TRANS-GENERATIONAL EFFECTS OF LOW PROTEIN DIET ON FERTILITY AND THE AMELIORATIVE POWER OF PROBIOTIC

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE,

KARNAL (DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF

THE REQUIREMENT FOR

THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

ANIMAL BIOCHEMISTRY

BY

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B.Tech. (Biotechnology)

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ICAR-NATIONAL DAIRY RESEARCH INSTITUE

(DEEMED UIVERSITY)

KARNAL 132001 (HARYANA), INDIA

2019

Regn. No. 17-M-AB-09

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This is to certify that the thesis entitled **"Trasgenerational effects of low protein diet on fertility and the ameliorative power of probiotic"** submitted by **Javed Ahmad** towards the partial fulfilment of the requirement for the award of the degree of Master of Technology in **Animal Biochemistry** of the **ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana), India,** is a bonafide research work carried out by him under my supervision and no part of the thesis has been submitted for any other degree or diploma.

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Dedicated
To
My Beloved family
And
My friends

ACKNOWLEDGEMENT

First of all I would like to thanks my almighty God for his blessings for giving me all the wonderful people who helped me to accomplish my venture. Formal words of acknowledgement will hardly fulfill the end of sentiments while expressing the deep sense of gratitude to many known and unknown hands which pushed me forward, lips put elixir in my heart, learned souls put me on the right path and enlightened me with their knowledge and experience. I shall ever remain grateful to all of them.

I wish to express my deepest sense of gratitude to my respected guide Dr. Gautam Kaul, Principal Scientist, Animal Biochemistry Division for his benevolence, sagacious guidance, persistent help, constant encouragement, care and all kinds of painstaking help extended to me that led to successful completion of my research. Any success that I have or will receive in my journey through science will perpetually be dedicated, in part, to you. His unflinching courage and conviction will always inspire me. Personally, I feel above all his merits he is a great human being, who helped and supported me during my tough times and buttressed me to perform my work comfortably. I have truly been very fortunate to have worked under his precious guidance.

I also wish to express my profound sense of gratitude and heartful thanks to Director Dr. R.R.B Singh and Vice-Chancellor of N.D.R.I. (deemed university), and Dr. Dheer Singh, Head, Animal Biochemistry Division for providing necessary facilities that helped me during my work.

I owe my sincere thanks to the members of my advisory committee Dr. Sadeesh E.M Scientist, Animal Biochemistry Division, Dr. Sudheer K.Tomar, Senior Scientist, Dairy Microbiology Division, Dr. Pradip V Behare, Scientist, Diary Microbiology Division, Dr. Pawan Singh, Principal Scientist, Live Stock and Production Management Division for their valuable suggestions and encouragement throughout the research period. Their critical evaluation along with prolific ideas and time to time suggestion helped me in the successful execution of my work. I am highly thankful for providing the financial assistance from National Diary Research Institute, Karnal.

I consider it a great privilege to record my deepest sense of gratitude to Dr. Sudheer K.Tomar and Dr. Pradip V Behare for providing free access to work in their lab throughout my research. I want to thanks them for their kindness. I am thankful to my entire senior for their love, encouragement, invaluable moral support and being there for me throughout. I am indebted towards their contributions. I wish to pay my special thanks to my loving juniors Deepak and Mohit for their love, care, affection as well as brotherly support. My sincere thanks to them for their ready incorporation and willing help to overcome my miner and major difficulties to whatever extent they could. Extremely thankful to Sandeep, Ahmed and Karmveer sir for their great contributions in proof reading and formatting of my thesis. I also thank my other lab members Deepti, Akaram, Sanjana, Poonam and Kavita for their support and help.

I am also thankful to my batch mates and friends: and my loving hostel friends Mohit, Yuvraaj, Parshant and Nancy for their love, moral support and good memories shared with me and providing family, healthy and amusing atmosphere during my Masters journey.

Thanks to all faculty member and staff of Animal Biochemistry division for their co-operation and

support, Raman Sir, Rajni mam, Ravikant sir, and other staff for their help. I am also very thankful to staff of small animal house especially Mahender sir for their kind help during my animal study. I also thanks to Dr. Gautam Kaul, Dr. Sudheer K,Tomar Singh sir and Pawan sir for their encouragement and support.

"Where emotions are involved, words cease to exist" and words become inadequate to express my profound sense of gratitude and love to my most admired Mummy, Papa (my strength) who brought me up to this stage and also the unforgettable support and affection showered on me. Thank you for understanding me and believing in me.

Not everyone is mentioned, but none is forgotten.

Javed Ahmad

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ABBREVIATIONS

%	:	Percent
^{o}C	:	Degree centigrade
α	:	Alpha
β	:	Beta
μg	:	Microgram
μl	:	Microlitre
μM	:	Micromole

IFN	:	Interferon
Kb	:	Kilo base pairs
KDa	:	Kilo Dalton
L	:	Litre
M	:	Molar
Mg	:	Milligram
mg/ml	:	Milligram per milliliter
Min	:	Minute
Ml	:	Milliliter
Mm	:	Millimeter
Ng	:	Nanograms
17βHSD	:	17β-Hydroxysteroid dehydrogenase
Abs	:	Absorbance
AO	:	Acridine orange
BSA	:	Bovine Serum albumin
BW	:	Body weight
Ca2+	:	Calcium ions
CAT	:	Catalase
CO2	:	Carbon dioxide
DF	:	Dilution factor
DNA	:	Deoxyribonucleic acid
DTPA	:	Diethylene Triamine Pentaacetic acid
DTT	:	Dithiothreitol
EDTA	:	Enzyme-linked immunosorbent assay

EtBr	:	Ethidium bromide
FSH	:	Follicle-stimulating hormone
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GD	:	Gestation day
GSH	:	Glutathione
LH	:	Luteinizing hormone
LHr	:	Luteinizing hormone receptor
Ltd	:	Limited
LP	:	Low protein
mRNA	:	Messenger RNA
NDRI	:	National Dairy Research Institute
No.	:	Number
OD	:	Optical density
P450scc	:	Cytochrome P450 side-chain cleavage enzyme
PBS	:	Phosphate buffer solution
PCR	:	Polymerase chain reaction
pH	:	Negative log of hydrogen ion concentration

PLG :Poly lacto-co-glycolide
PVA :Poly vinyl alcohol
PVP :Poly vinyl propyl
Pvt. :Private
qPCR :Quantitative PCR
RNA :Ribose nucleic acid
ROS :Reactive oxygen species

Rpm	:rotations per minute
RT	:Room Temperature
SDS	:Sodium dodecyl sulphate
Sec	:Second
SEM	:Standard error of mean
SOD	:Super oxide dismutase
StAR	:Steroidogenic acute regulatory protein

ABSTRACT

Infertility defined as the inability to conceive a child, having unprotected sexual intercourse for one year. There is growing evidence that paternal diet, physiology, and environmental factors negatively impact on sperm quality. Probiotics have proven its beneficial effects on various metabolic disorders like obesity, diabetes, hormonal disorder. Previous studies shown that, low protein diet impairs testicular spermatogenesis, reduced gonadal weight, sperm DNA damage in male. So, the current study was aimed to evaluate the beneficial effects of probiotics on male reproductive system in Swiss albino mice. In current study the animals were randomly divided in to 6 groups: LPD+PBS, LP+RSI3, LP+FER, NP+PBS, NP+RSI3 and NP+FER. The male mice were being fed either a control normal protein diet (NP 18% casein) or low-protein diet (LP 9% casein) for a minimum of 8 week period. Two probiotics namely, *Lactobacillus fermentum* (NCDC-400) and *Lactobacillus rhamnosus*-RSI3 (NCDC-610) were used (10^9 cfu/day/animal).. After 8 weeks, the female mice having same age mated with low and normal protein fed paternal mice to see the trans generational effects on their offsprings. The body weight was gained in all probiotic supplemented groups and the feed intake is remain unchanged in all the groups. Both the probiotic supplemented groups improved the testosterone levels (4.21±1.10;3.6±0.66) when compared to the low protein diet group. Subsequently, increase in sperm count and viability in case of LP+RSI3, LP+FER groups, on the contrary less sperm count and viability in LP+PBS group. Furthermore, the antioxidative enzyme super oxide dismutase in testes significantly increased in LP+RSI3(31.66 ±0.23U/mg of protein) as compared to LP+PBS (21.66 ± 0.53 U/mg of protein). The 17-β HSD expression in testes was found to be increased.These findings suggest that*Lactobacillus rhamnosus* NCDC 610 and *Lactobacillus fermentum* NCDC400 are readily adapted by the animals in their diet and improve the male fertility problems caused by low protein diet.

सार

एक वर्ष के लिए असुरक्षित संभोग के बिना, एक बच्चे को गर्भ धारण करने में असमर्थता के रूप में बांझपन को परिभाषित किया गया है। इस बात का प्रमाण है कि पैतृक आहार, शरीर विज्ञान और पर्यावरणीय कारक शुक्राणु की गुणवत्ता पर नकारात्मक प्रभाव डालते हैं, जिनमें से एक निम्न प्रोटीन आहार है। पिछले अध्ययनों से पता चला है कि कम प्रोटीन आहार वृषण शुक्राणुजनन को कम करता है, पुरुष में गोनैडल वजन, शुक्राणु डीएनए की क्षति को कम करता है। तो, वर्तमान अध्ययन का उद्देश्य स्विस एल्बिनो चूहों में पुरुष प्रजनन प्रणाली पर प्रोबायोटिक्स के लाभकारी प्रभावों का मूल्यांकन करना था। वर्तमान अध्ययन में चूहों को 6 समूहों में विभाजित किया गया: LPD + PBS, LP + RSI3, LP + FER, NP + PBS, NP + RSI3 और NP + FER। कम से कम 8 सप्ताह की अवधि के लिए नर चूहों को या तो एक नियंत्रण सामान्य प्रोटीन आहार (एनपी 18% कैसिइन) या कम-प्रोटीन आहार (एलपी 9% कैसिइन) खिलाया जा रहा था। दो प्रोबायोटिक्स, लैक्टोबैसिलस फेरमेंटम (NCDC-400) और लैक्टोबैसिलस rhamnosus-RSI3 (NCDC-610) का उपयोग किया गया (10^9 cfu / दिन / पशु), 8 सप्ताह के बाद, कम उम्र और सामान्य प्रोटीन वाले समान प्रोटीन वाले मादा चूहों को खिलाया जाता है। पैतृक चूहों को उनकी संतानों पर ट्रांस जनरेशनल प्रभाव देखने के लिए। शरीर का वजन सभी प्रोबायोटिक पूरक समूहों में प्राप्त किया गया था और सभी समूहों में फ़ीड का सेवन अपरिवर्तित रहा है। दोनों प्रोबायोटिक पूरक समूहों में कम प्रोटीन आहार समूह की तुलना में टेस्टोस्टेरोन का स्तर (4.21; 1.10; 3.6±0.66) में सुधार हुआ। बाद में, एलपी + आरएसआई 3, एलपी + एफईआर समूहों के मामले में शुक्राणुओं की संख्या और व्यवहार्यता में वृद्धि, इसके विपरीत एलपी + पीबीएस समूह में शुक्राणुओं की संख्या और व्यवहार्यता कम हो जाती है। इसके अलावा, एलपी + पीबीएस (21.66±0.53 यू / मिलीग्राम प्रोटीन) की तुलना में वृषण में एंटीऑक्सीडेंट एंजाइम सुपर ऑक्साइड डिस्चार्ज एलपी + आरएसआई 3 (31.66 mg 0.23U / मिलीग्राम प्रोटीन) में काफी वृद्धि हुई है। वृषण में 17-to एचएसडी की अभिव्यक्ति में वृद्धि देखी गई। इन निष्कर्षों से पता चलता है कि लैक्टोबैसिलस रम्नोसस एनसीडीसी-610 और लैक्टोबैसिलस फेरमेंटम एनसीडीसी-400 को जानवरों द्वारा अपने आहार में आसानी से अनुकूलित किया जाता है और कम प्रोटीन आहार के कारण पुरुष प्रजनन समस्याओं में सुधार होता है।

CHAPTER - 1

Introduction

INTRODUCTION

1. **INTRODUCTION**

Fertility is the natural capability of producing offspring's is measured by fertility rate. Fertility rate is defined as the number of children born per couple, person or population. The total fertility rate (TFR) is the average number of children born to each woman in a country. Male factor is responsible for the infertility of a large part of population. More than 30 million male are infertile in the world (Agarwal *et al*., 2015). There are enumerable number of factors that affect the fertility and reproductive health for both men and women. Such as reproductive system disorders, reproductive system diseases, hormonal disorders, aging, stress, obesity, nutrition malnutrition, alcohol consumption, and smoking reducing the chance of successful pregnancy. Male fertility affect by numerous Chemical and physical agents in the environment introduced by human activity (Oliva *et al*., 2001).

Proteins are substances that are part of cells, tissues and organs throughout the body. According to the Centers for Disease Control protein deficiency is common among people who live in developing countries. those who live in impoverished communities in developed countries and in the elderly who lack access to nutritious food. Protein deficiency also affects people who are born with a genetic disorder to produce certain proteins, and people with diseases that cause them to lose appetite and experience muscle breakdown. Underneath nutrition results in the loss of body weight and body condition, delays the onset of puberty, interferes with normal ovarian cyclicity by decreasing gonadotropin secretion, malnutrition and increases infertility (Boland *at al*., 2001 and Capuco *at al.*, 1990). High in fat and/or protein diet (28% protein) have been shown to impair testicular steroidogenesis, increasing rates of apoptosis and sperm-DNA damage in rodents (Zhao *at al.,* 2014). Paternal fed low protein diet (LPD), developed relative hypotension and elevated heart rate in adult male offspring. Whereas both male and female offspring displayed vascular dysfunction and impaired glucose tolerance relative to normal protein diet (NPD) offspring (Watkins *at al.,* 2014). Increased adiposity, glucose intolerance, perturbed hepatic gene expression symptomatic of nonalcoholic fatty liver disease, and altered gut bacterial profiles in offspring from low protein paternal diet compared to normal protein diet offspring (Watkins *at al*., 2018). In response to LPD, we observed increased mean testicular seminiferous tubule and epithelium area.

Various approaches are being used to improve fertility related problems such as testosterone replacement therapy (TRT), use of various drugs for example Clomiphene, Letrozole etc. But these drugs and TRT also exerts various kinds of side effects in the body like allergy, prostate cancer, stroke, heart attack etc. Functional foods like Probiotic and their metabolites may be the best alternative. Because these are free from side effects and confer health benefits to host. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). The modulations of gut microbiota by the probiotic have systematic effects on the immune system which activate the metabolic pathways to restore the tissue homeostasis. Dietary supplementation of probiotic controls body weight gain, fat accumulation plasma insulin level, Leptin level, total cholesterol and lipotoxicity in mice fed with obesogenic diet (Park *et al*., 2013). Probiotic administration in aged mice increased seminiferous tubule cross-sectional profiles, spermatogenesis, Leydig cell numbers and testosterone level (Poutahidis *et al*., 2014). There is no report available about the effects of probiotics on reproductive health affected by low protein diet. Keeping all these aspects in mind, we designed our experiments with the following two objectives:-

Objectives:

1. To study the effect of probiotic on low protein diet induced changes in male fertility.
2. To assess the fertility in male offspring after paternal supplementation of probiotic fed low protein diet.

CHAPTER – 2

Review of Literature

2. REVIEW OF LITERATURE

Current lifestyle trends in modern society, characterized by caloric abundance, reduced physical activity, use of more electronic devices, alcohol consumption, Smoking, and malnutrition which directly or indirectly affects fertility as well as reproductive performance. Intense research efforts are being made to understand the underline mechanisms by which excess fat and low protein results in the reduction of fertility potential. Further research was being utilized to find more effective, therapeutic and preventive measures to prevent infertility problems. Low protein diet induced oxidative stress which causes obesity, inflammation, stress are the subject of much interest because these are more related to life style and directly responsible for alteration in the level of various fertility markers.

2.1. Fertility and reproductive hormones

The etiology of the male infertility is multi-factorial and little is known about the causative factors leading to decrease in spermatogenesis. The primary reproductive organs of the male are the testis, or male gonads, which have both an exocrine (sperm producing) as well as endocrine (testosterone producing) function. Spermatogenesis represents the entire process of germ cell development within the seminiferous epithelium of the adult testis. The testis consists of convoluted seminiferous tubules called as interstitium. Interstitium contains mixture of blood and lymph vessels, nerves, fibroblast cell, macrophages and Leydig cells. The epithelium of seminiferous tubules consists of continually dividing germ cells that produce sperm cells and supporting sertoli cells.

Reduction in the male fertility is indicated as an alteration in the level of reproductive hormones such as Testosterone, Luteinizing Hormone (LH) and Follicle stimulating Hormone (FSH).

2.2. Testosterone:

Testosterone is a male sex hormone, secreted by Leydig or interstitial cells of the testis in the male and by the follicular theca and interstitial cells of the ovaries in the female. Testosterone secretion is regulated by negative feedback of testosterone on the release of LH from the pituitary gland. Testosterone is highly protein-bound. In males, 98% of the testosterone in circulation is bound; the value is slightly lower in females. The majority of the steroid is bound to a specific binding protein, sometimes referred to as sex

hormone binding globulin (SHBG) or testosterone binding globulin (TeBG), and to serum albumin (Dunn et al., 1981).

