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BY
FERESHTEH DARDMEH

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CV

Fereshteh Dardmeh, graduated as a Doctor of Veterinary Medicine (D.V.M.) from Urmia University, Iran in 2011. She then joined the "Center **for Sensory-**Motor Interaction (SMI)" **and** "Biomedicine group" in the "Faculty of Medicine; **Department of Health, Science and technology**" **of Aalborg University (Aalborg, Denmark) as a PhD student in 2013.**
Since, she has been actively involved in both teaching and research in the field of reproductive health and medicine, with a focus on translational investigations on the possible associations between pain, obesity and fertility, while assessing Probiotic supplements as a novel strategy in the management of pain and fertility potential.

ENGLISH SUMMARY

Obesity is one of the most obvious manifestations of the global epidemic of sedentary lifestyles and excessive energy intake. Obesity can increase the risk of developing musculoskeletal pain and negatively affects hormones, and fertility potential. Very limited is known about the triangle of obesity, pain, and fertility and further investigations are crucial to offer better management strategies. This study aimed at finding the possible effect of separate and co-existing conditions of obesity and pain on male fertility potential while assessing the ability of the *Lactobacillus rhamnosus* PB01 probiotic supplementation as a novel strategy to reverse the negative effects of the mentioned complications. This PhD project comprised of both human and animal studies. The human study was based on chronic pain patients and pain-free healthy matched controls, select-recruited to create two equal subgroups of normal weight (lean) and overweight. Sperm concentration, motility groups, kinematic parameters, DNA fragmentation index and morphology were assessed and compared by computer aided sperm analysis (CASA). Pressure pain thresholds (PPTs) were measured by a handheld pressure algometer in pre-defined points. The animal study was performed on normal weight, and diet-induced obesity (DIO) models of C57BL/6NTac male mice, randomly divided into two sub-groups continuing the same diet but receiving either a single daily dose ($1x10^9$ CFU) of *L. rhamnosus* (test group) or physiological saline (control group) for 4 weeks. Sensitivity to mechanical stimulation was assessed by an electronic Von Frey device every second week. Serum total antioxidant capacity (TAC), reproductive hormones levels, and lipid profiles were assessed by enzyme linked immunosorbent assay (ELISA). Results of the human phase demonstrated that PPT values were generally lower in the overweight chronic pain patients group compared to the respective control groups; however, the deference remained insignificant. Lean men with chronic musculoskeletal pain demonstrated a significantly lower percentage of progressively motile sperm and insignificantly lower concentration, lower normal morphology, and higher DNA fragmentation levels. The overweight chronic pain group had a tendency towards a lower concentration and percentage of progressively motile sperm and significantly lower kinematic

parameters (VCL: curvilinear velocity, STR: straightness, and WOB: wobble) compared to the overweight control groups. In the animal phase, the DIO group had a reduced serum TAC, while probiotic supplementation was found to increase TAC in both DIO and normal control groups. The DIO group demonstrated a clear reduction in several kinematic parameters (VCL, STR, VSL: straight-line velocity, VAP: average path velocity and LIN: linearity) including the percentage of progressive motile sperm, which were reversed proportionally in the DIO probiotics supplemented group. Collectively, the human study demonstrated a negative effect of chronic pain and obesity on the sperm characteristics as a marker of male fertility potential in lean humans. Due to the overlapping effects of obesity and chronic pain on the sperm characteristics, the statistical analysis of the results in this study cannot conclude a solid opinion on the possible effect of chronic pain on sperm quality in co-existing conditions of pain and overweight. Further research in overweight and lean chronic pain patients is required to confirm this hypothesis.

This PhD project aimed to find the possible effect of separate and co-existing conditions of obesity and pain on male fertility potential, taking into consideration Lactobacillus rhamnosus PB01 probiotic supplements as a new strategy to counter the negative effects of the mentioned complications.

Chronic musculoskeletal pain demonstrated slightly altered hormonal balance in normal weight and proved to have a negative effect on sperm concentration and kinematic parameters (VCL, VSL, VAP, BCF), expressed as a lower percentage of non-progressive, progressive and hyper activated spermatozoa. The mouse models demonstrated that oral supplementation of *Lactobacillus rhamnosus* PB01 may well be a potentially innovative approach to the regulation of weight and nociception, while also positively affecting male fertility, especially in cases of obesity. This study supports the hypothesis of "the potentially positive effect of probiotics on both weight and pain as well as sperm quality" which can naturally be followed up as a possible strategy for treatment of sub-fertile obese men.

