V. DISCUSSION

In the present study, the pathology of staphylococcal mastitis was studied in rabbits by experimental induction. *Staphylococcus aureus* strains were isolated from bovine mastitis cases. Comparative efficacy of a biofilm vaccine and free cell vaccine in reducing the intramammary infections in rabbits was also investigated. The study was designed in two phases. In the first phase, six rabbits each were infected with *S. aureus* strains 50 and 06 to establish the pathology of mastitis in rabbits. In the second stage, 12 rabbits each were vaccinated with the free cell and biofilm vaccines against *S. aureus* 50 and were later challenged with either a homologus or heterologus strain of *S. aureus*. The results of the present study are discussed as hereunder.

Mastitis is 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 as $2 billion per 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 cause either clinical or subclinical or chronic bovine mastitis with high economic losses to the farmers in particular and dairy industry in general. In cows, intramammary infections (IMI) due to *S. aureus*, which accounts for 25 to 30 per cent of total IMI, are generally subclinical (Leitner *et al.*, 2000 and Dego and Tareke 2003). *S. aureus* is a common pathogen 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., 2002 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 are 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 are: (i) present on the bacterial surface; (ii) confer upon bacterial capacity to form a biofilm; (iii) play a relevant role in bacterial infection 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 S. aureus mastitis, is being commercially used in Israel since 2004 (Leitner et al., 2008). Many other conventional vaccines are also commercially available against 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 are unable to prevent new intramammary infections. Further, the efficacy of conventional S. aureus vaccines has yet to prove their effectiveness with respect to Indian perspective.
5.1 Pathology of experimentally induced Staphylococcal mastitis in rabbits

In the first stage of the experiment, the mastitis was induced in six rabbits by inoculating *S.aureus* 50 strain and in another six rabbits mastitis was induced by inoculating 06 strains of *S.aureus* isolated from bovine mastitis cases. Both the strains of *S.aureus* successfully induced clinical 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 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).

5.1.1 Clinical Evaluation

The clinical signs observed in the rabbits infected with either *S.aureus* strains included rise in the body temperature, dullness, depression and decrease in feed consumption at 24 hr post infection. The inflammation of mammary glands was characterized by hyperemia, enlargement, firm to hard consistency, heat and pain on palpation, bluish discoloration (Blue breast appearance) by 48 hr post infection. Similar observations have been made by several workers in their experimental induction of mastitis in rabbits using *S.aureus* (Adlam et al., 1976, Reinoso et.al, 2002,..) whereas, small nodular lesions were recorded in cows due to *S.aureus* mastitis (Jones 1998, Shibahara and Nakamura, 1999). The clinical signs in rabbits started as early as 6 hr post infection and persisted for 48-72 hr PI. The rise in the body temperature could be
attributed to the effect of exotoxins elaborated by the *S.aureus*. The exotoxins and capsular polysaccharide, produced by *S.aureus*, could be responsible for the acute clinical symptoms associated with staphylococcal mastitis as observed by Yancey *et al.*, (1993) in cows.

5.1.1.1 Mean number of mammary gland involvement during infection

In the present study, the mammary glands exhibited lesions at various intervals after *S.aureus* challenge. It was observed that both the strains produced lesions in all the glands at higher infective doses in 24 to 48 hr PI. However, the infection with *S.aureus* 06 strain revealed involvement of higher number of glands compared to *S.aureus* 50 strain. The percentage of glands involved progressively decreased for both the strains from 96 hr PI. The higher percentage of mammary glands involvement could be attributed to the higher infective dose of *S.aureus*. The infected glands showed hyperemia, induration, bluish discoloration, heat and pain on palpation. 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 the macroscopic lesions such as swelling and induration were observed at 24 and 48 hr 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 *S.aureus* from bovine mastitis into normal lactating mouse mammary glands. They observed rapid multiplication of bacteria with production of alpha toxin, necrosis and death of the inoculated mice. Beytut *et al.* (2002) observed small nodular lesions,
induration and oedema in the lobes of cows when the investigations were carried out in Kars region. The results in the present study clearly showed that both the strains were able to induce mastitis in rabbits, which may be used for challenging in vaccinated animals. Further, the lesions in the mammary gland were more pronounced at infective doses \(10^6\) and \(10^7\) cfu *S.aureus*. However, there was no significant difference among the various infective doses. Based on the present observations it could be inferred that, \(10^6\) cfu / ml of bacterial suspension was an optimum infective dose to produce appreciable gross lesions and also for challenge studies in vaccinated animals.

### 5.1.1.2 Haematology

In the present experimental studies, inoculation of either *S.aureus* 50 or 06 strains into the rabbits showed an increase in the total leucocyte count along with increased heterophil count at 48 hr post challenge. The mean TLC value in the rabbits infected with *S.aureus* 50 strain was 8630±527 cells/ml at 48 hr post infection whereas the mean TLC values of the *S.aureus* 06 infected rabbits was 12170±294 cells/ml at 48 hr PI. The mean TLC of rabbits infected with 06 strain was significantly \((P \leq 0.05)\) increased at 24, 48, 72, 96 and 120 hours PI when compared to normal control rabbits. The mean heterophil percentage increased significantly from 32.50±0.50 in the pre infection samples to 59.00±2.53 at 48 hr PI in the 06 strain infected rabbits and it continued till 96 hr PI. Similar findings were observed in the 50 strain infected rabbits also. The increase in heterophils was characterized by an increase in number of band cells and other immature forms of polymorphonuclear neutrophils. McDowell *et al.*, (1971) studied the changes in blood leucocyte parameters during experimental *S.aureus* infection in ewes and observed a marginal decrease in the blood leukocyte count after intramammary *S.aureus* infection.
up to 48 hr post infection, but the TLC values were attained normal by 62 hr PI. The authors attributed that the decreased TLC might be due to rapid migration of PMN cells into the infected udders within 6-12 hr PI. On the contrary, the present study indicated a marginal increase in the blood leukocyte counts along with a profound increase in the milk SCC at 24 and 48 hr PI. The TLC and DLC of the infected rabbits were attained almost normal from 96 to 144 hr PI. However, the results are in accordance with Bramley et al. (1989) who observed increased number of PMN cells in circulation as early as 6hr PI which persisted some days PI following an intramammary S.aureus challenge in mice and the increase in TLC could be attributed to combat systemic S.aureus infection.