2.3. Follicle stimulating hormone (FSH) and luteinizing hormone (LH):

FSH and LH are consists of two non-covalently associated subunits designated α and β (Pierce and Parsons., 1981).The α-subunit of FSH contains 92 amino acids and is very similar to the α-subunits of LH. The β-subunit of FSH is unique and confers its immunological and functional specificity. FSH and LH control growth and reproductive activities of the gonadal tissues (Catt and Pierce, 1978; Daughaday *et al.,* 1985). FSH promotes follicular development in the ovary and gametogenesis in the testis (Franchimont *et al.,* 1973; Catt and Pierce, 1978). The gonadotroph cells of the anterior pituitary secrete both FSH and LH in response to gonadotropin releasing hormone (LHRH or GnRH) from the medial basal hypothalamus (Bonnar *et al.,* 1973). Both FSH and LH are secreted in a pulsatile manner, with rapid fluctuations over the normal range (Catt and Pierce, 1978; Crowley *et al.,* 1987; Beastall *et al.,* 1987). The pulsatility of FSH is less pronounced than that of LH. Release of both FSH and LH from the pituitary is under negative feedback control by the gonads (Bonnar *et al.,* 1973). In males, FSH, LH and testosterone regulate spermatogenesis by the Sertoli cells in the seminiferous tubules of the testes. FSH is less sensitive to feedback inhibition by testosterone than is LH and is thought to be regulated independently by the inhibitory peptide inhibin produced by the Sertoli cells (Jeffcoate *et al.,* 1975). Due to the negative feedback mechanism regulating gonadotropin release, elevated concentrations of LH and FSH are indicative of gonadal failure when accompanied by low concentrations of the gonadal steroids. In males, these observations suggest primary testicular failure or anorchia. FSH may also be elevated in Klinefelter's syndrome (seminiferous tubule dysgenesis) or as a consequence of Sertoli cell failure (Franchimont *et al.,* 1973). The successful initiation of testicular function is dependent on the hypothalamic secretion of GnRH which in turn stimulates FSH and LH to act on the testis (Schanbacher *et al.,* 1982). These actions initiate spermatogenesis and testosterone production. The testis in turn, through the secretion of hormones produced in the sertoli and leydig cells, exerts a negative feedback control on the production of gonadotropins (Griswold *et al.,* 1998).

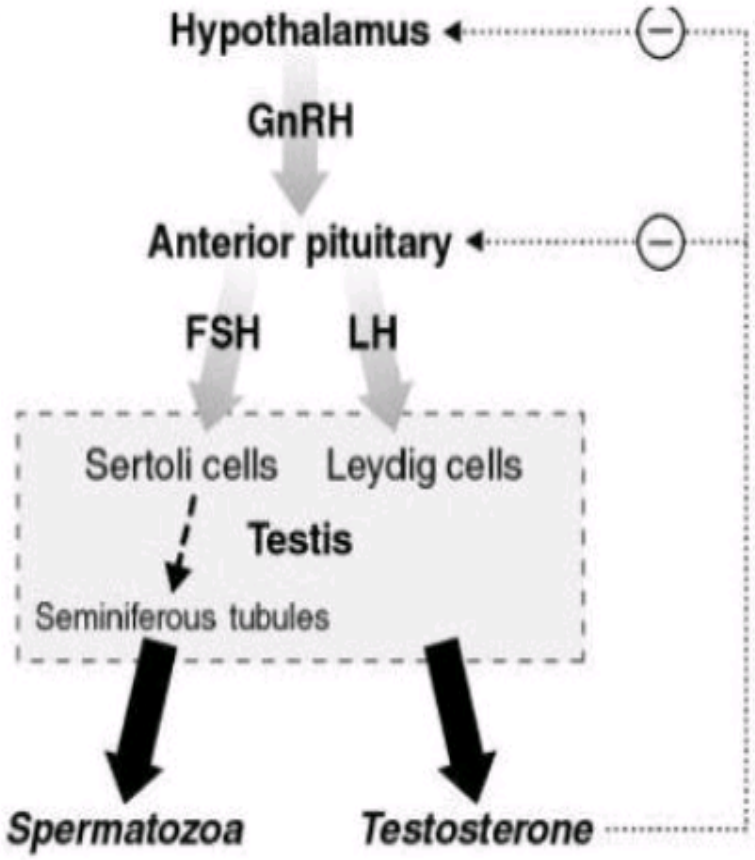


Fig. Schematic representation of Hypothalamic-pituitary-gonadal axis in men (Dandona and Rosenberg, 2010).

2.4. Factor affecting male fertility

There are many factors that may be attributed to the gradual decrease in fertility, including lifestyle habits, environmental factors, occupational exposure, and medical causes. Lifestyle habits refer to those habits that are mostly within our control and those that are resulting from our daily habits and rituals, behavior, and personal choices that invariably affect reproductive parameters in men as well as women. Some of the factors responsible for decreasing fertility in male are discussed below.

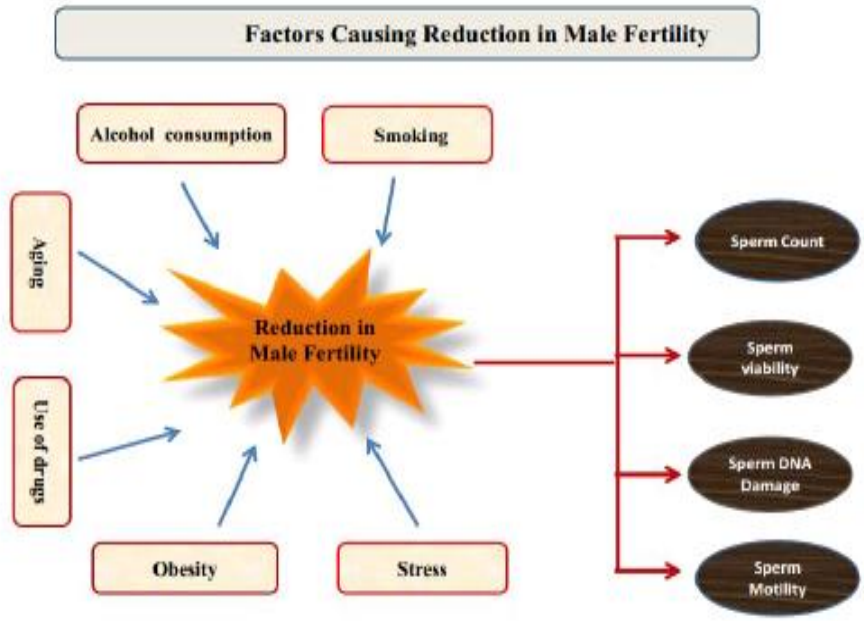


Fig. Schematic representation of various factors reducing male fertility.

2.4.1. Aging:

The age at which a couple decides to pursue conception is an important factor as fertility reduces with time. In men, testosterone levels decrease with age and result in hypogonadism (Stewart *et al*., 2011). Demographic data from a study suggests that male fertility begins a steady decline from 35 to 39 years onward, with a decline of about 22% per annum (Matorras *et al*., 2011). Semen quality deteriorates with aging, as sperm displays age-related reduction in motility and viability (Tubman *et al*., 2013). Advanced male age is associated with lower semen volume, sperm motility, and morphologically normal sperm (Kidd *et al*., 2001). Infertile men aged 40 and above were found to have increased levels of DNA damage (Varshini *et al*., 2012).

2.4.2. Smoking:

Cigarette smoke contains more than 4,000 chemicals, arises the occurrence of a number of potential health complications such as cardiovascular disease, lung cancer, prostate cancer. According to Practice Committee of American society for reproductive medicine, more than 35% of reproductive-aged males have been found as smokers. Smoking exerts a negative effect on sperm parameters (Hull *et al*., 2000). Chewing

tobacco or the use of smokeless tobacco also caused a reduction in sperm count, motility, viability, and morphology (Said *et al*., 2005). In fact, smoking worsens sperm quality in both fertile and infertile men. Regardless of fertility status, men who smoke demonstrated lower total sperm count and sperm motility, and a higher percentage of sperm with abnormal morphology (Li *et al*., 2011).

2.4.3. Alcohol consumption:

The effect of alcohol consumption on human fertility depends on the quantity alcohol consumed. There is no current threshold dose to indicate how much alcohol consumed will increase the risk of infertility. However, not all types of alcoholic drinks may have a similar negative effect on sperm parameters. For example, beer and spirits were found to have a negative association with sperm concentration and total sperm count, but not wines (Hansen *et al.,* 2010). In general, most studies find that alcohol intake affects male fertility by reducing semen volume, increasing the number of sperm with abnormal morphology, and by causing leukocytospermia. Smokers, who also drink alcohol, have abnormal sperm morphology compared to smokers who do not take alcohol (Karmon *et al.,* 2013). Men who consume high amounts of alcohol (>8 drinks/week or >40 g/day) are reported to have disrupted spermatogenesis (Pajarinen *et al.,* 1996). Men whose alcohol intake was >80 g/day displayed partial or complete spermatogenic arrest or Sertoli-only cell syndrome (Pajarinen *et al.,* 1996).

2.4.4. Use of drugs:

Amongest the illicit drugs that have a negative impact on male fertility are marijuana, opioid narcotics, cocaine, methamphetamines and ecstasy and anabolic-androgenic steroids (testosterone derivatives) (Fronczak *et al.,* 2012). Marijuana is one of the most commonly used drugs around the world (Battista *et al.,* 2008) and it acts both centrally and peripherally to cause abnormal reproductive function. Marijuana contains cannabinoids which bind to receptors located on reproductive structures such the ductus deferens. Cannabinoids have been reported to reduce testosterone released from leydig cells, modulate apoptosis of sertoli cells, decrease spermatogenesis, decrease sperm motility, decrease sperm capacitation and acrosome reaction (Lafuente *et al.,* 2013).

Another commonly used recreational drug is cocaine, a stimulant for both peripheral and central nervous systems which causes vasoconstriction and anesthetic effects. Long term use of cocaine claim that it can decrease sexual stimulation; men found it harder to achieve and maintain erection and to ejaculate (Gold *et al.,* 1997). Cocaine has been demonstrated to adversely affect spermatogenesis, which may be due to serum increases

in prolactin, as well as serum decreases in total and free testosterone (George *et al.,* 1996; Ragni *et al.,* 1988). Opiates comprise another large group of illicit drugs. Opiates, such as methadone and heroin, are depressants that cause both sedation and decreased pain perception by influencing neurotransmitters (Peugh *et al.,* 2001). In men taking heroin, sexual function became abnormal and remained so even after cessation (Wang *et al.,* 1978). Sperm parameters, most noticeably motility decreases with the use of heroin and methadone (Ragni *et al.,* 1985).

2.4.5. Physical and psychological stress:

Stress is an uncomfortable emotional state accompanying behavioral, biochemical and physiological changes (Nargund *et al.,* 2015). Stress; whether social, psychological or physical is an attention seeking element of society (Sharma *et al.,* 2013). Different studies have suggested that psychological stress plays a key role in male infertility (Collodel *et al.,* 2008; Sharma *et al.,* 2013). In males, it increases glucocorticoid level which suppresses testosterone concentration in testes (Whirledge and John, 2010) that rigorously affecting spermatogenesis (Nargund 2015). Stress may also induce structural and meiotic changes in sperm and make it difficult to target ovum (Collodel et al. 2008; Sultan and Tahir, 2011). Infertility may result in stress, depression, low self-esteem, negative thoughts and marital problem (Rauf and Salma, 2015).

2.4.6. Western diet:

According to WHO 2017 report "Obesity is a metabolic condition in which body starts to accumulate excessive or abnormal fat that stimulate various metabolic disorder and complications." Body mass index measurement suggests if a person has BMI more than 30 kg/m^2 so person will be referred as obese and if the person the BMI between 25-30 kg/m^2 so person will be referred as overweight not obese. Obesity leads to several metabolic complications like cardiovascular diseases, diabetes, reproductive complications, musculoskeletal disorders, and some cancers including endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon. Various scientific studies help to reveal the relationship between the obesity and female infertility and reproduction failure. Obese women are associated with higher waiting time to pregnancy (Nohr *et al.,* 2009).Obesity has stronger correlation with female fertility and fecundity because in obesity basal metabolic rate (BMI) get increased and this increased BMI causes reduction in conception in women and affect fertility (Pandey *et al.,* 2010). Male obesity is associated with increased incidence of low sperm concentration and low progressively motile sperm count (Hammoud *et al.,* 2008). Obesity may induce oxidative stress and

sperm DNA damage as well as decreased fertilizing ability linked to poor reproductive outcomes (Bakos *et al.,* 2011). Obesity can also affect sperm quality, sperm motility (Fernandez *et al.,* 2011). Increased BMI was found to be associated with occurrence of obesity (Paasch *et al.,* 2011). The chances of oligozoospermia or azoospermia were also observed more in obese men when compared normal weight men (Sermondade *et al.,* 2012).

2.4.7. Malnutrition:

There is an instinctive relationship between reproduction and nutrition (Triunfo and Lanzone 2015; Wong *et al.,* 2000). Nutrition has a vital role in sperm quality (Gaskins *et al.,* 2012). However, the effect of nutrition on male infertility needs an extensive research. Malnutrition might be an imperative cause of male infertility (Wong *et al.,* 2000). It adversely harms sperm functioning (Harris *et al.,* 2011). Prolonged starvation and excessive exercise can affect sperm count, motility and even can stop sperm production (Gaskins *et al.,* 2012; Sharma *et al.,* 2013). Excessive restriction in food intake can reduce the level of protein, Zinc (Zn), vitamin C, vitmin A, vitamin E, selenium (Se), folic acid and other nutrients that are necessary for proper functioning of body and spermatogenesis (Wong *et al.,* 2002; Kobori *et al.,* 2014). Deficiency of these elements can affect various semen parameters (Kobori *et al.,* 2014).

2.4.8. Protein:

Underneath nutrition results in the loss of body weight and body condition, delays the onset of puberty, interferes with normal ovarian cyclicity by decreasing gonadotropin secretion, malnutrition and increases infertility (Boland *et al.,* 2001 and Capuco *et al.,* 1990). High in fat and/or protein diet (28% protein) have been shown to impair testicular steroidogenesis, increasing rates of apoptosis and sperm-DNA damage in rodents (Zhao *et al.,* 2017). Paternal fed low protein diet (LPD) developed relative hypotension and elevated heart rate in adult male offspring. Whereas both male and female offspring displayed vascular dysfunction and impaired glucose tolerance relative to normal protein diet (NPD) offspring (Watkins *et al.,* 2014). Increased adiposity, glucose intolerance, perturbed hepatic gene expression symptomatic of nonalcoholic fatty liver disease and altered gut bacterial profiles in offspring from low protein paternal diet compared to normal protein diet offspring (Watkins *et al.,* 2018). In response to LPD, we observed increased mean testicular seminiferous tubule and epithelium area.

2.5. Prevention and treatment of fertility related problems

Various approaches are being used to improve fertility related problems such as testosterone replacement therapy, use of various drugs for example Clomiphene, Letrozole etc. But these drugs and TRT also exerts various kind side effects in the body like allergy, prostate cancer, stroke, heart attack etc. Functional food components may be the best alternatives. Several methods to obtain functional foods include the addition or removal of a component, modification of the food processing, genetic engineering, etc. which is allowing the food industry to develop new products with additional value for the market. So far, the most important components that can be added to food are.

2.6. Gonadotrophin releasing hormone (GnRH)

Idiopathic infertility is most commonly treated with GnRH. GnRH stimulates the release of LH and FSH by estrogen receptors blockade in hypothalamus (Safeer zaman *et al.,* 2009). Pulsatile treatment with GnRH, substitutes GnRH deficiency in infertile individuals suffering from hypogonadotropic hypogonadism (HGH) and lack of hypothalamus secretions. Individuals with HGH have reduced fertility status that is restored by FSH stimulation (Dabaja and Schlegel 2014).

2.6.1. Gonadotropins (GTs):

Human chorionic gonadotropin (rec-hCG), LH (rec-hLH), FSH (rec-hFSH) and purified urinary GTs are used for the treatment of infertile men with pituitary inefficiency. They persuade spermatogenesis in around 80% treated individuals (Dabaja and Schlegel 2014). Gonadotropins are self-administered via subcutaneous injections. Treatment duration differs from 6 to 24 months until sperm appearance in ejaculate or until pregnancy (Burgues and Calderon 1997).

2.6.2. Non-pharmacological treatment (surgical treatment):

Treatment of obstructive and non-obstructive infertility includes surgical involvement. In non-obstructive infertility, surgical sperm retrieval is mandatory for effective treatment with intracytoplasmic sperm injection (ICSI). There is 100% retrieval rate of sperm in obstructive infertility in surgical treatment. Typical surgical treatment is cost effective than alternative forms of treatment like assisted reproduction procedures (ART) (Cho and Seo 2014).

2.6.3. Assisted reproductive technologies (ART):

Male infertility can be managed by ART. These include laboratory techniques in which there is manipulation of male and female gametes for reproduction. These include ICSI, in vitro fertilization (IVF) and Intra-uterine insemination (IUI) (Sabarre *et al.,*

2013). Most ART centers use ICSI as priority option for infertility. In ICSI, oocytes in metaphase-II phase are prepared by removal of corona radiata and cumulus mass with hyaluronidase. Then one sperm from epididymis, ejaculate or testis is injected by a micropipette into oocyte cytoplasm, which has already been immobilized under oil. During injection the cytoplasm is aspirated and injected, to activate oocyte and improve fertilization. As spermatozoa influences oocyte activation, spermatozoa immobilization is persuaded via distorting sperm tail between injection micropipette and Petri dish bottom (Khorram *et al.*, 2001). IVF with ICSI (IVF/ICSI) also allows couples to become fertile. Literature has published offspring safety of IVF/ICSI. However, more study is required to determine risks associated with ART offspring's (Alukal and Dolores 2008).

2.6.4. Varicocelectomy:

Various techniques are available for varicocele treatment. These are divided into two types: surgical and radiological method (Inci and Gunay 2013). Surgical techniques are classified into microsurgical, laparoscopic and conventional open methods. Meanwhile, others areinguinal, retroperitoneal, scrotal and subinguinal approaches according to access level. Radiological treatment is alternatively used with less invasiveness and significance to control small collaterals which are not detected while surgery (Cho and Seo 2014; Binsaleh and Lo 2007). Laparoscopic varicocelectomy is an effective technique for the disease. Robotic surgery is another varicocelectomy option that has recently introduced (Chan 2012). However, microsurgical varicocelectomy is more reputed procedure among surgeons and is considered a gold standard owing to its greater surgical outcomes (Chan 2012; Cho and Seo 2014).

