Dissertation Ref. No. _____

ASSOCIATION OF SUBCLINICAL MASTITIS AND LAMENESS WITH REPRODUCTIVE PERFORMANCE AND SUBCLINICAL ENDOMETRITIS IN ZEBU X FRIESIAN CROSSBRED DAIRY COWS IN AND AROUND JIMMA TOWN DAIRY FARMS, ETHIOPIA

PhD Dissertation

By

Nuraddis Ibrahim Ababulgu (DVM, MSc)

Addis Ababa University, College of Veterinary Medicine and Agriculture,
Department of Clinical Studies
PhD program in Veterinary Obstetrics and Gynaecology

June, 2023
Bishoftu, Ethiopia

ASSOCIATION OF SUBCLINICAL MASTITIS AND LAMENESS WITH REPRODUCTIVE PERFORMANCE AND SUBCLINICAL ENDOMETRITIS IN ZEBU X FRIESIAN CROSSBRED DAIRY COWS IN AND AROUND JIMMA TOWN DAIRY FARMS, ETHIOPIA

A dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfilment of the requirements for the Doctor of Philosophy in Veterinary Obstetrics and Gynaecology

By

Nuraddis Ibrahim Ababulgu (DVM, MSc)

June, 2023,

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical studies

Title: ASSOCIATION OF SUBCLINICAL MASTITIS AND LAMENESS WITH REPRODUCTIVE PERFORMANCE AND SUBCLINICAL ENDOMETRITIS IN ZEBU X FRIESIAN CROSSBRED DAIRY COWS IN AND AROUND JIMMA TOWN DAIRY FARMS, ETHIOPIA

Submitted by: Nuraddis Ibrahim Ababulgu _____ _____

Name of Student Signature Date

Approved *for submittal to* dissertation assessment committee

1. Prof. Fekadu Regassa _____ _____

Major Advisor Signature Date

2. Dr. Tefera Yilma (PhD) _____ _____

Co- Advisor Signature Date

3. Prof. Tadele Tolosa _____ _____

Co-Advisor Signature Date

5. Dr. Haileleil Negussie _____ _____

Department chairperson Signature Date

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As members of the Examining Board of the final PhD open defence, we certify that we have read and evaluated the Dissertation prepared by Nuraddis Ibrahim Ababulgu titled: '**ASSOCIATION OF SUBCLINICAL MASTITIS AND LAMENESS WITH REPRODUCTIVE PERFORMANCE AND SUBCLINICAL ENDOMETRITIS IN ZEBU X FRIESIAN CROSSBRED DAIRY COWS IN AND AROUND JIMMA TOWN DAIRY FARMS, ETHIOPIA'** and recommend that it be accepted as fulfilling the thesis/dissertation requirement for the degree of Philosophy in Veterinary Obstetrics and Gynecology.

Dr. Hailelul Negussie	_____	_____
Chairman	Signature	Date
Prof. Alemayehu Lemma	_____	_____
Internal Examiner	Signature	Date
Dr. Tamirat Degefa	_____	_____
External Examiner	Signature	Date

Final approval and acceptance of the dissertation is contingent upon the submission of its corrected copy to the CGC through the concerned departmental graduate committee.
I hereby certify that I have read the revised version of this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Professor Fekadu Regassa	_____	_____
Dissertation advisor	Signature	Date
Dr. Hailelul Negussie	_____	_____
Department chair	Signature	Date

DEDICATION

This dissertation manuscript is dedicated to my father Ibrahim Ababulgu and my uncle Jemal Ababulgu who passed away few years ago and my mother Tejitu Abasimel for nursing me with affection towards success in my life.

STATEMENT OF AUTHOR

First, I declare that this thesis/dissertation is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfilment of the requirements for a PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Nuraddis Ibrahim Ababulgu Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _____

ACKNOWLEDGMENTS

Above all, I thank the Almighty Allah for giving me health, strength and support for the completion of my study.

First of all, I would like to express my sincere gratitude and respect to my major advisor Professor Fekadu Regasa and co-advisors Dr. Tefera Yilma and Prof. Tadele Tolosa for their keen and much needed support, supervision, friendly treatment, critical remarks, encouragement and inspiration from problem identification to the final write-up of the dissertation. They shared with me their accumulated professional experiences and were cooperative from the beginning of proposal writing to the completion of the dissertation work.

I would like to acknowledge Addis Ababa University, College of Veterinary Medicine and Agriculture for the funding this research. I would also like to express my earnest appreciation to the Jimma University for offering me the chance to pursue my PhD study at Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu. I would also like to express my deep thanks to Jimma town administration agricultural desk, the artificial insemination technicians, smallholder cattle owners, the staff of Jimma University College of Agriculture and Veterinary Medicine dairy farm, Jimma University Kito Furdisa dairy farm enterprise for their cooperation during the survey study and allowed me to collect samples. Without the support of them and extension staff and farmers the on-farm research wouldn't have been carried out this study. And their contribution is gratefully acknowledged.

I extend my special thanks to the staff of Jimma University College of Agriculture and Veterinary Medicine for allowing me to conduct the laboratory work and facilitating to access the microbiology laboratory. My deepest appreciation also goes to Prof. Manzoor

Ahmad Kirmani for his contribution of cytobrush from India and encouragement during my study.

The authors would like to thank the Jimma specialized hospital, clinical chemistry laboratory staffs and management staff of the farms for their support. Special thanks to the farm workers for their help and patience during animal examination and collection of samples.

Finally, my greatest appreciation goes to my family. I find it hard to express in word of my family encouragement, assistance and tolerance. Special acknowledgment of gratitude and appreciation is directed to them. I owe all of them more than a simple expression of thanks.

LIST OF ABBREVIATIONS

AI	Artificial Insemination
BCS	Body Condition Score
CFSI	Calving to First Service Interval
CCI	Caving to Conception Interval
CI	Caving Interval
CL	Corpus Luteum
CM	Clinical Mastitis
CMT	Californian Mastitis Test
CNS	Coagulase Negative Staphylococcus
CR	Conception Rate
CRFS	Conception Rate at First Service
DD	Digital Dermatitis
DIM	Days In Milk
DO	Days Open
EMB	Eosin Methylene Blue
GDF-9	Growth and Differentiation Factor-9
GnRH	Gonadotropin Releasing Hormone
IDD	Inter Digital Dermatitis
IFN	Interferon
IL	Interleukin
IMI	Intra Mammary Infections
LS	Locomotion Scoring
LH	Luteinising Hormone
LPS	Lipopolysaccharides
n	Number examined
NO	Nitric Oxide
NSPC	Number of Services Per Conception

LIST OF ABBREVIATIONS (Continued)

P_4	Progesterone
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
PGFM	Prostaglandin F-M
PMN	Polymorphnuclear cells
PR	Pregnancy Rate
SC	Service Per Conception
SCC	Somatic cell count
SCM	Subclinical Mastitis
TNFα	Tumor Necrosis Factor α

TABLE OF CONTENTS

LIST OF TABLES

Table **Pages**

LIST OF TABLES (Continued)

LIST OF FIGURES

Figures **Pages**

LIST OF APPENDICES

Appendices **Pages**

ASSOCIATION OF SUBCLINICAL MASTITIS AND LAMENESS WITH REPRODUCTIVE PERFORMANCE AND SUBCLINICAL ENDOMETRITIS IN ZEBU X FRIESIAN CROSSBRED DAIRY COWS IN AND AROUND JIMMA TOWN DAIRY FARMS, ETHIOPIA

Nuraddis Ibrahim Ababulgu
PhD Thesis
Addis Ababa University (2023)

ABSTRACT

A longitudinal observational study was conducted to assess the association of subclinical mastitis and lameness with reproductive performance and subclinical endometritis in crossbred dairy cows. The California Mastitis Test and cytobrush technique were used to screen for subclinical mastitis and subclinical endometritis, respectively. Samples positive for subclinical mastitis were subjected to bacteriological analysis. Lameness in cows was also assessed using a 5-point locomotion scoring system. Analysis of progesterone and cortisol concentrations in blood serum of cows was performed using electrochemiluminescence immunoassay "ECLIA". A total of 84 clinically healthy cows were tested for subclinical mastitis using the California Mastitis Test. The prevalence of subclinical mastitis at cow level was 51.2% (43/84). The prevalence of subclinical mastitis in 108 lame cows was 53.7% (58 of 108). Based on the results, the 192 cows according to the study were divided into the following four groups: Group I (n=43) cows with subclinical mastitis, Group II (n=50) are lame cows, Group III (n=58) lame cows with subclinical mastitis and Group IV (n=41) healthy cows (control group). Lame cattle are also classified as no lameness, mild lameness, moderate lameness, lameness, and severe lameness. Mean number of days from calving to first mating interval was highly significantly longest in lame cows with subclinical mastitis (122.71±28.6) than for lame cows (120.98±31.3), subclinical mastitis cows (120.51±24.5) and healthy cows

(85.15±28.3) (P<0.05). Mean number of services per conception was significantly highest in lame cows with subclinical mastitis (3.66±1.31) than lame cows (3±1.6), subclinical mastitis cows (2.51±0.83) and healthy cows (1.59±0.81) (P<0.05). Cows clinically lame had a longest calving to first service interval when compared with cows which were never lame and those mildly lame. The difference was significant (P<0.05). Lowest conception and pregnancy rates at first services were observed in lame cows with subclinical mastitis. Cows that were clinically lame had a lowest conception rate at first services than cows that were never lame and mildly lame, although the difference was not statistically significant (P>0.05). Clinically lame cows had a lowest pregnancy rate at first services than cows that were never lame and mildly lame (P<0.05). Risk factors analysis revealed that prevalence of subclinical mastitis, lameness and in lame cows with subclinical mastitis significantly differed with the parity and body condition score (P<0.05). The present study revealed that subclinical mastitis and lameness were significantly and directly associated with subclinical endometritis (P<0.05). Progesterone concentrations highly decreased in lame cows with subclinical mastitis than in subclinical mastitis and lameness alone while the cortisol concentrations also highly increased in lame cows with subclinical mastitis. The major bacterial isolate was Staphylococcus aureus. In conclusion these results provide further evidence that combined occurrence of subclinical mastitis and lameness inflict harmful effects on fertility and hormonal profiles of dairy cows than those diagnosed with mastitis and lameness alone, emphasizing the relevance of mastitis and lameness control programs in dairy farms.

Key words: *California Mastitis Test, Cortisol, Conception rate, Cytobrush, Lameness score, Pregnancy rate, Progesterone*

1. INTRODUCTION

Reproductive performance is an important factor in dairy farm profitability. Fertility is affected by many factors, but mastitis is of particular importance (Wang *et al.,* 2021). Kumar *et al.* (2017) also reported that mastitis affects fertility during the breeding period.

Mastitis is an inflammation of the mammary glands that affects the health cattle (Risco and Dahl, 2018). Mastitis not only affects the mammary glands, but also causes immune responses and hormonal profile changes that adversely affect fertility. Mastitis causes delayed oestrus, lower pregnancy rates (PR), and higher risk of abortion (Wang *et al.,* 2021). Mastitis affects cattle reproduction by destroying follicles, impairing oocyte growth or function, and decreasing ovulatory capacity (Boujenane *et al.,* 2015).

Mastitis causes prolonged oestrous intervals and reduced luteal phases in cows, impairs pregnancies, and inhibits embryonic development (Edelhoff *et al.,* 2020). Occurrence of mastitis significantly increased mean interval between calving and first service, days open and number of services per conception (Dolecheck *et al.,* 2019). Bovine mastitis is categorized into two types: clinical mastitis (CM) and subclinical mastitis (SCM).

Clinical mastitis is evidenced by changes in the milk appearance, swelling, redness, and increased udder temperature, whereas animals with SCM shows no significant changes in milk or udders on clinical examination and is detected only by laboratory tests and California Mastitis Test (CMT) (Reza *et al.,* 2011). Both clinical mastitis and subclinical mastitis reduce reproductive efficiency in dairy cows (Kumar et al., 2017). Nava-Trujillo et al. (2010) recorded that clinical mastitis increased days to first service (DFS) and days to conception in primiparous cows.

Mastitis is caused by contagious and environmental mastitis (Cervinkova *et al.,* 2013). Contagious pathogens are those whose primary host is the udder of infected cows. They transmitted from cow to cow mainly during milking and tend to cause chronic subclinical

infections with recurrent clinical episodes. Infectious agents include: *Streptococcus agalactiae, Staphylococcus aureus, Corynebacterium bovis* and *Mycoplasma sp.* (Radostitis *et al.,* 2007). Environmental pathogens include *Klebsiella species, Escherichia coli, Streptococcus. dysgalactiae* and *strept. Uberis* (Harmon, 1994).

Inflammation caused by mastitis occurs when bacteria invade the mammary gland, proliferate, and produce toxins, enzymes, and cell wall components that stimulate the production of various inflammatory mediators (Risco and Dahl, 2018). Infection of the mammary glands initiate an immune response, causing an abnormal secretion of cytokines and hormones, and abnormal functioning of the reproductive system, including the ovary, corpus luteum, uterus, and embryo. Mastitis caused cytokines and bacterial endotoxins can also cause delayed oestrus, hormonal imbalance, and other related problems, leading to decreased fertility (Wang *et al.,* 2021).

Lameness is defined as any abnormality that alters the gait or posture of an animal (Olechnowicz and Jaskowski, 2011). A negative relationship with fertility has been demonstrated for lameness (Hultgren *et al.,* 2004). Loss of fertility is influenced by many factors, but lameness is particularly significant (Bicalho *et al.*, 2007).

Lameness decreased pregnancy rate to first service (Melendez *et al.,* 2003) and protracted calving to first service interval and to conception (calving to conception interval, CCI) (Bicalho *et al.,* 2007). Huxley (2013) recorded a longer calving to first AI interval, calving to conception interval, lower conception rate and higher services per conception.

Lameness delays the onset of oestrus compared to non-lame cows (Garbarino *et al.,* 2004). Walker *et al.* (2010) reported that reduced sexual activity during oestrus in lame animals is caused by declined progesterone. Lameness in dairy cows is related to an increased risk of ovarian cysts due to suppressed LH surge (Garbarino *et al.,* 2004). Lameness have reduced fertility, limited mobility, and reduced oestrous behaviour. Higher blood levels of cortisol associated with pain and stress caused by lameness (Coetzee *et al.,* 2008) may interfere with the release of luteinizing hormone. Walker *et al.*

(2010) reported that the reduced oestrus activity in lame animals was not due to decreased serum oestrogen levels, but rather to decreased progesterone release during the luteal phase and thus inadequate sensitization to oestradiol rather than by lower levels of serum oestrogens.

Lameness impairs fertility and enhances the occurrence of mastitis in dairy cows (Kiliç *et al.,* 2007). Lameness is considered as a risk factor for the prevalence of mastitis in cattle (Vladimír *et al.,* 2020). Both lameness (Hudson *et al.,* 2014) and mastitis (Kumar *et al.,* 2017) are common problems associated with overall fertility. Poor management and unhygienic farm conditions have resulted in high rates of lameness and mastitis in dairy cows.

The association between lameness and mastitis affects fertility in cattle (Cook and Nordlund, 2003). Mitev (1998) recorded cows with subclinical mastitis also suffered from hoof disease. Prolonged lameness increases the risk of bacterial contamination. All factors that predispose to mastitis, such as increased herd size, extended barn duration, etc., also contribute to the development of lameness (Logue and Bergsten, 2007).

Previous studies reported that clinical lameness delays the onset of the ovarian cycle and the onset of oestrus compared to non-lame cows (Garbarino *et al.*, 2004). The exact value of side effects depends on the cost of the opening day or the value of the pregnancy. Bacha and Regasa (2009) reported that there was a relationship between subclinical mastitis and subclinical endometritis in the study carried out in Debre Zeit dairy farms, Ethiopia.

Ethiopia has the largest livestock population in Africa, with cattle population of approximately 58 million heads (CSA, 2017). Despite these large quantities, milk production often does not meet national requirements due to many factors, such as the presence of subclinical mastitis (Nibret *et al*., 2012, Tolosa *et al*., 2013; Abebe *et al*., 2016 and Fesseha *et al*., 2021) and lameness has great prominence (Sulayeman and Fromsa, 2012; Abunna *et al*., 2017 and Mulatu, 2018).

1.1. Statement of the problem

Many authors have attempted to assess the prevalence of mastitis on dairy farms in and around the town of Jimma. Here, the disease is considered a major impediment and identified as the main cause of poor milk production in the study area. There is not a single study of the prevalence of lameness and its associated risk factors in and around Jimma town. Hoof trimming was not exercised in the present study area.

The incidence and importance of lameness continue to be underestimated by farmers and veterinarians. In this regard, it is necessary to examine the farm, identify the scale of the problem and implement a comprehensive corrective program. In the study area there is no available literature that described the association between subclinical mastitis, lameness with subclinical endometritis and fertility of cross breed dairy cows. In addition no information is available regarding hormonal profile of these diseases; there is a requirement of detection of cortisol and progesterone hormones in relation to mastitis and lameness.

1.2. Hypothesis

The hypothesis of this study was that Subclinical mastitis and lameness affects reproductive performance and uterine health of dairy cattle by reducing progesterone and cortisol hormones.

1.3. Objectives

1.3.1. General objectives

This study was conducted with general objectives of assessing the association of subclinical mastitis (SCM) and lameness with reproductive performance and subclinical endometritis in crossbred dairy cows.

1.3.2. Specific objectives

The study included the following specific objectives:

1. To determine the effect of subclinical mastitis and lameness on fertility of crossbred cows,
2. To evaluate the relation between subclinical mastitis, lameness and subclinical endometritis.
3. To analyse progesterone and cortisol hormones to link with subclinical mastitis and lameness.
4. To isolate and identify bacteria causing subclinical mastitis.
5. To assess the risk factors and their association with subclinical mastitis and lameness.

2. LITERATURE REVIEW

2.1. Association of subclinical mastitis with reproductive performance and hormonal profile of dairy cows

2.1.1. Effect of mastitis on reproductive functions

Mastitis is associated with bacterial intramammary infections (IMIs). Mastitis is classified into clinical mastitis (CM) and subclinical mastitis (SCM). Clinical mastitis is with visual signs of inflammation in the udder or milk while subclinical mastitis (SCM) is without visual signs inflammation. Factors influencing the development of mastitis are poor milking management, poor milking hygiene, mechanical faults of the milking machine, pathogen loads on the cows' skin, epithelia and in environment, teats and udder injuries (Wang *et al.,* 2021).

Both clinical and subclinical mastitis are associated with reduced reproductive performance (Ahmadzadeh *et al.,* 2009). Declined fertility in mastitis affected cows with could be due to altered hormone profile, altered egg size, failure to fertilize and an unfavourable uterine environment for embryo development. Although the causes of early post-fertilization or pre-implantation embryonic loss are multifactorial, previous studies have suggested that infections and activation of the immune response influence early embryo survival. Poor reproductive performance due to mastitis resulted in longer calving to first postpartum artificial insemination (AI) interval, lower pregnancy rate at first insemination, increased days open and, increased late embryonic losses after pregnancy diagnosis (Santos *et al.*, 2004).

Subclinical mastitis declines the function of the preovulatory follicle and affects fertility. The main cause of this disease is delayed ovulation associated with reduced follicular steroid production in approximately one third of cows with subclinical mastitis; the remaining two thirds respond normally (Wolfenson *et al.,* 2015). A large epidemiological study of the effect of mastitis on fertility has been conducted, determined by the pattern

and level of SCC around the first AI (Lavon *et al.*, 2011a). Samples from 287,000 Israeli cattle were examined and analysed to determine the association between conception and elevated SCC with the timing of artificial insemination (AI). A SCC cutoff of 150,000 cells per ml of milk was set to differentiate between uninfected and mastitis cows. Therefore, cows with high SCC had chronic, possibly subclinical mastitis before and after artificial insemination (AI).

The subclinical mastitis had lower pregnancy rates (31.5%) compared to uninfected cattle (39.4%). In chronic subclinical mastitis, decreased fertility was associated with the increased SCC. The subgroup with mildly elevated SCC (150,000-450,000 cells/ml of milk) had a 14.5% of lower conception probability and moderately elevated SCC (450,000-106/ml of milk) and a 20.5% increase in cows with high SCC (more than 106 cells/ml milk) compared to the uninfected group. Another analysis was related to clinical mastitis: it reduced the chance of conception by 24% when it occurred 10 days before AI, but not when it occurred earlier.

Pinedo *et al.* (2009) investigated the effect of a high linear SCC score (greater than 4.5) on reproductive performance during early lactation and found that calving to first service interval were delayed by 21.8 days in cows with at least one high linear SCC before first artificial insemination (AI) and cows with at least one high linear SCC before fertile breeding had 48.7 days to conception and 0.49 more services to conception. Pinedo *et al.* (2009) also reported that when high linear SCC occurred before breeding, a 44% reduction in the chance of conception during the first 90 days of pregnancy and an increased risk of abortion (1.22). Lavon *et al.* (2011c) documented an association between conception rate and the pattern and degree of SCC elevation compared to time of insemination, and found that even mild SCC elevations prior to artificial insemination (AI) were associated with significantly lower conception rate.

Hertl *et al.* (2010) documented that the occurrence of clinical mastitis within 30 days after artificial insemination reduced conception rate by 23%. Hertl *et al*. (2010) also recorded the occurrence of clinical mastitis between 14 days before and 35 days after AI was associated with reduced conception probability. Cows with clinical mastitis during the first 45 days of pregnancy have a 2.7 times higher risk of abortion within the next 90 days than uninfected cows. Hudson *et al.* (2012) documented a negative association between clinical mastitis and fertility over a wide time compared to the risk period (28 days before the risk period to 70 days after the risk period) and reported that subclinical mastitis (SCC > 399,000/ml) was associated with declined fertility following a risk period or service.

According to previous research, the time of CM's occurrence determines how it affects reproductive performance of dairy cows. After 62 days post-calving (i.e. after voluntary waiting period), cows diagnosed with clinical mastitis before the first artificial insemination were less fertile than cows with clinical mastitis early postpartum (Nava - Trujillo et al., 2010). Mastitis-induced premature luteal regression after artificial insemination may lead to abortion. The elevation of PGF2α and possibly TNFα in mastitis has been shown in several studies to be associated with regression of CL (Malinowski and Gajewski, 2010). However, the evidence for luteal dysfunction in cattle with mastitis is sketchy, mainly due to differences in how G^- or G^+ toxins induce IMI and determine effects in clinical or subclinical mastitis.

2.1.2. Period of the occurrence of mastitis affects fertility

The period of mastitis occurrence affects a cow's postpartum reproduction. Schrick *et al.* (2001) investigated the effects of clinical mastitis at different time points and found that pre-AI clinical mastitis increased calving to first AI interval, while post-first AI clinical mastitis increased open days and number of services per conception. Clinical mastitis impairs fertility in early lactating cows. The incidence of mastitis prior to first AI resulted in longer DO in cattle (Manimaran *et al.,* 2014). Clinical mastitis after AI is associated with reduced fertility (Konig *et al.,* 2006) regardless of whether the IMI-inducing bacteria

are G^+ or G^- (Schrick *et al.*, 2001; Santos *et al.*, 2004). Other researchers reported that high SCC before AI had some effect on non-return rates (Miller *et al.*, 2001). In another study, Schrick *et al.* (2001) found that cows with clinical or subclinical mastitis prior to first AI had longer open days and fertility was affected by pathogen type. Santos *et al.* (2004) also agreed that conception rates are declined by pre-AI clinical mastitis caused by G+ or G- bacteria. König *et al.* (2006) documented that increased SCC during lactation reduced pregnancy rates. Several researchers have also studied the adverse effects of mastitis on cattle fertility (Table 1).

Table 1: Effects of mastitis on fertility in dairy cows

References	Reproductive parameters	CM b/n the 1st AI and pregnancy confirmation	Uninfected/control
Gunay and Gunay (2008)			
	CFSI	77.4 ± 8.2	75.9 ± 6.3
	DO	141.7 ± 14.0	94.1 ± 10.3
	NSPC	3.4 ± 0.9	1.8 ± 0.8
Nava-Trujillo *et al.* (2010)			
	CFSI	NA	98.53 ± 4.52
	DO		143.95 ± 7.45
	NSPC		2.21 ± 0.16
	CRFS		56.10%
Yang *et al.* (2012)	CFSI	58.19 ± 1.69b	54.73 ± 0.34c
	DO	133.31 ± 11.36	89.74 ± 2.17
	NSPC	2.19 ± 0.16b	1.53 ± 0.03c
	CRFS	27.8%a	54.9%b

Values with different lower case letters within row differ at $P<0.05$

a The control (healthy) group also includes CM-affected cows after pregnancy confirmation

b Both CM and SCM cows

c CM before first AI

Lomander *et al.* (2013) reported that cows with high postpartum SCC had a lower pregnancy at first insemination and a higher number of inseminations per animal than cows free of mastitis. Hocket *et al.* (2005) documented reduced estrous expression, shorter estrus duration, and lower pregnancy rates in preovulatory CM cows. Hocket *et al.* (2005) investigated the effects of experimentally induced preovulatory *Str. uberis* mastitis found that it was associated with reduced LH pulsatility and LH surges in cattle. Finally, estrous behavior was reduced and the onset of pregnancy during the estrous cycle was delayed.

