well as heterologous strains of *S.aureus*, and thereby its elimination. Similar cross reactivity to heterologous strains was also reported by Watson and Colditz, (1985) and Hogan *et al.* (1992) on using conventional *S.aureus* and *E. coli* J5 vaccine for bovine mastitis respectively.

Earlier studies by Kavitha (2008) and Jyothi (2009) have shown that vaccination of pregnant rabbits with bovine mastitis causing *E.coli* BF vaccine was superior than FC vaccine in terms of serum IgG response as well as milk IgA and IgG response and higher cross protection against homologous and heterologous challenge. Similarly, Chandrashekharan (2009) has reported that vaccination of lactating cows with *E.coli* BF vaccine was superior to FC vaccine as serum IgG level was significantly high in BF vaccinated than FC vaccinated and control cattle. The ability of the *S.aureus* BF vaccine to induce a significant serum IgG response and cross protection against homologous and heterologous challenge infection in rabbits as demonstrated in this study, further confirm the earlier findings.

### 5.6 Conclusion

In conclusion, analysis using *bap* specific PCR for bovine mastitis causing *S.aureus* isolates indicated 40 per cent of isolates were positive for the ‘*bap*’ gene which
encodes biofilm associated protein responsible for the development of the biofilms. The
ability of *S.aureus* to form biofilm is considered to be a major virulence factor
influencing its pathogenesis in mastitis.

Immunoblot analysis indicated that proteins extracted from *S.aureus* grown under
BF mode expressed immunogenic cross reactive proteins compared to FC proteins and
that BF cells could be most valuable immunogens for prophylaxis and diagnosis.

In the current trials, comparison between BF and FC vaccinated rabbits indicated
the superiority of BF vaccine as the CMT positivity, SCC values and percentage of
mammary glands showing lesions upon challenging, were significantly less in case of BF
vaccinated groups. Further, specific serum IgG levels detected by ELISA was
significantly high in BF vaccinated than FC vaccinated and control rabbits.

Analysis of data with respect to CMT positivity, SCC values, Percentage of
mammary glands showing lesions and specific serum IgG levels obtained from BF and
FC vaccinated and challenged with homologous and heterologous strains indicated cross
protection against challenge infection by BF vaccine.

The evaluation of BF vaccine in pregnant animals indicated that the animals
immunized with *S.aureus* BF vaccine in their last few weeks of pregnancy are better
protected against new *S.aureus* intramammary infection during their subsequent lactation
period.