5.1.2 Somatic Cell Count (SCC)

In the present study, the SCC of normal rabbits ranged from 2–3X10^5 /ml of milk. There was a drastic increase in the SCC of rabbits infected with either 50 or 06 strain of S.aureus by 24 hr PI, which reached a peak at 48 hr PI. The increased SCC values persisted consistently till 144 hr PI. The peak SCC values observed in the infected rabbits at 48 hr PI at the infective dose of 10^7 CFU/ml were 18.13 X10^5 and 30.76 X10^5 /ml in the rabbits infected with S.aureus 50 and 06 strains respectively. This increase in SCC was characterized mainly by a phenomenal increase in the PMN cells up to 72 hr PI and later along with the PMN cells, mononuclear cells were also observed in the milk samples of infected rabbits. Reinoso et al. (2002) reported increased SCC in milk samples collected from mastitis induced rabbits with S.aureus organisms.
The increase in the SCC following experimental staphylococcal mastitis in cows has been reported by Ebling et al., (1992) and Leitner et al., (2003). Somatic cell count has been widely implemented as a screening test to identify intramammary infections in lactating cows (Schalm and Noorlander 1957, Miljkovic and Milojevic 1962, Sharma and Rajani 1965, Chakraborty and Hazarika 1977, Okello 1992, Mohinikumari and Janakiramagupta, 2002 and Mdegala et al., 2004).

Following detection of the pathogen invasion into the mammary gland, the resident macrophages and the epithelial cells release several chemoattractants which trigger the migration of leukocytes, mainly PMN cells, from blood towards the inflammed mammary gland (Zhao and Lacasse, 2007). This phenomenon results in the increase in PMN cell population from their basal level of 5 to 25 percent to approximately 90 percent of total cells in the milk (Leitner et al., 2000). The migrated PMN cells are considered as first line of defense in the mammary gland, but the presence of functional PMN in milk is crucial to host defense against bacterial pathogens (Paape et al., 2003). Bramley et al. (1989) studied the viability of PMN cells in the mammary glands following an experimental S.aureus infection in mice. They observed that the viability was more pronounced in the milk PMN cells than the blood PMN. Anderson et al. (1977) and Ward et al. (1979) observed intense PMN infiltration in the udder tissue and phagocytosis of S.aureus by PMN cells.

The main function of the PMN cells in the infected mammary glands is to phagocytose pathogens and destroy them via oxygen dependent and oxygen independent systems. At the same time, PMN cells can potentially harm the mammary gland by
promoting tissue injury via reactive oxygen species generation and by granular enzyme release i.e., degranulation (Zhao and Lacasse, 2007). Further in *S. aureus* infection PMN cells engulf bacteria where they become inactive and damage of neutrophils by bacterial products. Antiphagocytic nature of protein A plays important roles (Jones 1998, Watson *et al.*, 1985) in the development of mastitis.

### 5.1.3 Gross lesions of mammary glands in the infection trials

The rabbits infected with either *S. aureus* 50 or 06 strains showed development of mammary gland inflammation by 24 hr post inoculation which progressed in severity during 48 hr PI and thereafter gradually inflammation reduced in severity from 72-96 hr PI to 144 hr PI. The lesions were more severe with *S. aureus* 06 strain than the 50 strain and persisted till 72 hr PI. The gross lesions observed were swelling, congestion and firm or hard to palpate. Upon incision thick, slightly discolored milk was oozed out. There was lot of difficulty in cutting tissues. Abscessations and cavities were also observed on the cut surfaces at 48 hr PI to 144 hr PI with both the strains. These findings are in agreement with Reinoso *et al.* (2002) and Adlam *et al.* (1976) who reported varying degrees of macroscopic lesions *viz* swelling, edema, abscessation and necrosis of the quarters in rabbits inoculated with $10^8$ cfu/ml of virulent and avirulent strains of *Staphylococcus aureus* isolated from the bovine mastitis cases, but Beytut *et al.* (2002) described lesions as small nodules in udder of a cow associated with hyperplastic changes. The abscesses and cavities were observed more in $10^6$ cfu and $10^7$cfu/ml. It clearly indicated that the development of gross lesions depends on the dose of the organisms in which exotoxin and bacterial byproducts play an important role in the development of gross lesions. The variation in the occurrence of lesions indicated the
species affected could be an important factor in development of mastitis. Based on these findings it could be construed that the number of organisms and the toxins elaborated by them are the deciding factors in the development of lesions even in naturally infected cattle.

In the present study, the gross lesions were also observed in visceral organs at various intervals of time after *S. aureus* infection. The systemic gross lesions in case of both *S. aureus* 50 and 06 strains were restricted to liver, lungs and heart. The lesions included mild to moderate swelling and congestion of the liver and mild congestion of the heart which persisted till 144 hr post infection. These lesions could be due to the systemic effect of the exotoxins and bacterial byproducts released by the inoculated *S. aureus*. The Toxic shock syndrome toxin (TSST-1) produced by *S. aureus* is well established and it is mainly because of absorption of toxins into circulation resulting in toxin mediated tissue damage (Gyles and Thoen, 1983).