Perrin et al. (2007) documented that conception rate decreased when CM occurred 0-3 weeks before the first AI, while conception rate (CR) did not change when CM occurred 3-6 weeks before, 0-3 weeks after, or 3-6 weeks occurred after AI, suggested that CM primarily affects ovulatory factors and oocytes, rather than products of conception. Maizon *et al.* (2004) reported that clinical mastitis was one of the key factors in prolonged days open when clinical mastitis was diagnosed after 45 DIM. Filho *et al.* (2012) also recorded that cows diagnosed with clinical mastitis up to 60 days after parturition had a significantly shortest time from calving to conception intervals (132.4 ± 72 days), then diagnosed 60–120 days postpartum (153.9 ± 8.0 days) and diagnosed 120 days postpartum (231.3 ± 9.9 days). Cows diagnosed up to 60 days after parturition are likely to have recovered without affecting fertility.

2.1.3. Mechanisms by which mastitis affects fertility

Recently, more attention has been paid to the association between mastitis and reproductive disorders. The link between udder health and fertility in cattle is based on a close functional relationship that leads to disruption of the endocrine and immune systems, leading to disruption of the estrous cycle, ovarian dysfunction and early embryonic death (Roth et al., 2013; Isobe *et al.,* 2014). These changes depend on the etiology of mastitis, the time of its occurrence, its course and on the type of inflammation (Hertl *et al.,* 2010; Lavon *et al.,* 2010, 2011b; Roth *et al.,* 2013; Asaf *et al.,* 2014; Wolfenson *et al.,* 2015).

There are probably numerous factors at play when it comes to the negative consequences of mastitis on reproductive efficiency. According to Hansen *et al.*, (2004) there is a comprehensive mechanism by which mammary gland infection reduces embryonic viability. Hansen *et al.*, (2004) also reported that increased body temperature (hyperthermia), disruption of oocyte maturation and embryonic development, altered uterine function, and disruption of the hypothalamic-pituitary axis were all caused by an increase in cytokine concentration (Figure 1).

According to Soto *et al.* (2003), immune/inflammatory reactions brought on by bacterial challenge resulted in decreased gonadotropin secretion, increased cortisol concentrations and disrupted reproductive processes during mastitis. The inflammatory process increases body temperature, which may have an impact on the viability of oocytes and embryos. Decreased feed intake due to inflammation can also alter energy metabolism and affects reproductive function. According to Rahman *et al.* (2012), effects on the ovary that alter the dynamics of the folliculogenesis, such as a reduced vascular bed and lower levels of the growth and differentiation factor-9 (GDF-9), result in reduced fertility in cows with chronic mastitis.

Cortisol, cytokines, $PGF_2\alpha$, GnRH, LH, FSH, P_4, estradiol-17, prolactin, immunoglobulins, and reactive oxygen metabolites all change in concentration or activity as a result of mastitis (Pinedo *et al*., 2009; Lavon *et al*., 2010, 2011 b; Furman *et al*., 2014; Wolfenson *et al*., 2015). Studies have shown that Gram-negative bacterial liposaccharide (endotoxin) delays the preovulatory LH surge and ovulation in one-third of follicular or early oestrous cows (Wolfenson *et al.*, 2015). Liposaccharides or cytokines have also been shown to decrease the production of steroid hormones (including estradiol) in ovarian and granulosa cells (Lavon *et al.*, 2011b; Furman *et al.*, 2014 and Wolfenson *et al.*, 2015). It has also been suggested that endotoxin may induce luteolysis through the release of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and other inflammatory mediators, thereby affecting early embryonic survival.

The production of steroid hormones such as estradiol in ovarian theca and granulosa cells has also been shown to be reduced by liposaccharides or cytokines (Furman *et al.,* 2014; Lavon *et al.,* 2011b; Wolfenson *et al.,* 2015). Additionally, it was hypothesized that endotoxin would cause luteolysis by releasing $PGF_2\alpha$ and other inflammatory mediators, which would have an impact on the early embryonic survival. Another adverse effect of mastitis is altered effectiveness of the uterus to the activity of prostaglandins $F_2\alpha$ and E2, or oxytocin (Hertl *et al.,* 2010; Rahman *et al.,* 2012). In addition, the adverse effects of subclinical mastitis, especially during flares, have been well documented (Pinedo *et al*., 2009; Lavon *et al*., 2010 and Lomander *et al.,* 2013).

This situation is especially common during oestrus, when the increased oestrogen concentration significantly reduces the concentration of macrophages and impairs the function of granulocytes, which inhibits the ability of somatic cells to undergo phagocytosis. Due to its duration, subclinical forms of mastitis may interfere with long-term processes such as follicular growth and development even more strongly than the acute form of this disease (Lavon *et al.,* 2011b and Roth *et al.,* 2013). Chronic subclinical mastitis, as a short-term acute inflammation, can delay ovulation in up to 30% of cows (Lavon *et al.,* 2010). Chronic SCM characterized by a small (150,000 to 450,000) or moderate (450,000 to 1 million) increase in SCC requires special attention, since the fertilization rate can reduce almost equally in both cases (Lavon *et al.,* 2011a).

2.1.4. Pyrexia and Pregnancy rate

There are several possible mechanisms by which mastitis may adversely affect fertility. One mechanism is that elevated body temperature in mastitis affects reproductive processes. Pyrexia or fever has been reported in both G- and G+ mastitis. The adverse effects of heat stress on reproductive function are well known, and indeed several studies in vitro (Krininger *et al.,* 2002) and in vivo (Gendelman *et al.,* 2010) showed that fever can directly change oocyte and embryo developmental competence or indirectly reproductive capacity due to decreased food intake and body condition. It is known that heat stress affects fertility. Adverse effect of heat stress on embryo survival is due to

effect of elevated temperature on oocyte and embryo function (Krininger *et al.*, 2002) reduced development to the blastocyst stage. It is believed that elevated body temperature can directly alter oocyte and embryo function, developmental capacity or have an indirect effect on reproduction due to reduced food consumption and body condition.

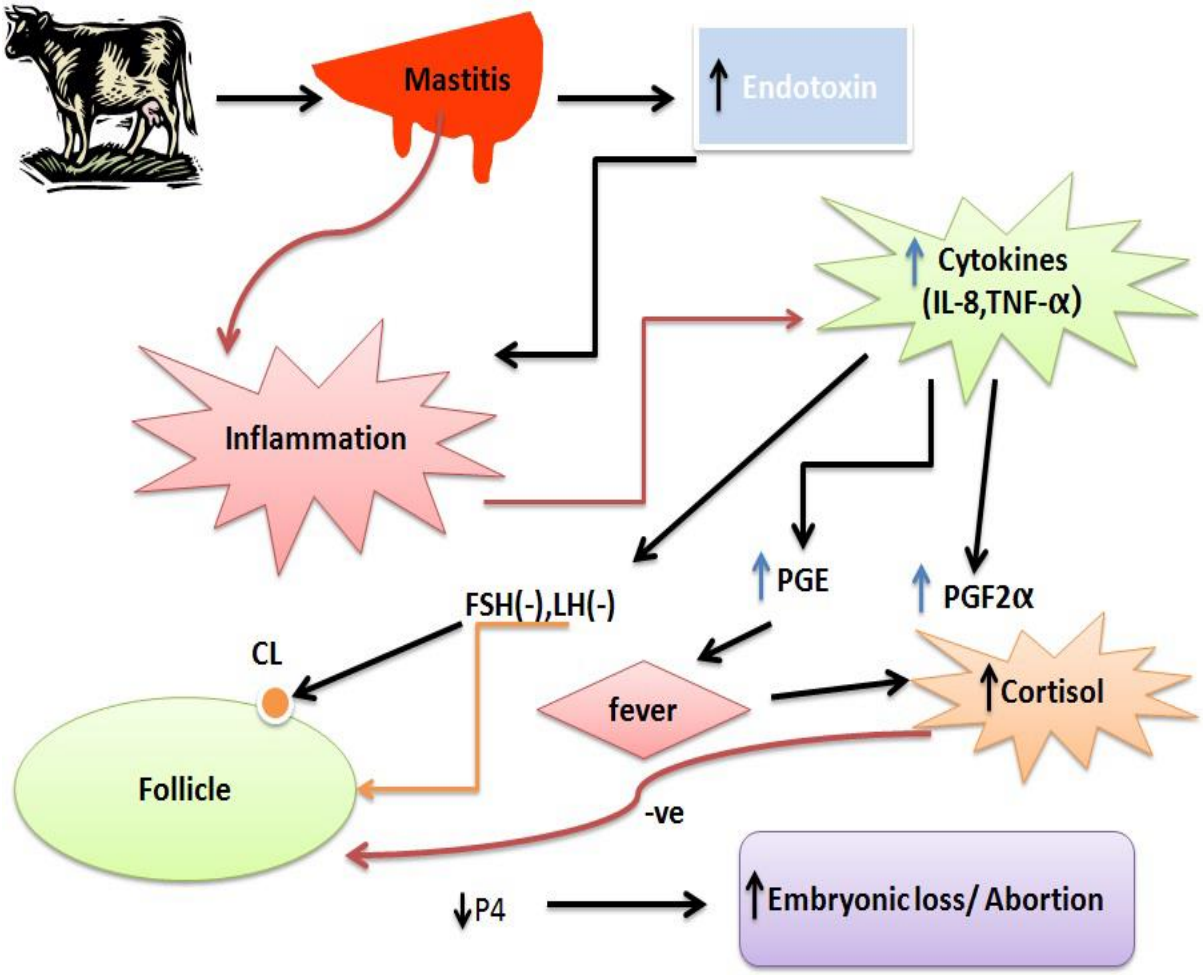

Figure 1: Possible mechanism by which mastitis affects the reproductive functions in the mastitis

Source: (Sharma *et al.*, 2017)

2.1.5. Inflammatory mediators disrupts oocyte maturation and embryonic development

Although mastitis-associated fertility decline does not appear to be related to the ovulatory process (Morris *et al*., 2009), inflammatory and immune response activation within the mammary gland can lead to oestrus cycle abnormalities and loss of oestrus. This reproductive disorder is thought to result from the activation of multiple pathways that disrupt reproductive function in the hypothalamic-pituitary axis, ovaries, oocytes, and embryos (Hansen *et al.,* 2004). Various bioactive molecules (e.g., TNF-α, NO, PGF2-α and many others from infected mammary glands are secreted into the bloodstream and have the ability to affect reproductive tissues such as the ovaries, hypothalamus, and others (Schrick *et al*., 2001) and the endometrium. In addition, follicle growth, oocyte and embryonic development (Soto *et al*., 2003) are also vulnerable to endotoxins.

High levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the serum and milk of cows with coliform mastitis suggest that these cytokines are important in mastitis induction. Waller *et al*. (2003) found the dynamics of neutrophils, cytokines (IL-8, TNF-α, IL-1β) and interferon-γ in milk and lymph of supramammary lymph nodes after intramammary injection of endotoxin, IL-8 found to play an important role in neutrophil recruitment in the mammary gland. They reported that IL-1β and TNF-α were not required for IL-8 production and release in response to endotoxin. Elevated levels of these molecules in mastitic cows are detrimental to embryonic development. For example, addition of TNF-α to bovine oocytes before and after fertilization increased apoptosis and thus decreased embryonic development (Soto *et al*., 2003). Similarly, adverse effects of TNF-α on embryonic development under bovine in vitro or in vivo conditions have been reported (Seals *et al*., 1998; Soto *et al*., 2003). Deb *et al*. (2011) reported that exposure of bovine oocytes to TNF-α in vitro reduced the number of oocytes developing to the blastocyst stage by day 8.

Jackson *et al*. (2012) reported that the detrimental effects of TNF-α on bovine embryonic development in vitro may be mediated by prostaglandins. The elevated PGF2α levels in

the endometrium may cause premature luteolysis. For example, induction of mastitis by *Streptococcus uberis* increased serum concentrations of PGF2α metabolite (PGFM) in response to oxytocin treatment and intrauterine injection of *E. coli*, causing regression of the corpus luteum and shortened estrous cycle in heifers (Hockett *et al*., 2000)). Several researchers studied the effect of immune or inflammatory stress induced by intravenous or intrauterine administration of lipopolysaccharides (LPS) in cows (Suzuki *et al*., 2001) during the follicular phase and found suppressed pulsatile LH secretion and delayed or blocked preovulatory LH surge. The suggested mechanisms include depression of pituitary pulsatile LH release resulting in disruption of gonadotropin support for follicular function; preovulatory oestradiol secretion, which subsequently reduces the oestrus expression; LH secretion; preovulatory LH surge; and ovulation.

Several researchers have investigated the effects of immune or inflammatory stress induced by intravenous or intrauterine administration of lipopolysaccharide (LPS) in follicular stage cattle (Sukki *et al*., 2001) and LH secretion was suppressed and the LH surge before ovulation was delayed or blocked. The suggested mechanisms involve inhibition of pituitary pulsatile LH release, leading to disruption of gonadotropin support for follicular function. Estradiol secretion before ovulation decreases estrus expression, LH secretion and then LH surge before ovulation. Lavon *et al*. (2008) explored the impact of intramammary infusion of lipopolysaccharides (LPS) on reproductive function during oestrus and at the time of ovulation. Similarly, Soto *et al*. (2003) also studied the effect of LPS on oocytes before and after fertilization and found that LPS had harmful consequences on both oocyte function and embryonic development. The LH surge was delayed in cows exposed to LPS during oestrus resulting in delayed ovulation, which ultimately reduced the chances of successful fertilization (Soto *et al*., 2003; Lavon *et al*. (2008).

Intramammary infusion of LPS and *Str*. *uberis* caused an increase in $PGF_2\alpha$, TNF-α, and NO levels in blood or milk and early luteal regression in cows. The effect of CM on early embryo development was due to elevated inflammatory mediators like cytokines leading to elevated levels of NO and $PGF_2\alpha$ (Hansen *et al*., 2004). Intramammary infusion of

LPS is also associated with increased synthesis of molecules which can be activated by specific cytokines. In particular, mastitis and endotoxin treatment increased concentrations of nitric oxide (NO) in milk and intramammary infusion of *E. coli* endotoxin resulted in increased milk concentrations of $PGF_2\alpha$. Cows with mastitis had a higher concentration of 13, 14-dihydro-15-keto $PGF_2\alpha$ (the major $PGF_2\alpha$ metabolite, PGFM) in blood following oxytocin challenge compared with healthy cows (Hockett *et al.*, 2000).

Molecules like TNF-α, NO, and $PGF_2\alpha$ act either on the oocyte or on the developing embryo thereby affect embryonic development. Addition of TNF-α to bovine oocytes matured in vitro did not change subsequent cleavage when oocytes were fertilized but the proportion of oocytes that became blastocyst was reduced when TNF-α is added to bovine embryos after fertilization, increasing the proportion of blastomeres becoming apoptotic. TNF-α when overexpressed leads to embryonic apoptosis resulting in embryonic death in diabetic rats (Pampfer, 2001). Prostaglandin $F_2\alpha$ has an adverse effect on embryo development in cattle: administration of $PGF_2\alpha$ to cows receiving supplemental progesterone compromised embryonic development and reduced pregnancy rate. Increased concentrations of NO have also been associated with early embryonic death. Culture with sodium nitroprusside dihydrate, a NO donor, inhibited development to the blastocyst stage of bovine embryos (Chen *et al.*, 2001).

Inflammatory mediators such as $PGF_{2\alpha}$ added to the culture medium during oocyte maturation decreased the percentage of blastocyst formation (Soto *et al.*, 2003). Similarly, TNFα added during maturation reduces the proportion of blastocysts formation (Soto *et al.*, 2003a). Addition of TNFα after the oocyte was fertilized caused an increase in the number of blastomeres that caused apoptosis (Hansen *et al.*, 2004). The addition of TNFα decreases cell number in the inner cell mass. In another approach follicular fluid from mastitic cows was used as oocyte maturation medium. Follicular fluid from G- or G+ toxin-induced mastitic cows reduced the rates of cleavage and blastocyst formation (Asaf *et al.*, 2014).

The toxic effects of NO occur through interaction between NO and O_{2-} to form the oxidant peroxynitrite. Soto *et al.,* 2003 recorded the concentration of LPS required to interfere with oocyte function was too high (1 ng/mL), which is irrelevant to the situation in mastitis and even higher concentrations (1000 ng/mL) of LPS had no effect on blastocyst development when added to the embryo culture after fertilization. Therefore, the major reproduction-disrupting role that LPS plays during mastitis is to cause release of cytokines and other molecules that disrupts reproduction and directly interferes with oocyte and embryo function.

2.1.6. Effect of mastitis on hormonal profiles

Altered fertility in mastitis-affected cows was due to changes in the release of hypothalamic-pituitary axis hormones responsible for oocyte maturation, ovulation and luteal function. The increased NSPC in cows with mastitis is due inhibition of gonadotropin secretion leading to reduced gonadotropin support for, folliculogenesis, ovulation, oocyte maturation and luteal function. Certain cytokines can reduce LH release (McCann *et al.,* 2000). Wolfenson *et al.* (2015) reported that clinical mastitis was associated with activation of the glucocorticoid system, resulting in a sharp rise of blood cortisol, known to be involved in depression of GnRH and LH secretion (Lavon *et al.,* 2010) exhibiting delayed ovulation that is caused by low secretion of oestradiol and a low or delayed preovulatory LH surge. Low oestradiol in the circulation close to oestrus is associated with interference of its positive effect on GnRH secretion, consequently leading to disruption of normal secretion of the preovulatory LH surge.

In cattle IFN-α secretion of LH can be blocked by a cortisol hormone whose secretion can be elevated during mastitis or after endotoxin exposure. Cytokines released during mastitis have direct effects on the ovary. IL-6 blocks FSH-induced oestradiol secretion from bovine granulosa cells, particularly from cells isolated from small follicles. Mastitis induced early regression of the corpus luteum (CL) post-AI can potentially causes pregnancy termination. Mastitis increases $PGF_{2\alpha}$ and possibly TNFα have been associated

with CL regression (Malinowski and Gajewski, 2010). Both TNF-*a* and IFN-c are cytotoxic to bovine luteal cells. In support to finding, McCann *et al*., 1997 indicated that cytokines released following endotoxin challenge blocked the pulsatile secretion of LH or inhibition of gonadotropin releasing hormone (GnRH) pulse amplitude. Consequently, insufficient follicular development and oocyte maturation could lead to insufficient oestrogen production and thus lack of behavioural oestrus, anovulation, and failure of conception.

Lavon *et al*. (2010) found that naturally occurring chronic subclinical or acute clinical mastitis resulted in delayed ovulation associated with low plasma oestradiol and a low or delayed preovulatory LH surge. Consequently, they evaluated the function of preovulatory follicle in cows experiencing subclinical mastitis or a past event of acute CM and found abnormal steroidogenesis in one third of animals without any alteration of follicular growth dynamics. They also recorded no carryover effect of past CM on follicular function (Lavon *et al*., 2011a). The immediate and carryover impacts of mastitis caused by G^- endotoxin (*E. coli,* LPS) and G^+ exosecretions (*S. aureus*) on preovulatory follicle function (Lavon *et al*., 2011b). LPS induced immediate, short-term, but not long-term impairment of follicular responses, while *S. aureus*-induced mastitis was showed both immediate and carryover disruptive effects on preovulatory follicle function (Lavon *et al*., 2011b).

Administration of lipopolysaccharide near the oestrus was shown to inhibit the LH surge leading to anovulation, delayed ovulation, and cyst formation in heifers. Another study showed that lipopolysaccharide causes a decline in CL size and a decrease in plasma progesterone level (Herzog *et al.*, 2012).

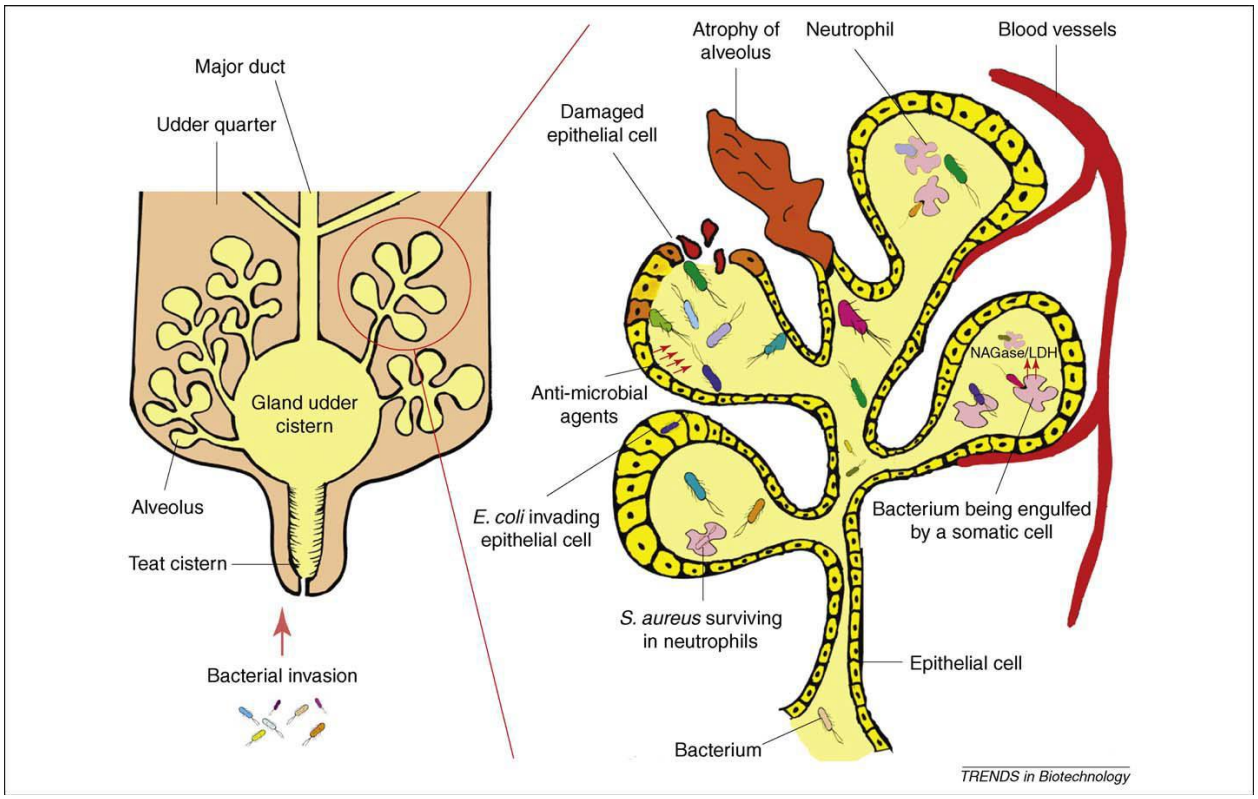

Figure 2: **Mastitis development in an infected udder**

Source: (Viguier *et al*., 2009)

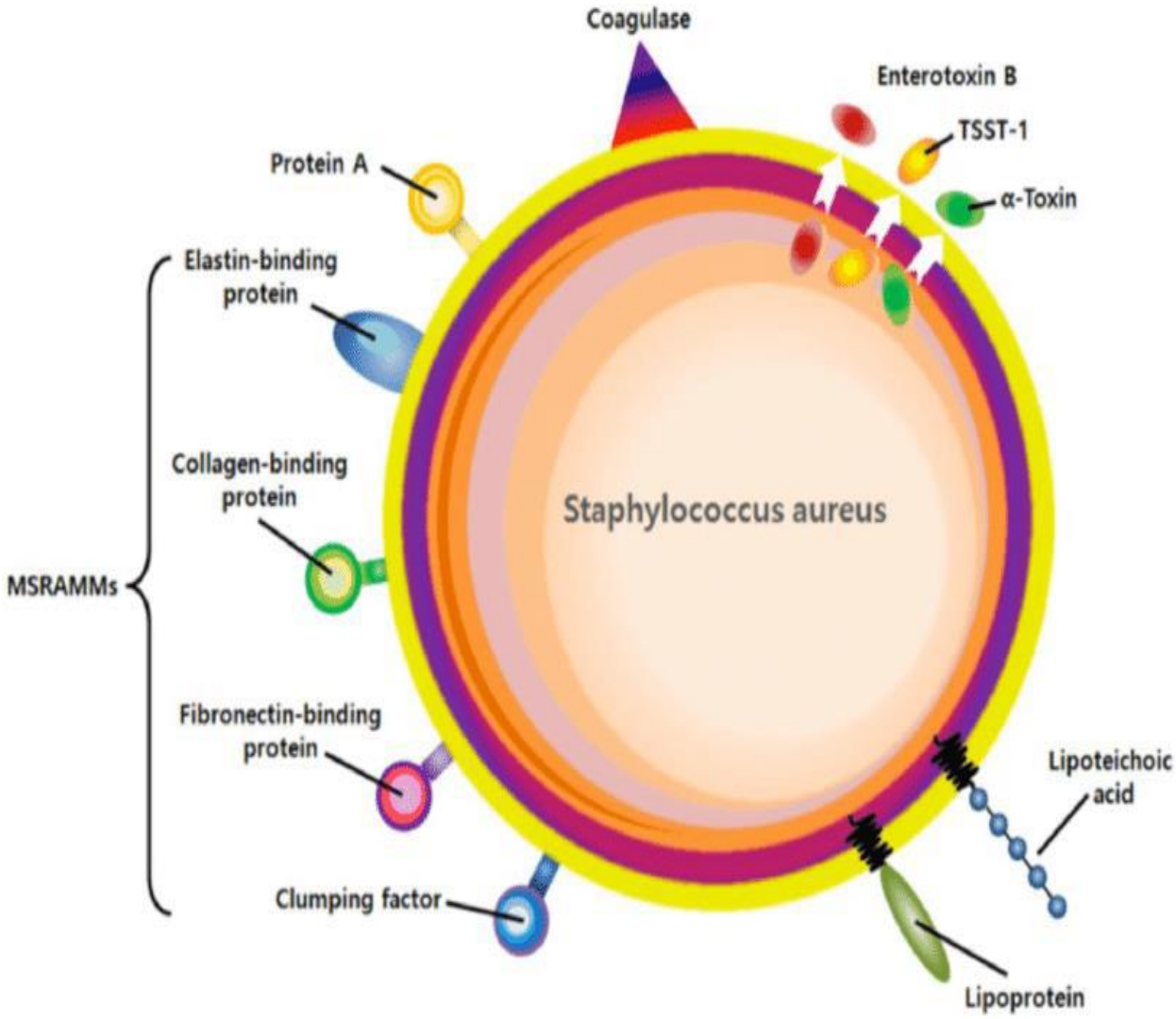

Figure 3: Virulence factors of *S. aureus*

Source: (Choi *et al*., 2014)

2.1.7. Combined effect of mastitis and other stress factors

Maizon *et al*., 2004 reported that, other than mastitis, dystocia, retained placenta, displaced abomasum, ketosis, milk fever, metritis, and pyometra adversely influenced the reproductive performance of dairy cows. Vacek *et al*. (2007) evaluated the relationship

among several health disorders (milk fever, retained placenta, metritis, endometritis and pyometra, ovarian cysts, and lameness) including CM and reproductive performance in dairy cows. They found that retained placenta, ovarian cysts, and metritis had a significant effect on CFSI, days open, and NSPC, while milk fever delayed CFSI and lameness increased DO and NSPC.