2.7. Probiotics and Gut micro-biota health:

Microbiologically sterile human fetus is colonized at birth by bacteria from the mother and the surrounding environment, and the complexity of resulting gut microbiota increases until weaning to solid foods. The human gut microbiota is dominated by bacteria belonging to three major phyla: Firmicutes, Bacteriodes and Actinobacteria. Several factors such as diet, genetic background and immune status affect the composition of gut microbiota (Turnbaugh *et al.,* 2009; Benson *et al.,* 2010), and both its composition and transcriptome are rapidly altered in response to dietary shifts (Hildebrandt *et al.,* 2009; Walker *et al.,* 2011). When mice were switched to a diet rich in fat, the gut microbiota composition altered within 24 hours and number of Firmicutes increased (Fleissner *et al.,* 2010). The gut microbiota has been suggested to be an environmental factor that affects adiposity and obesity (Backhed *et al.,* 2004). Obese

individuals have been reported to have an altered gut microbiota compared to lean controls and this was characterized by reduced numbers of Bacteriodes (Furet *et al.,* 2010). Germ free mice were leaner than conventionally raised counterparts and do not develop diet induced obesity (Backhed *et al.,* 2007). Increasing number of evidences suggests that the gut microbiota is involved in the pathophysiology of stress-related disorders. Chronic stress can cause behavioral, cognitive, biochemical, and gut microbiota aberrations. Gut bacteria can communicate with the host through the microbiota–gut–brain axis (which mainly includes the immune, neuroendocrine, and neural pathways) to influence brain and behavior. Also, there are reports showing that modulation of gut microbiota towards healthier composition (more bifidobacteria and lactobacilli) can prevent obesity and disease related to obesity (Aronsson *et al.,* 2010) and there are three approaches by which gut microbiota can be modulated towards positive balance: Probiotics, Prebiotics & Synbiotics.

2.7.1. Health benefits of probiotics:

Probiotics are defined as viable microbial dietary supplements which when administered in adequate amount exert beneficial effect on host health (FAO/WHO, 2012). These have attracted public attention because of their potential effectiveness for the prevention/treatment of cardiovascular diseases, certain cancers and immune disease. In addition, recent experimental studies have demonstrated the preventive effects of some bacterial strains on obesity. Among commensal bacteria, the lactic acid bacteria, bifidobacteria and lactobacilli are the most abundant probiotics in mammalian gut.

Response of energy metabolism to administration of probiotic has been reported to be strain dependent (Yin *et al.,* 2010). Induced obesity in mice by feeding 45% high fat diet for 8 weeks. Administration of *L. rhamnosus* PL60 resulted in significant body weight loss with specific reduction in white adipose tissue (Lee *et al.,* 2006). Various strains of *Lactobacilli, including L. casei, L. planatrum and L. reuteri,* have been shown to prevent diabetes (Matsuzaki *et al.,* 1997). *L. acidophilus* NCDC 13 supplementation on the progression of obesity in diet induced obese (C57BL/6) mice were evaluated and found to reduce the obesity in mice (Arora *et al.,* 2012). In a scientific experiment, supplementation of *Bifidobacterium breve* strain B-3 along with high fat diet for 8 weeks to diet-induced obese male C57BL/6J mice has been reported to significantly lower body weight in dose dependent manner, with significant differences beginning from 6 week. A significant difference in epididymal fat weights was also observed between control and B-3 administered group. Serum levels of total cholesterol, glucose, insulin and HOMA-IR

were found to be reduced whereas expression of fiaf gene in small and large intestine increased in B-3 groups in dose dependent manner (Kondo *et al.*, 2010).

Consumption of probiotics increases Testicular weight, increases the cross-sectional profile area of seminiferous tubules and their germ cell population, Counteracts Age-associated Testicular Atrophy, Induces Elevation of Serum Testosterone, increases the size of interstitial Leydig cell areas and Leydig cell numbers, ultimately recapitulates reproductive fitness (Poutahidis *et al.*, 2014).

Experimental subjects	**Fertility based disorders**	**Probiotics species alleviating fertility based disorders**	**References**
Human	Age related infertility	*Lactobacillus reuteri*	Poutahidis *et al.*, 2013 Levkovich et al., 2104
Human, Rat, Mice	Stress related infertility	*Lactobacillus helveticus* *Bifidobacterium longum*	Diop *et al.*, 2008 Zareie *et al.*, 2006; Messaoudi *et al.*, *2011* Kekkonen *et al.*, 2007; Ait-Belgnaoui *et al.*, 2014
Human, Rat, Mice	Hyperlipidemia related infertility	*Bifidobacterium sp.* *Lactobacillus sp.*	Korkmaz *et al.*, 2015 Kumar et al., 2007; Mann *et al.*, 1974
Rat, Mice	Obesity related infertility	*Lactobacillus plantarum* *Lactobacillus gasseri* *Bifidobacterium breve*	Lee et al., 2007; Kang *et al*; Kondo *et al* 2013; Million *et al.*, 2012
Mice, Rat	Diabetes related infertility	*Lactobacillus rhamnosus GG* *Lactobacillus johnsonii* *Lactobacillus casei*	Kim *et al*., 2013; Tabuchi *et al*., 2003 Honda *et al* 2012 Andersson *et al*., 2010; Yamano *et al*., 2006

CHAPTER – 3

Materials and Methods

MATERIALS AND METHODS

The present study was undertaken with the aim to evaluate beneficial effect of probiotics on male fertility induced changes by low protein diet.

3.1 MATERIALS

3.1.1. Probiotic Strain

Lactobacillus species were procured in lyophilized form from National Collection of Dairy Culture, (NCDC) Dairy Microbiology Division of National Dairy Research Institute, Karnal, Haryana (India). *Lactobacillus fermentum* NCDC-400 and *Lactobacillus rhamnosus RSI3* NCDC-610 were used for the present investigation.

3.1.2. Plastic wares and Glass wares

All the glass wares used in the present investigation were made of high-grade Pyrex glass were procured from Borosil Glass Works Ltd., India. Cover slips, latex gloves, filter papers, tissue paper were purchased from SD Fine Chem. Ltd., Mumbai. The glasswares, wherever used, were thoroughly cleaned, rinsed with ultrapure water and then heat sterilized at 250°C for 4 h. All the plastic ware purchased from Tarsons Pvt. Ltd. and Imperial Biomedics, India. Disposable plastic syringes were non-toxic and non-pyrogenic procured from Sigma Aldrich Chemicals (Norm-Ject, Henke-Sass Wolf GmbH, Germany). Autoclavable disposable tips for micropipettes were obtained from Labware, USA. Real time PCR plates, were purchased from Nunc Rosklide, Denmark or Thermo Fisher Scientific.

3.1.3. Chemicals

Chemicals used in this work were purchased from varies number of companies. Reagents such as NaCl, KCl, MgCl2.6H2O, NaH2PO4, KH2PO4, Na2HPO4, NaHCO3, CaCl2.2H2O, CuSO4, K2SO4, H2O2, citric acid, Low melting point agarose (LMP), fructose, EDTA, disodium salt, sodium pyruvate, glycerol and Tris-HCl were obtained from SISCO Research Laboratories Pvt. Ltd., India. Sodium lactate solution (60%), absolute ethanol (99.9%), boric acid, triton X- 100, and HEPES were purchased from Sigma-Aldrich, USA. SDS was purchased from Loba Chemie. Eosin and nigrosine stain were purchased from Merck Company Pvt. Ltd. *Lactobacillus* MRS Broth (cat no.-GM369) and *Lactobacillus* MRS Agar (cat no.-GM641) were purchased from HiMedia laboratories LLC, Pennsylvania, USA. TRIzol were purchased from invitrogen and Maxima SYBR Green/ROX qPCR Master Mix was purchased from Fermantas, Thermo

Fisher Scientific. Rodent testosterone ELISA kit was purchased from Endocrine technologies (cat no.- ERK R7016). All other chemicals purchased were of highest analytical and molecular biology grade available.

3.1.4 Equipment's

All the equipment used in experiments have been listed in Appendix-III

3.1.5 Reagents and buffers

Detail description of the media composition, buffers, reagents and stock solutions used are cited in Appendix I, II and III.

3.1.6 Media

A Modified Tyrodes's bicarbonate-buffered medium (sp-TALP) described in Parrish *et al.*, 1988 further as modified by Galantino-Homer *et al.*, 1997 was used in all experiments. The media (2X-stock) was initially prepared in the absence of Ca2+, BSA, pyruvate and bicarbonate, filter-sterilized and stored at 4^0C. Later sp-TALP medium was prepared by adding Ca2+ (2 mM), pyruvate (1 mM), NaHCO3 (25 mM) to 2X stock diluted in the ratio of 1:1 with distilled water. The volume was made up to 100 mL. For washing spermatozoa, 1 mg/mL BSA was added into it. After addition of the entire constituent, pH was adjusted to 7.5 and media was kept for 1 h in CO2 incubator to equilibrate, at the end of which again the pH was adjusted to 7.4.

3.2. METHODS

3.2.1. Objctive-1

3.2.1.1. Probiotic strains and growth media

Lactobacillus species were procured in lyophilized form from National Collection of Dairy Culture, (NCDC) Dairy Microbiology Division of National Dairy Research Institute, Karnal, Haryana (India). *Lactobacillus fermentum* NCDC-400 and *Lactobacillus rhamnosus RSI3* NCDC-610 were used for the present investigation. Propagation of culture was done in aseptic conditions with well sterilized equipment. *Lactobacillus* MRS Broth is recommended for cultivation of all *Lactobacillus* species and *Lactobacilli* MRS media are based on the formulation of De Man, Rogosa and Sharpe with slight modification. It supports luxuriant growth of all *Lactobacilli* from oral cavity, dairy products, foods, faeces and other sources. Protease peptone and beef extract supply nitrogenous and carbonaceous compounds, yeast extract provides vitamin B complex and dextrose the fermentable carbohydrate and energy source. Polysorbate 80 supplies fatty acids required for the metabolism of *Lactobacilli*. Sodium acetate and ammonium citrate inhibit Streptococci, moulds and many other microorganisms.

3.2.1.2. Maintenance of Probiotic Culture

The probiotic *L. rhamnosus* and *L. fermentum* were propagated in De Mann–Rogosa–Sharpe (MRS) broth and maintained in it by using 1% of pure probiotic culture inoculums and incubated for 18 hours at 37ºC. The probiotic were sub-cultured at least thrice prior to experiment and were stored at 4ºC. Finally the probiotics were concentrated by centrifugation, washing and finally suspended in saline to obtain desired colony forming units per millilitre (cfu/ml) which were confirmed by pour plate counting methods and adjusting the O.D to 0.9 (10^9cfu/ml) for *L. rhamnosus* and 1.1 (10^9cfu/ml) for *L. fermentum* at 600nm.

3.2.1.3. Bacterial purity confirmation

3.2.1.4. Gram's staining:

The microbial cells were gram stained examined by using under light microscope (1000X magnification). A loopful probiotic culture was placed on a clean glass slide to prepare a thin smear followed by heat fixed for 2-4 sec. Using spirit lamp in LAF. Fixed smear was flooded with crystal violet stain (10%) and allowed to remain for 1 minute. Crystal violet stain was washed off with distilled water and the slide was flooded with 1% iodine solution and allowed to remain for 1 minute and then washed off with distilled water. Smear was then flooded with 95% ethanol or acetone as decolorizer for 5 seconds and rinsed off with distilled water. In last step smear was counter stained with safranin (2.5%) for 45 seconds and gently rinsed off with distilled water. Slides were allowed to dry at room temperature and examined using light microscope under oil immersion (1000X magnification).

3.2.1.5. Enumeration of viable microorganism

The probiotic strain *L. Fermentum* and *L. rhamnosus* was propagated in De Mann–Rogosa–Sharpe (MRS) broth (Himedia Laboratories, Mumbai India) was maintained by using 1% inoculums and incubating it for 18h at 37°C. One ml of the activated MRS culture was centrifuged at 1800–2000g and the pellet obtained was washed three times with saline (0.9% NaCl) prior to use. Bacterial culture was serially diluted in 9 ml of pre-sterile saline tubes 10^1,10^2,10^3,10^4,10^5,10^6,10^7,10^8,10^9 from which took 200 µl of each diluted suspension was spread on petri-dish containing MRS agar (HiMedia Laboratories Pvt. Ltd) and incubated it for 24-48 hours at 37°C in incubator. Later, viable numbers of bacterial colonies were counted. Colony forming units of bacteria was adjusted accordingly to obtain 10^9,10^{10} and10^{11} CFU/ml finally by adding sterile saline before orally intubated to mice.

Table. 3.1 Composition of diet

Sr. No.	Component	NPD(g/kg)	LPD(g/kg)
1.	Starch Maize	425	485
2.	Sucrose	213	243
3.	Corn Oil	100	100
4.	Casein	180	90
5.	Cellulose	50	50
6.	Mineral mix (1)	20	20
7.	Vitamin mix (2)	5	5
8.	DL-methionine	5	5
9.	Choline Chloride	2	2

3.2.1.6. Animal model and grouping:

Male Swiss albino weanling mice were procured from the small animal house of National Dairy Research Institute (NDRI), Karnal, Haryana, India. All animals were of about 3 weeks (13-15g) when obtained from a small animal house. Experiments were initiated after one week of acclimatization when mice obtained about similar weight. All mice were housed in polypropylene cages (3 mice per cage) at the condition. The animal study was approved of the institutional animal ethics committee (approval no. 41-IAEC-18-59; dated, 27.01.2018). Animals were distributed into six groups NP+PBS (Normal protein+ phosphate buffer saline), NP+RSI3 (Normal protein + *L. rhamnosus* NP+FER. (Normal protein +*L. fermentum),* LP+PBS (Low protein+ phosphate buffer saline), LP+RSI3 (Low protein + *L. rhamnosus),* LP+FER.(Low protein+*L. fermentum).* Under the first objective total 36 animals (Mice) were dividing them in six groups of six animals each and housed in polypropylene cages. Animals were maintained on the pellet diet (BD) and tap water was provided *ad libitum* during the period of acclimatization for one week available in the Small Animal House, NDRI. The control group of the animal was fed with self-prepared control diet and orally intubated with 200µl sterile saline. Whereas in case of experimental groups, each animal was orally intubated with 200µl of saline containing 10^9 CFU of *L. fermentum* and *L. rhamnosus* respectively with 20–22G gavage needle along with self-prepared normal protein and low protein diet for 56 days continuously. At the end of the experimental feeding schedule, allow to mating assay by taking 1:2 ratio male and female mice same aged group to get male offspring for 2nd objective. Maintain the all-male offspring on chow diet all mice euthanized with diethyl ether overdose then blood and tissue samples were collected aseptically for further analysis.

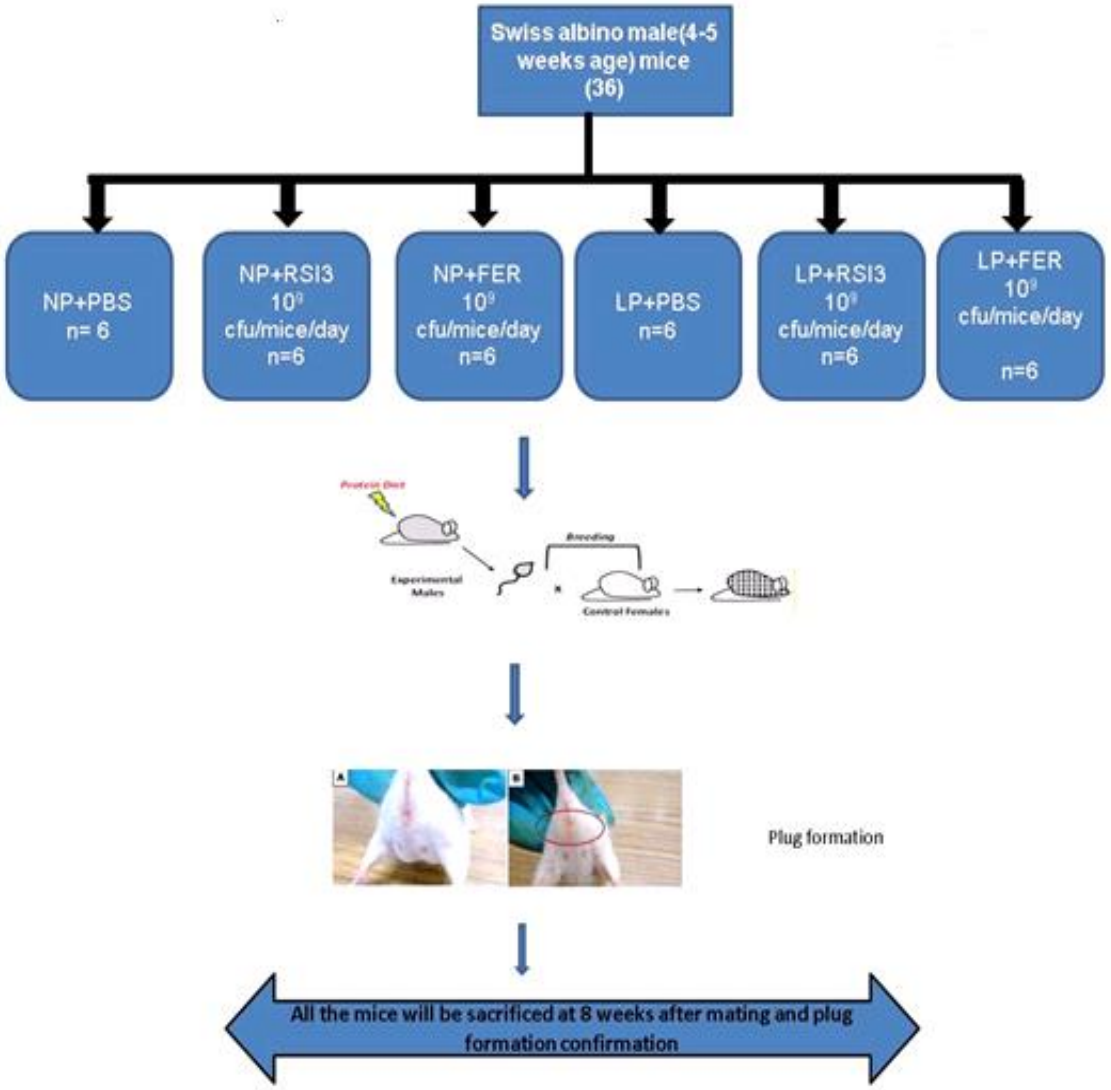


Fig.3.1 Grouping of animals

3.2.1.6.1. Assessment of Body Weight and Food Intake

Body weight and food intakes by collection of spillage, were measured once in a week.