DANSK RESUME

Fedme er en af de mest åbenlyse manifestationer af den globale tendens til stillesiddende livsstil og overdrevent energiindtag. Fedme kan øge risikoen for at udvikle smerter i bevægeapparatet og negativt påvirke hormoner og fertilitetspotentiale. Der er en meget begrænset viden om sammenhængen mellem fedme, smerte og fertilitet. Yderligere forskning er derfor afgørende for at kunne tilbyde bedre strategier.
Dette studium er et forsøg på at finde den mulige effekt af separate og sammenhængende tilstande af fedme og smerte på det mandlige fertilitetspotentiale. Her afprøves Lactobacillus rhamnosus PB01 probiotisk supplement som en ny strategi til at forhindre den negative effekt af de nævnte komplikationer.
Nærværende studium er sammensat af en nærværende stadium er sammensat af/baseret op humane studier samt dyremodeller. Den humane fase var baseret på patienter med kroniske smerter og smertefri og sunde matchede kontrolpersoner udvalgt med henblik på at oprette to lige store undergrupper med henholdsvis normal vægt og overvægt. Koncentrationen af sædceller, motilitet, kinematiske parametre, DNA fragmentation index og morfologi blev undersøgt og sammenlignet ved hjælp af med computerstøttet sædanalyse (CASA). Smertegrænser ved mekanisk tryk (PPT) blev målt ved hjælp af et håndholdt tryk-algometer i præ-definerede punkter.
Den animalske fase blev udført på modeller af C57BL/6NTac hanlige mus med henholdsvis "normal vægt" eller "kost-induceret fedme (DIO)". Disse to grupper af mus var hver tilfældigt opdelt i to lige store undergrupper, som fik henholdsvis en enkelt daglig dosis (1 x 109 CFU) af L. rhamnosus (testgruppen) eller fysiologisk kogsaltopløsning (kontrolgruppen) og den samme kost gennem 4 uger. Sensitiviteten over for mekanisk stimulation blev vurderet af en elektronisk Von Frey hver anden uge. Serum total antioxidant kapacitet (TAC), niveauet af reproduktionshormoner og lipid profiler blev vurderet vha. ELISA (enzyme linked immunosorbent assay).
Resultaterne af den menneskelige fase viste, at PPT værdier var generelt lavere i gruppen af overvægtige patienter med kroniske smerte sammenlignet med de respektive kontrolgrupper, omend ikke statistisk signifikant. Slanke mænd med kronisk musculoskeletal smerte havde en signifikant lavere procentdel progressivt motile sædceller og signifikant lavere koncentration og normal morfologi og et højere

DNA-fragmentations niveau. Gruppen af overvægtige patienter med kroniske smerter havde en betydeligt lavere procentdel af ikke-progressive motile og statiske sædceller ($P<0,05$) og en ubetydeligt lavere tendens i kinematiske parametre (progressiv motilitet, VCL, VSL, VAP og BCF) i forhold til de overvægtige kontrolgrupper.

I den animalske fase havde DIO gruppen en nedsat serum TAC, mens probiotisk tilskud blev fundet for at forhøje TAC i både DIO og normal kontrolgrupper. DIO-gruppen viste en klar reduktion i flere kinematiske parametre (VCL, VSL, VAP, STR og LIN) herunder procentdelen af progressivt motile sædceller, der var forholdsmæssigt omvendt i den probiotika supplerede DIO-gruppe.

Alt i alt viste den humane fase en negativ effekt af kronisk smerte og fedme på mandlig fertilitetspotentiale hos slanke mænd. P. gr. af den overlappende effekt af fedme og kronisk smerte på sperm karakteristika, kan en statistisk analyse af resultaterne i dette studium ikke føre til en klar konklusion vedr. den mulige effekt af kronisk smerte på sædkvalitet. Yderligere forskning i overvægtige og slanke patienter med kroniske smerter er nødvendig, før man kan bekræfte denne hypotese.