Ribeiro *et al*. (2013) reported that clinical and subclinical periparturient diseases showed a negative effect on reproduction. Peake *et al*. (2011) reported that the combined incidence of lameness, SCM, and body condition loss caused delayed onset of first luteal phase from calving and had synergistic effects on progesterone concentrations in Holstein-Friesian cows. Morris *et al*. (2009) observed reduced fertility in dairy cows that suffered with three production stressors. Ahmadzadeh *et al*. (2009) suggested that the effect of mastitis and other diseases is additive in nature, and thus, reproduction was affected to a greater extent when cows suffered both mastitis and other diseases than an independent event of diseases.

Vacek *et al*. (2007) assessed the influence of repeated episodes of CM and found that cows that experienced two or more incidence of CM had more DO and NSPC compared to healthy cows, while cows that suffered CM once did not differ significantly with those that had two or more incidences of CM during lactation. Moussavi *et al*. (2012) reported that increased number of mastitis episodes in early lactation significantly increased the NSPC with no apparent impact on the DO in heifers. Heringstad *et al*. (2006) found that mastitis was genetically associated with reduced fertility.

2.2. Association of lameness with reproductive performance and hormonal profile of dairy cows

2.2.1. Effect of lameness on reproductive functions

Lameness is defined as any abnormality that alters the gait or posture of an animal. Huxley (2013) reported that lameness is a symptom of various diseases. Hoof injuries are

the leading cause of lameness in most dairy farms. Contagious hoof lesions (e.g. toe and interdigital dermatitis and foot rot), hoof lesions (e.g. digital and interdigital dermatitis, and foot rot), horn lesions (e.g. sole hemorrhage, sole and toe ulcer, and white line disease), and other lesions (e.g. korn, fissures, thin soles, and corkscrew claw) are the major hoof lesions that causes lameness. With respect to effects on fertility, protracted CFSI, (Orgel *et al.,* 2016), CCI and increased NSPC (Sprecher *et al.,* 1997; Alawneh *et al.,* 2011) have been reported as consequences of lameness in cattle (Table 2). These results would disrupt dairy operations, as milk normally cannot be produced without pregnancy.

Lame cows are in pain, have reduced milk production and fertility (animal function), and have reduced mobility and oestrous behaviour. The sexual cycle in lame cows may pause because they are no longer able to compete for food. Elevated blood cortisol concentration is an indicator of pain induced distress (Coetzee *et al.,* 2008), associated with interference of luteinising hormone release by the pituitary gland. Lameness has an adverse effect on fertility in dairy cows (Garbarino *et al*., 2004; Hultgren *et al*., 2004). Cows diagnosed with clinical lameness during the first 70 DIM were 25% less likely to become pregnant than non-lame cows (Bicalho *et al*., 2007). The proportion of cows with above average fertility was lower for lame cows depending on the type of hoof disease) than for healthy cows. Similarly, the average CR was lower in lame cows than in non-lame cows. In nonlame cows compared with lame cows, PRFS was higher and NSPC was lower.

Lameness adversely affects fertility in dairy cows (Garbarino *et al.*, 2004; Hultgren *et al.*, 2004). Cows diagnosed with clinical lameness were reported less likely to become pregnant than non-lame cows (Bicalho *et al.,* 2007). The fertility of cows was lower in lame cows than in healthy cows. Also, the mean CR was lower in lame cows than in nonlame cows. Non-lame cows had higher PRFS and lower NSPC than lame cows. As lame cows lie down longer than healthy cows (Walker *et al.,* 2008), sexual behaviour hampered that lame cows cannot fully express oestrous behaviour (Walker *et al.,* 2010). Lame cows were less likely to come into oestrus, had lower oestrus and reduced

conception rates (Walker *et al* (2008). Lame cows were more likely not to return to cycle compared to non-lame cows (Garbarino *et al.,* 2004).

Kilic *et al.* (2007) found that lame cows had 82-92 days calving to first service and a 55-41% reduction in pregnancy compared to non-lame cows. Compared to healthy herd mates, lame animals usually require longer CCI (Bicalho *et al.,* 2007; Alawneh *et al.,* 2011). Cows with a good LS and free of foot disease reproduced much faster than diseased cows (Hernandez *et al.,* 2005). Melendez *et al.* (2003) reported a higher incidence of cystic ovary disease in lame cows compared to nonlame cows. Melendez *et al.* (2003) also recorded that cows that became lame had a lower conception rate than cows that nonlame. Overall pregnancy rates were lower in lame cows than in nonlame cows. A higher incidence of cystic ovarian disease was observed in lame cows than in non-lame cows.

An increase in days from CFSI in lame animals can be ascribed to a delayed onset of ovarian activity in the period after parturition (Kilic *et al*., 2007; Archer *et al*., 2010). Along with a prolonged CFSI, impaired reproduction includes a greater amount of inseminations and thus a longer time until conception (Alawneh *et al*., 2011). Lame cows begin normal postpartum activity more slowly than non-lame cows. Even when ovarian activity has resumed, lame cows have compromised oestrous behaviour. The increased number of days from CFSI in lame animals can be attributed to delayed onset of postpartum ovarian activity (Kilic *et al.,* 2007; Archer *et al.,* 2010). In addition to the longer time from CFSI, reproductive disorders are associated with increased NSPC and longer time to conception (Alawneh *et al.,* 2011). Even ovarian activity resumes, lame cows have impaired oestrous behaviour.

Lame cows have significantly delayed CCI, higher NSPC and lower CR (Hernandez *et al.,* 2005). For normal conception, a coordinated series of follicular changes must culminate in the ovulation of a healthy follicle. Lame cows with claw lesions and cows with multiple lesions were less likely to conceive than healthy cows. The CCI was 100 days in non-lame cows, but in lame cows with lesions and lame cows with multiple

lesions, this interval was longer at 140 and 170 days, respectively. Both the time to conception and the number of litters per conception increased in lame cows. Previous findings showed a significant association between increased levels of lameness and time to conception (Hernandez *et al.*, 2005).

Non lame cows were pregnant earlier than moderately lame or lame cows. Among cows classified as lame became pregnant earlier than cows with medium or high scores. Clinically lame cows had longer CFSI and CCI (Melendez *et al.*, 2003). The CR was reduced in cows treated for lameness (Morris *et al.*, 2009). Similarly, a significant increase in CCI was observed in cows diagnosed as lame compared to cows not classified as lame (Bicalho *et al.*, 2007). Data reported by many authors indicate that sole ulcers are the leading cause of clinical lameness in cattle and are associated with reduced fertility (Hultgren *et al.*, 2004). PRFS was significantly lower in lame than healthy cows, with more NSPC and higher culling rates (Table 2). Within the mean fecundity parameters of lame and non-lame cows, Kiliç *et al.* (2007) found significant differences in overall PR. A cow that was clinically lame less likely to become pregnant than a cow that was not lame (Bicalho *et al.*, 2007) (Table 2). Morris *et al.* (2009) reported a combined effect of lameness and increased SCC in cow's milk, reducing the likelihood of ovulation. High SCC in lame cows was associated with lower oestrus intensity and lower fertility in cows (Morris *et al.*, 2011; 2013).

Lame cows are less likely to sniff other cows. The purpose of sniffing is to detect chemical signals or pheromones within herd mates during oestrus. They not only increase self-attraction, but also induce sexual behaviour in others. Severely lame cows receive less positive feedback from other cows, so oestrus is less intense. On the other hand, lame cows are less likely to stand for long periods of time and are therefore less pheromoneically attractive to other cows (Walker *et al.*, 2008).

Table 2: Impact of lameness on reproductive indices

Study	Effect of lameness
Bicalho *et al.* (2007)	Less likely to become pregnant
Walker *et al.* (2008)	Lower P_4 concentration before oestrus
	Reduced oestrus intensity
Machado *et al.* (2010)	Lower CI
	Shortens period when herd-mates attempt to mount
	Mounted less frequently
	Oestrus expression of low intensity
Kara *et al.* (2011)	Increased likelihood of repeat breeding
Peake *et al.* (2011)	Longer CFSI (severe lameness)
Chapinal *et al.* (2013)	Longer CCI,
	Longer CI

2.2.2. Critical period in which lameness adversely affects reproduction

The increased vulnerability to diseases in the early postpartum period is mainly due to weakened of the immune competence of dairy cows during this period. Postpartum nutritional status and associated metabolic status weaken immune cell function and increase vulnerability to opportunistic microbial infections (Sordillo, 2016). In addition, the enlarged uterus postpartum contains placental remnants and lochia, which increases microbial proliferation and uterine infections (Sheldon *et al.*, 2009). The timing of postpartum recovery of ovarian function is one of the most important factors affecting subsequent fertility in dairy herds. Any delay in resuming ovarian function impairs subsequent fertility. Several factors are involved in the postpartum ovarian cycle in dairy cows, including diet, season, management and disease (Walker *et al*., 2008).

Hoof problems are considered to be one of the most important factors affecting fertility in dairy cows, as they are acute and painful conditions that usually occur after parturition. In a study that examined the association between lameness and delayed ovarian cycle in Holstein cows after parturition, the ovarian cycle was longer in lame cows than in non-lame cows. A previous study (Edmundson *et al.,* 1989) found that lame cows had a lower CRFS and a higher incidence of ovarian cysts than non-lame cows.

The occurrence of lameness within 30 days after parturition was 2.2% (Melendez *et al.,* 2003). Lameness is more likely to occur within the first 60 days postpartum (Hoedemaker *et al.,* 2009). Cows with clinical lameness during the first 70 DIM were 25% less likely to conceive than non-lame cows (Bicalho *et al.,* 2007). Major risk factors for hoof disease include significant changes during parturition and the transitional period (21 days before and after parturition) and changes caused by new environmental conditions such as floor type, housing and nutrition (Melendez *et al.*, 2003).

2.2.3. Mechanisms how lameness affects reproduction

The observed mechanisms associated with ovarian function may be related to pain and stress. Lameness is a painful and distressing process characterized by disturbances in body homeostasis, hyperalgesia and a catabolic state (Whay *et al.,* 1997). Plasma levels of adrenal-produced catecholamines, glucocorticoids, and stress-induced progesterone increase in cattle suffering from stress or pain. Persistent ovarian follicles formation is associated with elevated levels than normal levels of adrenocorticotrophic hormone (cortisol) and progesterone hormone.

These changes are associated with suppression of the GnRH and/or LH surge. Lying cows were more likely to cycle before insemination (Garbarino *et al*., 2004) and had a higher occurrence of ovarian cysts (Melendez *et al*., 2003), received more hormonal treatments, and were more likely to get anestrus treatment (Hultgren *et al*., 2003 and Morris *et al*., 2004) and ovulated less (Morris *et al*., 2009). Lame cows had a delayed

cyclicity prior to insemination (Garbarino *et al.*, 2004) and an increased prevalence of ovarian cysts (Melendez *et al.*, 2003), receive more hormonal reproductive therapy, and they have a higher anoestrus treatment (Hultgren *et al.* and Morris *et al.,* 2004) and were less likely to ovulate (Morris *et al*., 2009).

Lame cows reacted more slowly to the mounting attempts of herd members (Walker *et al.,* 2010), oestrus was observed less often. Lame cows had significantly lower oestrus intensity and engaged in mounting less frequently (Walker *et al.,* 2008). According to a study conducted in India (Sood and Nanda, 2006), lame animals had a significantly less probability of to stand to be mounted. Reproductive hormone profiles and follicular dynamics are disrupted in lame cows compared to healthy animals (Walker *et al.,* 2008; Sood *et al.,* 2009; Morris *et al.,* 2011). This might be due to the association between lameness and feeding. If the changes in feeding behaviour lead to a reduction in dry matter intake, the resulting effects on energy levels may lead to infertility through reduced folliculogenesis and ovulation.

Contrary to the levels in healthy control cows, the affected cows had lower levels of the minerals (calcium, copper, iodine, selenium, and iron) and higher levels of the blood acids (glucose and urea nitrogen), haptoglobin, histamine, and IgG. Negative energy balance and weight loss have been shown in studies to hinder ovarian follicular waves. According to Morris *et al.,* 2009; 2011 the follicle size of the lame cows was smaller than that of the non-lame cows. Cows that were exhibited reduced mineral levels (calcium, copper, iodine, selenium, and iron), elevated levels of glucose, blood urea nitrogen, haptoglobin, histamine, and IgG in contrast to the levels in healthy (control) cows. Research has demonstrated that negative energy balance and loss of weight can hinder ovarian follicular waves. The lame cows had smaller follicles when compared to those that were not lame cows (Morris *et al*., 2009; 2011).

Lack of LH pulses brought on by a more pronounced negative energy balance may be the cause of the smaller follicular diameter in lame cows. Unfavorable reproductive efficacy may also be explained by negative energy balance caused by lame cows' reduced feed

intake (Garbarino *et al.*, 2004). Endotoxins may contribute to impaired reproductive performance in cases of nutritionally related lameness (Seesupa *et al.*, 2016). Furthermore, rumen acidosis, which is caused by endotoxins released by the lysis of Gram-negative bacteria in the rumen, related to claw lesions caused by laminitis, which could affect reproductive cyclicity.

The smaller follicle diameter in lame cows was due to the lack of LH pulses and stronger negative energy balance. A negative energy balance caused by reduced feed intake in lame cows can be another explanation for the unfavorable reproductive performance (Garbarino *et al.*, 2004). In feeding-related lameness, endotoxins may partially cause reduced reproductive efficiency (Seesupa *et al*, 2016). In addition, hoof lesions associated with laminitis may be associated with rumen acidosis, where endotoxins released as a result of lysis of gram-negative bacteria in the rumen may interfere with the reproductive cycle.

In cows that lost weight, there is a decline in LH pulses and plasma levels of insulin-like growth factor 1 (IGF-1) (Lucy, 2001). Furthermore, when there is negative energy balance, the larger dominant follicles take longer to grow and reach a concentration of oestradiol that is sufficient to trigger ovulation (Beam and Butler, 1999). It is plausible to propose that if cows that are lame lose more weight and experience a greater negative energy balance, the inhibition of the ovarian follicular waves is more significant than that observed in healthy cows.

2.2.4. Heat stress and Pregnancy loss

Heat stress has been related to an increased occurrence of lameness (Spencer, 2001). Heat stress (Roth *et al.*, 2000) is one of the chronic stressors that affect follicular growth during the wave. Heat stress increases the breathing and heart rate of the animals, weakens the immune response and changes the behaviour of cows. Cows stand for a long time to dissipate the heat, which promotes blood pooling in the digits. In addition, high temperatures reduce feed intake, favour concentrate feed over forage, and reduce total

electrolyte buffering capacity. All these factors increase the likelihood of developing subacute ruminal acidosis (SARA) in hot and humid conditions. This in turn negatively affects the immunity and increases the possibility of foot problems (Vermunt, 2004).

High temperatures can negatively affect the number of oocytes in the ovaries. Increased heat can decline oocyte growth by affecting the progesterone secretion, the release of LH and FSH, and the general progression of oestrus. As oocytes mature during follicular development, disturbances caused by heat stress can impair proper oocyte development. Oocytes obtained in summer from Holstein cows were delayed in the first two embryonic divisions (Gendelman *et al.*, 2010).

Heat stress is related with declined embryogenesis and increased embryonic death in cattle (Hansen, 2007). In addition, heat stress reduces fertility in dairy cows due to poor thermal development due to decreased oestradiol secretion from dominant follicles growing in a low LH environment. Fertility can drop by about 20-27% during the summer (Chebel *et al*., 2004). Heat stress in cattle has also been linked to reduced embryo development and elevated embryo mortality (Hansen, 2007). In addition, heat stress may impair oestrus expression in dairy cows during the summer due to decreased oestradiol secretion from the dominant follicle that developed in a low LH environment. According to Chebel *et al.* (2004), CR can decrease by 20–27% during the summer.

Heat stress during pregnancy can delay foetal growth and increase foetal loss. Amundson *et al*. (2006) examined the impacts of environmental conditions during the breeding season on PR and found that the average daily minimum temperature and total humidity increased and decreased PR. Heat stress can greatly increase the risk of laminitis and hooves Lesion. The number of cows lying in loose barns on sandy soil reduces significantly as the daily ambient temperature increases. Cows stand in a well-ventilated, shaded area near the feeding path to improve heat exchange. After Scharko (1998) studied cow behaviour on hot summer days, he reported that cows panted and exhibited increased salivation.

According to the author, this contributed to the development of respiratory alkalosis and increased excretion of bicarbonates in the urine, which in turn may have contributed to the development of subacute gastric acidosis. To maintain dry matter intake and high milk production during heat stress, the protein content of the ration is increased. On hot days, cows eat rarely, but eat larger amounts, which, together with respiratory alkalosis, lead to the risk of acidosis and laminitis.

2.2.5. Inflammatory mediators disrupts oocyte maturation and embryonic development

Gilbert (2019) described the postpartum period in cattle as a prolonged inflammation accompanied by excessive oxidative stress and fatty acid release, probably indicating inflammatory damage to the developing oocyte and resting oocyte leading to a chronic decrease in fertility (Sheldon *et al.,* 2017).

Immune cells produce inflammatory mediators in injured tissues and reach other organs through the systemic circulation, including the brain, ovaries and uterus. These molecules disrupt GnRH and LH secretion, oocyte developmental competence and embryo survival (Hansen *et al.,* 2004). Lipopolysaccharide was infused in the uterus, mammary gland or intravenously decreased GnRH and LH release (Lavon *et al.,* 2008). Incubation of granulosa cells with LPS or TNFα decreased oestradiol production (Williams *et al.,* 2008). Incubation of immature oocytes in maturation medium with TNFα decreased blastocyst development after fertilization in vitro (Soto *et al.,* 2003). In addition, incubation of bovine embryos with TNFα five days after fertilization increased blastomere apoptosis in vitro (Soto *et al.,* 2003).

Several anovulatory situations are related to increased expression of pro inflammatory cytokines in the granulosa (IL1α, IL6 and TNF α) in the cow (ovulatory failure and follicular persistence, follicular cyst) (Baravalle *et al.,* 2015). Inflammation mediates alters in the follicular fluid that impair the oocyte ability to complete meiosis, fertilize and sustain conception. Activation of granulosa pattern recognition receptors can disrupt

steroidogenesis and the interaction between oocyte and cumulus. Inflammatory mediators also reported to cause abnormal spindle formation and meiosis abnormalities (Bromfield and Sheldon, 2011; Banerjee *et al.*, 2012).

Since inflammation affects the function of thecal cells and granulosa and luteal cells, it is associated with insufficient CL function and insufficient circulating progesterone levels, one of the main causes of infertility in cows (Diskin *et al.*, 2011; Ribeiro *et al.*, 2016). Lameness affects embryo survival through its adverse effects on oocyte quality and CL function, as well as through inadequate uterine microenvironment and through direct cytokine effects on embryo/placental cells. Hill and Gilbert (2008) demonstrated the direct effects of inflammation on the embryo by documenting non-infectious endometrial inflammation; after culture in conditioned uterine medium, the number of blastocyst cells was reduced, affecting the trophectoderm but not the inner cell mass.

Other authors consistently reported impaired elongation and decreased interferon-tau secretion. Inflammation interferes with maternal recognition of pregnancy and later, if pregnancy continues, reduces placental weight from day 42 of pregnancy (Lucy *et al.*, 2016; Ribeiro *et al.*, 2016). Maternal inflammatory diseases induced inflammatory changes in the transcription of conceptus cells (Ribeiro *et al.*, 2016). Lameness in cows after calving was associated with a higher incidence of ovarian cysts, lower PR and lower fertility than non-lame cows (Melendez *et al.*, 2003). Cows that lamed more likely to develop an ovarian cyst before first mating than healthy cows.

2.2.6. Effect of lameness on hormonal profile

Pain caused by hoof disease affects the release of reproductive hormones during the follicular phase (Dobson and Smith, 2000). As a result of stress, the frequency and amplitude of GnRH and LH pulses decrease. Decreased GnRH/LH secretion subsequently deprives supply of gonadotropin, leading to decreased oestradiol production by the slower growing follicles. Hence the negative effect of stress on ovarian function.

Lameness either delays the formation of a good follicle or delays optimal growth and function of the corpus luteum (Garbarino *et al.*, 2004). Despite the emergence of a good follicle, subsequent poor function eventually leads to low progesterone production. The presence of an immature corpus luteal cell leads to lower progesterone priming at a critical time for the hypothalamic oestradiol response (Gumen and Wiltbank, 2005).

Progesterone levels were lower in all groups of lame cows than in healthy animals. In addition, follicles luteinized with GnRH synchronization treatment did not produce a fully functional luteal cell before prostaglandin treatment in lame cows. Before exposure to progesterone during the luteal phase is important for normal oestrous behaviour and ovulation, but the lame ovulatory and nonovulatory groups had similar, although lower, prior progesterone values than healthy cows. Reduced sexual activity during oestrus in lame animals was due to reduced progesterone levels initiated during luteal phase and hence by an insufficient sensitisation to oestradiol rather than by lower levels of serum oestrogens (Walker *et al.*, 2010). Regarding P_4, concentrations of P_4 were reduced in lame cows than in normal cows (Garbarino *et al*., 2004; Walker *et al*., 2010).