3.2.1.6.2. Collection and Processing of Blood:

Overnight fasted mice were first anesthetized by slight exposure of diethyl ether in a glass chamber for a brief period. Blood was collected from the orbital venous plexus using a heparinized capillary tube and storage vials wetted with EDTA solution (2 U/μL). Immediately after collection, one drop of blood used to determine the blood glucose level and remaining blood was centrifuged at 4000 x g for 20-25 min at 4°C in a refrigerated centrifuge. Plasma was separated and stored at -70°Cuntil further analysis.

3.2.1.6.3. Estimation of Superoxide Dismutase (SOD) Activity in Testicular and in Liver tissues

The enzymatic activity of SOD was assayed by using the method of Marklund and Marklund,(1974). Firstly tissue was homogenized in phosphate buffer and centrifuged at 8000g for 20 minutes after centrifuged separated supernatant and discarded debris containing centrifuge tube. Total volume was made to 3 mL by adding Tris-HCl buffer (50 mM, pH 8.2; Appendix 2.4) containing 1 mM diethylene triamine penta-acetic acid and 0.2 mL of 2mM pyrogallol (Appendix 2.5). A blank was prepared without the addition of the sample. The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm against a reference cuvette containing 3 mL Tris-HCl buffer. The increase in absorbance was 0.02 per min in the absence of SOD. The inhibition of pyrogallol auto-oxidation was brought about by SOD, which was employed for the determination of enzyme activity. A unit of the enzyme was defined as the amount of enzyme that inhibits the reaction by 50%.

3.2.1.6.4. Estimation of catalase activities in testicular and in liver tissues

The catalase enzyme activity was estimated spectrophotometrically using the method of Aebi,(1984). The reaction mixture in the total volume of 3 ml contained 2 mL of homogenized tissue sample inappropriate dilution with phosphate buffer (50 mM, pH 7.0; Appendix 2.2) and 1 mL of H_2O_2 (30 mM; Appendix 2.3) at 25°C (room temperature) against a blank containing 1 mL phosphate buffer instead of substrate (H_2O_2) and 2 mL appropriately diluted tissue sample. The reaction was started by addition of H_2O_2 and decomposition of H_2O_2 was shown by the decrease in absorbance at 240 nm using Specord 200 double beam UV/visible spectrophotometer (Analytik Jena, Germany). The initial absorbance was approximately A =0.500 and the decrease in absorbance was followed for 1 min.

3.2.1.6.5. Histological examination

Testes were surgically removed from the mice and washed with pre ice-cold normal saline (0.9% NaCl) and 20 mM EDTA to remove blood. Testis was cut into small pieces, and fixed immediately in 10% phosphate buffered formalin for 48 h. The testis tissues were then transferred to 70% ethyl alcohol and stored until processed. The tissue specimens were processed, embedded in paraffin, sectioned at 0.1 μm and stained with hematoxylin and eosin (H&E) for histological examination under a light microscope. At least 10 slides of each sample were scored for histology of testis.

3.2.1.6.6. Processing of spermatozoa

3.2.1.6.7. Assessment of sperm viability by Eosin-Nigrosine staining

The live spermatozoa were determined using eosin-nigrosin as a vital dye. 10% (w/v) nigrosin and 0.6% (w/v) eosin yellow water-soluble in 3% sodium citrate dihydrate; maintain to pH 7 by adding few drops of 0.1 M NaH2PO4 or 0.1 M Na2HPO4. According to previously illustrated method using bright-field optics Nikon, Eclipse 80, magnification 1000X (Tomar N.S., 1997). 10 µL of Sperm suspension (10^6cells/mL) was mixed with an equal volume of eosin and nigrosine stain on a fresh dried glass slide. Sperms were examined under oil immersion microscope using 100X objective and 200 sperms were counted per slide. The sperms which had taken dye were counted as dead and the one excluded the dye was counted as live.

3.2.1.6.8. Assessment of membrane integrity by hypo-osmotic swelling test:

The membrane integrity of sperm was evaluated by using the HOS test which was mention in Jeyendran *et al.*, 1984. The hypo-osmotic solution (150 mOsm/L) was prepared by dissolving 7.35 g sodium citrate and 13.5 g fructose in 1 L of distilled water. The solution was stored at 4^0C till used.10 µl of Sperm suspension was mixed with equal volume of HOST solution kept at 37^0C for 1.30 hours. A total 200 spermatozoa were counted in different fields at 400X under phase contrast microscope and percentage of spermatozoa positive to HOS test (having coiled tails) were counted.

3.2.1.6.9. Measurement of Testosterone

Testosterone concentration in serum and cell suspension was measured by Rodent Testosterone ELISA Kit (Endocrine Technologies) and following steps were used as manufacturer's instruction.

Principal of assay

The testosterone quantitative test is based on a solid-phase enzyme immunoassay based on competitive binding method. A sample containing unknown amount of testosterone to be assayed (unlabeled antigen) was added to a standard amount of a conjugated testosterone (labelled antigen). The labelled and unlabeled antigens were then allowed to compete for high affinity binding sites of anti-testosterone antibodies coated on the plate. The bio specific reaction takes place when incubated for 1 hour at 37ºC. After incubation, free antigen was washed away then TMB substrate solution was added and incubated for 20 min. A blue colour developed was stopped with stop solution (2N HCl). Absorbance was measured at 450 nm using ELISA plate reader. A standard curve

was prepared using values from standards by subtracting absorbance values for blank tubes. The amount of labelled antigen in the sample is reversibly proportional to the concentration of unlabeled antigens. As the concentration increases in the sample the colour intensity decreases proportionally. The results for unknown may be read directly from this standard curve **(Fig. 3.2)** using either manual calculation or by a suitable manual program.

Materials provided

1. Micro titer 96 wells plate coated with anti-testosterone antibody,
2. HRP conjugate,
3. Lyophilized standards, 0, 0.1, 0.5, 1.0, 2.0, 10, 20ng/mL (QC1 (0.5ng/mL) and QC2 (2.0ng/mL)/vial, reconstitute in 1 mL with standard/sample diluent
4. Standard/sample diluent, 20 mL
5. TMB colour reagents
6. Stopping solution
7. 20X wash buffer

Reagents preparation:

1. Wash buffer was prepared by diluting 1 part with 19 parts of distilled water, excess amount was stored at 2-8^{o}C for couple of weeks.
2. Lyophilized standards should be diluted in 1mL standard dilution buffer and stored at -20^{o}C for long term use.

Assay protocol:

1. All reagents should be allowed to reach room temperature (18- 25^{o}C) before use
2. 50µl of standard, samples and controls were pipetted into appropriate wells
3. A 100 µl of testosterone enzyme conjugate solution was added to each well, shaken well for 1-2 minutes and incubated at 37^{o}C for 1h
4. The content of the wells was discarded and washed 5 times with wash solution (250-300µl) per well. The plate was inverted and firmly tapped against absorbent paper to remove any residual moisture.
5. Including the blank, 100 µl of TMB colour solution was added into each well. Pipetting order should be remembered (room temperature should be 20-25^{o}C).
6. Plate was incubated for 20 minutes at room temperature.
7. To stop the reaction, 50 µl of stopping solution was added in the same sequence that the substrate solution was added and mixed gently.
8. The absorbance of blue coloured solution was measured at 450nm with a microplate

reader.

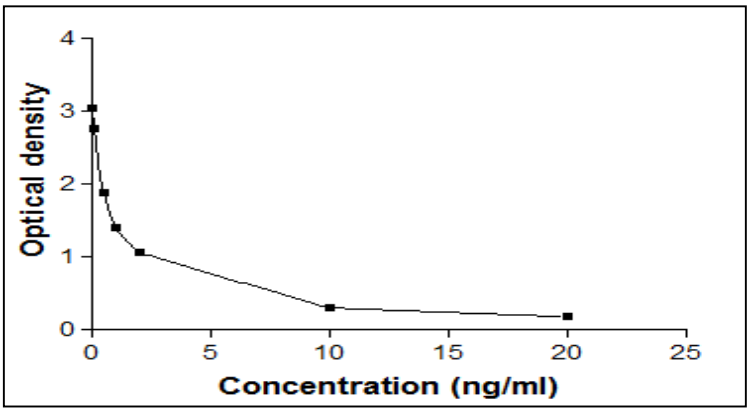


Fig 3.2: Standard curve of testosterone

Calculation

1) Mean absorbance values (A) were calculated for each set of reference standards, samples, controls, samples and blanks.

2) Values for blanks were subtracted from those for standards, controls and samples.

3) B/B0 values were calculated by dividing each value by the value for zero-standard.

4) For the standard, a graph was plotted on semi-log graph paper with B/B0% values on the ordinate and the testosterone concentrations (pg/mL) on abscissa.

5) Using the graph testosterone concentrations for unknown samples was read off.

3.2.1.6.9. Analysis of quantitative genes expression:

RNA isolation: Total RNA was isolated from- Mice testes

Homogenization: Tissue samples testis (50 to 100 mg) were homogenized in 1 ml of TRIZOL reagent in mortar and pestle per using a glass-Teflon or power homogenizer (e.g. Polytron, Tekmar's TISSUEMIZER). The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization.

Homogenized sample were vortexed thoroughly and incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes and centrifuged to remove cell debris and supernatant was transferred to a new tube.

Phase separation:

1. Molecular grade chloroform (200 µl per 1mL of TRIzol reagent) was added to the microcentrifuge tubes.

2. Samples were vortexed vigorously for 1-2 min and incubated at room temperature for 10 min followed by centrifugation at 12000 g for 10 min at 4^0C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an

interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. Aqueous phase was carefully pipetted out to a fresh tube without disturbing the interphase.

RNA precipitation:

1. RNA was precipitated from the aqueous phase by treating with 600 µl of isopropanol for 1mL TRIzol reagent.

2. The mixture was incubated at room temperature for 10 min followed by centrifugation at 12000 g for 10 min at 4^0C to obtain the RNA precipitate (often invisible before centrifugation) forms a gel-like pellet on the side and bottom of the tube.

3. The supernatant was removed and RNA pellet was washed with 1mL of 75% ethanol. Pellet was loosened by tapping and centrifuged at 8500 g for 5 min at 4^0C.

4. Ethanol was removed and pellet was air dried for 15-30 min.

5. RNA pellet was dissolved in 40 µl of DEPC treated water and immediately stored at -20^0C until further use.

Quantification, purity and integrity check of RNA

Isolated RNA was quantified by spectrophotometric analysis on nanodrop by checking UV absorption of the sample at 260 nm and 280 nm for the determination of RNA concentration and purity. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. Purity of RNA and concentration was displayed in the desktop computer attached with the machine. Samples with high purity and yield were further used for cDNA synthesis. RNA integrity was checked by agarose gel electrophoresis.

cDNA synthesis

Revert aid H Minus First strand cDNA synthesis kit (Fermentas, USA) was used for cDNA synthesis and following steps were used as manufacturer's instruction. RT-PCR was carried out in two steps: first strand synthesis (RT reaction) and PCR reaction (amplification of cDNA).

Scheme for cDNA synthesis from total RNA

Preparation of RNA-primer mix:

The RNA concentration taken for cDNA synthesis was 1.5 µg/ reaction. This required sample specific calculation for the volume of RNA to be taken. Based on the concentration of RNA (ng/µl) obtained from the nanodrop reading a volume of RNA which could deliver 1000 ng (1µg) was piptted. For example if nanodrop reading for a sample was 300 ng/µl, then a volume of 5 µl (1500/300=5) was used. Oligo dT primer

provided in the kit was added @ 1 µl. Total volume made upto 12 µl by adding DEPC treated water.

Reagents	Concentration	Volume (µl)
RNA	1µg	(X) sample dependent
Oligo dT primer	0.2µM	1
DEPC- treated water		12-(X+1)
Total volume		**12**

To denature the RNA and remove secondary structures, the RNA was heated at 65°C for 5 min for annealing and the reaction was slaked by placing the tube immediately on ice.

Preparation of Reverse Transcriptase reaction mix

Reagents	Concentration	Volume (µl)
5X Reaction buffer	1X	4
RibolockTM RNase inhibitor (20 U/µl)	1U	1
10mM dNTP mix	1µM	2
Revert AidTM M-MuL V Reverse transcriptase (200 U/ µl	10U	1

Finally the reaction mixture was incubated at 42°C for 1 hour (extension), followed by inactivation of enzyme at 70°C for 5 minutes and the cDNA was stored at -80°C till further use.

Control reactions for cDNA synthesis:

To exclude the chances of contamination, pipetting errors or to ensure the intactness of the kit components three different sets were employed.

Negative control-I (No Reverse transcriptase):

Reagents	Concentration	Volume (µl)
RNA (any sample)	1µg	X sample dependent
Primer	0.2 µM	1
5 X Reaction buffer	1X	4
RibolockTM RNase Inhibitor (20 U/µl)	1U	1
10mM dNTP mix	1µM	2
Water		20-(X+1+4+1+2)
Total volume		20

Negative control-II (No template):

Reagents	Concentration	Volume (µl)
Primer	0.2 µM	1
5 X Reaction buffer	1X	4
RibolockTM RNase Inhibitor (20 U/µl)	1U	1
10mM dNTP mix	1µM	2
Water		12
Total volume		20

Positive control:

Reagents	Concentration	Volume (µl)
GAPDH-RNA (provided with kit)	1µg	2
Primer	0.2 µM	1
5 X Reaction buffer	1X	4
RibolockTM RNase Inhibitor (20 U/µl)	1U	1
10mM dNTP mix	1µM	2
Water		12
Total volume		20

3.2.1.6.10. PCR amplification of GAPDH cDNA

Positive control reaction product obtained from the first strand cDNA synthesis was tested for presence of cDNA by amplifying the sample by PCR using GAPDH specific primers and protocol provided in kit following cycling condition :-

Conditions	Temperature (ºC)	Time	No. of cycles
Initial denaturation	95	5 min	1
Denaturation	98	20 Sec	35 cycles
Annealing	56	15 Sec	
Extension	72	15 Sec	
Final Extension	72	7 min	1

Primers for RT-PCR

The sequence of primers is given in Table 3.2 and annealing temperature of primer was optimized. Each of 10µl PCR reaction mixtures were subjected to different annealing temperatures. The reaction components were subjected to following cycling conditions:-

Cycle No.	Description	Temperature (ºC)	Time	No of cycles
Cycle1	Initial denaturation	95	5 min	1
Cycle2	Denaturation	98	20 Sec	35 cycles
	Annealing	55-60	15 Sec	
	Extension	72	15-30 Sec	
Cycle3	Final Extension	72	7 min	1

Table.3.2 Details of optimized primers used for real-time PCR amplification of genes under study

Gene name	Sense Primer (5'--------3')	Anti-sense Primer (5'--------3')	Primer concentration	Annealing Temp.	Template dilution		Product length
GAPDH mactin	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG	0.5 pM F x0.5 pM R	60°C	20 X	10 X	150
P450scc	GGAGGAGATCGTGGACCCTGA	TGGAGGCATGTTGAGCATGG	0.5 pM F x0.5 pM R	60°C	20 X	10 X	91
17 β HSD	AATGTGCTTTCCATTTGCAAGGT	ATGCCACTGGCAGAGGAGATG	0.5 pM F x0.5 pM R	60°C	20 X	10 X	98

3.2.1.6.11. Quantitative Real-Time RT-PCR

Quantitative Real-Time PCR (qRT-PCR) was carried out by two-step method. In the first step, first strand of cDNA was synthesized from the total RNA using reverse transcriptase system and in second step, PCR amplification was carried out using gene specific primers. The quantification of all gene transcripts was done by using Maxima SYBR Green qPCR Master Mix (Fermentas, USA). A primer matrix was performed for each gene to determine the optimal primer concentrations. The most consistent C_t values in duplicate samples and less standard error were chosen as optimized primer concentration for respective primers and were used for assaying the expression of genes under study. Details of optimized primer concentrations are mentioned in Table.3.2.

Different dilutions of the template were checked to optimize the template concentration. Each gene was performed in 4x dilution for cell samples and 20X dilution for tissue samples. Each reaction mixture consisted of 7.5 µl of Maxima SYBR Green qPCR Master Mix (Fermentas, USA), 0.5 µl of cDNA with the 0.25 µl of forward and 0.25 µl of reverse primers which were subjected to change according to the optimized primer concentration for respective genes and rest of the reaction mix volume was filled up with nuclease free water in a total reaction volume of 15 µl. Reaction was performed in duplicate for each sample in a Roche Light Cycler 480 II system. PCR conditions were 95°C for 10 min, then 40 cycles consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. To determine the specificity of the PCR reaction (a single specific peak and to detect primer/dimer formation), a dissociation curve was generated after completion of amplification using the machine's own programme.

For real-time PCR experiments the amount of mRNAs were normalized relative to the abundance of an endogenous control, GAPDH to account for the differences in total

RNA concentrations between samples. The mean sample threshold cycle (C_t) and mean endogenous control C_t for each sample were calculated from duplicate wells. The relative amount of target gene expression for each sample was calculated using the formula $2^{-(\Delta\Delta CT)}$ (Livak and Schmittagen, 2001). In order to check the DNA contamination, for each RNA sample, a control reaction was set up in which reverse transcriptase enzyme was omitted during cDNA synthesis. For each gene, a negative control was included for the real time PCR assay in which cDNA was not added.

3.3 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software and Excel. All the data were expressed as mean ± standard error of the mean (S.E.M.) and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A minimum of three independent experiments were performed for each experimental condition tested. The P-value of <0.05 was considered significant.

CHAPTER – 4

Results and Discussion

4. RESULTS & DISCUSSION

Nutritional habit is one of the major factors influencing the reproductive health (Giahi *et al*., 2015). Functional foods like Probiotics may be used as the best alternative because they confer health benefits to host and are free from side effects. A study on testicular tissue shows that mice consuming Lactobacillus reuteri in their drinking water have significantly increased seminiferous tubule cross-sectional profiles, increased spermatogenesis and Leyding cell numbers (Poutahidis *et al.,* 2014). The present study was carried out to assess in vivo ameliorative effect of Lactic acid bacteria *L.fermentum* (LF**:** *NCDC 400*) and *L. rahamanosus* (L.RSI3**:** *NCDC 610*) on dietary protein deficiency induced infertility in *swiss albino* paternal male mice.