Dette PhD-projekt havde til formål at finde den eventuelle effekt af separate og co-eksisterende forhold af fedme og smerte på mandlig fertilitetspotentiale og samtidig vurdere Lactobacillus rhamnosus PB01 probiotisk tilskud som en ny strategi til at modvirke de negative virkninger af de omtalte komplikationer. Kroniske smerter i bevægeapparatet i normalvægtige patienter ændrede hormonbalancen og viste sig at have en negativ effekt på koncentrationen af sædceller og de kinematiske parametre (VCL, VSL, VAP, BCF) som et udtryk for en lavere procentdel af ikke-progressive, progressive og hyperaktiverede spermatozoer. Musemodellerne demonstrerede, at orale tilskud af Lactobacillus rhamnosus PB01 meget vel kan være en potentielt innovativ tilgang til regulering af vægt og nociception, samtidig med, at det også positivt kan påvirke den mandlige fertilitet, især i tilfælde af fedme. Denne undersøgelse understøtter hypotesen om "den potentielt positive effekt af probiotika på såvel vægt og smertebehandling som sædkvalitet". Dette kan naturligt følges op som en mulig strategi til behandling af subfertile overvægtige mænd.

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I would like to dedicate this thesis to the memory of my mother whom I unfortunately lost while I was away from home during my PhD study and will miss forever.

Fereshteh Dardmeh
March 2017

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INTRODUCTION

1.1. OBESITY

The body mass index (BMI) or "Quetelet index" is defined as the body mass divided by the square of the body height, and universally expressed in units of kg/m^2, resulting from mass in kilograms and height in meters. Table 1 provides a classification for overweight and obesity based on BMI [1].

The prevalence of obesity is advancing. A systematic analysis has reported the number of overweight and obese individuals to have increased from 921 million in 1980 to 2.1 billion in 2013 worldwide [2]. The Danish society has also demonstrated an increasing trend in the prevalence of obesity [3]. The "Danish National Health Profile" by the "Danish Health Authority" in 2010 (den nationale sundhedsprofil 2010) has reported that 54.3% of the Danish population suffer from moderate to severe obesity with 14.3% falling in the severe obesity group [4].

Table 1. Classification of overweight and obesity based by Body Mass Index (BMI) ("WHO | Obesity: preventing and managing the global epidemic," 2015).

	Obesity Class	BMI (Kg/m^2)
Underweight		<18.5
Normal		18.5 – 24.9
Overweight		25.0 – 29.9
Obesity	I	30.0 – 34.9
	II	35.0 – 39.9
Extreme Obesity	III	≥40

An overwhelming number of medical conditions including insulin resistance, diabetes mellitus, glucose intolerance, dyslipidemia, hypertension, arthritis, hyperuricemia, gall bladder disease, sleep apnea and certain types of cancer have been associated to obesity [5]. Other diseases such as coronary heart failure, stroke, cardiac arrhythmia and artery disease have also been independently associated to obesity [5].

Obesity does more than produce alarming numbers; it also leads to damaged or negatively affected health and it is associated with several health conditions such as increasing the risk of heart attack, stroke, hypertension, diabetes, gallstones, cancer, osteoarthritis, obstructive sleep apnea, fatty liver, and depression [6–8]. BMI has also been noted as a decisive factor in infertility and consequently assisted reproductive technology (ART) treatment strategies [9].

1.2. PAIN AND NOCICEPTION

Nociception or pain is a large field of medical research and neuroscience and the distinction between the two is important to consider when performing clinical and preclinical research.

The International Association for the Study of pain has defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (International Association for the Study of Pain, 1994), whereas nociception is defined as "the neural processes of encoding and processing noxious stimuli" [10].

A recent study has described pain as an emotional result of several impulses, which originate from the nociceptors in somatic and visceral tissues [11]. These impulses travel in peripheral nerves, with a first synapse in the dorsal horn and a second synapse in the thalamus. They end up in the cerebral cortex and other supraspinal structures which results in a pain experience and activation of reflex and reflective behaviors, collectively aimed at eliminating further pain [11,12]. It is next expected, that the nociceptor-driven pain will be stopped successfully and further healed to recover and regain a pain-free state [11].

Chronic pain is classified as pain that exceeds 3 months duration [12] and has been categorized as localized, regional or widespread with a majority of cases associated with musculoskeletal pain [13]. It has been previously illustrated that chronic and acute pain have different underlying Mechanisms [14]. In chronic pain conditions, the

association between nociception and pain experience is incorrect or absent. Therefore, the expected recovery does not occur [11].