2.2.7. Combined effects of lameness on reproduction

Cows with lameness develop metabolic disease, gynaecological disorders and retained foetal membranes (Enting *et al*., 1997). Factors like nutrition, season and management have been associated with the postpartum ovarian cycle of dairy cows (Lucy, 2001). Milk fever, uterine infection, dystocia, mastitis and retained foetal membranes have been suggested to adversely affect fertility. Morris *et al.* (2011) showed a synergistic effect of lameness and high SCC, which reduced the probability of ovulation. High SCC in lame cows has been associated with reduced intensity of oestrus symptoms and cow fertility (Morris *et al.*, 2011; 2013). The CRFS and NSPC were similar for cows with different postpartum health histories after calving. All attempts must be done to ensure diseases which may act as confounding factors (James, 2012).

3. MATERIALS and METHODS

3.1. Study area description

The study was conducted in and around Jimma town, located in the South western state of Oromia region, Ethiopia. The town of Jimma is located about 352 km from Addis Ababa. Geographically it lies between 7°13′ and 8°56′ north latitude and 35°52′ and 37° east longitude. The altitude of the area varies from 880 to 3358 masl. Annual rainfall ranges from 1200-2000 mm. The annual temperature in this region varies between 7 °C and 30 °C. Farmers in the area practice mixed cropping and animal husbandry. This region is one of the largest coffee producing regions in south western Ethiopia. In addition, the area is known for its livestock production, which is estimated at approximately 2,212,962 cattle, 866,561 sheep, 457,311 goats, 96,782 horses, 17,644 mules, 77,767 donkeys, 1,951,129 poultry and 546,722 beehives (CSA, 2017). The intensive production system is practiced in urban areas of Jimma zone, where crossbred animals are mostly reared for their high productions. The main feeds are crop residues, hay and forages.

In Jimma town, the semi-intensive management system, the animals are mainly composed of crossbred cattle. They are kept indoors and graze in the field occasionally. They are often provided with some supplementary diet in addition to the natural pasture and crop by products and are maintained usually in separate stalls a short distance from each other in a house. They are supplemented with concentrates in addition to hay. On the other hand, the extensive production system that consists of local breeds that depend for feed on grazing at the field. Local dairy cows are managed under traditional and extensive husbandry systems.

The cattle are relatively smaller in size and have small udder and short teats. The average daily milk production from individual cows was very low. Pre-mating oestrous events are recorded by herd person using direct observation. Cows are observed for oestrous signs

by herd person in the field, before each milking, while they travel from the pasture to the milking parlour, and throughout each milking.

A cow is considered in oestrus if she showed typical behaviours of oestrus (standing to be mounted, mounting other cows, and head mounting). The AI service is performed by well-trained artificial insemination technician from ministry of agriculture. Pregnancy status is checked after AI by transrectal palpation, which is conducted by the AI technician. Cows are milked, two times daily.

Manure removal is made on a daily basis. Milking is done by hand. Many production constraints, mainly mastitis, lameness, anthrax, black leg, Pasteurellosis, mastitis, foot and mouth disease, liver fluke, gastro-intestinal nematodes, ticks, mange, babesiosis, dermatitis (ring worm) and form a bottle neck in the production process and productivity in the livestock subsector.

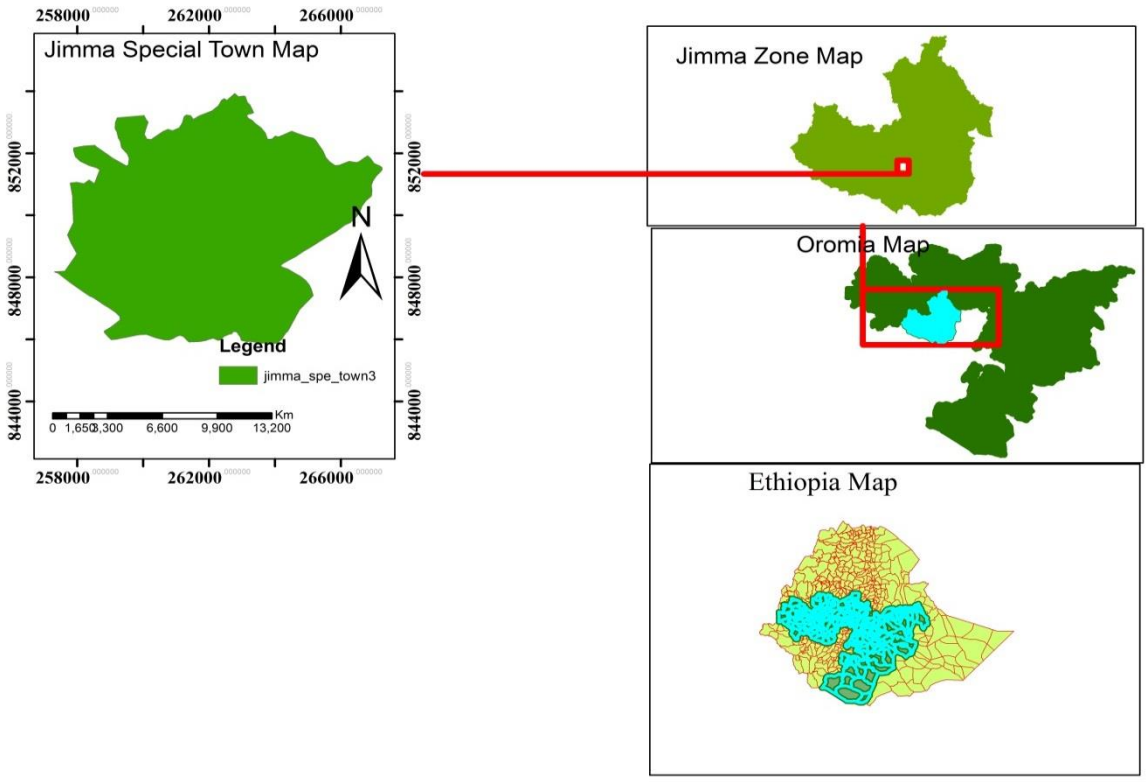

Figure 4: Map of Jimma zone of Jimma town

Source: (Tolosa *et al.*, 2013)

3.2. Study population

A total of 192 Zebu x Friesian crossbred dairy cows were selected for this study. The study was carried out on a total of 11 farms. Two of the farms were from Jimma University College of Agriculture and Veterinary Medicine and Jimma University dairy enterprise. The others 9 farms were from private dairy farms. Purposive sampling technique was employed to select 11 intensive farms out of 71 active dairy farms with the same environment, and management practices. All cows for observational study were kept under the same environment, feeding regime and husbandry system for the whole study period. The first AI in each herd did occur after the voluntary waiting period, which was 50 days after calving in all farms. The houses of all dairy farms were constructed

with ventilation, urinary disposal canal, feed store, feeding and watering canal. The floor is concrete in all farms and washed three times a week.

The intensively managed cattle were kept in doors and received concentrate feeds (Nug cake) in addition to hay and crop residues (such as corn stalks, wheat/barley straw and other leftovers from grain threshing). Dairy cows in all farms feed concentrate feed twice a day 0.5kg for 1liter milk yield and forage feed provided 2-3 times a day. Clean water available at all time. The dairy cows washed three times a week. Dairy cows exercise outdoor daily in the morning.

3.3. Study design

The longitudinal observational study was conducted on 192 crossbred dairy cows. Purposive sampling method was conducted to select these cows. All cows for this study were kept under the same environment, feeding regime and intensive husbandry system for the whole study period. Regular visits were carried out once per weeks for 330 days after calving to record their reproductive parameters on dairy farms.

The reproductive performance data included CFSI, days, NSPC, CRFS, % and PRFS, %. The CFSI was defined as the number of days between calving and the first insemination date. The NSPC was defined as the number of services that result in a conception. CRFS, %) defined as the number of known pregnant animals divided by the number of inseminated animals with known outcomes, considering only the first AI after calving, and multiplied by 100. PRFS, % defined as number of known pregnant animals divided by the number of cows eligible to become pregnant, considering only the first AI after calving, multiplied by 100.

A detailed history including animal number, parity, body condition score, date of calving and reproductive status was recorded.

3.4. Milk collection

Milk samples were collected from cows that had not been treated with early intramammary or systemic antimicrobials. The udders and especially the teats were washed and dried before sampling. Each teat ends was vigorously cleaned with a cotton or gauze sponge moistened with 70% ethyl alcohol. A separate sponge was used for each teat and scrubbing was continued until the new sponge surface remained clean.

Milk samples were collected from individual cows using sterile screw cap test tubes and the first milk stream from each quarter was discarded. After milk collection, all samples were clearly marked with the corresponding cow identification number. Milk samples were then stored in an ice box and transported to the microbiology laboratory at the School of Veterinary Medicine, Jimma University. In the laboratory, samples were cultured immediately or stored at 40°C (NMC, 1999).

3.5. Determination of subclinical mastitis

The California Mastitis Test (CMT) was used to screen cows for SCM. About 2 mL of milk from each quarter was placed in each of the four shallow cups of the CMT paddle, and an equal volume of CMT reagent was added to each cup. The mixture was subjected to gentle circular motion for 15 seconds in a horizontal plane. Test results were set on the basis of gel formation.

The CMT results were scored as 0 (negative), trace, 1(weak positive), 2 (distinct positive) and 3 (strong positive) depending on gel formation. All CMT scores of 0 and trace were negative while CMT scores of 1, 2, and 3 were indicators of subclinical mastitis. A cow considered positive if at least a quarter of the cows having at least one with CMT score of 1^{+}.

3.6. Bacteriological culture

Bacteriological culture was performed according to Quinn *et al*. (2002). For the primary isolation and identification of mastitis causing pathogens colony size, shape, colour, pigmentation, haemolytic characteristic, Grams reaction, Oxidase test [oxidase positive in modified oxidase test and oxidative in the oxidation and fermentation test (O-F test)] were performed. After these colonies were subcultured to different media, such as MacConkey agar (Oxiod, Hampshire, England), Manitol salt agar, Edwards medium (Oxiod Hampshire, England), Eosin methylene blue medium (EMB) (Oxiod, Hampshire, England), etc. to get a pure culture.

And the secondary biochemical tests such as, coagulase test, urease test, IMViC (indole, methyl red, Voges-Proskaur, and citrate) tests, sugar tests, etc. were done for bacterial species identification.

3.7. Determination of lameness

The five-point locomotion scoring system designed by Sprecher *et al.* (1997) was used once a day by repeatedly observing cows for affected gait. If the animal had a normal gait and a level posture while walking, its movement score was considered 1, which is normal. Similarly, when an animal has a normal gait and a level back posture when standing, but a curved back when walking, its movement score was considered 2, which is mildly lame. But animals with gait affected, short striding, back arched while standing and walking were determined as having locomotion score 3 (moderately lame) while animals with back always arched, only one deliberated step at a time and limping with one or more limbs were considered as having locomotion score 4 (Lame). Moreover, animals additionally with extreme reluctance to carry its own weight with one or more affected limbs were determined as having locomotion score 5 (severely lame).

3.8. Assessment of foot lesions

Since the locomotion scoring showed that the herd had a lameness problem, the second step of the study was to identify the types of foot lesions (Nordlund *et al.*, 2004). Thus, for any locomotion score ≥ 3, each type of foot and leg problem (lesions) was recorded on separate data sheets.

3.9. Screening of subclinical endometritis

Subclinical endometritis was determined using cytobrush at 56 ± 3 DIM postpartum as stated by Dubuc *et al*. (2010). In the cytobrush technique, a modified human cytobrush was connected to the plunger of an artificial insemination gun covered with plastic AI sheath.

A cytobrush assembly was inserted into the uterus as for AI. Inside the uterus the brush was slowly screwed in both directions. Cytobrush was smeared onto clean glass slides immediately after removal from the reproductive tract and stained with Giemsa stain. The slide was observed under microscope on 400X and oil immersion for endometrial cells and polymorphnuclear cells (PMN) cells). Samples with ≥ 4% PMN cells were categorized as subclinical endometritis (Dubuc *et al*., 2010).

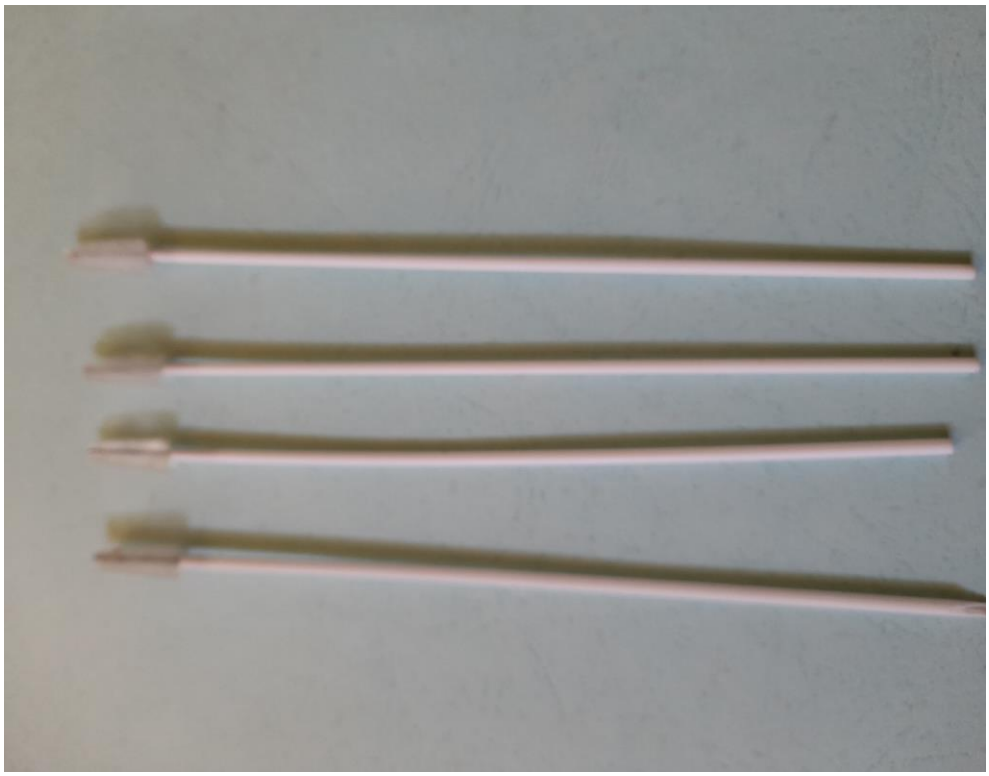

Figure 5: Cytobrush

3.10. Progesterone and cortisol analysis

Blood samples were collected from cows for plasma progesterone and cortisol profiling, 35 days post-first AI. Blood samples (10ml) were collected from the jugular vein using a vacutainer system. Blood samples were centrifuged at 4,000rpm for 10 min, plasma was separated and stored in plastic tubes and frozen at −20℃ until analysis.

Analysis of progesterone and cortisol concentrations in serum were performed using **e**lectro**c**hemi**l**uminescence immunoassay "ECLIA" at Jimma specialized hospital, clinical chemistry laboratory.

3.11. Data collection

The animals were classified into four groups as follows: group I (n=43) cows with subclinical mastitis; group II (n=50) lame cows; group III (n=58) lame cows with subclinical mastitis and group IV (n=41) healthy cows (control group). Based on a locomotion scoring system devised by Sprecher *et al*. (1997) study animals were classified into five categories of lameness: 1 – non-lame (41), 2 – mildly lame (8), 3 – moderately lame (27), 4 – lame (13) and 5- Severely lame (2). Cows which scored ≥ 3 points were classified as clinically lame (CL).

Data were collected from primiparous (n=66) and multiparous cows (n=126), that calved from January 2020 to July 2021. Body condition score was determined using a scale from 1 (emaciated), 2 (thin), 3 (average), 4 (fat) and 5 (very fat) as stated by Edmonson *et al*. (1989). The body condition score of animals was classified as poor (<3) and good (>3).

3.12. Statistical analysis

The raw data were collected and entered into MS excel program. Statistical analysis was conducted using SPSS version 20.0. Descriptive statistics were used to compute percentages, proportions and frequency distributions of the CRFS, PRFS and the prevalence of SCM, causing bacteria and lameness. Logistic regression was used to assess the strength of association among SCM, lameness, lame cows with SCM (dependent variable) and important predictor variables such as body condition score and parity.

To compute the relationship among the SCM, lameness, lame cows with SCM (independent variable) and subclinical endometritis (dependent variable) logistic regression was used.

The dependent variables, CFSI, days, NSPC and the independent variable, cow SCM, lame cows with SCM (0 = negative and 1 = positive) were compared by independent sample t-test.

The statistical comparison between means of progesterone (P_4), cortisol concentrations in subclinical mastitis, lame cows and lame cows with SCM status were performed by the independent sample t-test.

Mean days from CFSI, NSPC, P_4 and cortisol concentrations of the lame cows and healthy cows were compared by ANOVA.

The association between the dependent variables, progesterone (P_4) and cortisol concentrations and categorical independent variables such as subclinical mastitis, body condition scores and parity were assessed using multivariable linear regression analysis. In all the cases, 95% confidence level and $P < 0.05$ was used to determine statistical significance.

4. RESULTS

4.1. Association of subclinical mastitis with reproductive performance and subclinical endometritis in crossbred dairy cows

4.1.1. Prevalence of SCM

Eight four cows were tested for SCM using CMT, the prevalence of SCM at the cow level was 51.2% (43 of 84).

4.1.2. Impact of subclinical mastitis on fertility

The results of impact of SCM on fertility are presented in table 3. Perusal of table showed that the mean days from CFSI were longer in cows with SCM compared to negative cows (120.51±24.5 and 85.15±28.3 days, respectively) (P = 0.000). The mean NSPC was higher (P = 0.000) in cows with SCM (2.51±0.83) than in negative cows (1.59±0.81).

CRFS was reduced in cows with SCM (32.6%) compared to negative cows (56.1%) (P=0.030). SCM positive cows had a lower PRFS (23.3%) compared to SCM negative cows (46.3%) (P=0.026) (Table 3).

Table 3: Impact of SCM on fertility of dairy cows

Reproductive parameters	SCM	n	**mean ±SD**	**%**	**t-test**	**OR**	**95% CI**	**P-value**
CFSI (days)	Negative	41	85.15±28.3	-	6.112	-	-	0.000
	Positive	43	120.51±24.5	-		-	-	
NSPC	Negative	41	1.59±0.81	-	5.196	-	-	0.000
	Positive	43	2.51±0.83	-	-	-	-	
CRFS (%)	Negative	41	-	56.1	-	0.38	0.16-0.92	0.03
	Positive	43	-	32.6	-			
PRFS (%)	Negative	41	-	46.3	-	0.35	0.14-0.9	0.026
	Positive	43	-	23.3	-			

4.1.3. Factors associated with SCM

The results of factors associated with SCM are presented in table 4. The results showed that good body conditioned cows suffered less (35.90%), whereas poor body condition cows suffered more (64.4%) with SCM and these differences were highly significant (P=0.009). From primiparous cows examined, 30% were positive for SCM, and among multiparous cows examined, 70.5% of them were positive for SCM (P<0.05).

Table 4: Factors related to the incidence of SCM

Factor	**Category**	**n**	**Prevalence (%)**	**OR**	**95% CI**	**P-value**
BCS	Good	39	14(35.9)	0.31	0.13-0.76	0.009
	Poor	45	29 (64.4)			
Parity	Primiparous	40	12 (30)			
	Multiparous	44	31(70.5)	5.56	2.2-14.2	0.000

4.1.4. The relationship between SCM and subclinical endometritis

The results of association between SCM and subclinical endometritis (SCE) are presented in table 5. Perusal of results showed that the prevalence of subclinical endometritis in SCM positive cows was 41.9%, whereas it was 19.5% in SCM negative cows. The presence of SCM was significantly and directly associated with subclinical endometritis [2.97, CI (1.113-7.23), P=0.027].

Table 5: The association between subclinical endometritis (SCE) and SCM

SCM	SCE		OR	95% CI	P-value
	Negative (%)	Positive (%)			
Negative (%)	33 (80.5)	8 (19.5)	2.97	1.3-7.93	0.027
Positive (%)	25 (58.1)	18 (41.9)			
Total	58(69)	26 (31)			

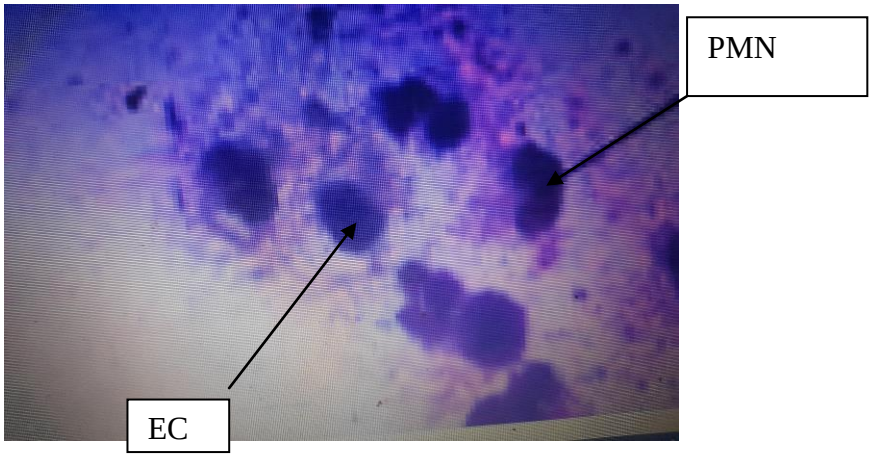

Figure 6: **Uterine cytology of cattle suffering from subclinical endometritis**

EC=endometrial cells PMN= Polymorphnuclear cells

4.1.5. Effect of subclinical mastitis on hormone concentrations

The mean concentration of serum progesterone ($P_{4)}$ at 35 days post-first AI cows that exhibited subclinical mastitis were 5.7±2.56ng/ml, while in subclinical mastitis negative cows was 8.74±0.95ng/ml (Figure 6). Cows stressed by subclinical mastitis had significantly higher mean concentrations of cortisol (65.9±21.2ng/ml) than subclinical mastitis negative cows (53.52±14.9ng/ml, Figure 6). Predictors like parity, body condition and presence of SCM were considered, only SCM was found to be significantly associated with level of progesterone hormone (P =0.000) and cortisol hormone (P =0.001) concentrations (Table 6 and 7). BCS was with P-value of 0.364 tells us that, adequate BCS has absolutely no impact on the level of progesterone, getting odds ratios of 1.51 (Table 6).

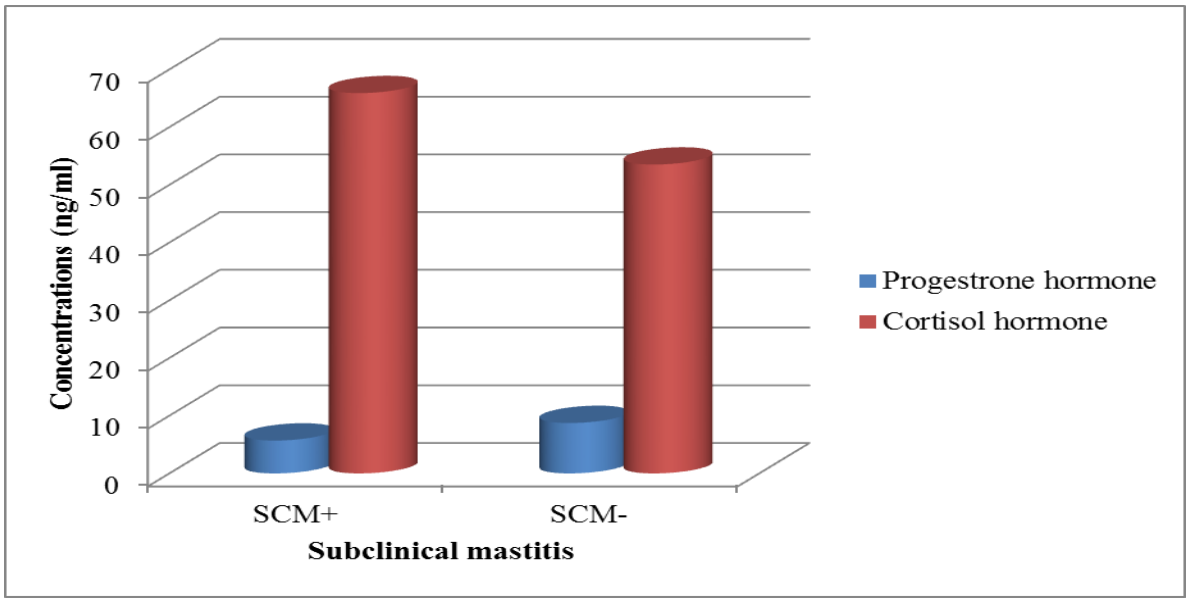

Figure 7: Plasma Progesterone and Cortisol concentrations in subclinical mastitis positive (SCM+) and subclinical mastitis negative (SCM-) cows

Table 6: Factors affecting the level of progesterone in the serum of study animals

Variable	Category	Coefficient	SE	P-value
SCM	Positive	2.659	0.484	0.000
	Negative	Ref		
Parity	Multiparous	-0.883	0.463	0.06
	Primiparous	Ref		
BCS	Good	0.417	0.456	0.364
	Poor	Ref		
Constant		8.687	0.502	0.000

Table 7: Factors affecting the level of cortisol hormone in the serum of study animal

Variable	Category	Coefficient	SE	P-value
SCM	Positive	15.993	4.57	0.001
	Negative			
Parity	Multiparous	-0.18	4.378	0.968
	Primiparous			
BCS	Good	4.41	4.313	0.31
	Poor			
Constant		53.62	4.741	0.000

4.1.6. Major pathogenic bacterial isolates from milk of SCM

The results are presented in table 8. These results showed that from 124 quarters of 31 cows with SCM, 27 milk samples yielded no bacterial growth and 19 samples yielded three or more different colonies which were contaminated and then rejected. Of the remaining 78 culture positive samples, a total of 101 bacteria were isolated; the most

prevalent being *S. aureus* (45.54%), coagulase negative staphylococcus (CNS) (20.8%) and streptococci (14.85%) (Table 8).