4.1. Purity confirmation of bacterial cultures

Purity of *L.fermentum* (LF: NCDC 400) and *L. rhamanosus* (L.RSI3: NCDC 610) was confirmed by gram staining under oil immersion microscope. The *L. fermentum cultures* and *L. rhamanosus* have rod shape bacteria (4.1A and B). Further, the morphological feature of *L. fermentum* was determined by plating on agar plates; colonies are generally flat, circular and rough. (Figures 4.1C and D) which indicates that *L. fermentum* and *L. rhamanosus* both are gram negative strains.

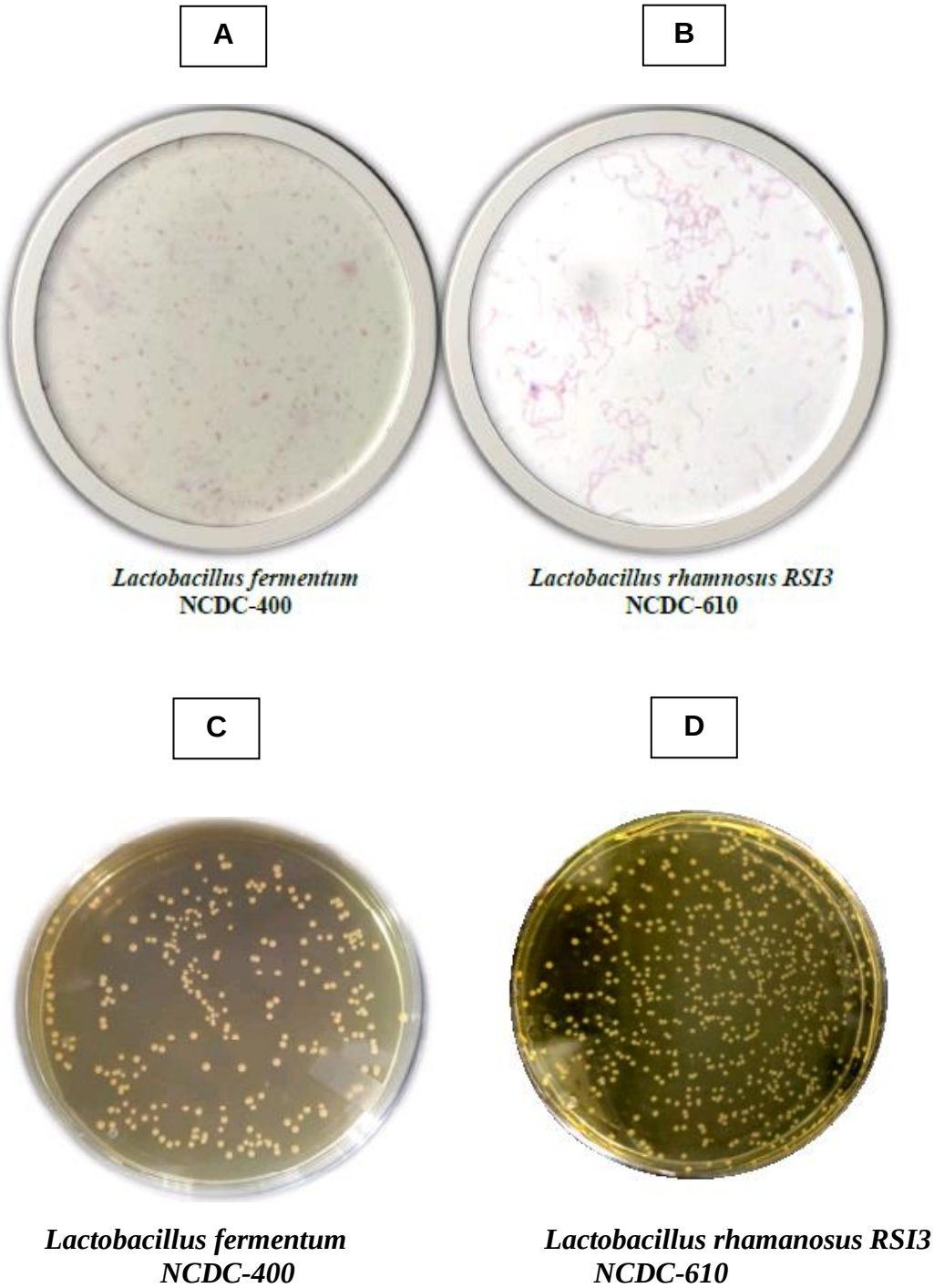
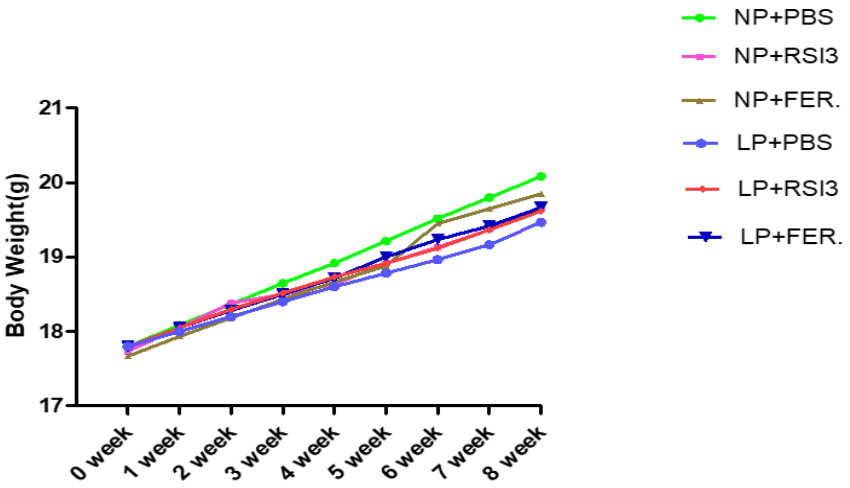


Fig. 4.1: Purity checks of *Lactobacillus fermentum* NCDC-400 *Lactobacillus rhamnosus* RSI3 NCDC-610 by gram staining (A, B) and colony morphology examination (C, D) under light microscope (1000X magnification).

4.2. Objective-1: To study the effect of probiotic on low protein diet induced changes in male fertility

4.2.1. Effect of probiotic on body weight and feed intake

In the experimental animals, the effect of feeding of probiotic *rhamnosus RSI3* and *fermentum* with normal protein (NP) and low protein (LP) diet on body weight and food intake have been expressed in Table 4.10. Results of this experiment indicates that the body weight of probiotic fed groups of animals increased as per the experimental period increased (P <0.05). Finally, the body weight gain was significantly higher (P<0.05) in NP+PBS 33.05±0.39 fed group and probiotics fed group of animals and 33.5 ± 2.331 g respectively than those of LP+PBS. When the results of body weight gain/loss were compared among NP+PBS, LP+PBS and all types of probiotic fed groups, it has been observed that the body weight gain was highest in NP +PBS and probiotics fed animals (group-3 and group-4) than LP+PBS. The body weights of LP+PBS of paternal male mice were progressively decreased with increase in feeding periods. The average food intake of animals were 5-7 g/day/mice in all the groups and this did not differ among all the groups as it is presented in table 4.2. (Yin *et al.*, 2010) also reported an increase in body weight after probiotic consumption. Our results also shows the amelioration in body weight of low protein diet fed mice after oral administration of probiotics.



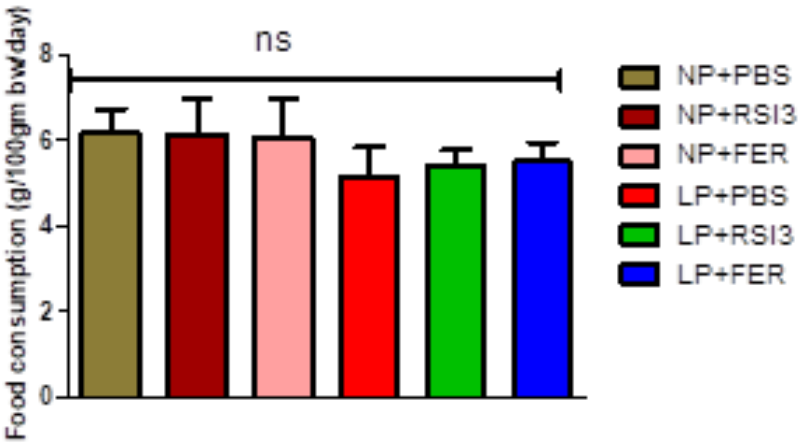


Figure 4.2.1 (A): Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on daily food consumption in paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8weeks. The values are represented as mean ± SEM (n=6).

4.2.2. Organ weight in male mice

Body condition indices (BCI) have been used to correlate the fitness parameters related to reproduction and survival in mammals and other taxa (Schulte-Hostedde *et al.*, 2005). The level of individual fitness called individual quality is often estimated by calculating different somatic indices, i.e., the relative size of internal organs (Norrdahl *et al.*, 2004).

The body organ weight of paternal male mice were recorded till 8 weeks of study and depicted in Table 4.2.1.We found significantly ($P < 0.05$) higher spleen weight in the NP+PBS and probiotic fed groups compare to LP+PBS. Similarly, significant increase in pair testes weight of NP+PBS and probiotic fed groups compare to LP+PBS. The decrease in the weight of testis of mice fed with low protein diet occurs due to protein deficiency which leads to the degeneration of testis tissues. We did not find any significant changes in the weights of liver and epididymal fat amongst all the groups.

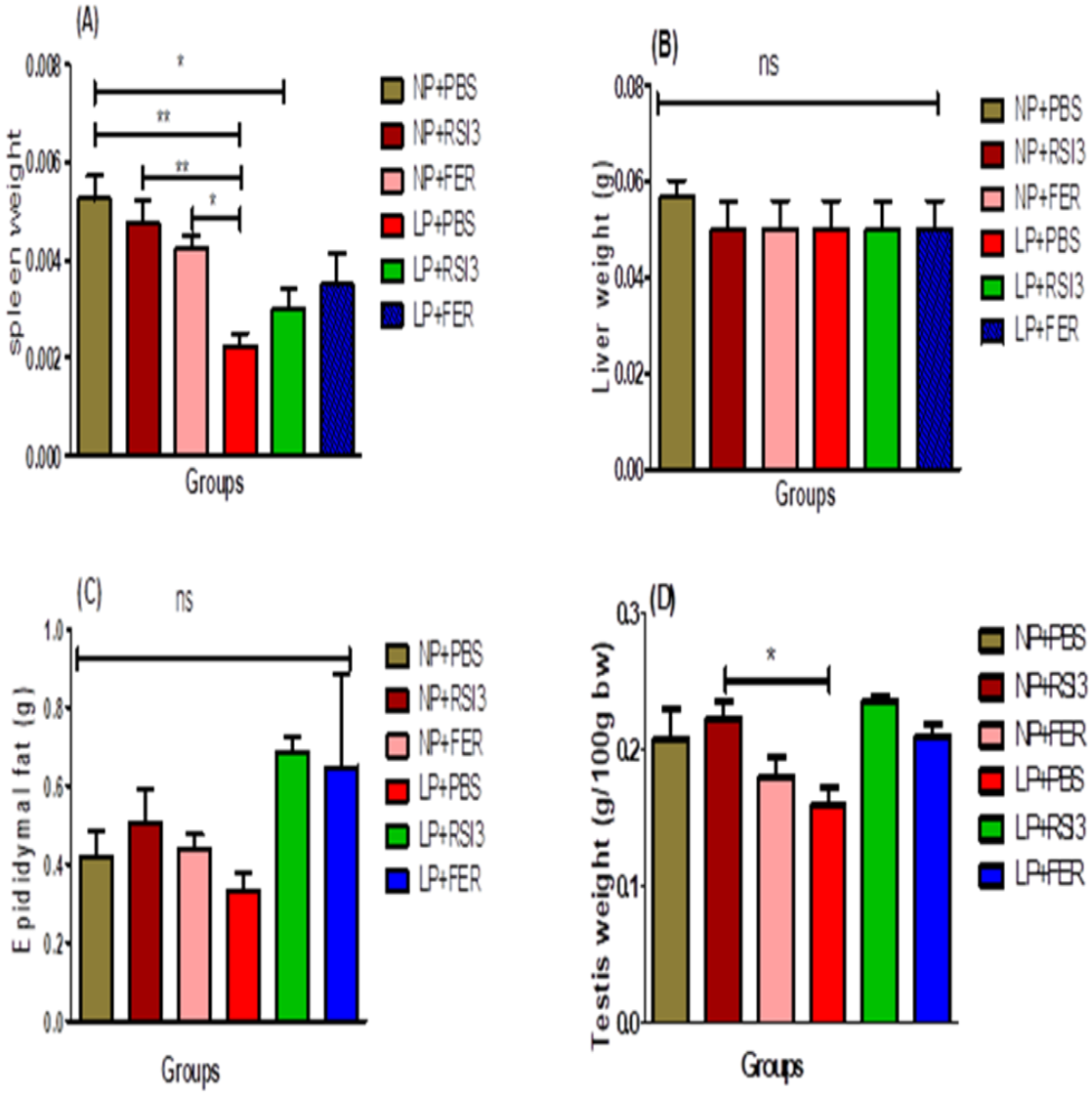


Figure 4.2.2: Effect of feeding of probiotic by oral gavaging and different protein diet (Normal and low) for eight weeks on (A) Spleen weight (B) Liver weight (C) Epididymal fat (D) Paired testis weight of paternal male mice. The values are expressed as means ± SEM (n = 6). The asterisk indicates the significant difference (* $p<0.05$, **$p<0.01$; One way ANOVA).

Table 4.2.1: Body and organ weight values of paternal male mice @ 8 weeks of age

Parameters measured	NP+PBS	NP+RSI3	NP+FER.	LP+PBS	LP+RSI3	LP+FER.
Body weight (g)	33.05±0.39	33.72±3.600	35.8±1.490	28.9±1.528	33.5±2.331	31.4 ± 1.52
Liver weight (g)	2.15±0.351	1.92±0.459	1.8±0.284	1.4±0.2708	1.8± 0.206	1.67 ± 0.17
Pair testes weight (g)	0.5±0	0.482±0.023	0.43±0.053	0.4±0.081	0.33± 0.15	0.37±0.095
Spleen weight (g)	0.12±0.053	0.1±0.03	0.1±0.078	0.01±0.05	0.1± 0.022	0.1± 0.037
Epididymal fat weight (g)	0.4±0.025	0.48±0.33	0.42±0.43	0.28±0.55	0.68±0.66	0.52±1.0

4.2.3. Fasting blood glucose level

Fasting glucose levels are used to distinguish diabetic mice from normal. Therefore under the present study, fasting blood glucose levels were measured to know sub-chronic oral toxicity of L. fermentum and L.rhamanosus to mice. The LPD suppressed the elevation of glucose level. (Kitada *et al.*, 2018) reported decrease in fasting blood glucose on feeding low protein diet to diabetic rats. The effect of feeding of NPD and LPD in comparison to probiotic diet. The results of overnight fasting animals on blood glucose level at 0 and 8 weeks of experimental period are shown in figure (4.2.3).

Statistically, there is no significant variation in fasting blood glucose level at day 0 among all the groups of paternal male mice. We found significantly lower values of fasting blood glucose level after 8 weeks feeding in the LP+PBS in comparison to NP+PBS and probiotic treatment groups. So, our results are in line with the results of earlier reports.

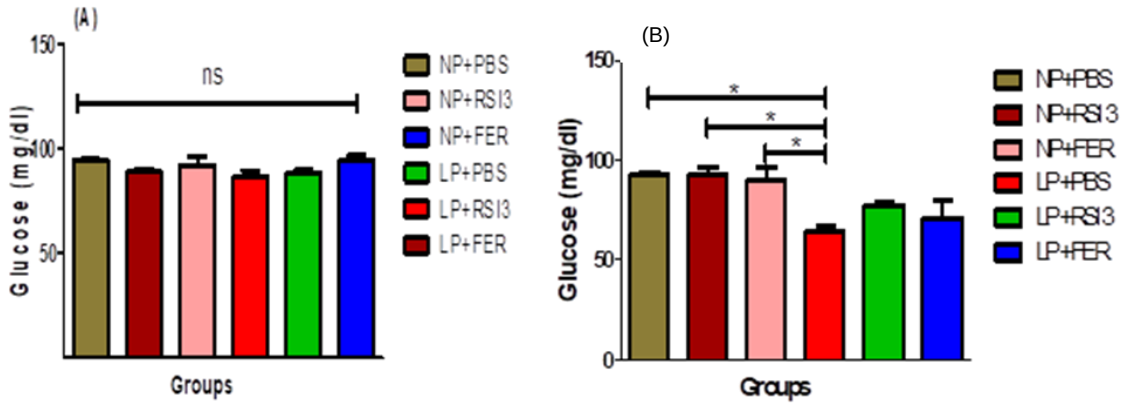


Figure 4.2.3: Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on fasting blood glucose level in paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8 weeks. The values are represented as mean ± SEM (n=6)

4.2.4. Sperm Count and Testosterone

Sperm count was determined by haemocytometer (Salisbury *et al.,* 1978) and was calculated as millions/ml of cauda epididymal fluid. Total number of sperm was counted by counting the number of sperm after suspension in per ml of media. The sperm count increased significantly in the NP+PBS, NP+RSI3, LP+FER and LP+RSI3 groups when compared to the LP+PBS group by the end of both the treatment periods (Figs 4.2.4). Serum concentrations of testosterone were estimated from six different groups of paternal male mice and three samples from each group were taken for analysis. The serum concentration of testosterone in paternal male mice of different treatment groups after 8 weeks of study have shown in Fig. 4.2.4. The results shown that the significant (P<0.01) increase in serum testosterone levels was in LP+ RSI3 (3.5±0.06ng/dl), LP+FER (2.9±0.08 ng/dl) and NP+PBS (7.76±0.02 ng/dl) groups in comparison with the LP+PBS (2.5±.001 ng/dl). The significant decrease in the testosterone level of low protein diet fed paternal male mice as a result of low formation of testosterone due to protein deficiency. The protein deficiency hampers the mechanism of testosterone formation (Hanai *et al*, 2007).

