VI. SUMMARY

In the present study, mastitis was induced experimentally in rabbits using *S. aureus* strains isolated from bovine mastitis cases and compared the efficacy of a biofilm vaccine with free cell vaccine in preventing the intramammary infections in rabbits. The study was designed in two phases. In the first phase, six rabbits each were infected with *S. aureus* 50 and 06 strains to study the pathology of mastitis in rabbits. In the second phase, 12 rabbits each were vaccinated with the free cell and biofilm vaccines against *S. aureus* 50 and were later challenged with either a homologus (50) or heterologus (06) strain of *S. aureus*.

In the first phase of the experiment, the rabbits were infected by inoculating 0.5ml of bacterial suspension at the base of the teat. The first pair of teats were inoculated with PBS and the subsequent pairs were inoculated with increasing concentrations of *S. aureus* viz., $10^4$ cfu/ml, $10^5$ cfu/ml, $10^6$ cfu/ml and $10^7$ cfu/ml. Blood and milk samples were collected from each rabbit at the interval of 24 hrs after inoculation and analyzed for TLC and DLC in blood and SCC in milk. One rabbit each was euthanized at the end of 24, 48, 72, 96, 120 and 144 hrs post inoculation and the mammary gland tissues were collected for histopathology after recording gross changes, electron microscopy and immunohistochemistry.

In the second stage, pregnant rabbits were divided into 7 groups: 1) Six rabbits immunized with free cell vaccine and challenged with the homologus strain of *S. aureus*; 2) Six rabbits immunized with free cell vaccine and challenged with the heterologous strain of *S. aureus*; 3) Six rabbits immunized with biofilm vaccine and challenged with...
the homologus strain of *S. aureus*; 4) Six rabbits immunized with biofilm vaccine and challenged with the heterologus strain of *S. aureus*; 5) Two unimmunized rabbits infected with *S. aureus* 50; 6) Two unimmunized rabbits infected with *S. aureus* 06 and 7) Two unimmunized rabbits which served as healthy controls. The vaccine was administered to the pregnant rabbits by subcutaneous route on 12th, 26th day of pregnancy and 3rd day of lactation. The rabbits were challenged on the 10th day of lactation and the blood, milk and tissue samples were collected on 0, 1, 2, 6, 14 and 21 post challenge and analyzed for TLC, DLC and SCC. The rabbits were observed for clinical signs and representative samples were for histopathology, transmission electron microscopy and Immunohistochemical analysis.

Both strains 50 and 06 strains produced clinical mastitis in rabbits. The clinical signs observed were rise in body temperature, dullness, depression and decrease in feed consumption at 24 hrs post infection. The inflammation of the mammary glands characterized by hyperemia, enlargement, firm to hard consistency, heat and pain on palpation, bluish discoloration (Blue breast appearance) were observed by 48 hr post infection. The severity of clinical signs was reduced from 4th day post inoculation. It was observed that the clinical signs induced by *S. aureus* O6 strain were more severe than the *S. aureus* 50 strain. The involvement of mammary glands with inflammation was 80 percent for both the strains in higher infective dose levels at 24 & 48 hrs PI.

The infected rabbits showed increase in TLC with a value of 8630±527 cells/cmm and 12170±294 cells/cmm for the strain *S. aureus* 50 and 06 strains respectively at 48 hr post inoculation. The DLC of the infected rabbits revealed a marginal increase in the
number of heterophils at all days post infection. The percent increase of heterophils were 53.20±4.40 & 59.00±2.53 at 48 hrs PI in 50 and 06 stains of *S.aureus*.

In the present study, the SCC of normal rabbits ranged from 2.00- 3.00X10^5 /ml of milk. There was a increase in the SCC of rabbits infected with either *S.aureus* 50 or 06 strain by 24 hr PI, which continued till 144 hr PI. The peak SCC values observed in the infected rabbits at 48 hrs PI at the infective dose of 10^7 CFU/ml were 18.13 X10^5 for *S.aureus* 50 strain and 30.76 X10^5 /ml for *S.aureus* 06 strains. This increase in SCC was characterized by an increase in the number of desquamated epithelial cells and PMNs at all days PI. The mean SCC values of milk samples from glands infected with various dose levels of either strain of *S.aureus* were significantly higher (P≤0.01) than control PBS glands. But no such significance was observed between the SCC values of milk samples from glands infected with different dose levels of *S.aureus*.