### 5.1.4 Histopathological lesions in the mammary glands of infected rabbits

The histopathological lesions observed in the infected mammary glands were very conspicuous at 24 and 48 hr PI infected with either *S. aureus* 50 or 06 strains. The lesions at 24 hr post inoculation revealed enlarged lobules with moderate degree of hyperemia and perilobular and interlobular edema. The acini were distended and lined by vacuolated and necrotic epithelial cells. The secretory epithelium was detached. The lumen of the acini was filled with varying amount of eosinophilic secretory material along with desquamated necrotic epithelial cells and a large number of heterophils. The heterophilic infiltration was also observed in the interlobular septa, perilobular areas and perivascula
spaces. The *S. aureus* organisms appeared as small group of bluish tinged cocci in several acini and interlobular areas. These findings were well correlated with the findings of Ward *et. al.* (1979) who recorded blue breast appearance, necrosis and heterophilic infiltrations in rabbits and Anderson *et. al.* (1977) who recorded infiltration of neutrophils, detached epithelial cells in alveoli, interalveolar cell infiltration, contracted alveoli, hyperplastic epithelium, cellular debris and fibrosis in mouse.

The lesions were more severe in glands infected with $10^4$, $10^5$, $10^6$ and $10^7$ cfu infective doses of *S. aureus* 50 and 06 strains. However, the lesions encountered in *S. aureus* 06 infected rabbits were much severe and characterized by loss of normal architecture of several lobules with complete loss of alveolar epithelial cells, accumulation of necrotic debris along with a large number of heterophils and occasional organisms in groups.

The glands inoculated with $10^7$ cfu of *S. aureus* in case of both 50 and 06 strains revealed more severe lesions compared to the other doses. The lesions observed were of severe degree with destruction of acinar epithelium and there was initiation of fibroblast proliferation in the interacinar and the interlobular septa.

The microscopical lesions observed at 48 hr PI were more severe than those observed at 24 hr post inoculation for both *S. aureus* 50 and 06 strains. The congestion of blood vessels and hemorrhage in the perilobular and interlobular along with connective tissue proliferation was noticed. There was diffuse involvement of the lobules which showed severe destruction of the acini due to necrosis of epithelial cells and infiltration of large number of heterophils. There was hemorrhagic necrosis. The secretions in acini
appeared severely reduced with scanty amount of eosinophilic material. The organisms in small groups were observed in scanty within the acini as well as in the interlobular septa. These findings were well correlated with observations of Adlam et. al. (1976) who recorded organisms in alveoli, desquamation of epithelium, PMN cells infiltration and necrosis in rabbits and Anderson et.al. (1977) who recorded interalveolar cell infiltration, contracted alveoli, hyperplastic epithelium, cellular debris and fibrosis in mouse.

At 72 hr PI, the histopathological changes in the 50 strain infected mammary glands of rabbits revealed continuation in the magnitude and severity of lesions. The lesions comprised severe interalveolar and interlobular septal fibrosis, atrophy of several acini consisting of necrotic granular material with occasional acini showing infiltrated heterophils and decreased secretions. The mononuclear cell infiltration was observed in the interalveolar septa as well as in the interlobular septa which appeared thickened due to connective tissue proliferation.

The glands infected with S.aureus 06 strain at 72 PI revealed microscopical changes similar to those observed at 48 hr PI. However, there was mononuclear cell infiltration into the interalveolar and interlobular septa along with thickening of the septa due to severe connective tissue proliferation.

At 96 post infection, the microscopical lesions observed in the mammary glands of both S.aureus 50 and 06 strain infected rabbits were similar to those observed at 72 hr PI but the lesions were very severe. There was massive fibrosis around alveoli and lobules leading to atrophy with decreased secretion. In some glands only some islands of alveoli were seen in completely fibrosered areas. Thickening of the interalveolar and
interlobular septa, infiltration of mononuclear cells and transformation of alveoli to large cystic spaces were also observed. These findings were in accordance with the observations of Ward et al. (1979) who studied the role of alpha and beta toxins of *Staphylococcus aureus* in the pathogenesis of mastitis in rabbits and compared with that of natural infection. In addition to the above lesions, the *S. aureus* 06 infected glands revealed persistence of multifocal abscesses consisting of homogenous, necrotic material leading to gangrenous mastitis. These findings are in complying with the findings of Adlam et.al. (1976) who studied both natural and experimental Staphylococcus mastitis in rabbits and recorded gangrenous mastitis upto two weeks. In the present study, there was an area of broad band of inflammatory tissue adjacent to mammary glands extended to subcutaneous tissue. This finding was adequately supported by the observations of Viana et.al (2008) who recorded spectrum of pathological lesions associated with natural and chronic staphylococcal mastitis in 130 rabbits.

The histopathological changes at 120 hr PI in the mammary glands of both *S. aureus* 50 and 06 infected rabbits showed cystic spaces with watery secretions and homogenous cellular debris. The interacinar septa, interlobular septa and the perilobular areas were thickened with fibrotic connective tissue and infiltrated mononuclear cell infiltration. In some glands, only some islands of alveoli were seen in complete areas of fibrosis. Calcification was observed in several acini. There was appreciable difference in the microscopical changes between the different infective doses.

At 144 hr PI, the glands infected with either *S. aureus* 50 or 06 strain revealed complete fibrosis in most of the lobules resulting in atrophy of lobules and acini with
infiltration of mononuclear cells. There were cystic spaces with complete loss of secretary epithelium. There were differences in the occurrence of lesions at various infective doses.

The results of the present study clearly indicate mammary tissue damage in the rabbits infected with either *S.aureus* 50 or 06 strains. The mammary tissue damage has been shown to be induced by either apoptosis or necrosis. Both bacterial factors and host immune reactions contribute to epithelial tissue damage. During infection of mammary glands, the tissue damage can be initially caused by bacteria and their products. Mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils, into the mammary gland. With more immune cells migrating into the mammary gland and breakdown of the blood-milk barrier, damage to the mammary epithelium worsens. Polymorphonuclear neutrophils can harm the mammary tissue by releasing reactive oxygen intermediaries and proteolytic enzymes (Zhao and Lacase, 2007). The oxidative stress can damage all types of biomolecules like DNA, proteins, lipids and carbohydrates, which perpetuates tissue injury.