Table 8: The relative isolation rate of subclinical mastitis causing bacteria

No	Bacterial species	Frequency	
		No isolated	Isolation (%)
1	*Staphylococcus aureus*	46	45.54
2	CNS	21	20.8
3	*Klebsella pneumonae*	4	3.96
4	*Corynebacterium species*	6	5.94
5	Bacillus	2	1.98
6	*Streptococcus species*	15	14.85
7	*Micrococcus species*	4	3.96
8	*Enterobacter aerogenes*	2	1.98
9	*Escherichia coli*	1	0.99
10	Total	101	100

4.2. Association of lameness with reproductive performance and subclinical endometritis in dairy cows

Lame cows classified as non-lame, mildly lame, moderately lame, lame, and severely lame. The mean days from CFSI in nonlame cows (n=41) was 85.15±23.6 days while in mildly lame cows (n=8) was 89.63±9.13 statistically with non-significant difference. The mean days from CFSI in moderately lame cows (n=27) was 118.2±29.8, in lame cows (n=15) was 142.67±25.4 (P=0.000) (Table 9).

Table 9: Descriptive statistics (mean ± SD) of CFSI in different degrees of lameness

Locomotion score	N	mean ± SD	P-value
		CFSI (days)	
1 (Nonlame)	41	85.15±23.6	0.000
2 (Mildly lame)	8	89.63±9.13	
3 (Moderately lame)	27	118.2±29.8	
4 (Lame)	15	142.67±25.4	

The mean NSPC in nonlame cows (n=41) was 1.59±0.81 while in mildly lame cows (n=8) was 1.75±0.9. The mean number of services per conception (NSPC) in moderately lame cows (n=41) was 2.85±1.54 and in lame cows (n=15) was 3.93±1.4 (P=0.000) (Table 10).

Table 10: Descriptive statistics (mean ± SD) of NSPC in different degrees of lameness

Locomotion score	N	mean ± SD	P-value
		NSPC	
1 (Nonlame)	41	1.59±0.81	0.000
2 (Mildly lame)	8	1.75±0.9	
3 (Moderately lame)	27	2.85±1.54	
4 (Lame)	15	3.93±1.4	

The CRFS in nonlame cows (n=41) was 23(56.1%) while in mildly lame cows (n=8) was 4(50%). The CRFS in moderately lame cows (n=27) was 9(33.3%), in lame cows (n=15) was 4(26.7%) (P=0.131) (Table 11).

Table 11: The relationship between CRFS and different degrees of lameness in crossbred cows

Locomotion score	n	CRFS n (%)	χ^2	P-value
1 (Nonlame)	41	23 (56.1)	5.08	0.131
2 (Mildly lame)	8	4 (50)		
3 (Moderately lame)	27	9 (33.3)		
4 (Lame)	15	4(26.7%)		

The PRFS in nonlame cows (n=41) was 19 (46.3%) while in mildly lame cows (n=8) was 2 (25%). The PRFS in moderately lame cows (n=27) was 5 (18.5%), in lame cows (n=15) was 1 (6.7) (P=0.006) (Table 12).

Table 12: The relationship between PRFS and different degrees of lameness in crossbred cows

Locomotion score	N	PRFS n (%)	χ^2	P-value
1 (Nonlame)	41	19 (46.3)	11	0.006
2 (Mildly lame)	8	2 (25)		
3 (Moderately lame)	27	5 (18.5)		
4 (Lame)	15	1 (6.7)		

4.2.1. Risk factors associated with lameness

Of the good body conditioned cows, 13 of 37 (35.1%) were lame cows and of the poor body condition cows, 37 of 54 (68.5%) were lame cows (P = 0.002). From 37 examined primiparous cows, 15 (40.5%) were lame cows, and among 54 examined multiparous cows, 35 (64.8) of them were lame cows (P = 0.022) (Table 13).

Table 13: Factors associated with the occurrence of lameness

Factor	Group	n	Prevalence (%)	OR	95% CI	P-value
BCS	Good (>3)	37	13(35.1)	0.25	0.103,0.6	0.002
	Poor (≤3)	54	37(68.5)			
Parity	Primiparous	37	15(40.5)			0.022
	Multiparous	54	35(64.8)	2.7	1.14,6.4	

The lesions that were found causing lameness were excess hoof growth (76.32%), sole ulceration (13.16%) and foot rot (10.52%) (Table 14).

Table 14: Clinical lameness in animals with locomotion score ≥ 3

No	Type	Frequencies	%	Quarters
1	Excess hoof growth	29	76.3]2	Front & rear feet
2	Foot rot	4	10.52	Rear feet
3	Sole ulceration	5	13.16	Rear feet
4	Total	38	100	

4.2.2. *Effect of lameness on hormone concentrations*

The Serum progesterone (P_4) concentration at 35 days post first artificial insemination (AI) in nonlame cows (n=41) was 8.74±0.95ng/ml while in mildly lame cows (n=8) was 6.2±1.35ng/ml. The Serum progesterone (P_4) concentration in moderately lame cows (n=27) was 5.7±2.1ng/ml, in lame cows (n=15) was 4.56 ± 1.95 ng/ml (P=0.000) (Table 15).

Table 15: Plasma progesterone concentration (Mean ±SD) in different degrees of lameness in crossbred cows

Locomotion score	Description	n	Plasma progesterone (ng/ml)
1	Normal (Not lame)	41	8.74 ± 0.95
2	Mildly lame	8	6.2 ± 1.35
3	Moderately lame	27	5.7 ± 2.1
4	Lame	15	4.56 ±1.95

The Serum cortisol concentration in nonlame cows (n=41) was 53.52±14.98ng/ml while in mildly lame cows (n=8) was 48.44±5.22ng/ml. The Serum cortisol concentration in moderately lame cows (n=27) was 63.2±17.2ng/ml and in lame cows (n=15) was 60.8±19.4 ng/ml (Table 16). Factors like parity, body condition and presence of lameness were considered, only lameness was found to be significantly associated with progesterone (P =0.000) and cortisol hormone (P =0.001) concentrations (Table 17 and 18).

Table 16: Plasma cortisol concentration (Mean ±SD) in different degrees of lameness in crossbred cows

Locomotion score	Description	n	Plasma cortisol (ng/ml)
1	Normal (Not lame)	41	53.52±14.98
2	Mildly lame	8	48.44±5.22
3	Moderately lame	27	63.2±17.2
4	Lame	15	60.8±19.4

Table 17: Factors affecting the level of progesterone in the serum of lame cows

Variable	Group	Coefficient	SE	P-value
Lameness	Positive	-3.25	0.4	0.000
	Negative			
Parity	Multiparous	-0.322	0.4	0.37
	Primiparous			
BCS	Good	-0.194	0.4	0.60
	Poor			

Table 18: Factors affecting the level of cortisol hormone in the serum of lame cows

Variable	Group	Coefficient	SE	P- value
Lameness	Positive	14.265	3.97	0.001
	Negative			
Parity	Multiparous	-6.3	3.96	0.116
	Primiparous			
BCS	Good	1.79	4.2	0.67
	Poor			

4.2.3. The association between lameness and subclinical endometritis

Uterine samples revealed an overall prevalence of subclinical endometritis infections of 30.8% (28/91). Of 91 cross breed dairy cows the prevalence of subclinical endometritis was 40% (20/50) and 19.5% (8/41) in lame cows and nonlame cows, respectively (Table 19).

Table 19: The association between lameness and subclinical endometritis

Lameness	Subclinical endometritis		OR	95% CI	P-value
	Negative (%)	Positive (%)			
Negative (%)	33 (80.5)	8 (19.5)			0.035
Positive (%)	30 (60)	20 (40)	2.75	1.1-7.2	
Total	63(69.23)	28 (30.8)			

4.3. Combined effect of lameness and SCM on fertility and hormonal profile of dairy cows

4.3.1. Prevalence of subclinical mastitis in lame cows

Hundred and eight lame cows were tested for SCM using CMT and the prevalence of SCM at the cow level was found to be 53.7% (58 of 108).

4.3.2. Combined effect of lameness and SCM on fertility

The mean days from CFSI were much extended in lame cows with subclinical mastitis (152.71±28.6) compared to lame cows (120.98±31.3). Lame cows with mastitis was highly associated with CFSI (P = 0.000). The mean NSPC was higher (P = 0.019) in lame cows with mastitis (3.66±1.31) than in lame cows (3±1.6) (Table 20).

Table 20: Mean ± SD of reproductive measures in the lame cows and lame cows infected with SCM

Category	n	mean ± SD	P-value
	CFSI (days)		
Lame cows	50	120.98±31.3	0.000
Lame cows with SCM	58	152.98±45.03	
	NSPC		
Lame cows	50	3±1.6	0.019
Lame cows with SCM	58	3.66±1.31	

CRFS was reduced in lame cows with SCM (17.2%) comparing to lame cows (34%) (P=0.001). Lame cows with SCM had a lower PRFS (3.4%) comparing to lame cows (16%) (P=0.007) (Table 21).

Table 21: Comparison of reproductive measures between lame cows and lame cows infected with SCM

Parameters	Category	n	Percent	P-value
CRFS	Lame cows	50	34	0.045
	Lame cows with SCM	58	17.2	
PRFS	Lame cows	50	16	0.025
	Lame cows with SCM	58	3.4	

4.3.3. Factors associated

Of the good body conditioned cows, 33.3% (7 of 21) were positive for lame animals with subclinical mastitis and of the poor body condition cows, 58.6% (51 of 87) were positive

for lame animals with subclinical mastitis (P = 0.037). From primiparous cows examined, 34.6% (9 of 26) were positive for lame animals with subclinical mastitis, and among multiparous cows examined, 59.8% (49 of 82) of them were positive for lame animals with subclinical mastitis (P = 0.025) (Table 22).

Table 22: Risk factors related to the incidence of SCM in lame animals

Factor	Group	n	Prevalence (%)	OR	95% CI	P-value
BCS	Good	21	7(33.3)			
	Poor	87	51(58.6)	0.353	0.13,0.96	0.037
Parity	Primiparous	26	9(34.6)			
	Multiparous	82	49(59.8)	2.81	1.12,7.04	0.025

4.3.4. The association between SCM and subclinical endometritis

The uterine samples were collected from lame cows and lame cows positive for SCM. The prevalence of subclinical endometritis in lame cows positive for SCM was 65.5% (38 of 58) and 40% (20 of 50) in lame cows, respectively (Table 23). The presence of SCM in lame cows was directly significantly associated with subclinical endometritis [OR=2.85, CI=1.3-6.2, P=0.008] (Table 23).

Table 23: **The relationship between subclinical endometritis (SCE) and Lame cows with SCM**

Lameness	**Subclinical endometritis**		**OR**	**95%CI**	**P-value**
	Negative (%)	Positive (%)			
Lame cows (%)	30 (60)	20 (40)			0.008
Lame cows with SCM (%)	26 (58.1)	38 (65.5)	2.85	1.3, 6.2	
Total	56 (51.9)	52 (48.1)			

4.3.5. Hormonal concentrations of cows

The Serum progesterone (P_{4}) concentrations after first insemination of cows that exhibited lameness were 7.21±2.51 ng/ml and in lame cows with subclinical mastitis were 5.56±2.2 ng/ml (P =0.000). Lame cows with mastitis had significantly higher concentration of cortisol (78.2 ±21.99ng/ml) than in lame cows (66.78±20.74ng/ml) during the experimental period, statistically significant difference was observed in relation to cortisol concentration (P=0.004). Parity, body condition and presence of subclinical mastitis in lame cows were considered as risk factors, only lame cows with subclinical mastitis was found to be significantly associated with level of progesterone hormone (P =0.001) and cortisol hormone (P =0.004) concentrations (Table 24 and 25).

Table 24: Factors affecting the level of progesterone in the serum of study animals

Variable	Category	Coefficient	SE	t	95 % CI	P-value
Lameness	lame cows with SCM	1.6	0.5	3.5	0.68, 2.5	0.001
	Lame cows	Ref				
Parity	Multiparous	-0.15	0.53	-0.29	-1.2, 0.902	0.776
	Primiparous	Ref				
BCS	Good	-0.56	0.57	-0.958	-1.7,0.571	0.327
	Poor	Ref				
Constant		5.72	0.52	11.1	4.7,6.7	0.000

Table 25: Factors affecting the level of cortisol hormone in the serum of study cows

Variable	Group	Coefficient	SE	t	95 % CI	P-value
Lameness	Lame cows with SCM	12.5	4.3	2.91	3.98, 81.4	0.004
	Lame cows	Ref				
Parity	Multiparous	-7.1	4.97	-1.42	-16.94, 2.78	0.158
	Primiparous	Ref				
BCS	Good	-1.26.	5.36	-0.235	-11.88, 9.36	0.815
	Poor	Ref				
Constant		71.8	4.84	14.84	62.21, 81.4	0.000

4.3.6. Results of bacteriological examination

From 148 quarters of 37 lame cows with SCM, 36 milk samples were free of bacterial growth and 29 milk samples were contaminated with 3 or more different colonies and discarded. A total of 104 bacteria were isolated from 83 culture positive specimens. The most common bacteria were *S. aureus* (39.42%), *E. coli* (24%), and CNS (13.46%) (Table 26).

Table 26: The relative isolation rate of subclinical mastitis causing bacteria in lame cows

No	Bacterial species	Frequency	
		No isolated	Isolation (%)
1.	*S. aureus*	41	39.42
2.	*S. intermedius*	2	1.92
3.	Coagulase negative staphylococci (CNS)	14	13.46
4	*Klebsella pneumonae*	6	5.8
5	*Corynebacterium species*	8	7.7
6	*Escherichia coli*	25	24
7	Streptococci	8	7.7
8	Total	104	100

5. DISCUSSION

5.1. Association of subclinical mastitis with reproductive performance and subclinical endometritis of dairy cows

The result of this study revealed that a prevalence of SCM of 52.6% based on CMT. Our finding agreed with reports of Zeryehun and Abera (2017), in the Eastern Harrarghe zone, Tuke *et al.* (2017) in Alage dairy farm, Abebe *et al.* (2016) in Hawassa town, Birhanu *et al.* (2013) in Asella dairy farms. Our finding was higher than that reported by Bedacha and Menghistu (2011), in Batu district, Belina *et al.* (2016) in North Shewa and Borana pastoral area and Kitila *et al.* (2021) in west Wollega, western Oromia, Ethiopia. The outcome of this study was lower than that of Fesseha *et al.* (2021) in Modjo town, Tolosa *et al.* (2013), in Jimma town and Dabash *et al.* (2014), in North Shewa Zone, Ethiopia. These differences in prevalence may indicate that the disease is multifactorial and interacts with a number of factors, such as lack of veterinary services, intramammary infusion drugs, and management methods.

CFSI was significantly longer in subclinical mastitis positive cows compared to SCM negative cows. Our finding was consistent with Kirk (2004) and Ranasinghe *et al.* (2021) who reported that cows with SCM had a longer CFSI. In addition, Linderoth (2003) also reported that cows that SCM increased CFSI compared to healthy herd mates.

The mean NSPC was higher in cows with SCM than in SCM negative cows. Our result was in agreement with Khokon *et al.* (2017) and Dolecheck *et al.* (2019), who reported that when mastitis occurs before insemination, NSPC increased by an average of 0.72 in AI compared to healthy cows. Hansen *et al.* (2004) reported that the increase in NSPC in mastitis cows was due to anovulation, failure to fertilize, and embryonic death.

The results of this study revealed that a conception rate at first service (CRFS) in SCM cows was lower than in negative cows. This finding was also agreed with findings of David *et al.* (2015) that SCM significantly lowers conception rate. Reduced fertility in

animals with mastitis related to production of several bioactive molecules that can inhibit the reproductive system function (Slebodzinski *et al*., 2002).

In the present study, SCM positive cows had a lower PRFS compared to negative cows. Studies have also shown an association between pregnancy loss and mastitis (Hansen *et al.*, 2004). In addition, Lavon *et al.* (2011) reported low PR in cows with high SCC before AI, this result indicates that high SCC is often associated with reduced fertility, indicating that the more severe the mastitis, the greater its impact on reproductive performance.

This finding was consistent with previous findings (Abebe *et al*., 2016 and Fesseha *et al.*, 2021) that the incidence of SCM increased with increasing parity and thus reduced fertility. This may be due to longer duration of infection where a mastitis control program is not implemented (Radostitis *et al*., 2007). Although previous studies have shown a combined effect of SCM exposure and parity ≥ 4 on pregnancy loss in dairy cows (Dahl *et al.*, 2019). The uterine environment of old mastitis cows is more sensitive to the systemic inflammatory response compared to younger cows due to high levels of inflammatory and IFN signaling and cell division dysfunction were recorded by Tanikawa *et al*. (2017).

In this study, poor BCS is a predictor for mastitis (Mungube *et al.*, 2004 and Sarker *et al.*, 2013) and had poor reproductive performance. When cows had lower BCS, cows with SCM were associated with an increased risk of pregnancy loss (Soto *et al.*, 2003 and Hernandez *et al.*, 2012).

In the current study, the odds of subclinical endometritis (SCE) prevalence was 2.97 times higher (OR=2.97) in cows with SCM compared to SCM negative cows. Cows with SCM and SCE have reduced fertility and more unstable pregnancies than those diagnosed with mastitis alone (Ribeiro *et al.,* 2013). Dosognen *et al.* (2002) reported plasma lipopolysaccharide (LPS) increased in mastitis cows. A direct relationship between SCM and SCE is due to the transfer of bacteria and bacterial products,

particularly LPS, from the udder to the uterus and vice versa (Bacha and Regassa, 2010; Eckel and Ametaj, 2016).

This study showed that the plasma progesterone concentrations in cows that exhibited SCM had significantly lower than SCM negative cows. In agreement with our finding David *et al*. (2015) and Pinedo *et al*. (2009) also reported that SCM was the factor of changes in the progesterone levels. The inflammatory factor such as TNF-α suppresses the LH receptors in granulosa cells, which reduces LH sensitivity, which leads to inhibition of progesterone secretion from the corpus luteum and disturbs uterine function (Wang *et al.*, 2021).

The current study revealed that the plasma cortisol concentration of SCM cows was significantly higher than that of SCM negative cows. Similarly, Hockett *et al.* (2000) described that cortisol levels were increased in SCM cows. During SCM cortisol levels increased, reducing gonadotropin release, and inflammatory responses associated with bacterial challenges (Soto *et al.,* 2003) have been implicated in interference of reproductive processes. SCM is the cause of changes in cortisol concentration (David *et al.,* 2015 and Pinedo *et al.,* 2009). During the luteal phase of mastitis cows, the blood cortisol concentration increases, by suppressing LH levels (Wang *et al.,* 2021).

The major bacterial isolates were *S. aureus*, CNS and streptococci are the predominant bacterial isolates. These findings are comparable to the outcomes reported by Abebe *et al*. (2016). The reason for the higher isolation of the pathogen *S. aureus* is the wide ecological distribution on the skins and in the mammary glands that are origins of infection in other healthy cows and transferred during milking process (Radostitis *et al.,* 2007).

Coagulase-negative staphylococcus (CNS) isolation rate was lower than Zeryehun and Abera (2017) and higher than Fessehan *et al.* (2021). This difference in the detection rate of coagulase-negative staphylococcus (CNS) in mastitis milk may be due to differences in laboratory analysis methods used and management practices.

Streptococci isolated from mastitis cows' milk were comparable to those of Birhanu *et al.* (2013). However, our result is lower than Fesseha *et al.* (2021). This lower isolation of streptococci was due to the extensive use of Penstrip in the treatment of mastitis. Intramammary infections (IMI) which caused by Gram-positive and Gram-negative bacteria decline fertility in dairy cows (Dalanezi *et al.*, 2020).

5.2. Association of lameness with reproductive performance and subclinical endometritis

In the current study the mean CFSI was longer in clinically lame cows compared with cows which were mildly and never lame cows, the difference was significant ($P<0.05$). In agreement to our study Orgel *et al*. (2016) and Niorn *et al.* (2020) also recorded lameness extended CFSI.

In the present study we obtained a higher NSPC in clinically lame cows when compared to cows which were mildly and never lame cows, the difference was significant ($P =0.000$). Similar to our finding, Niorn *et al.* (2020) also recorded 2.98 ± 2.4 NSPC in lame cows. Lower than our finding Olechnowicz and Jaskowski (2011) reported 2.14 for lame cows. Huxley (2013) also recorded 1.2 more services per conception. This difference may be due to managemental factors.

The current study showed that clinically lame cows had lower CRFS (31%) than the nonlame cows (56.1%) ($P=0.035$). Our finding was similar to Chapinal *et al.* (2013) who reported 36% of lame cows in the north eastern United States. Ferguson and Skidmore (2013) recorded a higher CRFS of 44% for lame cows in the USA, while Morton (2010) reported a CRFS of 47% in Australia. Serhat *et al.* (2005) also noted that a lower conception rate in lame cows.

Huxley (2013) reported a 20% lower conception rate due to lameness. Melendez *et al.* (2003) also found that lameness was associated with poorer conception rates at first

service. All these variations can be caused by pain, hormonal insufficiency and associated with a negative energy balance (Morton, 2010).

The current study revealed cows with lameness had a lower PRFS (14.3%) comparing with nonlame cows (46.3%) (P=0.021). In accordance to our finding Jan and Jędrzej (2015) found that lame cows had a significantly lower PR than non-lame cows. Similarly, Chapinal *et al.* (2013) also observed 20% in the north eastern United States. Our finding concurs with the report of De Vries and Risco (2005) (12%), but lower than the 32% reported by Ferguson and Skidmore (2013). This difference may be due to management factors affecting reproduction.

This study revealed that the prevalence of lameness was strongly associated with the occurrence of subclinical endometritis, and lameness cows had significantly higher rates of subclinical endometritis than healthy cows. Garbarino *et al.* (2004) found that lameness negatively affects ovarian function in Holstein cows during the postpartum period. Lameness in cows was associated with a higher occurrence of ovarian cysts and uterine inflammation (Seesupa *et al.*, 2016).

The Serum progesterone (P_4) profile after first insemination of cows that exhibited lameness was 4.7±1.7ng/ml whereas in lameness free cows were 8.47±0.95ng/ml. Our finding was in agreement with reports of Walker *et al*. (2008) and Walker *et al*. (2010), who recorded that lameness reduced progesterone concentrations.

In the current study cows stressed by lameness had significantly higher concentrations of cortisol (79.6±20.4ng/ml) than in nonlame cows (53.52±14.98ng/ml). Consistent with our study, Almeida *et al.* (2008) also reported that elevated serum cortisol was observed in cows with foot lesions. Endo *et al.* (2003) also recorded significantly higher plasma cortisol levels in cows with lameness compared to controls. Elevated plasma cortisol levels are a consequence of the pain and stress of lameness, which has affected the normal release of reproductive hormones (Dobson and Smith, 2000) and affected oestrus behaviour and oocyte production.