The release of neuropeptides from the nerve endings can sensitize nociceptors. This may lead to hyperalgesia and central sensitization of dorsal horn neurons, which are expressed as prolonged neuronal discharges.

As mentioned above, acute pain is the result of a strong unpleasant stimulus, to express area of tissue damage. There is a clear alteration from acute pain with a distinct change in the damage area, to chronic pain, where there might be permanent tissue damage. However, chronic pain may remain due to central pain, even though the tissue has been healed [15].

Pain can also be caused by the direct stimulation of nociceptors due to release of inflammatory mediators such as histamine, bradykinin, chemokines and cytokines[16,17]. The development of inflammatory pain is linked to the activation of intracellular signaling pathways and increased levels of proinflammatory cytokines. Inhibiting the action of the proinflammatory cytokines has also been suggested a possible strategy in the treatment of inflammatory pain [18,19].

Acute inflammation is a natural physiological response to infection or tissue injury. One of the primary characteristics of inflammatory states is allodynia in which a normally innocuous stimuli can produce pain [16]. Thus, chronic inflammation is maladaptive and can also cause considerable pain. In response to chronic inflammatory pain, the chemical mediators accountable for tissue inflammation affect the nociceptive nerve endings to lower the neuronal excitation threshold [20]. As a result of released inflammatory substances associated with primary tissue damage, nerve cells, pain receptors and the surroundings of the nerve signal will change [11,21]. This phenomenon (neuroplasticity) refers to alterations in synapses and neural pathways due to changes in environment, behavior, and neural process as well as changes resulting from physical injury like obesity [21,22]

1.3. INFERTILITY

Infertility in general, refers to a couple having failed to conceive a baby after 12 months of regular unprotected sexual intercourse. About 15% of couples worldwide are affected by infertility with male factor contributing to 50% of cases overall [23].

Fertility is a significant component of reproductive health in general and infertility expands beyond the psychological entity and reaches to a social concept [24]. It can have serious implication on physiological, physical, economic, and social wellbeing for both spouses [24].

Today, infertility can be well-thought-out as a public health issue [25]. Reproductive health and success is affected by many lifestyle factors including obesity, inappropriate diet, not exercising or exercising excessively, psychological stress, delayed child bearing, smoking, alcohol and/or caffeine consumption, and exposure to environmental pollutants and chemicals [26,27].

Reduced sperm quality is one of the main causes of male infertility. The sperm quality is dependent on several parameters including the volume, concentration, and total count of spermatozoa, motility, vitality, and morphology [28].

Causes of male infertility can include the defective spermatogenesis due to pituitary disorders, germ cell aplasia, varicocele, testicular cancer and environmental factors or to flawed sperm transfer as a result of congenital abnormalities or immunological and neurogenic factors [29]. In 15% of infertile men, the infertility is caused by genetic factors which may be hereditary or a consequence of environmental factors, leading to DNA damage in precursors of the spermatozoa [30]

In 2012, Jørgensen et al. demonstrated that only 23% of Danish men, have an acceptable sperm quality, which is the lowest sperm quality amongst the European countries [31].

1.4. CO-EXISTING CONDITIONS

1.4.1. OBESITY AND PAIN

One of the associate conditions to obesity is pain, and musculoskeletal pain. Obesity has been, for example, implicated in the development or progression of low back pain [32]. The mechanism of action by which obesity leads to lumbar back pain is not completely understood [33].
Obesity has been suggested to be affecting the discs directly through mechanical stress on the intervertebral discs or by the indirect effects on blood flow to the lumbar spine, subsequently leading to low back pain [33,34]. Further research to elucidate the exact mechanism is needed. However, theoretically, obesity over time seems to contribute to low back pain and that brings an idea that weight loss may help prevent the onset, severity, and pain intensity of low back pain in obese people. The link between obesity and knee osteoarthritis (OA) has also been demonstrated [35], but potential factors underlying the association of obesity with OA have not entirely been elucidated. However, previous studies have demonstrated that obesity leads to an excess load on the joint, increased cartilage turnover, increased collagen type 2 degradation products, and increased risk of degenerative meniscal lesions. Although all of these factors have been theorized to lead to knee OA, no causal relationships have been demonstrated [36,37].
Results from some studies suggested that treatment of obesity lead to reduced weight and subsequently pain, while other studies observed pain and weight as primary outcomes; however, variæøability in measurements makes comparisons and conclusions rather difficult [38].