The PMN cells have primary, secondary and tertiary granules which contain bactericidal peptides, proteins and enzymes such as elastase, proteinases and myeloperoxidases which are released into the extracellular environment and cause tissue destruction during mastitis (Paape *et al.*, 2003).

The mammary tissue damage could also be caused by the proteinases and collagenolytic enzymes which degrade the extracellular matrix components (Haddadi *et al.*, 2005). The toxins elaborated by *S.aureus* could cause direct damage to the cell
membrane and milk producing glands (Jones, 1998). The tissue damage was induced either by apoptosis or necrosis in the mammary epithelial cells indirectly through induction of proteases or proinflammatory cytokines (Zhao and Lacase, 2007). Jain (1979) pointed out that the toxin and toxin products are involved in mastitis and gangrene. Alpha toxin is most potent factor in the pathogenesis of mastitis through vasoconstriction leading to ischaemic necrosis and gangrene. Coagulase and other bacterial products are involved in enhancing infection and phagocytosis. Gamma toxin is also most irritating bacterial toxin produced by staphylococcus organisms. Peptidoglycan fraction of cell wall is involved in hypersensitivity reaction of the gland. Further, Cucarella et al. (2001) stated that pathogenesis of *Staphylococcus aureus* is attributed to the combined effect of extra cellular factors and toxins, together with the invasive properties of the organism such as adherence, biofilm formation and resistant to phagocytosis. Watson et al. (1996) reported that the virulence of *S.aureus* depends on alpha toxin, beta toxin and leucocidin. The *S.aureus* could trigger white blood cells and epithelial cells in the mammary gland to secrete cytokines which can bring about tissue damage by recruiting PMN cells that function as phagocytes at the site of infection. These cytokines also promote a wide variety of functions of the PMN cells, including adhesion, surface receptor expression, free radical production and release of lysosomal constituents (Paape et al., 2003). Levels of cytokines increase during *S.aureus* mastitis and they could also induce apoptosis in the bovine mammary epithelial cells (Bannerman et al., 2004). Gamma toxin, the most irritating bacterial toxin that causes activation of laminin and collagen IV. Coagulases, cell surface proteins like fibrinogen, fibronectin,
laminin, collagen and vitronectin binding proteins deposits fibrin may be the possible causes for fibrosis.

Though the pathogenesis of mastitis is very complex, both the host and etiological factors play a major role on the mastitis development. Therefore the tissue damage in the present investigation might be perpetuated by all these factors and substantially comply with the previous findings.

5.1.4.1 Histopathological lesions in the Visceral Organs

The lesions observed in the visceral organs at 48 and 72 hr PI in case of both S.aureus 50 and 06 infected rabbits were similar to those observed at 24 hr PI but severity of the lesions were increased. In addition, the liver showed infiltration of mononuclear cells, while the heart showed loss of normal architecture and infiltration of occasional heterophils. The lungs showed moderate degree of congestion, edema and hemorrhages, while kidneys showed infiltration of cells in the interstitium and pelvis.

The lesions observed in the visceral organs of the infected rabbits could be due to the systemic spread of organisms and their exotoxins as well as the exopolysaccharide of bacterial cell wall. The earlier researchers have not described the histopathological changes in the visceral organs of experimentally infected animals. However Shibahara and Nakamura (1999) observed centrilobular degenerations in the liver of cow with S.aureus organism. Further, in the present study, it can be explained that these lesions were well correlated with the clinical signs exhibited by the infected rabbits. Hence, it is hypothesized that the lesions in visceral organs were due to systemic spread of organism and their toxins.
5.1.5 Ultrastructural Changes

It was established that in the present study the optimal tissue damage occurred at 48 hr PI in case of both *S.aureus* 50 and 06 infected rabbits. Hence the mammary gland tissues collected at 48 hr PI were subjected for transmission electron microscopy to study the ultrastructural changes in the infected glands.

The epithelial cells showed varying degrees of degeneration and necrosis characterized by disruption of the endoplasmic reticulum, degeneration of mitochondria with electron dense bodies and loosening of the inter epithelial cellular junction. The loss of cellular organelles and vacuolations were evident in epithelial cells. Loss of endoplasmic reticulum and other organelles dilated Golgi apparatus and condensed mitochondria with electron dense bodies were noticed. Electron dense particles were seen in vesicular structures indicative of phagocytosis. Similar findings were noticed by Almeida et al. (1996) who studied transmission electron microscopy of bovine mammary cells invaded by *S. aureus* and showed intracellular replication of the bacterium within membrane-bound vacuoles.

The interstitium was thickened with the presence of electron dense bacteria like structures. The capillary endothelium was distorted with more number of PMN cells and RBCs. Capillaries were surrounded by fibrous like structures. Cohesive nature of outer and inner nuclear membrane giving thick appearance was recorded. The infiltrated cells comprised predominantly of heterophils along with few lymphocytes and occasional macrophages. It could be postulated that the ultrastructural changes might have produced because of multiple virulent factors of *S.aureus*. Bacilli are known to adhere to plasma
membrane and enter in to the cells i.e, epithelial cells, endothelial cells, macrophages and neutrophils. Finely granular aggregates at sides of bacterial localization induce structural changes in the affected cells. The pathogenic versatility results from production of a large number of extracellular proteins, some of which are direct virulence factors and others function as accessory factors (cheville, 1994).

5.1.6 Immunohistochemical demonstration of the bacterial antigens

The tissue sections taken on APES coated slides were subjected to Immunohistochemistry to detect the bacterial antigens. The bacteria were demonstrated in the infected mammary glands as golden yellow spots in small groups. The reaction was very specific at 24 hr and 48 hr post infection in both S.aureus 50 and 06 infected rabbits. The reaction was evident upto 96 hr post infection but absent at 120 hr and 144 hr post infection.