Lameness prevalence in the current study was significantly associated with BCS and parity that agrees with previous authors (Green *et al*., 2002; Solano *et al*., 2015), however contradicts with Sadiq *et al*. (2017) and Mulatu (2018). Differences may be due to the sample size system, breeds and corresponding milk yielding capacity.

In the present study cows with clinical lameness were with 76.32% of excess hoof growths hat was in agreement with the reports of Sulayeman and Fromsa (2012). Similarly, Sadiq *et al.* (2017) reported that lameness in cows associated with increased hoof growth in Selangor, Malaysia. According to Shearer and van Amstel (2011) guidelines, increased hoof growth leads to increase the affected nails.

5.3. Combined effect of subclinical mastitis and lameness on reproductive performance and its association with subclinical endometritis

Cows with lameness were tested for SCM using CMT and the prevalence of SCM at the cow level was found to be 53.7% (58 of 108). Our result was lower than finding of Dogra *et al.,* 2020. Previous studies have shown that lame animals have a higher incidence of SCM in lame cows, which was due to a longer period of lying down, which exposed the udders to various intramammary infections (IMI). In other study also reported that elevated SCC levels in milk predispose to mastitis in lame cows (Peeler *et al.,* 1994; Coulon *et al.,* 1998 and Arvidson, 2000).

The results of this study revealed the mean days from CFSI were much significantly extended in lame animals with subclinical mastitis compared with lame cows (P = 0.000). According to the current investigation, the mean NSPC was longer (P = 0.019) in lame animals with subclinical mastitis than in lame cows. CRFS and PRFS were reduced in lame cows infected with subclinical mastitis comparing with lame cows. Walid *et al.* (2017) and Sogstad *et al.* (2006) reported that claw and hook lesions are associated with lower reproductive performance and mastitis.

This study confirmed a progressive increase in the presence of subclinical mastitis in lame animals with increased parity and thus had poor reproductive performance. This was due to longer duration of infection, especially in a herd where a mastitis control program is not implemented (Radostitis *et al.,* 2007). Although previous studies have shown a combined effect of exposure to SCM during pregnancy and parity ≥ 4 on pregnancy loss (PL) (Dahl *et al.,* 2019). The uterine environment of older mastitis cows is more sensitive to the systemic inflammatory response compared to younger cows (Tanikawa *et al.,* 2017).

In this study, poor BCS is a risk factor for lame animals with subclinical mastitis and had poor reproductive performance. Peake *et al.* (2011) recorded that the synergistic incidence of lameness, subclinical mastitis and loss of body condition results in a delay in the first postpartum luteal phase from calving.

In the current study the odds of subclinical endometritis (SCE) presence was 2.85 times higher (OR=2.85) in lame cows with subclinical mastitis (SCM) compared to lame cows. Reports from Dosognen *et al.* (2002) showed that plasma lipopolysaccharide (LPS) increased during mastitis. A direct link between combination of lameness with SCM and SCE was due to the transfer of bacteria and bacterial products, particularly LPS, from the udder to the uterus and vice versa (Eckel and Ametaj, 2016).

The current study revealed that the plasma progesterone concentrations in lame cows that exhibited subclinical mastitis had significantly lower concentration than in lame animals (P =0.000). Our finding was in agreement with Eckel and Ametaj (2016) that lameness and mastitis reduce GnRH and thus LH pulse frequency, resulting in reduction of follicular oestradiol production and a delay and reduction of the LH surge. Consistent with our finding, Peake *et al.* (2011) also noted that progesterone concentrations were lower in subclinical mastitis. The inflammatory factor TNF-α impedes the number of LH receptors in granulosa cells, thus decreasing LH reactivity. Decreased LH secretions also inhibit progesterone secretion from the corpus luteum and affect uterine function (Wang *et al.,* 2021).

This study also demonstrated that cortisol concentration in blood serum of lame cows stressed by subclinical mastitis had significantly higher concentration of cortisol than lame cows (P=0.004). Our finding agrees with Hockett *et al.* (2000) that cortisol concentrations were increased in cows with mastitis. Increased cortisol levels reduced gonadotropin release during mastitis associated with bacterial challenges (Soto *et al.*, 2003) have been implicated in disruption of reproductive processes. SCM caused changes in cortisol concentrations (David *et al.,* 2015 and Pinedo *et al,* 2009). In cows with mastitis the blood cortisol concentration increases during the luteal phase suppresseing LH levels (Wang *et al.,* 2021).

Our results confirmed that the most common intramammary pathogens in positive milk were *S. aureus*, *E. coli* and CNS. It has been studied that lameness combined with a longer period of lying down increases the risk of bacterial contamination of the mammary ducts, and cows lying down immediately after milking would have a higher risk of intramammary infection (Vladimír *et al.,* 2020).

S. aureus is the most common pathogen isolated during the current study period, accounting for 39.42% of all. Our result was comparable to the finding of Fesseha *et al* (2021). The reason for the higher isolation of *Staphylococcus aureus* bacteria was the wide environmental distribution on the skin and in mammary glands, which was also transfer to other healthy cows during milking process (Dahl *et al.*, 2019).

In this study, 24% of *E. coli* bacteria were isolated from milk of mastitis cows, which agrees with report of Iqbal *et al.* (2004). But our result was higher than the report of Mekibib *et al.* (2010) at Holeta and Sori *et al.* (2005) in and around Sebeta. The prevalence of *E. coli* was due to the most common hygiene-related environmental contaminants. In addition, high levels of *E. coli* of milk indicate poor milk quality associated with poor farm hygiene.

In this study, coagulase negative staphylococcus (CNS) (13.46%) *was* also responsible for the presence of mastitis. Similar result reported by Fesseha *et al.* (2021). Our result

was much lower than the finding of Zeryehun and Abera (2017). This variation in the frequency of CNS was due to differences in laboratory analysis technique.

6. CONCLUSION AND RECOMMENDATIONS

This study demonstrates that SCM is a highly prevalent disease in the dairy farms of Jimma town and the subclinical occurrence of the disease remains a substantial problem for dairy farms. In this study the occurrence of SCM delayed CFSI, elevated NSPC, reduced CRFS and decreased PRFS. The present study revealed that subclinical endometritis (SCE) was associated with SCM. The SCM declined the concentration of progesterone and increased the cortisol concentration. The bacteria most commonly related with SCM were *S. aureus,* coagulase negative staphylococcus (CNS) and streptococci.

This study confirmed consequence of lameness on reproductive performance by reducing CRFS and PRFS in dairy cows. The outcome of present study revealed that lameness was highly associated with subclinical endometritis. The result of this research demonstrates lameness causes change of the concentration of progesterone and cortisol. In the current study clinically lame (CL) cows had a longer CFSI when compared with cows which were never lame and with mildly lame cows. Similarly**, t**he mean NSPC was longer in clinically lame cows than in nonlame cows. Parity and body condition score can be considered as important factors which influence the incidence of lameness in dairy cows. The lesions that were found causing lameness were excess hoof growth with vertical and horizontal fissure, sole ulceration and foot rot. Significant associations were found in this study between various degrees of lameness and fertility.

The present study has shown that the combined occurrence of lameness and subclinical mastitis highly delayed the CFSI and elevated the NSPC. In addition in lame cows with SCM the CRFS was highly reduced and a PRFS was also highly decreased. This study also indicated that parity and body condition are found to be important predictors that influence the occurrence of SCM in lame cows. The current study indicated that subclinical endometritis (SCE) was associated with lame animals with SCM. The lame animals with subclinical mastitis highly declined the concentration of progesterone and increased the cortisol concentration. The bacteria most commonly associated with

subclinical mastitis in lame animals were *Staphylococcus aureus*, *Escherichia coli* and Coagulase negative staphylococci (CNS).

In general, combined occurrence of SCM and lameness inflicts harmful effects on reproductive performance and hormonal profiles of dairy cows than those diagnosed with mastitis and lameness alone.

Based on the findings, the following recommendations could be drawn:

- The present study justifies the need to implement a feasible intervention strategy for mastitis, giving special attention to SCM. These include a durable extension service focused on awareness and hygienic milking practices
- Animal health care should focus on regular screening of dairy cows for subclinical mastitis and treatment of lactating and dry cows, and advice on culling chronically infected animals.
- One of the main areas where information is lacking is the extent to which farmers perceive lameness. Therefore, there is a need to raise awareness about the impact of lameness in the dairy industry. Training sessions on locomotion scoring should be organized for farmers and animal health workers.
- Hoof trimmings should be used.
- Emphasis should be given to flooring quality of the barn and to the hygiene of dairy cattle. Therefore, cracked and slippery floor types should be avoided.
- This study provided further evidence of combined effect of lameness with subclinical mastitis can negatively affect fertility of dairy cows and therefore, emphasis should be given to both lameness and subclinical mastitis control programs in dairy farms.
- The results of this study suggest that reduction of SCM and lameness should be part of an overall management plan used to improve reproductive performance.
- The health of the feet and udder should be regularly monitored to ensure early detection and rapid measures to control SCM in dairy cows should be planned and

implemented.

- The owners should practice monitoring for chronic disease and culling of older cows with repeated attacks.
- A national SCM and lameness control strategy should be implemented.
- A research should be carried out to assess the relationship between the bacteriological examinations of milk with suppression of fertility in cows.
- The statistics of the prevalence of SCM and lameness in this study have mainly been focused at cow level. Therefore, most of our results are considered broadly generalizable to other farms with similar management systems and environments; further research is needed to validate this..
- Further studies should be done with large samples size to understand the accurate effects of SCM and lameness on fertility crossbred dairy cattle.

7. REFERENCES

Abebe R., Hatiya H., Abera M., Megersa B., Asmare K.. (2016): Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia, *BMC Veterinary Research* (2016) **12**:270,

Abera, M., Demie, B., Aragaw, K., Regassa, F., Regassa, A. (2010): Isolation and identification of Staphylococcus aureus from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *J. Vet. Med. Anim. Heal.,* **2**, 29–34.

Abunna Fufa, Blen Merid, Gebeyehu Goshu, Hika Waktole and Gezahegn Mammo, (2018): Assessment of major reproductive health problems, their effect on reproductive performance and association with brucellosis in dairy cows in Bishoftu town, Ethiopia, *J Dairy Vet Anim Res* 2018, **7**(1): 00183.

Abunna, F., Zeben, I., Waketole, H. and Mamo, G. (2017): Epidemiology of lameness in extensive and intensive dairy farms in Bishoftu town, central Oromia, Ethiopia. *Biomirror*, **8**: 9-17.

Ahmadzadeh, A., Frago, F., Shafii, B., Dalton, J.C., Price, W. J. and McGuire, M. A. (2009): Effect of clinical mastitis and other diseases on reproductive performance of Holstein cows. *Animal Reproduction Science*, **112**: 273–282.

Alawneh J. I., Laven R. A., Stevenson M. A. (2011): The effect of lameness on the fertility of dairy cattle in a seasonally breeding pasture-based system. *J. Dairy Sci.*, **94**, 5487-5493.

Albaaj A., Foucras G., Raboisson D. (2017): High somatic cell counts and changes in milk fat and protein contents around insemination are negatively associated with conception in dairy cows. *Theriogenology;* **88***:*18–27.

Almeida P.E., Weber P.S., Burton J.L., Zanella A.J. (2008): Depressed DHEA and increased sickness response behaviours in lame dairy cows with inflammatory foot lesions. *Domest Anim Endocrinol.;* **34**:89–99.

Amene, F. (2006): Studies on reproductive performance and major reproductive health problems of HF cows at Alage dairy farm. MSc thesis, Addis Ababa University, Faculty of Veterinary Medicine. DebreZeit, Ethiopia.

Amundson, J.L., Mader, T.L., Rasby, R.J., Hu, Q.S. (2006): Environmental effects on pregnancy rate in beef cattle. *J. Anim. Sci.*, **84**, 3415–3420.

Archer, S.C.; Green, M.J.; Huxley, J.N. (2010): Association between milk yield and serial locomotion score assessments in UK dairy cows. *J. Dairy Sci.*, **93**, 4045–4053.

Arvidson, A. (2000): Environmental and management factors associated with the incidence of clinical mastitis in high yielding dairy herds with a low incidence of subclinical mastitis. Graduate Report 136. Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, p. 72. Uppsala, Sweden.

Asaf S., Leitner G., Furman O., Lavon Y., Kalo D., Wolfenson D., Roth Z. (2014): Effects of *Escherichia coli*- and *Staphylococcus aureus*-induced mastitis in lactating cows on oocyte developmental competence. *Reproduction*, **147**: 33–43.

Bacha Belachew and Fekadu Gudeta Regassa (2010): Subclinical endometritis in Zebu x Friesian crossbred dairy cows: its risk factors, association with subclinical mastitis and effect on reproductive performance, *Trop. Anim. Health Prod.,* **42** (3): 397–403.

Banerjee J., Sharma R., Agarwal A., Maitra D., Diamond M.P., Abu-Soud H.M. (2012): IL-6 and mouse oocyte spindle. *PLoS One*, **7**(4):e35535.

Baravalle M.E., Stassi A.F., Velazquez M.M.L., Belotti E.M., Rodriguez F.M., Ortega H.H., Salvetti N.R.. (2015): Altered expression of proinflammatoru cytokines in ovarian follicles of cows with cystic ovarian disease. *J Comp Patho*l, **153**:116-130.

Beam S.W., Butler W.R. (1999): Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *J Reprod Fertil Suppl.*; 54:411–424.

Bedacha B.D., Menghistu H.T. (2011): Study on prevalence of mastitis and its associated risk factors in lactating dairy cows in batu and its environs, Ethiopia. *Glob Vet*.; 7(6):632–637.

Belina D., Yimer M., Adem H., Tamerat N., Tadesse K., Tarekegn W., Jelalu K. (2016): Prevalence, isolation of bacteria and risk factors of mastitis of dairy cattle in

selected zones of Oromia regional states, Ethiopia. *Glob J Med Res*.; **16** (1):39–42.

Benti, A.D. and Zewdie, W. (2014): Major reproductive health problems of indigenous Borena cows in Ethiopia. *Journal of Advanced Veterinary and Animal Research*, **1**(4): 182-188.

Bicalho R.C., Vokey F., Erb H.N., Guard C.L. (2007): Visual locomotion scoring in the first seventy days in milk: impact on pregnancy and survival. *J. Dairy Sci.,* **90:** 4586-4591.

Birhanu Abera, Diriba Lemma and Iyob Iticha (2013): Study of bovine mastitis in Asella government dairy farm of Oromia Regional state, South Eastern Ethiopia, *Int.J.Curr.Res.Aca.Rev.,* **1**(2):134-145.

Birhanu, M., Leta, S., Mamo, G., Tesfaye, S. (2017): Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. *BMC Res Notes*, **10**, 767.

Blen Merid Woldeamanuel (2016): Assessment of major reproductive health problems, their effect on reproductive performance and association with brucellosis in dairy cows in Bishoftu town, Ethiopia, MSc thesis, Addis Ababa University, college of veterinary medicine and agriculture, department of animal producton studies, Bishoftu, Ethiopia.

Boujenane, I., El Aimani, J. and By, K. (2015): Effects of clinical mastitis on reproductive and milk performance of Holstein cows in Morocco. *Trop. Anim. Health Prod.,* **47**(1):207–11.

Bromfield J.J.**,** Sheldon I..M. (2011): Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. *Endocrinology*, **152**(12):5029-40.

Carlos A. Risco, Mohammad O. Dahl (2018): Effect of inflammatory response related to mastitis on dairy cattle reproduction. *Clinical Theriogenology*, **10**(3): 253-257.

Cervinkova D., Vlkova H., Borodacova I., Makovcova J., Babak V., Lorencova A., Vrtkova I., Marosevic D., Jaglic Z. (2013): Prevalence of mastitis pathogens in milk from clinically healthy cows, *Vet Med.*; **58**(11):567–75.

Chapinal N., Von Keyserlingk M.A.G., Cerri R.L.A., Ito K., LeBlanc S.J. and Weary D.M. (2013): Short communication: Herd level reproductive performance and its relationship with lameness and leg injuries in free stall dairy herds in north eastern United States, *J. Dairy Sci.*, **96**, 7066–7072.

Chebel, R. C., Santos, J. E. P., Reynolds, J. P., Cerri, R. L. A., Juchem, S. O. and Overton, M. (2004): Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. *Animal Reproduction Science*, **84**: 239–255.

Chen, H.W., Jiang, W.S. and Tzeng, C.R. (2001): Nitric oxide as a regulator in preimplantation embryo development and apoptosis. *Fertil Steril*., **75**: 1163–1171.

Choi, J.H., Seo, H.S., Lim, S.Y., Park, K. (2014): Cutaneous immune defenses against *Staphylococcus aureus* infections. *J. Lifestyle Med*.**, 4**, 39–46.

Coetzee J.F., Lubbers B.V., Toerber S.E., Gehring R., Thomson D.U., White B.J., Apley M.D. (2008): Plasma concentrations of substance P and cortisol in beef calves after castration or simulated castration. *American Journal of Veterinary Research*; **69**: 751-62.

Cook, N. B. K. Nordlund (2003): Interrelationships between housing and herd health. *Advances in Dairy Technol.*, **15**, 85-95.

Coulon, J.B., Pradel, P., Cochard, T. and Poutrel, B. (1998): Effect of extreme walking conditions for dairy cows on milk yield, chemical composition, and somatic cell count. *J. Dairy Sci.* **81(4)**: 994-1003.

CSA (Central Statistical Agency) (2017): Report on livestock and livestock characteristics. The Federal Democratic Republic of Ethiopia, Private peasant holdings, Statistical bulletin 570. Addis Ababa, Ethiopia.

Dahl Mohammad O., Albert De Vries, Klibs N. Galvão, Fiona P. Maunsell, Carlos A. Risco, Jorge A. Hernandez (2020): Combined effect of mastitis and parity on pregnancy loss in lactating Holstein cows, *Theriogenology*, **143**:57-63.

Dalanezi F. M., S. F. Joaquim, F. F. Guimarães, S. T. Guerra, B. C. Lopes, E. M. S. Schmidt (2020): Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows, *J. Dairy Sci.*, **103**:3648–3655.

David Wolfenson, Gabried Leitner & Yanir Lavon (2015): The Disruptive Effects of Mastitis on Reproducton and Fertility in Dairy cows, *Italian Journal of Animal Science,* **14**:4, 4125.

Deb, G. K., Dey, S. R., Bang, J. I., Cho, S. J., Park, H. C., Lee, J. G. and Kong, I. K. (2011): 9-cis retinoic acid improves developmental competence and embryo quality during maturation of bovine oocytes through the inhibition of oocyte TNF gene expression. *Journal of Animal Science*, **89**:2759–2767.

De Vries, A., and C.A. Risco (2005): Trends and seasonality of reproductive performance in Florida and Georgia dairy herds from 1976 to 2002. *J. Dairy Sci.*, **88**:3155–3165.

Dereje, K., Kebede, A., Abebe, N., Tamiru, Y. (2018): Isolation, Identification and Antimicrobial Susceptibility Test of Mastitis Causing Bacteria at Holeta Agricultural Research Center Dairy Farms. *Int. J. Anim. Sci. Technol.***, 2**, 6–13.

Diskin M.G., Parr M.H., Morris D.G. (2011): Embryo death in cattle: an update. *Reprod Fertil Dev*, **24**:244-251.

Dobson H., Smith R. F. (2000): What is stress, and how does it affect reproduction? *Anim. Reprod. Sci.*, **60-61**, 743-752.

Dobson, H., Walker, S.L., Morris, M.J., Routly, J.E., Smith, R.F. (2008): Why is it getting more difficult to successfully artificially inseminate dairy cows? *Animal*, **2**, 1104–1111.

Dogra S., R. Singh1, A. K. Panday1 and R. Agrawal (2020): Impact of lameness on udder health and reproduction in crossbred cattle, *Haryana Vet.*, **59**(2), 248-250.

Dolecheck K.A., García-Guerra A., Moraes L.E. (2019): Quantifying the effects of mastitis on the reproductive performance of dairy cows: A meta-analysis, *J. Dairy Sci*., **102 (**9) pp. 8454–8477.

Dosogne, H.; Meyer, E.; Struk, A.; van Loon, J.; Massart-Leen, A.M.; Burvenich, C. (2002): Effect of enrofloxacin treatment of plasma endotoxin during bovine *Escherichia coli* mastitis. *Inflamm. Res*., **51**, 201-205.

Dubuc J., Duffield T.F., Leslie K..E., Walton J.S., LeBlanc S.J. (2010): Definitions and diagnosis of postpartum endometritis in dairy cows, *J. Dairy Sci*., **93:**5225-5234.

Eckel, E.F., Ametaj, B.N. (2016): Invited Review: Roles of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. *J. Dairy Sci*., *99*, 1-24.

Edelhoff I. N. F., Pereira M. H. C., Bromfield J. J., Vasconcelos J. L. M. and Santos J. E. P., (2020): Inflammatory diseases in dairy cows: Risk factors and associations with pregnancy after embryo transfer, *J. Dairy Sci.* **103**:11970–11987.

Edmonson, A.J., I.J. Lean, L.D. Weaver, T. Farver, and G. Webster (1989): A body condition scoring chart for Holstein dairy cows. *J. Dairy Sci.* 72:68–78.

Edmundson I.J., Lean I.J., Weaver L.D., Farver T.., Webster G. (1989): A Body Condition Scoring Chart for Holstein Dairy Cows. *J Dairy Sci.*; 72(1):68–78.

Elemo, K.K., Bedada, B.A., Kebede, T. (2018): Prevalence, Risk Factors and Major Bacterial Causes of Bovine Mastitis in Smallholder Dairy Farms in and around Sinana District, Bale Zone, South Eastern Ethiopia. *Glob. J. Sci. Front. Res.*, **18**, 1–10.

Endo H., Ogata Y., Takahashi K., Fujikura T., Konno M. (2003): Management for claw diseases in free-stall typedairy farms using evaluation data on pain stress and metabolic profile test. *J Livestock Med*; **50**: 241–248.

Enting, H., D. Kooij, A.A. Dijkhuizen, R.B.M. Huirne, E.N. Noordhuizen-Stassen (1997): Economic losses due to clinical lameness in dairy cattle." *Livestock Production Science***, 49**(3): 259-267.

Food and Agriculture organization (FAO) (2018): Africa sustainable livestock 2050: Livestock production systems spotlight Cattle sectors in Ethiopia. Pp 1-11. FAO, 2018I8271EN/1/01.18.

Ferguson, J.D., and A. Skidmore (2013): Reproductive performance in a select sample of dairy herds. *J. Dairy Sci.* **96**:1269–1289.

Fesseha Haben, Mesfin Mathewos, Saliman Aliye, Amanuel Wolde (2021): Study on Prevalence of Bovine Mastitis and Associated Risk Factors in Dairy Farms of Modjo Town and Suburbs, Central Oromia, Ethiopia, *Veterinary Medicine: Research and Reports*, 12: 271–283.

Filho, V. B. S., Schiavon, R. S., Gastal, G. D. A., Timm, C. D. and Lucia Jr, T. (2012): Association of the occurrence of some diseases with reproductive performance

and milk production of dairy herds in southern Brazil. *Revista Brasileira de Zootecnia*, 41(2): 467–471.

Fourichon C., Seegers H., Malher X. (2000): Effect of disease on reproduction in the dairy cow: a meta-analysis. *Theriogenology,* **53**:1729-59.

Fuenzalida, M. J. Fricke, P. M. and Ruegg, P. L. (2015): The association between occurrence and severity of subclinical and clinical mastitis on pregnancies per artificial insemination at first service of Holstein cows. *J Dairy Sci*., **98**, 3791–3805.

Furman, O., Leitner, G., Roth, Z., Lavon, Y., Jacoby, S., Wolfenson, D. (2014): Experimental model of toxin-induced subclinical mastitis and its effect on disruption of follicular function in cows. *Theriogenology*, **82**(8), 1165–72.

Garbarino E.J., Hernandez J.A., Shearer J.K., Risco C.A., Thatcher W.W. (2004): Effect of lameness on ovarian activity in postpartum Holstein cows. *J Dairy Sci*., **87**: 4123-31.

Garedew, L., Melese, B., Tesfaye, R., 2015. *Staphylococcus aureus* in mastitic crossbreed cows and its associated risk factors in Addis Ababa City, Ethiopia. *Ethiop. Vet. J.***, 19**, 107–116.