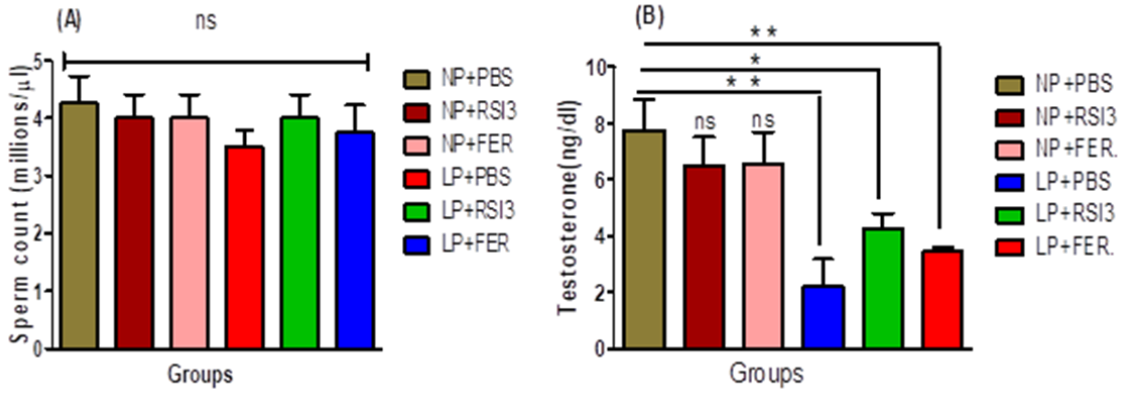


Figure 4.2.4: Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on total sperm Count (millions/ul) (A) and serum testosterone (ng/dl) (B) in paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8 weeks. The values are represented as mean ± SEM (n=6)

4.2.5. Eosin-Nigrosin assay

Eosin-nigrosin stain is used to count the percentage viability of sperm cell. Nigrosin form a dark background on which cells are visualized. Eosin stains dead cells as dark whereas live cells are visualized as bright against a dark background. By visualizing number of

viable cell per hundred counted sperm, There was significant reduction (*p<0.05) in the percentage of live sperms of cauda epididymides of paternal male mice fed with LPD+PBS (46.67±1.2) as compared to the NPD+PBS (60.00±2.887) LP+FER and LP+RSI3 (Fig.4.2.5) . Statically, there was no significant difference in the percentage of dead sperms amongst different treatment groups. The significant decrease in percentage of live spermatozoa in groups fed with low protein diet is due to the change in their functionality as a result of nutrient deficiency.

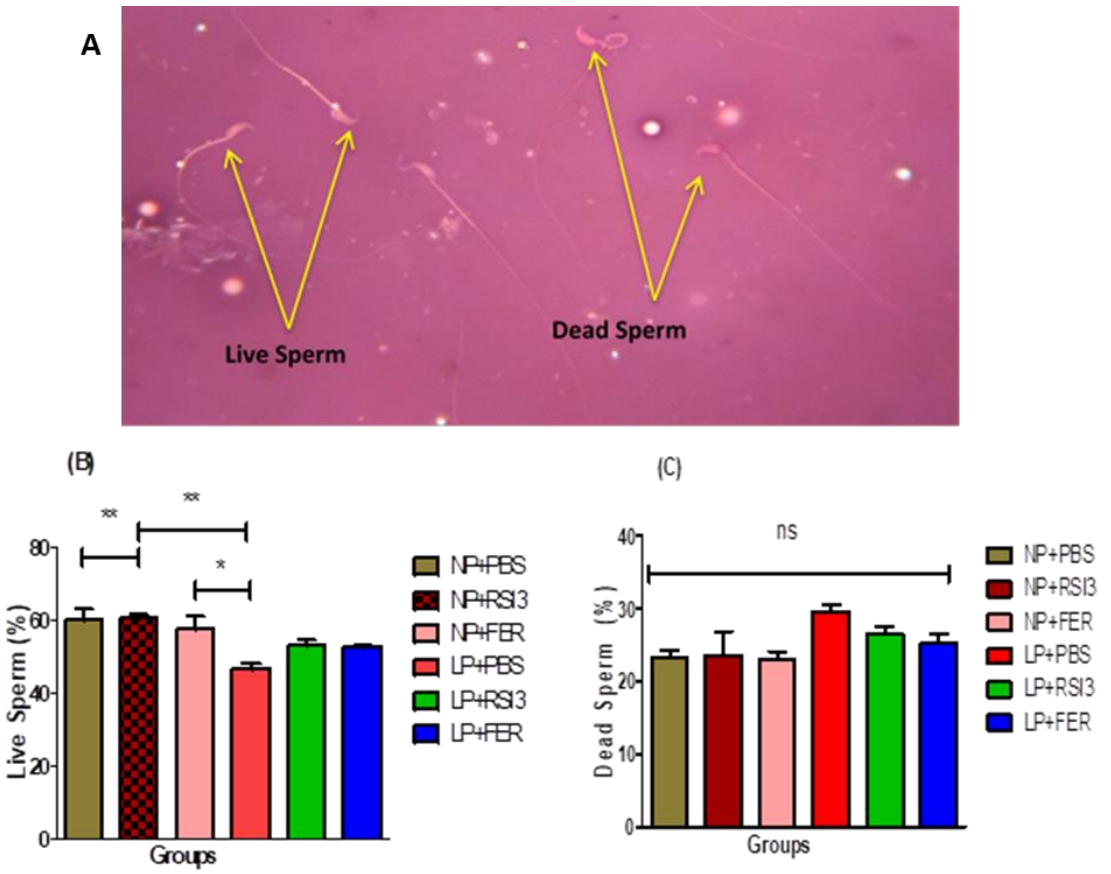


Figure 4.2.5: Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on sperm viability in paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8 weeks. Paternal male mice sperm cells are stained with eosin and nigrosin dye. (A) Dark pink coloured sperm represent dead sperm; (B) Live sperm excluded the stain, hence appears light in color. (B) And (C) Percentage live sperm and dead sperm. The values are represented as mean ± SEM (n=6)

4.2.6. HOST assay

Hypo-osmotic swelling test (HOST) is a WHO recommended test for the assessment of membrane integrity of sperm cell. When placed in a hypo-osmotic solution sperm

membrane get swelled due to osmosis of solution inside the cell. The swelling is prominent in the tail of sperm leading to its coiling. Viable cells have intact membrane thus form coiled tails whereas dead cells with non-intact membrane remains straight.

In our study, the percentage of coiled sperm decreased significantly (*p<0.05) in low protein diet group (35.50±1.285) as compared to group fed with NP diet (48.33±3.676) and LP diets with probiotics (Fig. 4.4). There was no significant change in membrane integrity of cell of remaining group compared to control group.. As depicted by decrease in coiled tails the change in membrane protein composition become unbalanced after low protein diet. Our results are in connection with previous report of change in membrane integrity on low protein diet consumption.

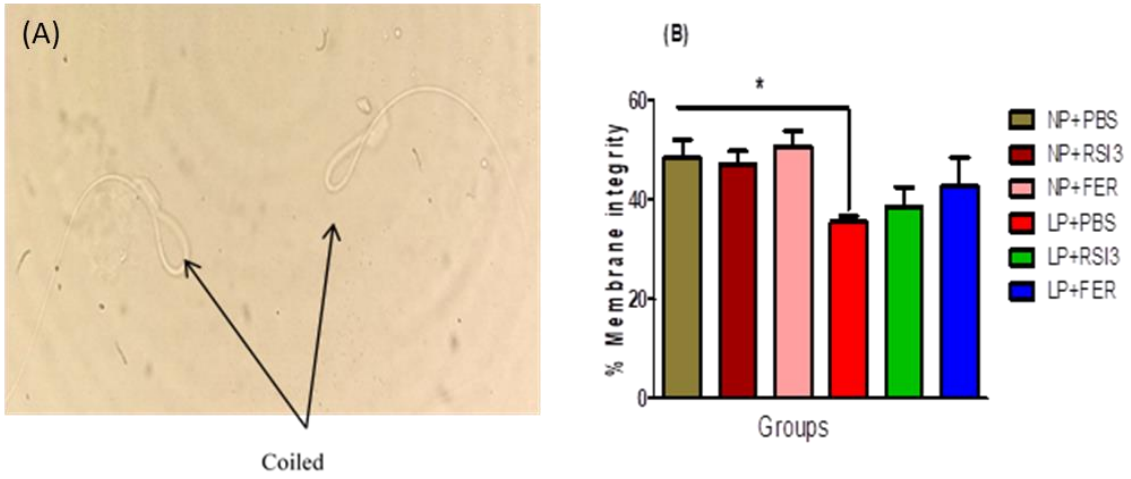


Figure 4.2.6: (A) Membrane integrity of father mice spermatozoa by Hypo-osmotic swelling Test (HOST). (1) Straight tail represents lost membrane integrity, while (2) Coiled tail represents maintained membrane integrity; (B) Effect of Percent Membrane integrity in dietary incorporation of NP and LP alone and in combination with L. *fermentum* and L. RSI3.

Table 4.2.4. : Effect of LP and NP along with probiotics on sperm parameters of father mice.

Parameters measured	NP+PBS	NP+RSI3	NP+FER.	LP+PBS	LP+RSI3	LP+FER.
Testosterone conc. (ng/mL)	6.81±0.023	6.12±0.15	6.10±0.25	2.05±0.33	4.21±1.10	3.6±0.66

4.3. Histological examination

4.3.1. Histology of testis

Influence of different dietary interventions on till 8 weeks, the testis of *Swiss albino* male mice was evaluated after H&E staining, viewed at 200x magnification (Figure 4.3.1).We found irregularities in the seminiferous tubules normal morphology in different treatment groups. The visible distorted histology can be seen clearly in the group fed with LP+PBS. We further quantified histological sections using Image-J software which is freely available image processing platform by N.I.H. (National Institute of Health U.S.A.)

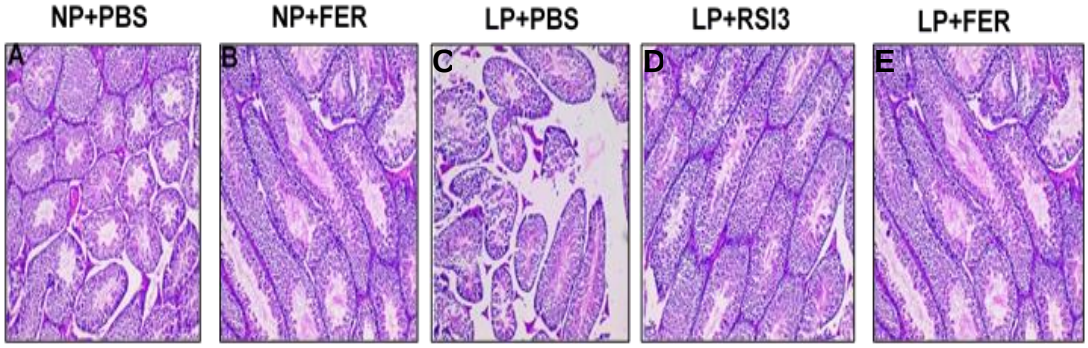


Figure 4.3.1: Hematoxyline and eosin stained testicular tissues of male mice. (A) Control (exposed with Normal protein diet with phosphate buffer saline); (B) exposed with NP+FER (normal protein with *L.fermentum)*; (C) exposed with LP+PBS (low protein diet with phosphate buffer saline); (D) exposed with LP+RSI3 (low protein diet with *L. rahamanosus*) (E) exposed with LP+FER (low protein diet with *L. fermentum).*

4.3.2. Seminiferous tubule cross sectional and leydig cell area

Image-J analysis of light microscopic images of histological sections suggested significant decrease in the seminiferous tubule cross sectional area in paternal male mice fed with LP+PBS for 8 weeks. However other mice groups fed with low protein, normal protein with probiotics for 8 weeks duration did not show any significant changes in the seminiferous tubule cross sectional area. Further, microscopic examination of histological images did not show any significant changes in the leydig cell area in paternal male mice fed with different combination of diets (i.e. NP+PBS, NP+RSI3, NP+FER, LP+PBS, LP+RSI3, LP+FER) for 8 weeks.

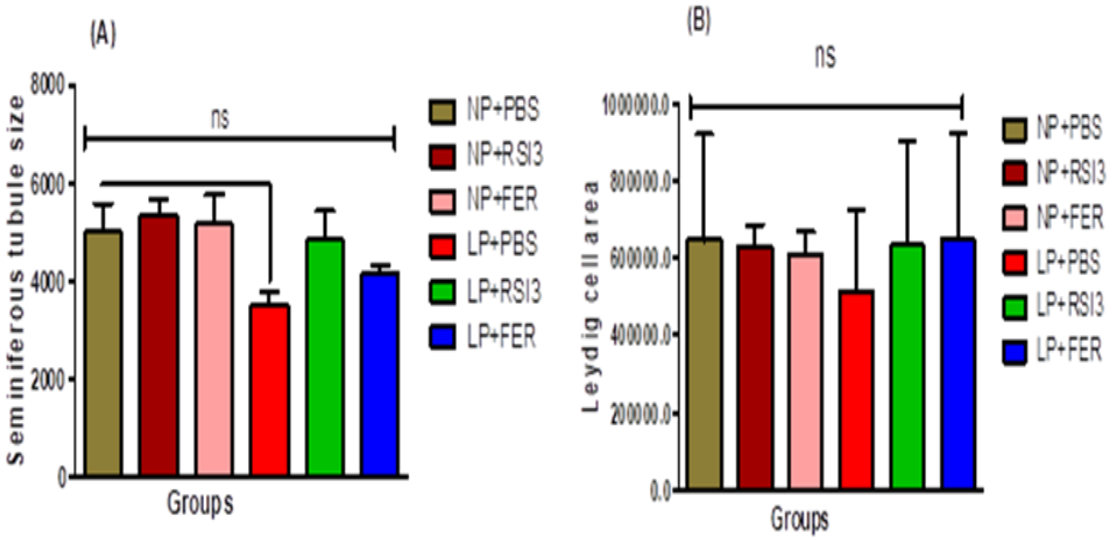


Figure 4.3.2: Effect of dietary incorporation of NP and LP alone and in combination with *L. fermentum* and *L. RSI3* on (A) Mean seminiferous tubule cross sectional area and (B) Leydig cell area. Values are expressed as mean ± SEM (n=6) at 8 weeks.

4.4. Oxidative stress markers enzymes

In order to alleviate oxidative damages in the cell, organisms have evolved enzymatic and non-enzymatic antioxidant system for maintaining delicate intracellular redox balance. The major endogenous antioxidant enzyme and non-enzymatic systems includes superoxide dismutase (SOD) and catalase (CAT).

4.4.1. Superoxide dismutase activity in testicular tissues

The superoxide dismutase (SOD) activity in testis tissue at 8 week study was found to decrease in LP+PBS (20.21±0.33) in comparison with NP+PBS (32.1±0.2), NP+RSI3 (29.3±0.23), NP+FER (30.14±0.11). LP+RSI3 (25.14±0.22), LP+FER (26.05±0.02) (Fig.4.3.4.). It is pertinent to mention that generation of free radicals under oxidative stress may be the reason behind alteration in SOD activity.

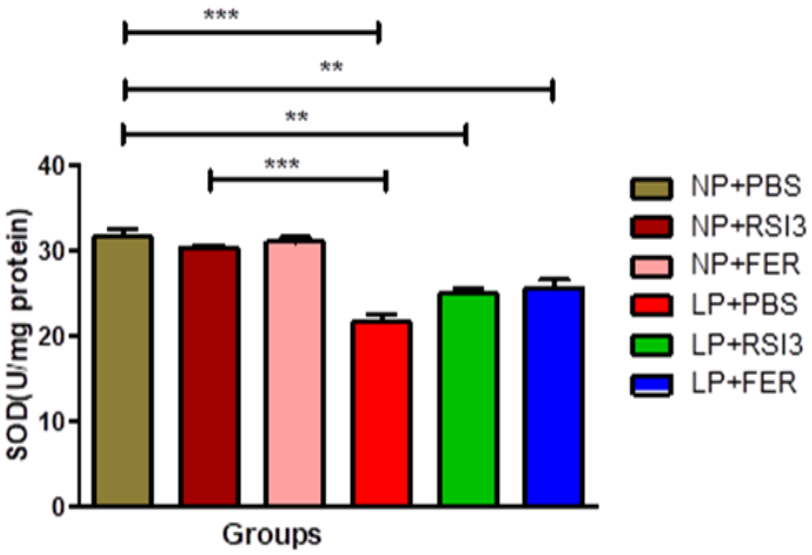


Figure 4.3.4: Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on super oxide dismutase (SOD) enzyme activity in testes of paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8 weeks. The values are represented as mean ± SEM (n=6)

4.4.2. Superoxide dismutase (SOD) and Catalase (CAT) activity in liver tissues

The superoxide dismutase (SOD) activity in liver tissue at 8 week study was found to decreased but no significant difference between LPD and NPD groups was identified the data is presented in Fig. 4.3.5. Catalase (CAT) causes molecular conversion of H_2O_2 into H_2O and O^2. The catalase activity in liver tissue at 8 week study was found to decrease in LP+PBS (43.4±.033) in comparison with NP+PBS (48.1±3.2), NP+RSI3 (50.0±1.12), NP+FER (55.4±0.23). LP+RSI3 (48.0±0.66), LP+FER (46.1±0.21) (Table: 4.3.1. and Fig: 4.3.5.). The decrease in the level of catalase activity of liver mice fed with low protein diet may be due to free radical generation leads to degeneration in the tissues.