The immunostained bacterial antigens were also appreciable in hair follicles of skin in both S.aureus 50 and 06 strain infected rabbits at 24 and 48 hr PI.

There was paucity of literature regarding the histochemical detection of bacteria in mammary glands. But immunohistochemistry effectively demonstrated bacterial antigens and is widely employed. However, the technique has failed to detect bacterial antigens during the later stages of infection. This could be due to clearance of bacteria from the site of infection. Hence, it could be construed that the bacterial antigens are very well demonstrated initial stages of infection.
5.2 Western blot analysis

In the study carried out at the Dept. of Microbiology (Isloor, 2009,DBT report) the findings of western blot analysis of *S.aureus* proteins revealed six immunogenic proteins in *S.aureus* 50 BF proteins. These proteins were not detected either in BF proteins or FC proteins when analysed by FC HIS. This indicates the superiority of BF proteins which are capable of inducing better antibody response compared to FC proteins. Further, in this study the immunogenic proteins of 30.59, 34.02 and 37.05 kDa were observed in *S.aureus* 06 BF proteins, 28.60, 25.25, 54.39 and 62.45 kDa in FC proteins with an additional protein of 38.09 kDa in *S.aureus* 50 FC proteins alone, when probed with *S.aureus* 50 BF HIS indicating the cross reactivity of BF proteins. But, such cross reactivity was not observed when blot was probed with FC HIS. These findings are in line with observations of Naveen Kumar (2005) who analysed bovine mastitic *S.aureus* BF and FC proteins by western blotting. The proteins of BF and FC when probed with BF hyperimmune 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 hyperimmune serum indicating immunogenicity and cross reactivity of BF OMPs.

Antibody response detected against proteins of both the strains with recognition of extra proteins as immunogenic was confirmed the superiority of BF-based antigen with respect to cross protection. Additional immunogenic proteins were recognized by
S. aureus 50BF protein HIS in antigen grown under BF mode compared to FC protein HIS probing. Similar findings were also made in BF of S. Gallinarum (Prakash and Krishnappa, 2002), P. multocida A: 1 (Arun, 2002), and bovine mastitic E. coli (Sumathi (2005) BF cells. Kavitha (2008) made the western blot analysis of bovine mastitis E.coli BF and FC OMPs and reported that an additional 24.4 and 28.5 kDa polypeptides in case of OMPs of both E.coli O9 and O147 grown under BF mode and 34.5 kDa polypeptide in case of E.coli O147 grown under BF mode were detected when probed by E.coli O9 BF HIS indicating the immunogenicity and cross reactivity of novel proteins expressed when E.coli was grown under BF mode.

These studies established that the BF cells expressing some unique proteins which are highly immunogenic are absent in FC. Thus these proteins may play an important role in imparting protection. The findings of the present work amply support the earlier findings of Naveen Kumar (2005) who reported on immunoblot analysis of S. aureus isolated from bovine mastitis cases and then grown under BF mode differed from their FC counterparts. It can be concluded that antigens of BF express cross-reactive proteins which can be subsequently incorporated in the bovine mastitis vaccine to elicit cross protection. Thus the occurrence of mastitis can be prevented by better usage of BF vaccine.

5.3 Challenge studies after immunization

5.3.1 Clinical evaluation

In the present study, out of the six rabbits immunized with free cell vaccine and challenged by the homologus strain of S.aureus 50, all the animals exhibited mild clinical
signs such as moderate rise in temperature, depression, hyperemic and swollen mammary glands. These clinical signs were evident at 24 and 48 hr post challenge. All the six heterologus challenged rabbits also developed similar clinical signs but were more severe. The clinical signs observed in the positive controls were similar to those observed in the clinical studies of respective strains.

Perusal of literature revealed that several researchers have studied the efficacy of whole cell staphylococcus and cell toxoid vaccines in goats against *S.aureus* mastitis. McDowell et al. (1971) observed the development of milder clinical signs such as mild elevation of rectal temperature, depression and inflammation of the mammary glands in the goats challenged with *S.aureus*. However, several research workers have also reported that the clinical severity of *S.aureus* mastitis did not differ between the vaccinated and unvaccinated cows after challenge (Giraudo et al., 1997 and Leitner 2008). But they recorded early resolution of infection in vaccinated animals than in the unvaccinated cows. The results of the present study in rabbits are in concurrence with the findings of the earlier researchers in cows.

The biofilm vaccinated rabbits challenged with either homologus or heterologus strain of *S.aureus* revealed the development of very mild clinical signs which included slight increase in body temperature upto 48 hr post challenge and slight hyperemia and congestion of mammary glands at 24 hr post challenge. The animals remained apparently healthy and active throughout the course of the experiment.

Perusal of literature revealed paucity of published reports on biofilm vaccination in *S.aureus* mastitis. However, Shivaraj and Krishnappa (2002) and Prakash (2006)
showed that the *E. coli* biofilm vaccine provided upto 100% protection with the *E. coli* challenge studies in broiler chicken. These observations clearly indicated that the biofilm vaccine provided better protection against *S. aureus* infection than the free cell vaccine and the findings are well supports the present findings in the rabbits wherein similar changes were noticed. This could be due to immunogenic potential of BF vaccine as it consists a unique fragment of protein, which was evident by Western blot analysis of BF, so the BF vaccine might have induced better immunity and protected from *S. aureus* infection in rabbits.

5.3.1.1 Mean number of mammary gland involvement during the challenge studies

In the present study, a marked reduction in the number of mammary glands involved with infection was observed in both homologus and heterologus *S. aureus* strain challenged rabbits after biofilm cell vaccination compared to free cell vaccinated group.