Gendelman, M., Aroyo, A., Yavin, S. and Roth, Z. (2010): Seasonal effects on gene expression, cleavage timing, and developmental competence of bovine preimplantation embryos. *Reproduction*, **140**:73–82.

Gilbert R.O. (2019): Mechanisms of disruption of fertility by infectious diseases of the reproductive tract. *J Dairy Sci*, **102**:3754-3765.

Green L.E., Hedges V.J., Schukken Y.H., Blowey R.W., Packington A.J. (2002): The impact of clinical lameness on the milk yield of dairy cows. *J. Dairy Sci.* **85**, 2250–2256.

Gumen A., Wiltbank M.C. (2005): Length of progesterone exposure needed to resolve large follicle anovular condition in dairy cows. *Theriogenology*, **63**:202–210.

Gunay, A. and Gunay, U. (2008): Effects of Clinical Mastitis on Reproductive Performance in Holstein Cows. *Acta Veterinaria Brno*., 77: 555–560.

Hagnestam-Nielsen C., Emanuelson U., Berglund B., Strandberg E. (2009): Relationship between somatic cell count and milk yield in different stages of lactation. *J Dairy Sci*., 92(7): 3124-3133.

Hansen, P.J., (2007): Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. *Theriogenology*, **68**: 242–249.

Hansen, P. J., Soto, P. and Natzke, R. P. (2004): Mastitis and fertility in cattle – possible involvement of inflammation or immune activation in embryonic mortality. *American Journal of Reproductive Immunology*, **51**, 294–301.

Harmon R.J. (1994): Symposium - Mastitis and Genetic Evaluation for Somatic Cell Count - Physiology of Mastitis and Factors Affecting Somatic Cell Counts. *J Dairy Sci*.; **77**:2103–12.

Heringstad, B., Chang, Y. M., Andersen-Ranberg, I. M. and Gianola, D. (2006): Genetic analysis of number of mastitis cases and number of services to conception using a censored threshold model. *J Dairy Sci*., **89**, 4042–4048.

Hernandez J.A., Garbarino E.J., Shearer J.K., Risco C.A., Thatcher W.W. (2005): Comparison of the calving-to-conception interval in dairy cows with different degrees of lameness during the pre-breeding postpartum period. *Journal of the American Veterinary Medical Association;* **227**: 1284-91.

Hernandez, J. A., C. A. Risco, F. S. Lima, and J. E. Santos, (2012): Observed and expected combined effects of clinical mastitis and low body condition on pregnancy loss in dairy cows. *Theriogenology* , **77**:115-121.

Hertl, J.A., Gröhn, J.A., Leach, J.A., Bar, D., Bennett, G.J., González, R.N., Rauch, B.J., Welcome, F.L., Tauer, L.W. and Schukken, Y.H. (2010): Effects of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. *J Dairy Sci*., **93**: 1551–1560.

Herzog K. Strüve K. Kasteli J.P. Piechotta M. Ulbrich S.E. Pfarrer C. Shirasuna K. Shimizu T. Miyamoto A. Bollwein H. (2012): *Escherichia coli* lipopolysaccharide

administration transiently suppresses luteal structure and function in diestrous cows. *Reproduction*, **144**:467–476.

Hill J, Gilbert R. (2008): Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. *Aust Vet J*, 86:312-316.

Hockett, M.E., Almeida, R.A., Rohrbach, N.R., Oliver, S.P., Dowlen, H.H. and Schrick, F.N. (2005): Effects of induced clinical mastitis during preovulation on endocrine and follicular function. *J Dairy Sci*., **88**: 2422–2431.

Hockett M.E., Hopkins F.M., Lewis M.J., Saxton A.M., Dowlen H.H., Oliver S.P., Schrick F.N. (2000): Endocrine profiles of dairy cows following experimentally induced clinical mastitis during early lactation. *Anim Reprod Sci*, **58**: 241-251.

Hoedemaker M., Prange D., Gundelach Y. (2009): Body condition change ante- and postpartum, health and reproductive performance in German Holstein cows. *Reprod. Dom. Anim.*, **44**, 167-173.

Hudson C. D., Bradley, A. J., Breen, J. E. and Green, M. J. (2012): Associations between udder health and reproductive performance in United Kingdom dairy cows. *J Dairy Sci*., **95**, 3683–97.

Hudson C.D., Huxley J.N., Green M.J. (2014): Using simulation to interpret a discrete time survival model in a complex biological system: fertility and lameness in dairy cows. PLoS One 2014 ;9:e103426. doi:10.1371/journal.pone.0103426.

Hultgren J., Manske T., Bergsten C. (2004): Associations of sole ulcer at claw trimming with reproductive performance, udder health, milk yield, and culling in Swedish dairy cattle. *Prev. Vet. Med.*, **62**, 233-251.

Huxley, J.N. (2013): Impact of lameness and claw lesions in cows on health and production. *Livest. Sci.* **156**, 64–70.

Iqbal M., Khan M.A., Daraz B., Saddique U. (2004): Bacteriology of mastitic milk and in vitro antibiogram of the isolates. *Pakistan Veterinary Journal*, **24**:161-164.

Isobe N., Iwamoto Ch., Kubota H., Yoshimura Y. (2014): Relationship between the somatic cell count in milk and reproductive function in prepartum dairy cows. *J. Reprod. Dev.*, ***60***: 433–437.

Jackson, J.A., Shuster, D.E., Silvia, W.L. and Harmon, R.J. (1990): Physiological responses to intramammary or intravenous treatment with endotoxin in lactating dairy cows. *J Dairy Sci.,* **73**: 627–632.

James Alex Willshire (2012): The use of mobility score to predict dairy cow reproductive performance, a dissertation of 8,667 words submitted as part of the requirements in the examination for the RCVS Diploma in Cattle Health and Production.

Kara, N. K., Galic, A. & Koyuncu, M. (2011): Effects of stall type and bedding materials on lameness and hygiene score and effect of lameness on some reproductive problems in dairy cattle. *Journal of Applied Animal Research,* **39,** 334-338.

Khokon Shohidul Islam Md., Azizunnesa, Md. Mazedul Islam, Kulsum Begum Chowdhury, Md. Lutfor Rahman and MdZulfekar Ali (2017): Effect of mastitis on post-partum conception of cross bred dairy cows in Chittagong district of Bangladesh, Journal of Advanced Veterinary and Animal Research, ISSN 2311-7710 (Electronic), http://doi.org/10.5455/javar.2017.d203.

Kiliç, N., Ceylan, A., Serin, I. and Gökbulut, C. (2007): Possible interaction between lameness, fertility, some minerals, and vitamin E in dairy cows. *Bull. Vet. Ins. Pulawy.* **51**: 425-429.

Kirk J.H. (2004): Effect of clinical mastitis on production and reproduction (E suppl)*:* E1-E2 (http://www.vetmed. ucdavis.edu/vetext/INF-DA/Mastitis-on-Produc.pdf), accessed December 7, 2006.

Kitila G., Kebede B., Wakgari M. (2021): Prevalence, aetiology and risk factors of mastitis of dairy cows kept under extensive management system in west Wollega, western Oromia, Ethiopia. *Vet Med Sci*.; 7(3):1–7.18.

Konig S., Hubner G., Sharifi R., Bohlsen E., Detterer J., Simianer H. & Holtz W. (2006): Relation between the somatic cell count and the success of first insemination in East Frisian dairy herds on the basis of logistic models analysis. *Zuchtungskunde,* **78,** 90-101.

Kriningер, C.E., Stephens, S.H. and Hansen, P.J. (2002): Developmental changes in inhibitory effects of arsenic and heat shock on growth of preimplantation bovine embryos. *Mol Reprod Dev.,* 63: 335–340.

Kumar N., Manimaran A., Kumaresan A., Jeyakumar S., Sreela L., Mooventhan P., Sivaram M**.** (2017): Mastitis effects on reproductive performance in dairy cattle: a review. *Trop Anim Health Prod*; **49:**663-673.

Lavon, Y., Ezra, E., Leitner, G. and Wolfenson, D. (2011): Association of conception rate with pattern and level of somatic cell count elevation relative to time of insemination in dairy cows. *J Dairy Sci.,* **94**(9), 4538–4545.

Lavon, Y., Leitner, G., Goshen, T., Braw-Tal, R., Jacoby, S. and Wolfenson, D. (2008): Exposure to endotoxin during estrus alters the timing of ovulation and hormonal concentrations in cows. *Theriogenology*, **70**, 956–967.

Lavon, Y., Leitner, G., Voet, H. and Wolfenson, D. (2010): Naturally occurring mastitis effects on timing of ovulation, steroid and gonadotrophic hormone concentrations, and follicular and luteal growth in cows. *J Dairy Sci.*, **93**, 911–921.

Lavon, Y., Leitner, G., Klipper, E., Moallem, U., Meidan, R. and Wolfenson, D. (2011a): Subclinical, chronic intramammary infection lowers steroid concentrations and gene expression in bovine preovulatory follicles. *Domestic Animal Endocrinology*, **40**(2), 98–109.

Lavon, Y., Leitner, G., Goshen, T., Braw-Tal, R. and Jacoby, S. (2011b): Immediate and carryover effects of Gram-negative or Gram positive toxin-induced mastitis on follicular functions in cows. *Theriogenology*, **76**(5), 942–953.

Lavon, Y., Ezra, E., Leitner, G. and Wolfenson, D. (2011c): Association of conception rate with pattern and level of somatic cell count elevation relative to time of insemination in dairy cows. *J Dairy Sci*., **94**(9), 4538–4545.

Linderoth S. (2003): Don't let subclinical mastitis zap reproductive efficiency. Dairy herd management (http://www.dairyherd.com/printFriendly.asp?ed_id=2332), accessed December 15, 2006.

Lobago, F., Lemma, A., Tegegne, A. and Tibbo, M. (2001): Lameness as a disease of intensification in dairy cattle under urban and peri-urban production system in the

Addis Ababa milk shed. AGRIS: International Information System for the Agricultural Science and Technology.

Logue, D., C. Bergsten (2007): Lameness in cows - a welfare and profit reducing problem. Animal Health: Management and Control of Infectious and Production Diseases. *B. Int. Dairy Fed.*, **416**, 54-66.

Lomander, H., Svensson, C., Hallén-Sandgren, C., Gustafsson, H. and Frössling, J. (2013): Associations between decreased fertility and management factors, claw health, and somatic cell count in Swedish dairy cows. *J Dairy Sci*., 96, 6315–6323.

Lucy, M.C. (2001): Reproductive loss in high-producing dairy cattle: where will it end? *J Dairy Sci*., **84**(6): 1277–93.

Lucy M.C., Evans T.J., Pock S.E. (2016): Lymphocytic foci in the endometrium of pregnant dairy cows: characterization and association with reduced placental weight and embryonic loss. *Theriogenology*, **86**:1711-1719.

Machado, V. S., Caixeta, L. S., Mcart, J. A. A. & Bicalho, R. C. (2010): The effect of claw horn disruption lesions and body condition score at dry-off on survivability, reproductive performance, and milk production in the subsequent lactation. *Journal of Dairy Science,* **93,** 4071-4078.

Maizon, D.O., Oltenacu, P.A., Grohn, Y.T., Strawderman, R.L. and Emanuelson, U. (2004): Effects of diseases on reproductive performance in Swedish Red and White dairy cattle. *Prev Vet Med*., 66: 113–126.

Malinowski E, Gajewski Z. (2010): Mastitis and fertility disorders in cows. *Polish Journal of Veterinary Sciences*, **13**: 555–560.

Manimaran, A., Kumaresan, A., Sreela, L., Boopathi, V., Arul Prakash, M. (2014): Effects of Clinical Mastitis on Days Open in Dairy Cattle and Buffaloes. *Indian Veterinary Journal*, **91**(12), 67–68.

McCann, S.M., Kimura, M., Karanth, S., Yu, W. H. and Rettori, V. (1997): Nitric oxide controls the hypothalamic-pituitary response to cytokines. *Neuroimmunomodulation*, **4**, 98–106.

McCann, S.M., Kimura. M., Karanth. S., Yu, W.H., Mastronardi, C.A. and Rettori, V. (2000): The mechanism of action of cytokines to control the release of hypothalamic and pituitary hormones in infection. *Ann NY Acad Sci*., 917: 4–18.

Mekibib, B., M. Furgassa, F. Abuna, B. Megersa and Regassa, A. (2010): Bovine mastitis: prevalence, risk factors and major pathogens in dairy farms of Holeta town, Central Ethiopia. *Veter. World,* **9**(3): 397 - 403.

Miller, R. H., Clay, J. S. and Norman H. D. (2001): Relationship of somatic cell score with fertility measures. *J. Dairy Sci.*, 84, 2543–2548.

Mitev J., Gergovska Z., Miteva T., Penev T. (2011): Influence of lameness on daily milk yield, lactation curve and body conditions score during lactation in black and white cows. *Bulg J Agric Sci* **17**: 704-711.

Mitev, J. (1998): Influence of nutrition and body condition score during dry period on the reproductive performance and health status of dairy cows after calving. Ph.D. Thesis. Trakia University, Stara Zagora.

Morris M.J. Walker, S.L. Jones, D.N. Routly, J.E. Smith, R.F. Dobson, H. (2009): Influence of somatic cell count, body condition and lameness on follicular growth and ovulation in dairy cows. *Theriogenology*, **71**: 801–806.

Morris M. J., Kaneko K., Walker S. L., Jones D. N., Routly J. E., Smith R. F., Dobson H. (2011): Influence of lameness on follicular growth, ovulation, reproductive hormone concentrations and estrus behavior in dairy cows. *Theriogenology*, **76**, 658-668.

Morris M. J., Kaneko K., Uppal S. K., Walker S. L., Jones D. N., Routly J. E., Smith R. F., Dobson H. (2013): Effects of high somatic cell counts in milk on repro- ductive hormones and oestrus behaviour in dairy cows with special reference to those with concurrent lameness. *Anim. Reprod. Sci.*, **141**, 20-25.

Melendez P., Bartolome J., Archbald L. F., Donovan A. (2003): The association between lameness, ovarian cysts and fertility in lactating dairy cows. *Theriogenology*, **59**, 927-937.

Morton J. M. (2010): Interrelationships between herd-level reproductive performance measures based on intervals from initiation of the breeding program in year-round and seasonal calving dairy herds. *J. Dairy Sci.*, **93**:901–910.

Moussavi, A. H. and Mesgaran, M. D. and Gilbert, R. O. (2012): Effect of mastitis during the first lactation on production and reproduction performance of Holstein cows. *Trop. Anim. Health Prod.*, **44**(7), 1567–1573.

Mulatu Woldehana Bachore (2018): Epidemiological investigation of lameness in selected dairy farms of Sululta town, central Ethiopia**,** MvSc thesis, College of Veterinary Medicine and Agriculture of Addis Ababa University.

Mungube, E. O., B. A. Tenhagen, T. Kassa, F. Regassa, M. N. Kyule, M Greiner, and M. P. O. Baumann (2004): Risk factors for dairy cow mastitis in Central Highlands of Ethiopia. *Trop. Anim. Health Prod.,* **36**:463-472.

Nava-Trujillo, H., Soto-Belloso, E. and Hoet, A.E. (2010): Effects of clinical mastitis from calving to first service on reproductive performance in dual-purpose cows. *Anim Reprod Sci*., **121**: 12–16.

Neelesh Sharma Neelesh, Azhar Shuaib Batoo, Zul huma, Sarabpreet Kour, Jyoti Misri and Kafil Hussain (2017): Impact of Mastitis on Reproductive Performance in Dairy Animals: A Review, *Theriogenology Insight*: **7**(1): 41-49.

Nibret Moges, Tekle Hailemariam, Tewodros Fentahun, Mersha Chanie and Achenef Melaku (2012): Bovine Mastitis and Associated Risk Factors in Small Holder Lactating Dairy Farms in Hawassa, Southern Ethiopia, Global Veterinaria 9 (4): 441-446.

Niorn Ratanapob, Wandee Thiangtum, Theera Rukkwamsuk, Smit Srisomrun, Supachart Panneum and Pipat Arunvipas (2020): The relationship between lameness and reproductive performance in dairy cows raised in small holder farms, Thailand, Songklanakarin *J. Sci. Technol.* **42** (4), 766-770.

NMC (1999): Current Concept in bovine mastitis National mastitis Council (NMC).3rd 1840 Wilsonblud, Arlinton, VA 22201.

Olechnowicz J., Jaskowski J.M. Behaviour of lame cows: A review. *Vet. Med.* (2011): **56**:581–588.

Orgel, C., Ruddat, I., & Hoedemaker, M. (2016): Prevalence and severity of lameness in early lactation in dairy cows and the effect on reproductive performance. *Tierarzti Prax Ausg G Grosstiere Nutztiere*, **17**, 207-217.

Pampfer, S. (2001): Dysregulation of the cytokine network in the uterus of the diabetic rat. *Am J Reprod Immuno*l., 45: 375–381.

Peake, K.A., Biggs, A.M., Argo, C.M., Smith, R.F., Christley, R.M., Routly, J.E. and Dobson, H. (2011): Effects of lameness, subclinical mastitis and loss of body condition on the reproductive performance of dairy cows. *Vet Rec*., **168**: 301–307.

Peeler E.J., Otte M.J., Esslemont R.J. (1994): Interrelationship of periparturient diseases in dairy cows. *The Veterinary Record*, **5** 129–132.

Perrin, L., Bostelmann, R.W. and Sheldon, I.M. (2007): Reduced conception rates associated with bovine mastitis during a 'window of opportunity'. *Vet Rec*., **161**: 61–62.

Pinedo, P. J., Donovan, A., Rae, O. and DeLapaz, J. (2009): Association between paratuberculosis infection and general immune status in dairy cattle, International Association for Paratuberculosis, Minneapolis, MN. 127.

Quinn, P.J., M.E. Carter, B.K. Markey and Carter, G.R. (2002): Clinical Veterinary microbiology. Harcourt publishers, Virginia, Pp.331-344.

Rahman, M.M.,Mazzilli,M., Pennarossa, G., Brevini, T. A. L., Zecconi, A. and Gandolfi F. (2012): Chronic mastitis is associated with altered ovarian follicle development in dairy cattle. *J. Dairy Sci.*, 95, 1885–1893.

Radostitis, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D. (2007): Mastitis in veterinary medicine: a text book of disease of cattle, sheep, pigs, goats, and horses 10th edition, Ballier, Tindall, London, Pp 674–762.

Ranasinghe, R.M.S. B.K., R.M.C. Deshapriya, D.I. Abeygunawardana, R. Rahularaj, C.M.B. Dematawew (2021): Subclinical mastitis in dairy cows in major milk-producing areas of Sri Lanka: Prevalence, associated risk factors, and effects on reproduction, *J. Dairy Sci.*, 104 (12): 12900-12911.

Refaai, W., Van, Aert, M., El-Aal, A. A., Behery, A. E. and Opsomer, G. (2013): Infectious diseases causing lameness in cattle with a main emphasis on digital dermatitis (Mortellaro disease). *Livestock Science*, **156**: 53-63.

Regasa, S., Mengistu, S., Abraha, A. (2019); Milk safety assessment, isolation, and antimicrobial susceptibility profile of staphylococcus aureus in selected dairy

farms of Mukaturi and Sululta town, Oromia region, Ethiopia. *Vet. Med. Int.* 2–10.

Reza V. H., F. M. Mehran, M. S. Majid, and M. Hamid (2011): "Bacterial pathogens of intramammary infections in Azeri buffaloes of Iran and their antibiogram," *African Journal of Agricultural Research*, **11**: 2516–2521.

Ribeiro ES, Lima FS, Greco LF, Bisinotto RS, Monteiro AP, Favoreto M, Ayres H, Marsola RS, Martinez N, Thatcher WW, Santos JEP. (2013): Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. *J Dairy Sci,* **96**:5682- 5697.

Ribeiro ES, Gomes G Greco LF, Cerri RLA, Vieira-Neto A, Monteiro PLJ Jr, Lima FS, Bisinotto RS, Thatcher WW, Santos JEP. (2016): Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. *J Dairy Sci*, **99**:2201-2220.

Rodgers J.C., Bird S.L., Larson J.E., DiLorenzo N., Dahlen C.R., DiCostanzo A., Lamb G.C. (2012): An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. *J Anim Sci*, **90**:4055-4062.

Roth Z., Dvir A., Kalo D., Lavon Y., Krifucks O., Wolfenson D., Leitner G. (2013): Naturally occurring mastitis disrupts developmental competence of bovine oocytes. *J. Dairy Sci.*, **96**: 1–7.

Roth, Z., A. Meidan, R. Brawtal, D. Wolfenson (2000): Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentrations in cows. *J. Reprod. Fertil.,* **120**, 83-90.

Sadiq, M. B., Ramanoon, S. Z., Mansor, R., Syed-Hussain, S. S. and Mossadeq, W. S., (2017): Prevalence of, claw lesions, and associated risk factors in dairy farms in Selangor, Malaysia. *Trop. Anim. Health Prod.*, **49**: 1741-1748.

Santos, J.E.P., Cerri, R.L.A., Ballou, M.A., Higginbotham, G.E. and Kirk, J.H. (2004): Effect of timing of first clinical mastitis occurrence on lactation and reproductive performance of Holstein dairy cows. *Anim Reprod Sci*., **80**: 31–45.

Sarker, S. C., M.S. Parvin, A. K. M. A. Rahman, M. T. Islam (2013): Prevalence and risk factors of subclinical mastitis in lactating dairy cows in north and south regions of Bangladesh. *Trop. Anim. Health Prod.,* **45**:1171–1176.

Sato K, Bartlett PC, Alban L, Agger JF, Houe H. (2008): Managerial and environmental determinants of clinical mastitis in Danish dairy herds. *Acta Vet Scand*, **50**: 1-8.

Scharko, P. B., and T. J. Davidson (1998): Impact of preventive hoof care on the performance of first lactation heifers. Page in Proc. 4th International Dairy Housing Conference, St. Louis, MO. ASAE, St. Joseph, MI.: 254 – 258.

Schrick, F.N., Hockett, M.E., Saxton, A.M., Lewis, M.J., Dowlen, H.H. and Oliver, S.P. (2001): Influence of subclinical mastitis during early lactation on reproductive parameters. *J Dairy Sci*., **84**: 1407–1412.

Seals, R. C., Lemaster, J. W., Hopkins, F. M. and Schrick, F. N. (1998): Effects of elevated concentrations of prostaglandin F2 alpha on pregnancy rates in progestogen supplemented cattle. *Prostaglandins & Other Lipid Mediators*, **56**, 377–89.

Seesupa, S., Kanistanon, K., Pilachai, R., & Aiumlamai, S. (2016): Prevalence of subclinical laminitis and its effects on reproductive performance in lactating cows in Thailand. *Thai Journal of Veterinary Medicine*, ***46***, 109-117.

Serhat Zsoy, Kemal Altunatmaz, Huriye Horoz, G.ven KAÞIKCI, Serhat Alkan, Tanay Bülal (2005): The Relationship between Lameness, Fertility and Aflatoxin in a Dairy Cattle Herd, *Turk J Vet Anim Sci* 29 (2005) 981-986.

Shearer, J. K. and van Amstel, S. R. (2011): Lameness in dairy cattle. *Dairy Production Medicine*, Pp.233-253.

Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth H. (2009): Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biol Reprod*, **81**:1025-1032.

Sheldon I.M., Owens S.E., Turner M.L. (2017): Innate immunity and the sensing of infection, damage and danger in the female genital tract. *J Reprod Immunol*, **119**:67-73.

Skarzynski, D.J., Miyamoto, Y. and Okuda, K. (2000): Production of prostaglandin $F_2\alpha$ by cultured bovine endometrial cells in response to tumor necrosis factora: cell type specificity and intracellular mechanisms. *Biol Reprod.*, **62**: 1116–1120.

Slebodzinski A.B., Malinowski E., Lipczak W. (2002): Concentrations of triidothyronine (T3), tumour necrosis factor-a (TNF-a) and interleukin-6 (IL-6) in milk from healthy and naturally infected quarters of cows. *Res. Vet. Sci.* **72**(1):17-21.

Smith K.L., Todhunter D.A., Schoenberger P.S. (1985): Environmental mastitis: cause, prevalence, prevention. *J Dairy Sci.*; 68:1531–53.

Sogstad, A.M., Østerås, O. and Fjeldaas, T. (2006): Bovine claw and limb disorders related to reproductive performance and production diseases. *J. Dairy Sci.*, **89**: 2519-2528.