While available evidence strongly suggests that comorbid obesity is common in chronic pain conditions [39,40] there is still much to learn about the nature of the co-occurrence between overweight/obesity and pain.

Identifying and clarifying the relationship between pain and obesity without being influenced by the many other confounding factors can further complicate the process of designing, running and interpreting the results of studies on overweight/obesity. Therefore, researchers need to use “state of the art” measurement and rigorous scientific methodology when focusing on expanding knowledge of the

relationship between pain and obesity. In addition, developing explanatory models that address co-occurrence of overweight/obesity and pain seem required to examine any subsequent effects on health-related outcomes. Finally, evaluating and testing treatments that most effectively target comorbidities and meet the needs of the ever-growing population of individuals struggling with both overweight and pain would be an ultimate goal [38].

1.4.2. OBESITY AND MALE INFERTILITY

Infertility (also referred to as subfertility in the literature) has been defined as "the inability to conceive after one year of regular unprotected sexual intercourse" [41].

Obesity in adult men has in recent years been associated with low semen quality [42–47], but not consistently [48–50]. Adult male obesity has also been linked to sub-fecundity, as measured by a prolonged waiting time to pregnancy [51–53]. Overweight men have lower concentrations of testosterone, sex hormone binding globulin (SHBG), and inhibin B, and higher concentrations of estradiol, but unaffected or only slightly lower concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [44,45,48,50,54–56].

Overweight men also frequently show T deficiencies possibly contributing to a decreased semen quality [57], seen as decreased total sperm count (TSC) [45], increased frequency of oligozoospermia [42], and a significantly negative relationship between BMI and total number of normal, motile sperm [46].
Many studies are demonstrated that the decrease in semen quality and male reproductive potential over the past half century may be lead to the increasing rate of obesity. According to Carlsen et al., there has been a substantial decrease in semen quality, and due to the effect of low semen quality on male fertility, plausibly an overall decline in male reproductive potential can occur [58].
Observations by some studies demonstrated that male sperm counts continue to decrease by as much as 1.5% per year in the United States [59]. These results are similar to those found in other Western countries and countries in which obesity is more prevalent, but do not exist in regions in which obesity is less common [60]. Furthermore, another

study also reported that patients with male-factor infertility show a significant growth in the incidence of obesity, and couples with obese male partners experience sub-fecundity more often, a correlation that seems necessary to be addressed [61].
Since this decline has occurred relatively in parallel with increasing rates of obesity, it is highly important to focus on the possibility of obesity as an etiology of male infertility and reduced fecundity. The 'obesity pandemic' seen in many countries is a serious threat to public health, and a reduced capacity to reproduce is a potential but less well-known health hazard attributed to obesity [59,62–64].

1.4.3. RESEARCH ON CO-EXISTENCE OF OBESITY, PAIN, AND INFERTILITY

As mentioned in the above sections, possible links between obesity, certain pain conditions and infertility have been shown to some extent. However, to the best of our knowledge, a possible association amongst obesity, pain and infertility in males or females has not been investigated.

Research on chronic pain and fertility has been mainly focused on painful conditions related to chronic pelvic pain e.g. endometriosis in women or prostatitis in men [65–67]. However, other chronic pain conditions and how those can alter the fertility have been less studied and it is still not clear whether pain alone, or under obesity condition can lead to infertility. In addition, the influence of different pain therapeutics on fertility or the effects of fertility treatments on pain have not been systematically investigated.

When it comes to the scenario where all these three conditions co-exist, it makes it even more complicated as the obesity condition and its related treatments might influence both pain and fertility and vice versa.

Since these three conditions of obesity, chronic pain, and infertility are common health-related problems and affect the quality of life of the affected individuals and pose a burden to the society and health care system, it would be highly valuable to find the association if any and to identify whether it would be possible to break the negative link between

these conditions to pave a new road for better management or treatment of obesity, chronic pain and fertility.

The Danish Fertility Society has administered a guideline in June 2013, suggesting BMI as a deciding factor for assisted reproductive technology (ART) treatment, which also emphasizes on the importance of this type of studies in the clinical prospective.