The mean percentage of mammary gland involvement at 48 hr PI in biofilm vaccinated rabbits were 14.55±1.1 and 24.95±4.8 for the homologus and heterologus challenges respectively which were significantly lesser compared to that of the free cell vaccinated rabbits which were 50.0±0.0 and 62.5±5.1. On the other hand, the unvaccinated control rabbits infected with 50 and 06 *S. aureus* strains showed a mean percentage mammary gland involvement of 82.5±3.5 & 84.5±4.5 respectively at 48 hr post challenge. These observations clearly indicated that the biofilm vaccine provided better protection against *S. aureus* infection than the free cell vaccine. It could be inferred that biofilm vaccine containing the unique immunogenic components of 67, 37, 26 and 20.8 kDa might be responsible for reduced number of mammary gland involvement.
5.3.1.2 Haematology

In the present study, the challenged rabbits after vaccination showed an increase in the total blood leukocyte counts up to six days post challenge. The mean TLC values of the rabbits challenged with S.aureus 50 (homologus challenge) and S.aureus 06 (heterologus challenge) strains after free Cell vaccination were 11270±460 and 12240±290 respectively at 24 hr post challenge and the values were 11037±434 and 12337±247 at 48 hr post challenge. These values were significantly higher (P≤0.05) than the TLC values of normal control animals. The TLC values of the homologus challenged rabbits after Biofilm vaccination were 9330±236 and 9550±314 at 24 and 48 hr PI and these values were significantly different (P≤0.05) from those of the homologus challenged rabbits after free cell vaccination. Schalm et al. (1964) stated that cows parentally immunized against Staphylococcus aureus shows marked leucocytosis in response to second intramammary challenge with killed whole Staphylococcus aureus bacterin. The leucocytosis may indicate CMI as a result of vaccination. Leucocytes may have increased Staphylococcus aureus multiplication due to mammary tissue damage from the immuno inflammatory response. The local inflammation in the mammary glands of free cell vaccinated and challenged rabbits could be responsible for the marginal increase in TLC values which corresponded with the intense clinical signs observed in the present study. It could be inferred from the above observations that the biofilm vaccine was more protective against S.aureus challenge than the free cell vaccine by virtue of biofilm chemical composition compared to free cells.
5.3.2 Somatic Cell Count in rabbits during the challenge trials

In 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 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 S. aureus vaccine (Pamela, 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 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).

Analysis showed that the difference in the SCC of milk collected on day ‘0’ i.e., before challenge was ‘non-significant’ in all the groups. The values varied from $3.2 \times 10^5$ cells/ml to $8.53 \times 10^5$ cells/ml (Table 14). Meanwhile, on day one i.e., 24 hr post challenge, there was sudden increase in SCC in all the four vaccinated groups which varied from $10.23 \times 10^5$ cells/ml to $37.29 \times 10^5$ cells/ml. This 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 *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 *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.

The mean SCC values in the milk of both FC and BF vaccinated rabbits challenged with either homologous or heterologous strains revealed a significant decrease (P≤ 0.001) in SCC values compared to that of the positive controls. The mean SCC values on day two post challenges were 22.04±1.68 X 10^5 cells/ml and 41.15±6.56 X 10^5 cells/ml respectively for the homologous and heterologous challenged rabbits after free cell vaccination. The mean SCC values were consistently increasing in free cell vaccinated rabbits. It was also observed that there was significant increase in the mean values (P≥ 0.05) of SCC in the FC homologus and heterologus group compared to normal group rabbits.

The BF vaccinated rabbits challenged with homologous or heterologous strains of *S.aureus* revealed the highest mean SCC of 12.96±0.26 X 10^5 cells/ml and 14.69±0.41 X 10^5 cells/ml respectively on day 2 post challenge. The mean SCC values of the BF vaccinated rabbits subjected to both homologous and heterologus challenge were significantly lesser (P≤ 0.001) compared to FC homologus group and FC heterologus group on all days of post 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.

The mean SCC values of the BF vaccinated rabbits subjected to either homologous or heterologous challenges were significantly lesser (P ≤ 0.001) compared to that of FC vaccinated rabbits. These observations indicated a milder degree of intramammary infection upon challenge in the biofilm vaccinated rabbits than the free cell immunized rabbits and asserted that the BF vaccine is superior to the free cell vaccine in protecting the animals against *S.aureus* mastitis during the early phases of infection.

In contrast to the observations of the present study, Giraudo et al., (1997) observed that the vaccine had no effect on the SCC values of the cows challenged with *S.aureus*.

In the present study, the SCC values of the FC immunized rabbits were higher than the healthy control rabbits suggestive of an intramammary infection. The SCC values of the biofilm vaccinated group in particular were far lower than either the positive
controls or the free cell vaccinated rabbits. These lowered SCC values in the biofilm vaccinated group of rabbits correlated with the reduced clinical signs of mastitis.

5.3.3 Gross pathological lesions observed in rabbits sacrificed during the challenge trials after vaccination

In the present study, the rabbits vaccinated with biofilm vaccine developed no gross pathological lesions involving the mammary glands after challenge with either homologous or heterologous strains of *S. aureus* compared to moderate gross lesions of free cell vaccine. The lesions observed were moderate congestion and enlargement of mammary glands with watery milk secretions. The gross lesions persisted up to Day six post challenge in FC vaccinated and challenged rabbits.

There are scanty reports with respect to the pathological aspects of mastitis after immunization. Though the earlier workers (Leitner *et al.*, 2003 and Giraudo *et al.*, 1997) have described the intensity of mastitis in the challenged cows and in rabbits based on the clinical observations and visible changes in the udder, they have not reported about the gross changes in the infected animals (Adlam *et al.*, 1977).

In the present study, we recorded the gross pathological alterations in the immunized and challenged rabbits at various stages of the study. The gross lesions in visceral organs of both FC and BF vaccinated & challenged rabbits included slight congestion of liver, heart, spleen and lungs and mild catarrhal enteritis only at 24 and 48 hr post challenge. No appreciable gross changes could be detected thereafter in the visceral organs of challenged rabbits. The gross lesions observed in the challenged rabbits could be due to the systemic effects of the exotoxins and toxin products
produced by the *S.aureus*. The toxins like alpha, beta, delta, gamma and leucocidin are well established to cause cytotoxicity of tissue and damage. It can be constructed that these toxins or products of *S. aureus* might be responsible for the development of gross lesions. These properties of toxins strengthens that *S.aureus* is capable of producing diversified lesions in the mastitis.