Solano, L., Barkema, H. W., Pajor, E. A., Mason, S., LeBlanc, S. J., Heyerhoff, J. Z., Nash, C. G. R., Haley, D. B., Vasseur, E. and Pellerin, D. (2015): Prevalence of lameness and associated risk factors in Canadian Holstein-Friesian cows housed in free stall barns. *J Dairy Sci*, **98**: 6978-6991.

Sood, P., & Nanda, A.S. (2006): Effect of lameness on oestrous behaviour in crossbred cows. *Theriogenology*, **66**, 1357-1380.

Sood, P., Nanda, A.S., Singh, N., (2009): Effect of lameness on follicular dynamics in postpartum anestrous crossbred cows. *Veterinarski Arhiv* **79**, 531-542.

Sordillo LM. (2016): Nutritional strategies to optimize dairy cattle immunity, *J Dairy Sci*, **99**:4967-4982.

Sori H, Zerihun A, Abdicho S. (2005): Dairy cattle mastitis in and around Sebeta, Ethiopia. *Intern J Appl Res Vet Med*.; **3**(4):332–338.

Soto, P., Natzke, R.P. and Hansen, P.J. (2003): Identification of possible mediators of embryonic mortality caused by mastitis: actions of lipopolysaccharide, prostaglandin F2a, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. *Am J Reprod Immunol.,* **50**: 263–272.

Soto P. Natzke R.P. Hansen P.J. (2003a): Actions of tumor necrosis factor-a on oocyte maturation and embryonic development in cattle. *Am. J. Reprod. Immunol.* **50**:380–388.

Spencer, E. (2001): Heat stress and lameness. Newsletter of the Hoof Trimmers Association Inc., 8–9.

Sprecher, D.J., Hostetler, D.E. & Kaneene, J.B. (1997): A lameness scoring system that uses posture and gait to predict dairy cattle reproductive performance. *Theriogenology*, **47**(6), 1179-1187.

Sulayeman, M and Fromsa A (2012): Lameness in dairy cattle: Prevalence, risk factors and impact on milk production. *Global Vet*., **8**: 1-7.

Suzuki, C., Yoshioka, K., Iwamura, S. and Hirose, H. (2001): Endotoxin induces delayed ovulation following endocrine aberration during the proestrous phase in Holstein heifers. *Domest Anim Endocrinol.,* **20**: 267–278.

Tanikawa, N., A. Ohtsu, R. Kawahara-Miki, K. Kimura, S. Matsuyama, H. Iwata, T. Kuwayama, and K. Shirasuna (2017): Age-associated mRNA expression changes in bovine endometrial cells in vitro. *Reprod. Biol. Endocrinol.***, 15**:63.

Tilahun, K.A., Mamo, Y.A., Mulatu, R.A. (2016): Isolation and identification of methicillin resistant S. aureus (MRSA) from mastitic cow's milk. *Int. J. Vet. Wildl. Sci*, **1**, 15–22.

Tolosa T., J. Verbeke, S. Piepers, K. Supré, S. De Vliegher (2013): Risk factors associated with subclinical mastitis as detected by California mastitis test in smallholder dairy farms in Jimma, Ethiopia using multilevel modelling, *Prev. Vet. Med.*, **112** (2013) 68-75.

Tuke Muluken, Dawit Kassaye, Yimer Muktar, Tsegaye Negese and Kifle Nigusu, (2017): Bovine Mastitis: prevalence and associated risk factors in alage ATVET College Dairy Farm, Southern Ethiopia. *J Vet Sci Tech*., **8**(04):462.

Vacek, M., Stádník, L. and Štípk, M. (2007): Relationships between the incidence of health disorders and the reproduction traits of Holstein cows in the Czech Republic. *Czech J Anim Sci.,* **52**(8): 227–235.

Vasilev, N., D. Dinev, Y. Mitev, M. Koleva, C. H. Miteva (2007): Hygiene status of dairy cows reared in a spacious building and resulting quality of produced milk. *Trakia J. Sci.* **5**, 47-51.

Vermunt, J. (2004): Herd lameness – a review. Major causal factors and guidelines for prevention and control of lameness. 13th Intl. Symp. and 5th Conf. on Lameness in Ruminants, 11–15 Feb. 2004, Maribor, Slovenia, 3–18.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R. (2008): Mastitis detection: Current trends and future perspectives. *Trends. Biotechnol.* **27**(8): 486–93.

Vladimír Hisira, Pavel Slovák, Paulína Marčeková, Marián Kadaši, Pavol Mudroň (2020): Assessment of udder health in lame cows, *ACTA VET. BRNO* 2020, **89**: 133–140.

Walid Refaai, Medhat Gad and Yasser Mahmmod (2017): Association of claw disorders with subclinical intramammary infections in Egyptian dairy cows, *Veterinary World, EISSN:* 2231-0916.

Walker S.L., Smith R.F., Routly J. E., Jones D. N., M. J. Morris, and H. Dobson (2008): Lameness, activity time-budgets, and oestrus expression in dairy cattle. *J Dairy Sci;* **91***:4552*–9.

Walker S., Smith R., Jones D., J. E. Routly, M.J. Morris,, Dobson H. (2010): The effect of a chronic stressor, lameness, on detailed sexual behaviour and hormonal profiles in milk and plasma of dairy cattle. *Reproduction in Domestic Animals*; **45**: 109-17.

Waller, K.P., Colditz, I.G. and Stensson, K. (2003): Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. *Res Vet Sci.,* **74**: 31–36.

Walsh S.W., Williams E.J., Evans A.C.O. (2011): A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci,* **123***:*127–38.

Wang Nan Changhai Zhou, Wangdui Basang, Yanbin Zhu, Xin Wang, Chunjin Li, Lu Chen Xu Zhou (2021): Mechanisms by which mastitis affects reproduction in dairy cow: A review, Reproduction in Domestic Animals, **56**(9), Pp 1165-1175.

Wodajo Muleta Tadeg, Alemayehu Lemma, Tefera Yilma, Hagos Asgedom, Abadi Amare Reda (2021): Seroprevalence of infectious bovine rhinotracheitis and

brucellosis and their effect on reproductive performance of dairy cattle, *J. Vet. Med. Anim. Health*, **13** (2):106-113.

Whay H.R., Waterman A.E., Webster A.J. (1997): Associations between locomotion, claw lesions and nociceptive threshold in dairy heifers during the peri-partum period. *Vet J.*; **154**:155–161.

Williams EJ, Sibley K, Miller AN, Lane EA, Fishwick J, Nash DM, Herath S, England GCW, Dobson H, Sheldon IM. (2008): The effect of Escherichia coli lipopolysaccharide and tumour necrosis factor alpha on ovarian function. *Am J Reprod Immunol*, **60**:462-473.

Wilson, D. J., Grohn,Y. T., Bennett, G. J., González, R. N., Schukken, Y. H. and Spatz, J. (2008): Milk Production Change Following Clinical Mastitis and Reproductive Performance Compared Among J5 Vaccinated and Control Dairy Cattle. *Journal of Dairy Science*, **91**, 3869–3879.

Wirtu, A., Abunna, F., Duguma, A., 2018. Isolation and Identification of Staphylococcus aureus from Dairy Farms in Bishoftu Town, Ethiopia. *JOJ Pub Heal.* 3, 001–003.

Wolfenson D., Leitner G., Lavon Y. (2015): The disruptive effects of mastitis on reproduction and fertility in dairy cows. Ital. *J. Anim. Sci.*, **14**: 650–654.

Yang, F. L., Li, X. S., Yang, B. Z., Zhang, Y., Zhang, X. F., Qin, G. S. and Liang, X.W. (2012): Clinical mastitis from calving to next conception negatively affected reproductive performance of dairy cows in Nanning, China. *African Journal of Biotechnology*, **11**(10), 2574–2580.

Yohannis, M., Molla, Y. (2013): Prevalence, risk factors and major bacterial causes of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia. *Glob. J.* Microbiol. Res. **1**, 107–111.

Zeryehun T., Abera G. (2017): Prevalence and bacterial isolates of mastitis in dairy farms in selected districts of eastern Harrarghe Zone, Eastern Ethiopia. *J Vet Med.*; 2017:7. doi:10.1155/2017/649861.

8. APPENDICES

Appendix I: Questionnaire survey format for mastitis and lameness

I. Personal information

District (Town):_____ Kebele _____

Type of farm: intensive ☐ semi-intensive ☐ extensive ☐

Farm owner's (attendant's) Full Name_____ sex___ Age_____

Education Level: Illiterate ☐ 1-4 grades ☐ 5-8 grades ☐ 9-12 grades ☐ diploma and above ☐

Marital status: Single ☐ Married ☐ Widow ☐ Widower ☐ Divorced ☐

Name of the farm_____

When was the farm established? 1-5 years ☐ 6-10 years ☐ >10 years ☐

II. Management and Housing conditions of the farm

1. What is your farm's herd size?

2. Housing management

A) Confined ☐ B) semi-open ☐ C) loose- barn ☐

D) House with concrete floors and block walls with rain proof roof ☐

E) House with mad floor, walls made of wood with rain proof roof ☐

F) Other…….

3. Total sanitary: Type of floor in animal house, clearing and daily removing manure

Good ☐ Fair ☐ Poor ☐

4. Type of feed cows or heifers are fed:

Hay ☐ Straw ☐ Concentrate ☐ Concentrate and hay or straw ☐

5. Do animals get water regularly? _____; how many times a day? _____

6. Do you have ration formulation practice based on any manual given to you by the experts?

A. Yes ☐ B. No ☐

7. How do you feed your cattle? A. Tie stall ☐ B. Free stall ☐ C. both ☐

8. If tie stall how many hours do your cattle stay at tie stall in a day? _____

9. For how many hours do your animals get laid? _____

10. Do you let your animals for exercise outside the barn? A. Yes ☐ B. NO ☐

11. If yes, for how many hours do your animals be out of the barn? _____

12. Do you have farm production record? A. Yes ☐ B. No ☐

13. Average milk yield per Cow per day _____litter.

14. Average Lactation length _____ in months.

15. What kind of beddings do you use on the farm?

A. Sand ☐ B. Straw ☐ C. No beddings ☐

16. Do you have any footbath practices? A. Yes ☐ B. No ☐

17. Do you have regular hoof trimming practices? A. Yes ☐ B. No ☐

18. Do you wash udder before milking? Yes ☐ No ☐

19. If question 18 is yes, do you use the same towel or cloth to dry teats for all cows?

Yes ☐ No ☐

III. Disease status of the farm

1. What are the main diseases of dairy cattle at your farm?

No	Local name of the diseases	Scientific name	Rank

2. Any condition of reproductive health problems occurred in your dairy farms?

A) Abortion B) dystocia C) Retained fetal membrane D) Uterine prolapse

E) Anoestrous F) repeat breeding ☐ G) not at all ☐

H) Other (specify)……

3. Is there blind teats? Yes ☐ No ☐

4. In the last 12 months has this animal experienced any periods of udder problem?

Yes ☐ No ☐ Unknown ☐

5. Teat lesion, present ☐ absent ☐

6. Gross milk quality:

Watery ☐ blood tinged ☐ clots/flakes ☐ normal ☐

7. Has this animal been treated by any animal health professional? Yes ☐ No ☐

8. If yes, in response to this period of ill health who gave treatment?

Myself ☐ Government Vet (AHA) ☐ Private Vet ☐ No treatment given before ☐

9. Is there problem of cure after therapy and has this animal received any more treatments, for this disease during that time? Yes ☐ No ☐

10. Is there problem of cure after therapy? Yes ☐ No ☐

11. Mention all drugs used for treatment of mastitis in this district a) ___

b) _____

12. Does that mastitis cow give similar amount of milk per day/lactation as they were healthy **(**not mastitis**)?** A. Yes ☐ B. No ☐

13. Is there any abnormal gaits observed before? A. Yes ☐ B. No ☐

14. If yes which animals did show such problems more?

A. Calves ☐ B. Pregnant ☐ C. Lactating ☐

D. Dry cow ☐ E. Bulls ☐ F. Heifers ☐

15. Do you have any locomotion scoring practice while the animals are at different positions (walking/or standing on different surfaces like concrete)? A. Yes ☐

B. No ☐

16. What were common signs of abnormal gait observed at your farm?

A. Arching Back ☐ B. Limping legs ☐ C. Abduction /Adduction of legs ☐

D. Nodding movement of the head in a vertical plane ☐ E. All ☐

17. Which legs do have gait problems predominantly?

A. Hind Legs ☐ B. Front legs ☐ C. Both ☐

186. What do you do with known lame animals at your farms?

A. Cull ☐ B. Leave as it with some medications ☐ C. Both ☐

19. Do those lame cows give similar amount of milk per day/lactation as they were healthy (not lame)? A. Yes ☐ B. No ☐

IV. **ANIMAL INFORMATION**

1. Cow ID _____

2. Date of birth_____

3. Age years: 3-6yrs ☐ 6-9 yrs ☐

4. Breed: local ☐ Exotic ☐ Cross ☐

5. Parity _____

6. Sire's name/ID_____ Breed_____

7. Dam's name/ID_____ Breed_____

8. BCS_____

9. Lactation stage (in months) Early (<2) ☐ Medium (3-6) ☐ Late (> 6) ☐

10. Presence of blind teats? Yes ☐ No ☐

11. If question 10 is yes, how many quarters are blind?

One ☐ Two quarters ☐ Three quarters ☐ All quarters ☐

12. In the last 12 months has this animal experienced any periods of udder problem?

Yes ☐ No ☐ Unknown ☐

13. Teat lesion, present ☐ absent ☐

14. Gross milk quality:

Watery ☐ blood tinged ☐ clots/flakes ☐ normal ☐

15. Has this animal been treated by any animal health professional?

Yes ☐ No ☐

16. If yes, in response to this period of ill health who gave treatment?

Myself ☐ Government Vet (AHA) ☐ Private Vet ☐

No treatment given before ☐

17. Is there problem of cure after therapy and has this animal received any more treatments, for this disease during that time? Yes ☐ No ☐

18. Is there problem of cure after therapy? Yes ☐ No ☐

19. Sample collected from: RH ☐ RF ☐ LF ☐ LH ☐

20. Type of Mastitis Clinical ☐ Subclinical ☐

21. Average milk yield per mastitic Cow per day _____litter.

22. Is there any abnormal gaits observed? A. Yes ☐ B. No ☐

23. Which legs do have gait problems predominantly?

A. Hind Legs ☐ B. Front legs ☐ C. Both ☐

24. Average milk yield per lame cow per day _____litter.

V. REPRODUCTIVE PERFORMANCE OF INDIVIDUAL DAIRY COW

1. Was she born in your herd or did you get her from somewhere else? _____

2. If born in herd, when was she born? (Give date as" year/season ") _____

3. If acquired, when did you get her? (Give date as" year/season ") _____

4. Did you buy her; get her as gift or as dowry?

5. Was she a calf, heifer or adult at that time?

6. How old was she at that time?

7. Why did you get/buy her?

8. At what age did your cow/heifer reach for mating? (age at puberty/AFS) _____

9. What was the age of your cow when she gave birth to the first calf? (AFC)_____

a. Birth date.....

b. First calving date.....

10. Date/ month/ year of pervious calving? _____

11. Date/ month/ year of recent calving? _____

12. Intervals from calving to first service? _____

13. Intervals from calving to conception? _____

14. Number of service per conception (how many times did you take the cow for service before got pregnant last time) _____

15. 1st PP AI (Days) _____

16. Pregnancy Rate (PR) _____

17. Conception Rate (CR) _____

Appendix II: Body condition score protocol of cattle

Score Condition futures

L- Marked emaciation (animals would be condemned at anti mortem examination).

L transverse processes prominently, dorsal spines appear sharply.

L+ individual dorsal spines are pointed to the touch, hips, pins, tails head ribs are prominent. Transverse processes visible, usually individually.

M- ribs, hips and pins clearly visible, muscle mass between hook and pins sharply concave slightly more flesh above the transverse processes that in L+

M ribs usually visible, little fat covered dorsal spines barely visible

M+ animal smooth and well covered, dorsal spines cannot be seen, but are easily felt.

F- Animal smooth and well covered, but fat deposits are not marked. Dorsal spines can be felt with firm pressure but feel rounded rather than sharp.

F fat covers in critical areas can be seen or felt. Transverse processes cannot be seen or felt.

F+ heavy deposits of fat clearly visible on tail, head, brisket and dorsal spines, ribs, hooks and spines fully covered and cannot be felt even with firm pressure.

Source: Nicholson and Butter worth (1986).

Appendix III: Procedures for Collecting and Storing of Milk Samples

Wash your hands and then put on new disposable gloves. Label the sample vial using a waterproof marking pen. Clearly record the date, the cow ID and the quarter from which the sample will be taken. RF = right front, LF = left front, RR = right rear, LR = left rear.

Brush off any loose manure, dirt or bedding particles from the udder and teats. Pre-dip with an effective germicidal teat dip, leaving the dip on for 30 seconds. *If the udder and teats are extremely dirty, thoroughly wash and dry the udder and teats before pre-dipping.

Wipe each teat dry with a single-use paper or cloth towel, paying particular attention to the teat end. Be sure there is no teat dip remaining on the teat, as it will kill bacteria in your milk sample.

Discard 3 to 4 streams of milk on the floor to minimize chances of contaminating the sample with bacteria in the teat canal.

Scrub teat ends vigorously for 10-15 seconds using a cotton ball or gauze pad soaked in 70% isopropyl alcohol. Scrub until the ball or pad comes away clean, using as many as

necessary. Scrub far teats first, followed by near teats to avoid recontaminating teats you have already scrubbed. Use a new swab/s for each teat. Teats should not be dripping with alcohol, as this will also kill any bacteria in your milk sample.

Open the sample vial immediately before the sample is taken, not before. Do not touch the inside of the vial or cap or let the teat end touch the vial. Hold the vial at an angle of 45o to keep loose dirt or hair from falling into it. Direct streams of milk into the vial without touching the teat end. Sample as quickly as possible, starting with near teats first, followed by far teats. Fill the vial approximately 1/3 full. Attempting to fill the entire sample vial increases the chance of contamination and the full vial may burst when frozen. Immediately close the sample vial so that it is airtight. Collect milk from each quarter into a separate vial (quarter samples). Immediately place the sample vial on ice or in the refrigerator. If you are not able to immediately get the sample to the lab for plating, store:

At room temperature (Less than 1 hour); at refrigerator temperature (More than 1 hour, less than 2 days) and at freezer temperature (More than 2 days, less than 60 days).

Sources: (Quine et al., 2004; NMC, 2004).

Appendix IV: Clinical Examination

During the routine follow up the reproductive tracts of each cow is examined using rectal palpation and vaginoscopy to determine the presence of clinical uterine infection. The cows are first inspected for abnormal vaginal discharge on the vulva, perineum and tail. Cows with vaginal discharge are diagnosed as affected by clinical endometritis and excluded from the study. When no discharge is visible externally sterile vaginal speculum will be lubricated and inserted into the vagina up to the level of the external os of the cervix and inspection is performed using illumination from the penlight. Cows which received systemic or intrauterine antibiotic therapy within 6 days prior to enrolment will not be selected for the study. Pregnancy diagnosis will be performed by rectal palpation of the uterus and its contents post insemination.

Appendix V: Selective and Differential Medias

Blood Agar

This media consists of Columbia agar base with 5% sheep blood added. It is a widely used general purpose media. The Columbia agar base is a basal medium to which blood may be added for isolating fastidious organisms, or to which other enrichments may be added for special purposes. It contains two types of peptones to obtain a fast and abundant growth; give sharply defined hemolytic reactions, typical colonial morphology and improved pigment production.

Mannitol Salt Agar (MSA)

Mannitol salt agar is both a selective and differential media used for the isolation of pathogenic Staphylococci from mixed cultures.

Components

7.5% NaCl – selects for species of Staphylococcus. This concentration of salt is too high for most other bacteria to withstand and therefore, inhibits their growth.

Mannitol – alcohol of the carbohydrate mannose. Mannitol fermentation produces acid end products which turn the medium yellow. Yellow indicates mannitol positive and no color change indicates mannitol negative.

Phenol red pH indicator – yellow in acid pH (The same indicator that is used in phenol red carbohydrate fermentation broths).

MacConkey's Agar (MaC)

MacConkey's Agar is both a selective and differential media; it is selective for Gram negative bacteria and can differentiate those bacteria that have the ability to ferment lactose.

Components: (a) Bile salts - Inhibits most Gram-positive bacteria, except Enterococcus and some species of Staphylococcus i.e. Staphylococcus aureus.(b) Crystal violet dye- Inhibits certain Gram-positive bacteria thus selecting for Gram negatives. (c) Lactose-

Some bacteria can ferment lactose acid-end products, others cannot. (d) Neutral pH red indicator - Stains microbes fermenting lactose: hot pink in acid pH, rose in neutral pH and tan in alkaline pH. (e) Peptone - a source of proteins, amino acids for microbial growth.

Eosin Methylene Blue (EMB) Agar (Levine)

Eosin methylene blue agar (EMB) is both a selective and differential medium used for the detection and isolation of Gram-negative intestinal pathogens. The medium have Lactose (a disaccharide which can be fermented by some bacterial enzymes to produce acidic end products) and Eosin and Methylene Blue (these are dyes which inhibit the growth of most Gram positive bacteria).

They also react with any acidic products resulted from lactose fermentation to color the colonies. Acid production from lactose fermentation causes precipitation of the dyes on the surface of the colony resulting in different colours: large amounts of acid (green metallic sheen); small amounts of acid (pink) and no fermentation (colourless).

Appendix VI: Gram Stain Procedures

Using a sterile inoculating loop, add 1 drop of sterile water to the slide. Prepare a mixed smear of culture.

Air dry and Heat fix.

Cover the smear with Crystal Violet (primary stain) for 1 min.

Gently wash off the slide with water.

Add Gram’s Iodine (mordant) for 1 min.

Wash with water.

Decolorize with 95% ethanol. This is the "tricky" step. Stop decolorizing with alcohol as soon as the purple colour has stopped leaching off the slide (time will vary depending on thickness of smear). Immediately wash with water. Be sure to dispose of all ethanol waste in the appropriately labelled waste container.

Cover the smear with Safranin for 30 seconds.

Wash both the top and the bottom of the slide with water.

Blot the slide with bibulous paper.

Using the 10X objective lens and using the 100X (oil immersion lens), focus first on the line and then on the smear.

Interpretation: Bluish purple colour indicate gram-positive and pinkish colour indicate gram-negative bacteria.

Appendix VII: Procedure for oxidation and fermentation test

1. Prepare O-F base medium and when it is cooled at 50^{o}c, add 10 ml of sterile glucose in to 100 ml of O-F base, for a final concentration of 10% glucose and dispense into a sterilised tube.
2. Heat two tubes of medium in boiling water for 10 minutes to drive off the oxygen, cool and inoculate by inserting a straight wire vertically
3. Incubate one tube aerobically and the second tube anaerobically or seal the surface with a layer of sterile liquid paraffin oil to create an aerobic condition
4. Incubate both tubes at 37°C for 24-48 hours or more, up to 7 days with the caps loose. Longer incubation may be required for slowly growing species.
5. Examine tubes daily for colour change.
6. Interpretation: Fermentation: Yellow colour in both tubes with or without gas.

- Oxidation: Yellow colour only in tube without oil.
- No oxidation/fermentation: No change in the colour of the tubes. The carbohydrates have not been fermented or oxidized.

Appendix VIII: Catalase and Coagulase test procedure

Catalase test procedures

1. Pick a colony from an 18-24 hours culture and place it on a clean glass slide.
2. Put one drop of 3% H_2O_2 over the organism on the slide.
3. Observe for immediate bubbling (gas liberation) and record the result.
4. Interpretation: A positive result is the rapid evolution of O_2 as evidenced by bubbling and a negative result is no bubbles or only a few scattered bubbles.

Coagulase test procedure

1. Three test tubes are taken and labelled “test”, “negative control” and “positive control”.
2. Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma. To the tube labelled test, 0.1 ml of overnight broth culture of test bacteria is added.
3. To the tube labelled positive control, 0.1 ml of overnight broth culture of known *S. aureus* is added and to the tube labelled negative control, only 0.1 ml of sterile broth is added.
4. All the tubes are mixed gently, incubated at 37^oC and observed up to four hours. If the test remains negative until four hours at 37^oC, the tube is kept at room temperature for overnight incubation.
5. Avoid shaking or agitating the tube during reading. Doubtful or false negative results may occur due to break down of the clot.
6. Result: Positive result is indicated from a loose clot suspended to a solid clot that is immovable, which remains in place even after inverting the tube. No degree of clotting is observed in negative result.