1.4.4. CURRENT OBESITY AND WEIGHT–LOSS TREATMENTS

Obesity and weight-loss treatments range from simple strategies such as increased physical activity and making diet and lifestyle changes to pharmacotherapy using medicines, which block fat absorption or increase fat-metabolism. Weight-loss (bariatric) surgery is another strategy for people incapable of making the mentioned lifestyle and behavioral changes [68].

Anti-obesity drug discovery has generally seen many false starts, failures in clinical development, and withdrawals due to adverse effects, which were not known or fully anticipated during the studies. Many preclinical studies have demonstrated some potential of the drugs aiming pathways in metabolic tissue (e.g. adipocytes, skeletal muscle, and liver) [69], but very few have reached clinical development (e.g. orlistat, liraglutide and metformin) [70].

Many studies have linked the intestinal microbiome with overall health including obesity; therefore, the metabolism regulating role of the gut microbiota has become a focus of study for the management of obesity and its associated comorbidities [71–74].

1.4.5. PROBIOTICS:

1.4.5.1 The weight-regulatory effect of probiotics

The microbial community of the gut is recognized as one of the most important factors connected to obesity and metabolic disorders [75–77]. Previous findings strongly suggest that gut microbiota contribute to the complications related to high-fat diet feeding. There are many studies reporting the anti-obesity effect of *Lactobacillus*, through mechanisms such as and regulation of leptin [78], regulation of lipid and glucose metabolism [76,79], increase of numbers of small adipocytes and decreasing of adipocyte size in white adipose tissue [80,81], and production of conjugated linoleic acid [82,83].
Moreover, probiotic supplementation can mimic key aspects of microbial symbiosis, which may enhance reproductive fitness in mammalian hosts [84].

It would thus be useful to develop specific strategies using probiotics to modify gut microbiota to favor a specific strain (i.e., lactobacillus) and to prevent the deleterious effects of high-fat or obesity-induced complications.
Studies have reported increased natural killer cell (NK) activity and inflammatory type responses with the administration of some probiotic strains [85]. The oral application of *L.casei* strain Shirota has also been reported to have increased the inflammatory immune response levels associated cytokine IL-2 [86].

1.4.5.2 The anti-inflammatory effect of probiotics

Different internal and external challenges can interfere with the normal balance of the healthy gut microflora [74] while the composition and function of a balanced normal microflora may also be directed by inflammation to become immunogenic and aberrant, leading to the continuation of the inflammation and subsequently gut barrier dysfunction.
However, the negative effect of this change may be reversed by oral supplementation of specific strains of the indigenous healthy gut microflora which have been reported to normalize the balance of the

intestinal microbiome, possibly eliminating the cycle leading to inflammation [74,87].
The reported strong anti-inflammatory capability demonstrated by the most common probiotics [88], may be mediated by neutralizing the inflammatory process through enhancing the degradation of enteral antigens, decreasing the secretion of inflammatory mediators, promoting the balance of the indigenous flora, and excluding the pathogens [87,89].
In 2008, Kekkonen et al. reported that probiotics have an anti-inflammatory potential seen as a decrease in serum C-reactive protein (CRP) levels and as a reduction in bacteria-induced production of pro-inflammatory cytokines in peripheral blood mononuclear cell (PBMC) in healthy adults [90].Several studies have demonstrated the capability of probiotics to reduce nociception [91], hypersensitivity and visceral pain in cases of colonic inflammation [92], irritable bowel syndrome [93–96] and diet-induced obesity [91] and non-inflammatory IBS-like models [97].
However, no previous study to our knowledge, has focused on the effect of probiotics for the management of somatic pain.

1.4.5.3 Probiotics and fertility

Some previous publications have pointed out the possible beneficial effect of probiotics on the vaginal flora [98] and female urogenital diseases [99,100] with more recent studies focusing on the biological control of vaginosis [101], female fertility and pregnancy [102,103].
The use of probiotics on male fertility has also been assessed in some recent studies. A completely randomized experimental design study on male murine models compromised by high-fat diet demonstrated significantly improved sperm indexes, reduced testicular tissue injury and increased serum testosterone levels [104].
Another study on the bacterial communities in semen from men of infertile couples has suggested that *Lactobacillus* might not only be a potential probiotic for semen quality maintenance, but also might be helpful in countering the negative influence of Prevotella and Pseudomonas [105].