5.3.4 **Histopathological lesions observed in rabbits sacrificed during the challenge trials after vaccination**

The severity of the microscopical changes observed in the mammary glands of FC vaccinated rabbits challenged with either the homologus or heterologus *S.aureus* strain were in consistent with those of the positive controls, but in BF vaccinated rabbits challenged with either the homologus or heterologus *S.aureus* strain, the microscopical changes were minimal.

Both homologus and heterologus challenge produced more severe lesions in the FC vaccinated rabbits. However, in biofilm vaccinated rabbits, the lesions were very less both in the homologus and heterologus challenge group of rabbits throughout the course of the study.

In rabbits immunized with biofilm vaccine and challenged with either homologus or heterologus strains of *S.aureus*, the microscopic lesions revealed very mild infection involving one or two glands. The histopathological changes observed at Day 1 post challenge were mild hyperemia, vacuolar degeneration and necrosis of lining epithelial cells in occasional lobules with infiltration of mild number of heterophils into the lumen of acini as well as the interalveolar septa. The lesions were moderate on Day two post
challenge. On Day 6, 14 and 21 post challenges, the mammary glands appeared normal with normal architecture and retained functional activity with no evidence of infection.

At 24 hr post challenge, the histopathological lesions in the rabbit challenged with the homologus strain (50) after FC vaccination revealed microscopically, total loss of architecture, degeneration, necrosis and desquamation of acinar epithelial cells, presence of necrotic cellular debris along with heterophils in the lumen, infiltration of heterophils into the interacinar and interlobular septa and presence of few of organisms in a few alveoli. There was evidence of septal thickening with loss of secretions. The lesions became more severe at 48 hr post challenge affecting more number of lobules. Fibroblastic proliferation and destruction of alveoli is salient feature.

On day six post challenge, the microscopic lesions in FC vaccinated homologus challenged rabbit were severe degree of interlobular septal thickening along with mononuclear cellular infiltration, atrophy of alveoli and formation of cystic spaces.

On Day 14 and Day 21 post challenge the mammary glands of the homologus challenged rabbit after FC vaccination revealed complete loss of architecture with severe fibrosis and cystic transformations. The teat canal fibrosis was well appreciated in most of the mammary glands.

The rabbits immunized with the free cell vaccine and subsequently challenged with the heterologus S.aureus strain (06) revealed similar microscopic lesions with that of homologus challenged rabbits on Days 1, 2, 6, 14 and 21 post challenge.
There is paucity of literature regarding the histopathology of mammary glands in the vaccinated and challenged animals. The earlier researchers evaluated the efficacy of their vaccines based on the clinical severity, SCC in milk and the levels of serum and milk immunoglobulin. In the present study, along with the other parameters, the gross and histopathological changes of the challenged and control rabbits were also included. The histopathological lesions were minimal in the biofilm vaccinated group of rabbits compared to the positive controls infected with either \textit{S.aureus} 50 or 06 strain in which the lesions were as severe as those observed during the infection trials with the respective strains. The biofilm vaccine effectively brought down the severity of histopathological changes in the mammary glands of challenged rabbits.

The histopathological changes in the visceral organs of free cell vaccinated rabbits sacrificed at 24hr, 48hr, 6\textsuperscript{th} day, 14\textsuperscript{th} day and 21\textsuperscript{st} day after challenge with both strains of \textit{S.aureus} showed the evidence of systemic spread of infection. The heart and liver showed severe vacuolar degenerative changes, congestion and oedema. The kidney showed mild fibrosis, varied areas of calcifications and infiltration of inflammatory cells in the interstitium.

In biofilm vaccinated groups the microscopic changes of visceral organs were very minimal. There was no evidence of systemic spread of infection. The spleen showed secondary lymphoid follicles indicative of immune stimulation. The lymph nodes showed increased number of follicles indicative of proliferative changes. The lymph nodes in the biofilm vaccinated group showed widening of the cortical follicular tissue and presence of varying sized vacuoles in the cortex and paracortex. Hyperplastic changes with
formation of secondary lymphoid follicles are a feature of the biofilm vaccinated rabbits. In some instances, the Peyer’s patches of intestines also showed hyperplastic changes. These proliferative changes in the lymph nodes and spleen are suggestive of immunostimulation which was evident in both the free cell and biofilm vaccinated rabbits but was more pronounced in the biofilm vaccinated rabbits. These changes in the lymphoid organs clearly indicated that the BF vaccine imparts better immunity against \textit{S.aureus} infection compared to free cell vaccine.

\textbf{5.3.5 Ultrastructural changes in the mammary glands of challenged rabbits}

The ultrastructural changes observed at 48 hr post challenge of FC homologous and heterologous rabbits showed complete loss of alveolar epithelial cells architecture, nuclear details, vacuolations in the cytoplasm, altered dense cells, dilated cisterna of endoplasmic reticulum, fazy nucleus, interstitium filled with fibrous like material with electron dense bodies indicative of bacteria, and loss of myoepithelial cells. The infiltrated cells comprised predominant heterophils and a few lymphocytes in varying stages of degeneration. At 14 days post challenge, the degenerative changes in the epithelial cells were more severe compared to 48 hr post challenge with fibrosis of interstium, loss of cellular details and medium to large vacuoles in both FC homo and heterologous mammary glands. The fibrous tissue proliferation with electron dense bodies was a consistent feature. The nuclei appeared indistinct in the epithelial cells with condensation of the nuclear membrane. Margination of chromatin, indistinct cell organelles, vacuolations of cytoplasm, electron dense bodies in cytoplasm and nucleoplasm indicate advanced apoptosis. However in biofilm vaccinated and challenged
the rabbits there were no appreciable ultrastructural changes except for mild vacuolations in the cytoplasm and occasional cells showing indistinct cell organelles.