The rabbits infected with either *S.aureus* 50 or 06 strains in the present study revealed grossly the inflammation of mammary glands by 24 hr PI and intensified by 48 hr PI. The gross pathological lesions reduced from 72 hr to 144 hr PI. The severity of lesions was more intense with *S.aureus* 06 strain than the *S.aureus* 50 strain and persisted till 72 hr PI. The gross lesions were characterized by swelling of affected mammary glands, congestion and firm or hard to palpate. Upon incision thick, slightly discolored milk oozed out. There was lot of difficulty in cutting tissues. Plenty of abscessations and cavities were also observed on the cut surfaces at 48 hr PI to 144 hr PI in both the strains.

The histopathological lesions observed in the mammary glands of rabbits infected with 50 or 06 strain were more severe at 24 and 48 hr PI with total loss of architecture
and the lesions continued in severity till 144 hr post infection. The lesions observed during 24 and 48 hr PI in the infected mammary glands included the congestion of blood vessels in the perilobular and interlobular connective tissue, diffuse involvement of the lobules with severe destruction of the acini due to necrosis of epithelial cells. There were infiltration of heterophils, hemorrhages and necrosis. The secretory activity of the acini was severely reduced with scanty amount of eosinophilic material in occasional acini. In addition to heterophils, a few mononuclear cells were also encountered in the interacinar and the interlobular septa as well as in the acinar lumen. Initiation of thickening of the interalveolar and interlobular septa and the organisms in small groups were observed within the acini as well as in the interlobular septa.

At 72 hr PI, the histopathological changes in the \textit{S.aureus} 50 strain infected mammary glands of rabbits revealed continuation in the 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 the interlobular septa.

The microscopical changes at 96 hr to 144 hr PI in the mammary glands of both \textit{S.aureus} 50 and 06 infected rabbits showed cystic spaces with watery secretions and bluish cellular debris. The interacinar septa, interlobular septa and the perilobular areas were thickened with fibrosis and infiltrated with mononuclear cells. In some glands, only some islands of alveoli were seen in areas of fibrosis. Calcification was observed in several acini.
The microscopic changes in the visceral organs showed severe vacuolation of hepatocytes and mild to moderate congestion of the lungs with mild fibrosis, Kidneys showed marked areas of calcification, infiltration of cells in the interstitium and pelvis with scarring and Heart showed myocardial degenerations. These lesions were observed consistently throughout the experimental period.

The ultrastructural changes in the mammary glands at 48 hr PI consisted of varying degrees of epithelial cell 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 several epithelial cells. Loss of endoplasmic reticulum, other organelles, dilated Golgi apparatus, condensed mitochondria with electron dense bodies were noticed. Electron dense particles were seen in vesicular structures indicative of phagocytosis. The interstitium was thickend with electron dense bacteria like structures. The infiltrated cells comprised predominantly of heterophils along with a few lymphocytes and occasional macrophages.

The immunohistochemical staining of the mammary gland tissue sections demonstrated the presence of *S. aureus* organisms as golden yellow cocci in small groups. The reaction was very specific at 24 hrs to 96 hrs post infection in both *S.aureus* 50 and 06 infected rabbits, but bacterial antigens were not detectable at 120 hr and 144 hr post infection indicating the clearance of bacteria from the site of infection.

In the second stage of the experiment, the rabbits were challenged with *S. aureus* after either free cell or biofilm vaccination.
The clinical signs observed in the free cell vaccinated homologus and heterologus challenged rabbits were comparable to those observed in the positive control rabbits infected with the \textit{S.aureus} 50 and 06 strains. On the other hand, the clinical signs were very mild in the biofilm immunized rabbits challenged with either \textit{S.aureus} 50 or 06 strain which included slight rise in body temperature and slight hyperemia and edema of the mammary glands up to 48 hr post challenge. The mean number of mammary gland involvement was significantly lesser in the biofilm vaccinated rabbits than the free cell vaccinated rabbits.

There was a marginal increase in the total leucocyte count up to 6 days PI in the rabbits vaccinated with either the free cell or the biofilm vaccine. The TLC values were reduced on the 21\textsuperscript{st} day post challenge. However, the increase in TLC was significantly higher in the positive controls and free cell immunized rabbits compared to that of the biofilm vaccinated rabbits. There was a marginal increase in the heterophil numbers of both the vaccinated and positive control group of rabbits upto Day 14 post challenge.