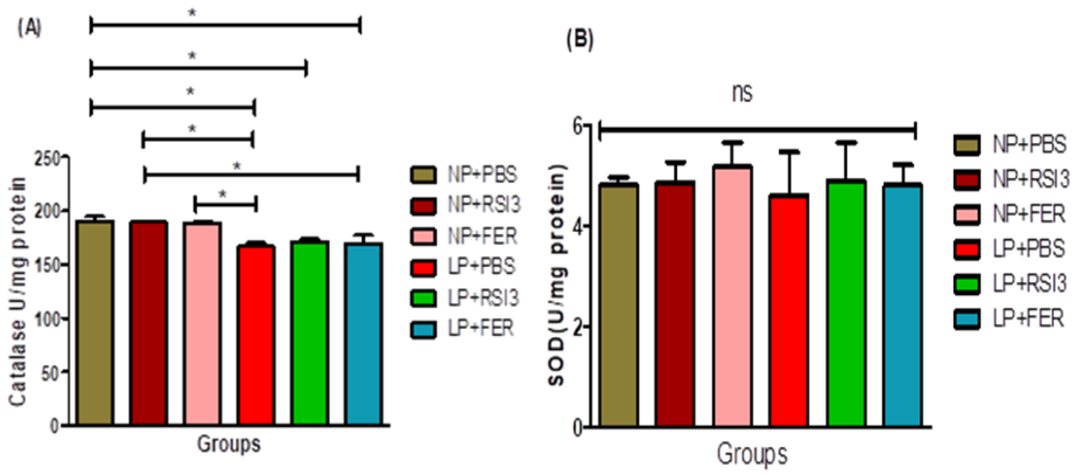


Figure 4.3.5: Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on super oxide dismutase (SOD) and catalase (CAT) enzyme activity in liver tissues of paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8 weeks. The values are represented as mean ± SEM (n=6). The asterisk indicates the significant difference (* $p<0.05$ One way ANOVA) as compared with the control (NP+PB).

Table 4.3.1. : Effect of dietary incorporation of *L. fermentum* & *L. rhamanosus* on super oxide dismutase (SOD) and catalase (CAT) enzyme activity in testis and liver tissues of paternal male mice fed with NP (Normal protein), LP (Low protein) diet for 8 weeks.

		NP+PBS	NP+RSI3	NP+FER	LP+PBS	LP+RSI3	LP+FER
Testis	SOD(U/mg)	32.1±0.2	29.3±0.23	30.14±0.11	20.21±0.33	25.14±0.22	26.05±0.02
Liver	SOD (U/mg)	48.1±3.2	50.0±1.12	55.4±0.23	43.4±.033	48.0±0.66	46.1±0.21
	Catalase (U/mg)	185.1±0.2	185.0±0.1	185.9±0.1	150.4±0.0	154.8±2.3	154.5±2.4

4.5. Objective-2: To assess the fertility in male offspring after paternal supplementation of probiotic fed low protein diet

4.5.1. Effect on birth weight, organ weight and feed intake in offspring

Birth weight and feed intake of the offspring's were recorded at 4 weeks after parturition as depicted in the fig. 4.4.1 (A, B). We did not report any significant change in the birth weights and feed intake amongst all the groups but we recorded that the average daily consumption of feed per animal per day was 4-5g/100g/day/mice in the entire groups. The organ weight of offspring's remained non-significant in all groups on oral administration of different probiotics along with dietary intervention of low and high protein diet to

paternal parent. We also calculated weights of liver, spleen, epididymal fat and paired testes in the offspring's. We found no change on the offspring due to indirect effect of treatment to paternal parent.

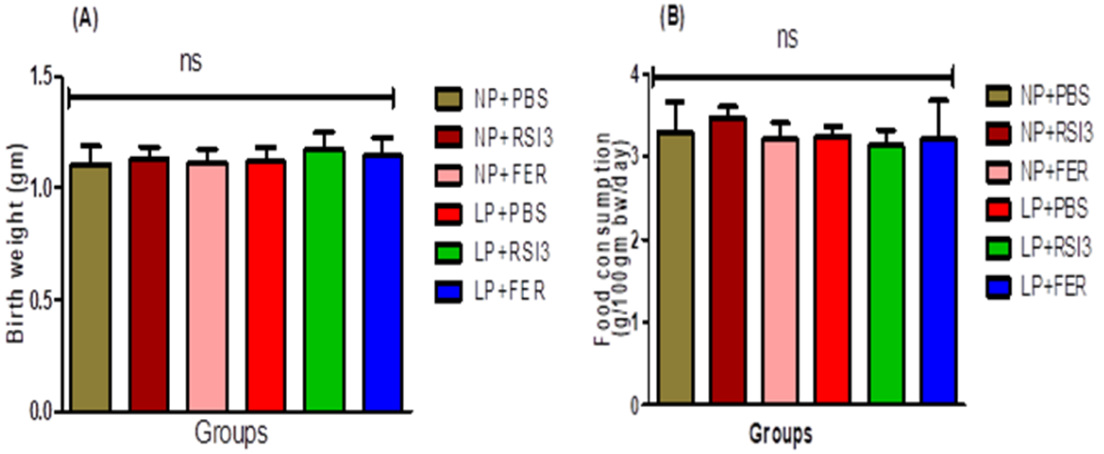


Figure 4.4.1 (A): (A) Birth weight of swiss albino male mice offspring; (B) Food consumption (g/100 gm/ bw/day). The values are expressed as mean ± SEM, (n=6).

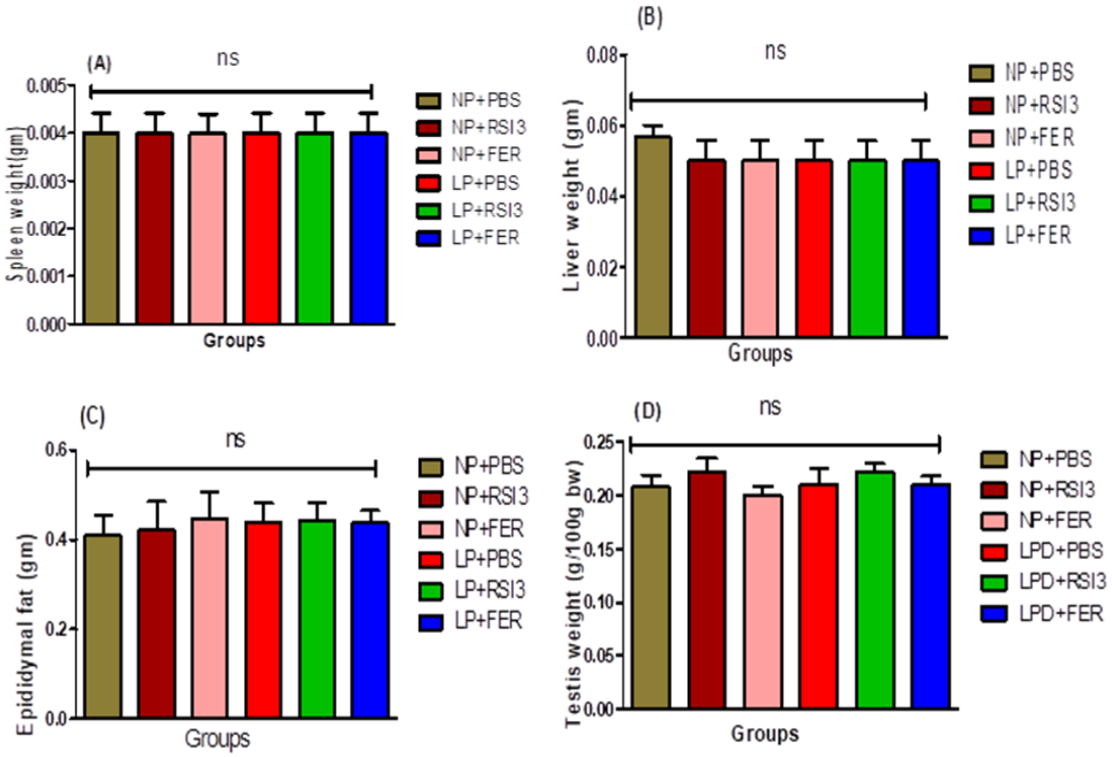


Figure 4.4.1 (B): Body organ weight; (A) Spleen weight (B) Liver weight (C) Epididymal fat (D) Paired testis weight in male mice offspring's fed with NP (Normal protein), LP (Low protein) diet for 8 weeks. The values are expressed as means ± SEM (n = 6).

4.5.2. Fasting blood glucose levels in the offspring's

Fasting blood glucose level in male mice offspring's at 4 weeks of experimental period shown in figure 4.4.2. The fasting blood glucose of offspring's remained non-significant in all groups on oral administration of different probiotics along with dietary intervention of low and normal protein diet to paternal parent. Hence, it shows that treatment of low protein diet to paternal parent does not affect the blood glucose levels in offspring's.

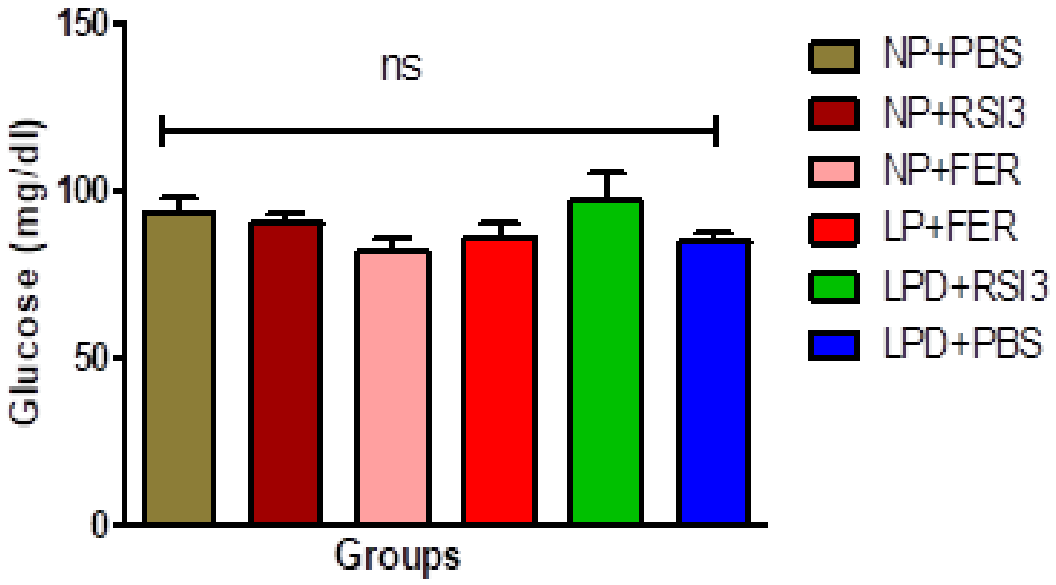


Figure 4.4.2. Effect of feeding of normal diet for 4 weeks on the fasting blood glucose level of swiss albino male mice offspring. The values are expressed as means ± SEM (n = 6).

4.5.3. Eosin-Nigrosin assay in offsprings

Eosin-Nigrosin assay is meant to assess the no. of live and dead spermatozoa in semen. The viability of sperm from offspring's after oral administration of different probiotics along with dietary intervention of low and normal protein diet to paternal parent remained non-significant in all male offspring's groups as shown in fig.4.4.4

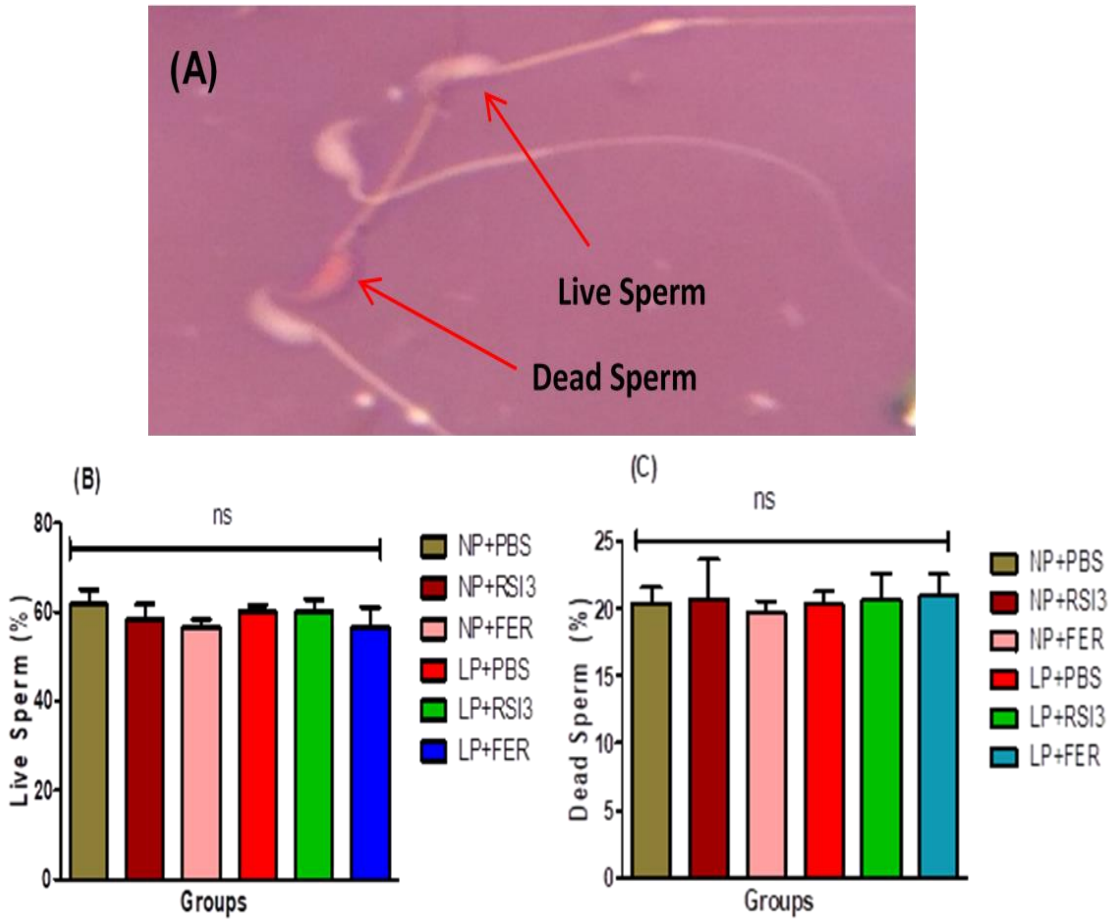


Figure 4.4.3: (A) Eosin and nigrosin staining of *swiss albino* male mice offspring spermatozoa (40 x magnifications). Dead spermatozoa appear as (colored) while live spermatozoa appear as (colorless); (B) and (C) Effect of Percent Live sperm and dead sperm. The values are expressed as means ± SEM (n = 6).

4.5.4. HOST assay

Hypo-osmotic test (HOST) confirms that membrane integrity of sperm from offspring's remained non-significant in all groups after oral administration of different probiotics along with dietary intervention of low and normal protein diet to paternal parent.

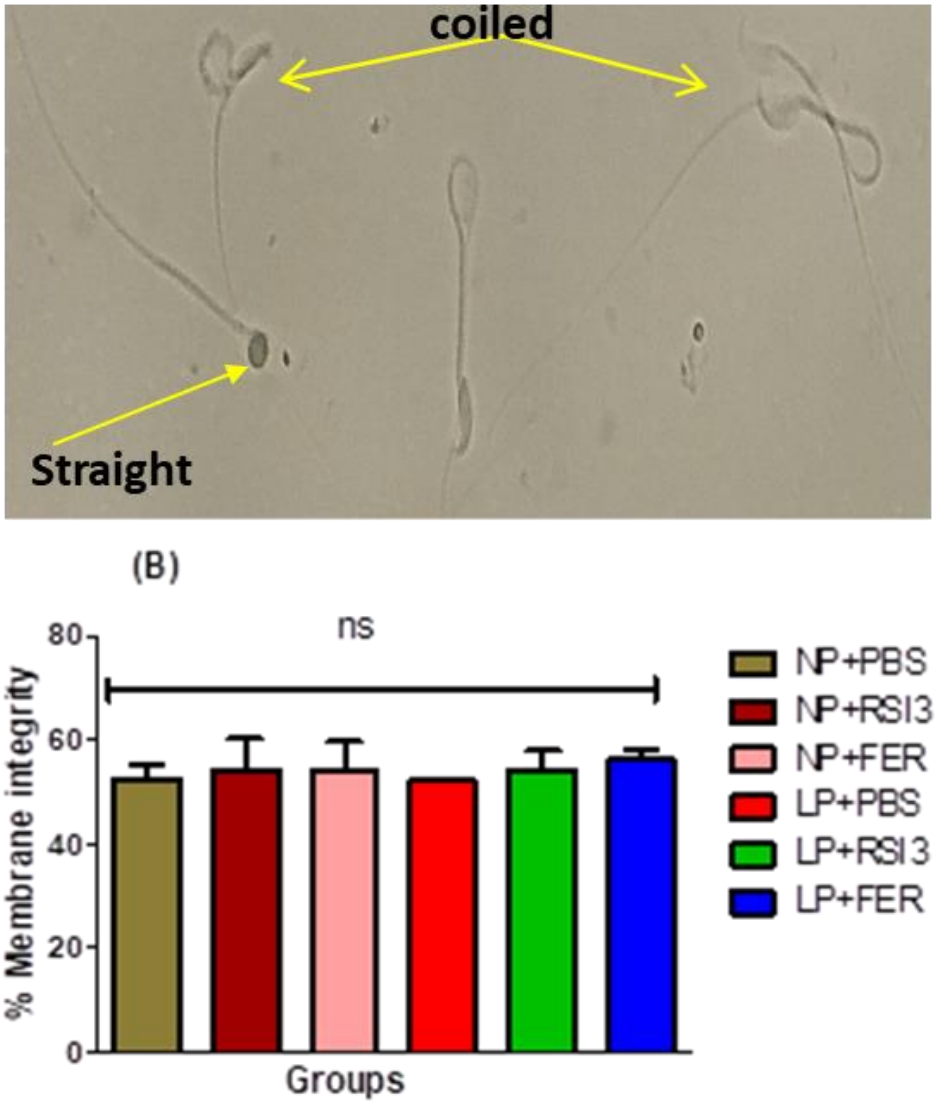


Figure 4.4.4: (A) Membrane integrity of male mice offspring's spermatozoa by Hypo-osmotic swelling Test (HOST). (1) Straight tail represents lost membrane integrity, while (2) Coiled tail represents maintained membrane integrity; (B) Effect of Percent Membrane integrity. The values are expressed as means ± SEM (n = 6).