The ultrastructural changes in the challenged rabbits were suggestive of reduced severity of lesions in biofilm vaccinated rabbits compared to free cell vaccinated rabbits.

**5.3.6 Immunohistochemistry**

5.3.6.1 Immunohistochemistry to detect bacterial antigen in mammary gland tissues

Immunohistochemistry was performed by immunoperoxidase technique on selected sections taken on APES coated slides to detect the presence of bacteria. In case of free cell vaccinated rabbits, bacteria could be demonstrated at 24 and 48 hr post challenge but the reaction was less intense compared to that seen in the infection trials. The reaction was not specific in any of the biofilm vaccinated rabbits indicating total evacuation of the infection. These results are suggestive of an early clearance of bacteria in the biofilm vaccinated group of rabbits than in the free cell vaccinated and control rabbits. In the present study the bacterial antigens could be very well demonstrable as early as 24 hr PIQ with immunohistochemistry.

5.3.6.2 Immunohistochemistry to detect CD4 and CD8 antigens in mammary gland tissues

Direct Fluorescent Antibody technique was employed to detect the presence of CD4 and CD8 positive T lymphocytes in the mammary gland tissues of the rabbits challenged with *S.aureus* after vaccination.
The CD8 T positive cells were more compared to the CD4 T cells in the normal mammary glands. The vaccinated rabbits showed considerably higher number of both CD4 T and CD8 T cells compared to the control animals on all the occasions. Among the free cell vaccinated group, rabbits showed higher number of CD4 T cells. Both homologous and heterologous challenged biofilm vaccinated rabbits showed profoundly increased number of both CD4 T and CD8 T cells on 6th, 14th and 21st days post challenge. Marisa et al (2002) recorded a significant increase in the percentage of IFN-γ producing CD4 and CD8 T cells after S. aureus Ima challenge in immunized mice compared to control mice. They opined that the IFN-γ producing CD4 and CD8 T cells could play a pivotal role in the eradication of intracellular staphylococci. Lee et al. (2005) also observed the increase of CD4 and CD8 T cells with the administration of adjuvants in association with the Staphylococcus aureus trivalent vaccine. The results of the present study draw adequate support from the previous findings.

Further, Chandrashekar (2009) observed an increase in both CD4 and CD8 T cells in the peripheral blood of cows immunized with either free cell or biofilm vaccines of E.coli. He opined that the CD4 cells or T helper cells produced a variety of cytokines which led to Th1 and Th2 immune responses and antibody production from B cells. CD8 T lymphocytes are also known as cytotoxic T cells which recognize and eliminate altered self cells via antigen presentation in conjunction with MHC class I molecules. Therefore, cytotoxic cells may act as scavengers, removing old or damaged secretory cells. Cytotoxicity, as a general mechanism for pathogen control, can involve apoptosis of infected cells through Fas/Fasl interaction or lysis/apoptosis of infected cell resulting from release of cytotoxic granule protein (Flynn, 2006).
The results of the present study also indicate that the biofilm vaccine triggered more active cell mediated immunity than the free cell vaccine in the rabbits.

5.3.7 Enzyme Linked Immuno Sorbent Assay: Seromonitoring of post vaccinal antibodies in vaccinated rabbits

The seromonitoring of the vaccinated rabbits by an indirect ELISA revealed that the biofilm vaccinated rabbits showed an increased PP values after vaccination indicative of better immune responses. The 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 indicated the superiority of BF vaccine.

Further, in post challenge period, there was a sudden drop in detectable antibody levels immediately after challenge during first two days (48 hr), followed by a sudden significant rise in the antibody levels, especially in the BF vaccinated group (Figure 16). The sudden drop in the level of IgG antibodies within 48 hr 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 might 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 mammary immunized cows, the observation of the present study supports 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 secretory 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 \textit{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 \textit{S.aureus}. Enhanced immunoglobulin level of sera may lead to increased opsonization of homologous as well as heterologous strains of \textit{S.aureus}, and thereby its elimination. Similar cross reactivity to heterologous strains was also reported by Watson \textit{et al.} (1996) and Hogan \textit{et al.} (1992) on using conventional \textit{S.aureus} and \textit{E. coli J5} vaccines for bovine mastitis respectively.

Earlier studies by Kavitha (2008) and Jyothi (2009) have shown that vaccination of pregnant rabbits with bovine mastitis causing \textit{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, Chandrashekhar (2009) reported that the vaccination of lactating cows with \textit{E.coli} BF vaccine was superior to FC vaccine as serum IgG level was significantly highest in BF vaccinated than FC vaccinated and control cattle. The ability of the \textit{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 the present study, further confirm the earlier findings.
In conclusion, biofilm vaccinated rabbits showed better humoral as well as cell mediated immune responses compared to the free cell vaccine. However, the BF vaccine showed better cross reactivity as revealed by the reduced intra mammary infection in the heterologus challenged rabbits also.

Conclusions:

- The *S.aureus* 50 and 06 strains isolated from bovine mastitis cases experimentally induced acute, clinical and subclinical mastitis in rabbits.

- The *S.aureus* 06 strain produced more severe mastitis than the *S.aureus* 50 strain in rabbits.

- Rabbits could be successfully used as a good model to study the pathology of bovine mastitis as well as to study the immune responses against *S.aureus* mastitis after immunization.

- Comparison between BF and FC vaccinated rabbits indicated the superiority of BF vaccine as the TLC, SCC values, mean number of mammary glands showing lesions, gross and histopathological lesions and ultrastructural changes were significantly less and immune cells, ELISA PP values were more in case of BF vaccinated groups.

- The results of the present study indicated that the biofilm vaccine conferred better protection against intramammary challenge with *S.aureus* than the free cell vaccine and the biofilm vaccine could be employed for protection against mastitis in bovines.