The SCC values of the challenged rabbits showed an increase after challenge but the values were significantly lower compared to that of the positive controls. The mean SCC values in the milk of both FC and BF vaccinated rabbits challenged with either homologus or heterologus strains revealed a significant decrease (P≤ 0.001) in SCC values compared to that of the positive controls. The reduction in the mean SCC values in the BF vaccinated rabbits was significant compared to the mean values of the FC vaccinated rabbits. The mean SCC values of the biofilm vaccinated groups were 12.96±0.26 X 10\textsuperscript{5} cells/ml and 14.69±0.41 X 10\textsuperscript{5} cells/ml respectively for the homologus
and heterologus challenge on day 2 post challenge. Whereas, the free cell vaccinated groups showed a mean SCC of $22.04\pm1.68 \times 10^5$ cells/ml & $41.15\pm6.56 \times 10^5$ cells/ml respectively for the homologus and heterologus challenged rabbits on day 2 post challenge. The mean SCC values of the BF vaccinated rabbits subjected to either homologus or heterologus challenge were significantly lesser ($P \leq 0.001$) compared to that of FC vaccinated rabbits subjected to respective challenges on day 1, 2, 6, 14th and 21st day post challenge.

In the present study, the rabbits vaccinated with biofilm vaccine did not show any gross lesions in the mammary glands after challenge with either homologous or heterologous strains of *S.aureus* compared to free cell vaccine in which moderate gross lesions were noticed. The lesions included moderate congestion and enlargement of mammary glands with watery milk secretions.

The microscopical changes observed in the mammary glands of FC vaccinated rabbits challenged with either the homologus or heterologus *S. aureus* strain were similar to 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.

The histopathological changes observed on day 1 and day 2 post challenge in the BF vaccinated and challenged rabbits 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. On the other hand the free cell vaccinated and challenged rabbits showed more severe histopathological changes on different days post challenge characterized by 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, presence of few of organisms in occasional alveoli, severe interlobular septal thickening along with mononuclear cellular infiltration, atrophy of alveoli and formation of cystic spaces and in some cases teat canal fibrosis was also noticed.

The ultrastructural changes in the challenged rabbits were suggestive of reduced severity of lesions in biofilm vaccinated rabbits compared to free cell vaccinated rabbits. The ultrastructural changes observed at 48 hr post challenge of FC homologous and heterologous rabbits showed complete loss of architecture of alveolar epithelial cells, loss of nuclear details, vacuolations in the cytoplasm, altered dense cells, dilated cisterne of endoplasmic reticulum, fazy nucleus, Interstitium filled with fibrous like material with electron dense bodies indicative of bacteria. At 14 days post challenge, the degenerative changes in the epithelial cells were more severe compared to 48 hr post challenge. Interstitium was enlarged and fibrosed. Loss of cellular details and medium to large vacuoles were commonly seen in both FC homologus and heterologous mammary glands. The ultrastructural changes observed at 48 hrs post challenge of BF homologous and heterologous rabbits showed intact nucleus with nucleolus.
The presence of CD4 and CD8 positive T lymphocytes in the mammary glands of immunized and challenged rabbits were detected by direct FAT. The CD8 T positive cells were more numerous in the rabbit mammary glands compared to the CD4 T cells in the control animals. The vaccinated rabbits showed considerably higher number of both CD4 T and CD8 T cells compared to the control animals throughout the experimental study. Among the Free Cell vaccinated group, rabbits showed more 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.

The seromonitoring of the vaccinated rabbits by an indirect ELISA revealed that the biofilm vaccinated rabbits showed increased IgG titers after vaccination indicative of better immune response. The peak PP value for the FC vaccinated homologous challenge group was 40.23±0.0 as against the peak PP value of 67.78±0.0 for the BF vaccinated homologous challenged group on day 21 post challenge. The PP values of the unvaccinated control rabbits ranged from 21.0±0.0 to 24.92±0.0 throughout the course of the experiment. The titers were significantly higher in the biofilm vaccinated rabbits than the free cell vaccinated rabbits particularly on day 29 and day 31 post challenge.