4.5.5. Superoxide dismutase (SOD) and Catalase (CAT) activity in testicular tissues of offspring's

Super oxide dismutase & catalase activities are assessed from the testicular samples of offspring's after 4 weeks of parturition presented in figure 4.4.6 we didn't find any significant changes in the super oxide dismutase & catalase activities in any of the male offspring's group

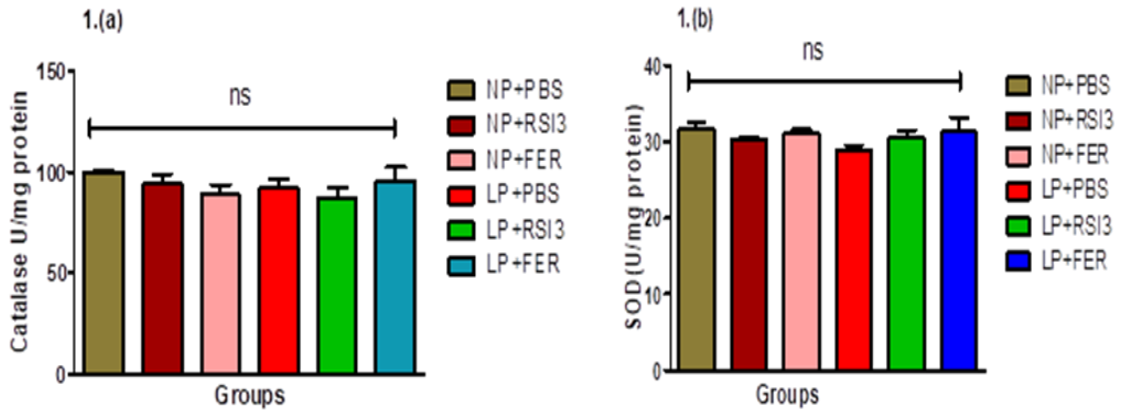


Figure 4.4.5: Catalase 1.(a) and super oxide dismutase 1.(b) activities are being assessed in male mice offspring's testicular tissue. The values are expressed as means ± SEM (n = 6).

4.5.6. Superoxide dismutase (SOD) and Catalase (CAT) activity in the liver tissues of offspring's

The super oxide dismutase and catalase enzyme activities in liver tissue of offspring are assessed, which are maintained on normal chow diet for 4 weeks of parturition are presented in figure 4.4.7. We didn't find any significant change in the super oxide dismutase and catalase activities in all male offspring's groups on oral administration of different probiotics along with dietary intervention of low and high protein diet to paternal parent. We found no change on the offspring's due to indirect effect of treatment to paternal parent.

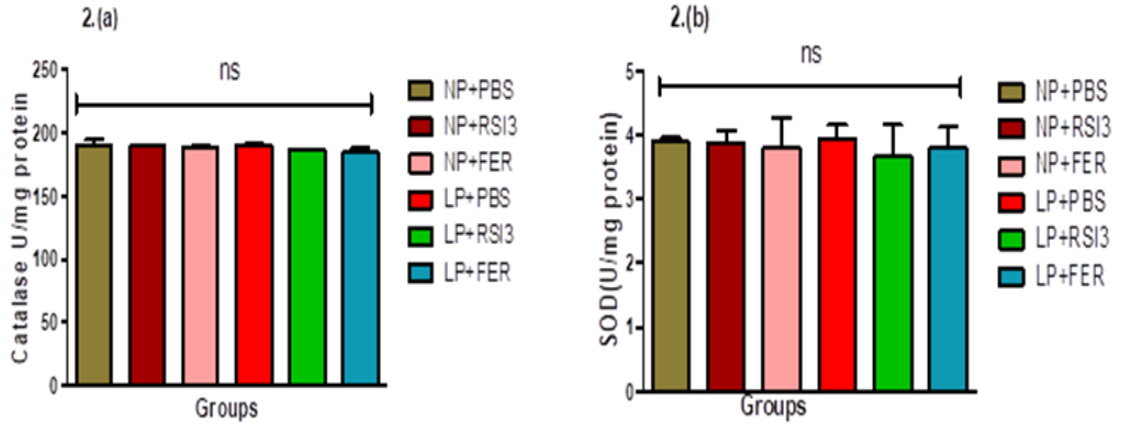


Figure 4.4.6: Catalase 2.(a) and super oxide dismutase 2.(b) activities are being assessed in male mice offspring's liver tissue. The values are expressed as means ± SEM (n = 6).

4.5.7. Histology of testis in the offspring's

Light microscopic examination of H&E-stained sections of male mice offspring's study have shown in the images (Figure 4.4.8).We found no irregularities in the seminiferous tubules and normal morphology in all groups after 4 weeks of parturition. The visible changes in the histology can be seen in different treatment groups. We further quantified histological sections using Image-J software.

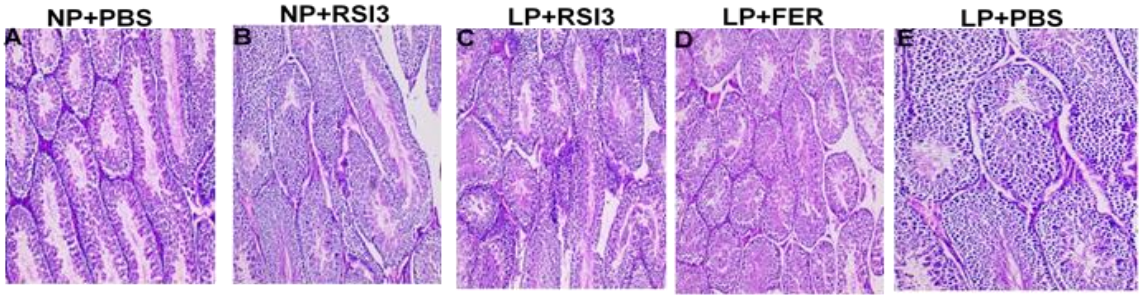


Figure 4.4.7: Hematoxyline and eosin stained testicular tissue of *swiss albino* male mice offspring's which are maintained on normal protein diet after 4 weeks of parturition.

4.5.8. Seminiferous tubule cross sectional and leydig cell area in the offspring's

Image-J analysis of light microscopic images of histological sections didn't suggest any significant change in the seminiferous tubule cross sectional area in mice offspring's after 4 week of parturition.

Image-J analysis of light microscopic images of histological sections did not show any significant changes in the leydig cell area in offspring's after 4 week of parturition of male mice offspring's fed with normal protein diet.

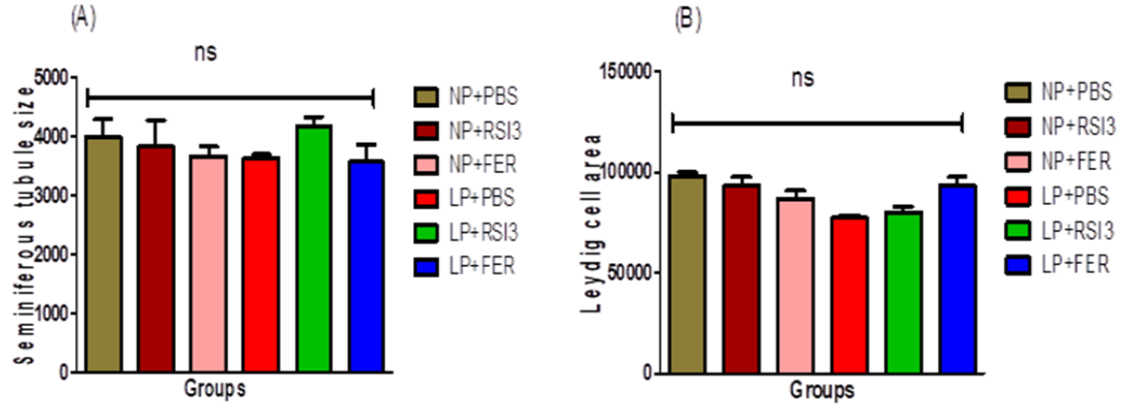


Figure 4.4.8: Effect of dietary incorporation of normal protein and low protein in *swiss albino* male mice offspring (A) Mean seminiferous tubule size (B) leydig cell area. Values are expressed as mean ± SEM (n=6 at 4 week.)

4.5.9. Quantitative real time PCR (qPCR) analysis of relative gene expression

We estimated quantitative real time PCR (qPCR) expression of hydroxyl steroid dehydrogenase (17-β HSD) and cytochrome P450 side chain cleavage (P450) genes from the testicular tissues after feeding with low protein, normal protein and along with probiotics after 8 weeks in different treatment groups of paternal male mice and in the male offspring's after 4 weeks of parturition expressed in figure 4.4.10 (A) & (B). We found that mRNA levels of 17-β HSD are significantly increased in LP+RSI3 group after 8 weeks. We also reported statistically significantly increase in the 17-β HSD mRNA levels in LP+RSI3 offspring group after 4 weeks of parturition. However, mRNA expression levels 17-β HSD in other groups were non-significant. Our estimation of cytochrome P450 mRNA expression levels revealed significantly increase in LP+FER group after 8 weeks and in the offspring's of mice fed with NP+RSI3 and NP+RSI3 after 4 weeks of parturition. However, mRNA expression levels cytochrome P450 side chain cleavage gene in other groups were non-significant. We confirmed amplification of cDNA by gel electrophoresis from qPCR amplified cDNA product. Our results showed that the amplified cDNA product length was approximately 450 bp in length confirmed by the DNA ladder run along-with amplified cDNA.

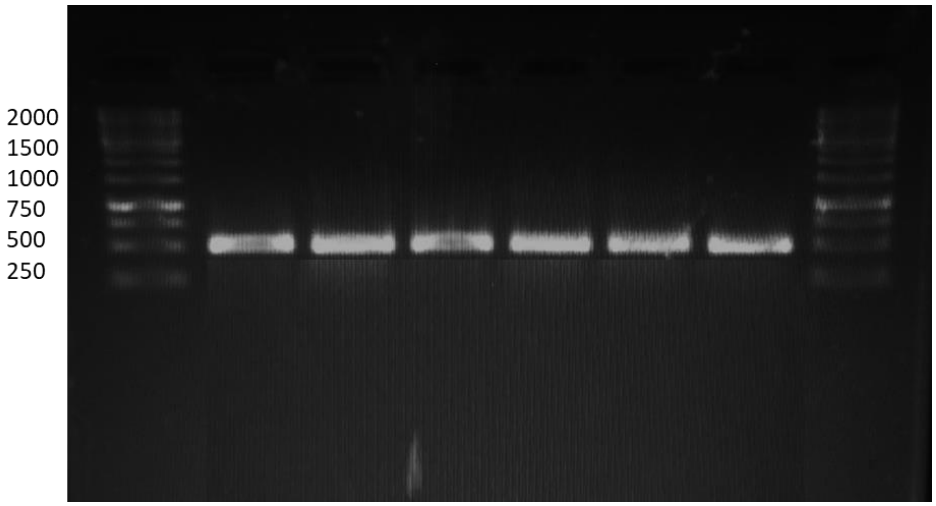


Figure 4.4.9 (A): Gel electrophoresis result of qPCR amplified cDNA. The representative image showing amplified cDNA product length which is approximately 450 bp in length.

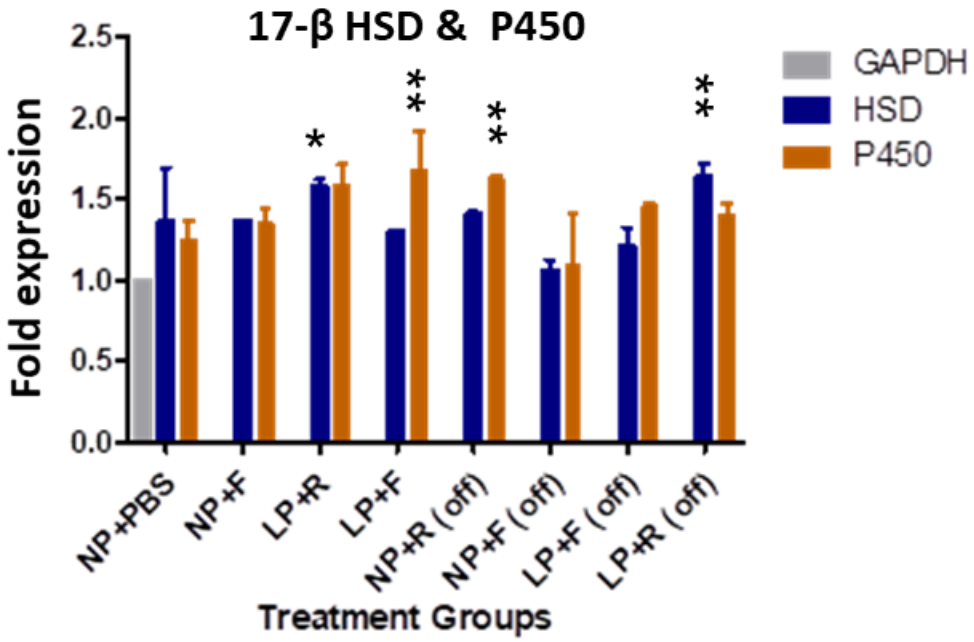


Figure 4.4.9 (B): Quantitative real time PCR (qPCR) analysis of relative gene expression of hydroxyl steroid dehydrogenase (17-β HSD) and cytochrome P450 side chain cleavage (P450) genes from the testicular tissues in male offspring as well as paternal male mice. The values are expressed as means ± SEM (n = 6). The asterisk indicates the significant difference (* $p<0.05$, ** $p<0.01$; Two way ANOVA) as compared with the control GAPDH (NP+PBS).

CHAPTER – 5

Summary and Conclusions

SUMMARY AND CONCLUSION

The current study was aimed to evaluate the beneficial effects of probiotics on male reproduction in Swiss albino mice. There is an ample of the literature available on how infertility was increasing gradually in day to day life. But, very rare literature is available to restore the fertility in a safe way. Therefore, our studywas much moreconcerned with preventative methods of infertility by using safe and secure live microorganisms. For which, two very well established probiotics namely, *Lactobacillus fermentum* (NCDC-400)and *Lactobacillus rhamnosus*-RSI3 (NCDC-610) were used in current study to evaluate their preventative effects on infertility in male mice along with their offsprings.

The primary parameters such as body weight, feed intake (weekly), fasting blood glucose levels (before dissection) and organ weights (after sacrifice of animals) were recorded. In our findings there was an increase in the body weights in probiotic supplemented groups LP+RSI3 and LP+FER groups compared with LP+PBS. But, after weaning we did not find any significant changes in the body weights of all offspring's after fed with normal diet up to four weeks period. There are no statistical significant changes in the feed intake of all the parental groups and their offsprings. Until 8 weeks, the fasting blood glucose levels were significantly lower in the LP+PBS in comparison to NP+PBS and normal in NP+RSI3 and NP+FER LP+FER and LP+RSI3. The organ weights, especially paired testes and spleen weights are normal in both the probiotic supplemented groups (NP+RSI3, NP+FER, LP+RSI3, LP+FER) and NP+PBS when compared to LP+PBS. Whereas, there are no significant changes in the liver and epididymal fat weights among all the groups.

The seminal parameters: sperm count, sperm viability and integrity were assessed after sacrificed the mice. From these we found the total sperm count was less in the LP+PBS; in contrast there was more in the number of sperms in case of LP+RSI3, LP+FER. A variety of methods are available to assess the viability of sperms and each has its advantages and drawbacks. In our study we used Eosin-Nirosin, HOST (Hypo Osmotic Swelling Test) assay, to investigate the abnormalities of sperms. The HOS test used to determine the membrane integrity of the sperm membrane by maintain equilibrium between the sperm cell and its environment. Those sperms are live; they have more membrane integrity and will not allow the solution around the environment. On the other hand those sperms are dead they lose their membrane integrity and they will swell.

In this HOST assay we find the percentages of coiled sperms (live sperms) were significantly less in LP+PBS group as compared to NP+PBS. In contrast, there were more number of sperms were coiled in treated groups LP+RSI3, LP+FER. It indicates that there is more viability of sperms in probiotics supplemented groups. Which implies that probiotics improves the viability of sperm cells even in the low protein diet conditions. Moreover, there is more number of sperms stained by Eosin-Negrosin stain observed in LPD+PBS group. On the contrary, less sperms stained in supplemented groups. Furthermore, the histopathology of testes showed that irregularities in the seminiferous tubules in case of LP+PBS. However, there were no significant changes in the seminiferous tubule cross sectional area and leyding cell area in all other groups and including all offsprings.

From the various group, three serum samples were used for the analysis of the primary sex hormone: testosterone concentrations through the ELISA method specifically outsourced on account of our previous experience with various available assay kits. From these assay, the results showed that there was a decrease in plasma testosterone levels in LP+PBS group (2.5±.001 ng/dl) which was expected as it being a low protein diet, whereas, higher testosterone concentrations in probiotic supplemented groups was observed. In testes: superoxide dismutase (SOD) was found to be significantly decreased in LP+PBS (21.66 ± 0.53 U/mg of protein) as compared to NP+PBS (31.66 ±0.23) and LP+RSI3. But, there is no significantly difference in case of other groups. In liver: catalase found to be less in LP+RSI3 and LP+FER than LP+PBS. We did not find any statistical significance in SOD in all the groups. At final the gene expression studies did by using quantitative real time PCR (qPCR) for hydroxyl steroid dehydrogenase (17-β HSD) and cytochrome P450 genes from the testicular tissues.We found 17-β HSD gene expression was more in LP+RSI3 than LP+PBS. But, there are no significant changes in the P450 gene expression in all groups.

Returning to the hypothesis at the beginning of this study, it is now possible to state that these two probiotics *Lactobacillus rhamnosus* NCD 610 and *Lactobacillus fermentum* NCDC400 are readily adapted by the small animals in their diet and improve the male fertility problems caused by the intake of nutritional deficient diets especially in the current low protein diet which was under investigation.

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