1.4.6. OBESITY AND WEIGHT–LOSS IN THE TREATMENT OF INFERTILITY (ART)

Obesity research, as it relates to reproduction and pain, requires a transdisciplinary approach because these are complex systems affected by environmental, biological and genetic influences to name a few [106]. Therefore, tackling the problem of reproduction in obese men will require cooperative efforts among experts in each dimension of these research fields.

There is a significant degree of doubt when choosing a strategy for treating obese patients suffering from infertility, where predictive decision making models may be helpful [107].

Ultimately, this type of research may help to form models of shared decision making in which physicians and patients mutually decide how to proceed with strategies for the treatment of patients suffering from pain and/or infertility. Such models which consider the potential risks and benefits of an individual (at a given age, certain weight range, pain level, etc.), could potentially offer a strategy including weight loss before or during fertility treatment. Current approaches to treat obesity-induced male infertility have been demonstrated in Figure 1.

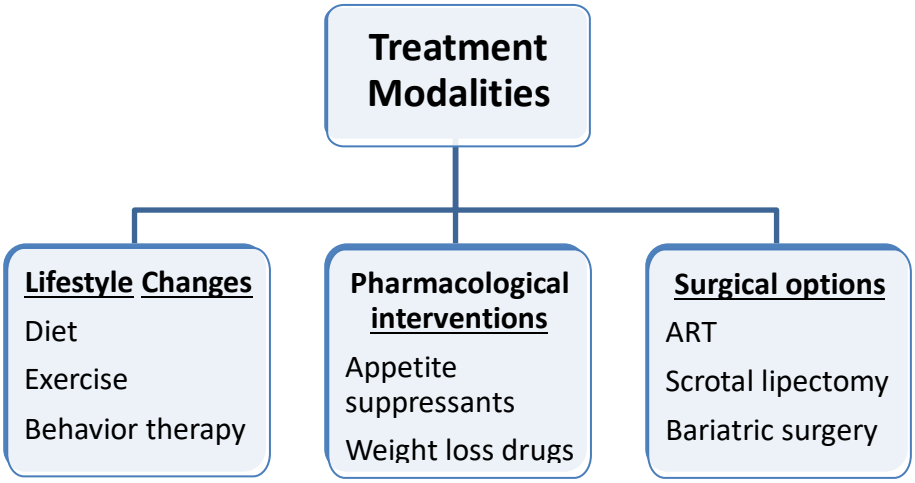

Figure 1 Approaches to treat obesity-induced male infertility (Cabler et al., 2010b)

1.4.7. PROBIOTICS IN CO-EXISTENCE OF OBESITY, PAIN, AND INFERTILITY

Despite the separate studies on the effect of probiotics on weight, pain and fertility management and improvement, no study to date has assessed the effect of probiotics in a triangle condition where all these problems co-exist.

1.5. AIMS AND HYPOTHESIS:

The first aim of this PhD study was to perform an investigational study to examine the possible link between obesity, pain, and fertility in the male gender in humans. The second aim was to assess the potential effect of probiotic *Lactobacillus rhamnosus* PB01 (DSM 14870) on weight management, nociceptive and mechanical sensitivity regulation, and sperm quality as a biomarker of male fertility in a diet-induced obesity murine model. The overall PhD study design, including the human and animal phases has been illustrated in figure 2.

Animal models have been demonstrated to provide insights into the pathophysiology of the phenomenon [108] while allowing for exploratory interventional studies. Accordingly, this PhD study was designed to apply a translational approach.

Paper I: Effects of chronic musculoskeletal pain on fertility potential in lean and overweight male patients.

Assessed hypothesis: "Obesity and chronic pain induce a negative effect on sperm quality (motility, morphology, DNA fragmentation index, etc.) as a biomarker of male fertility potential."

Paper II: Potential Nociceptive Regulatory Effect of Probiotic Lactobacillus rhamnosus PB01 (DSM 14870) on Mechanical Sensitivity in Diet-Induced Obesity Model

Assessed hypothesis: "Probiotic *lactobacillus rhamnosus* PB01induces weight-loss and reduces nociception in diet induced obesity mice models".

Paper III: Lactobacillus rhamnosus PB01 (DSM 14870) supplementation affects markers of sperm kinematic parameters in a diet-induced obesity mouse model.

Assessed hypothesis: Probiotic *lactobacillus rhamnosus* PB01induces weight-loss and reverses the negative effect of obesity on sperm motility as a biomarker of male fertility potential.

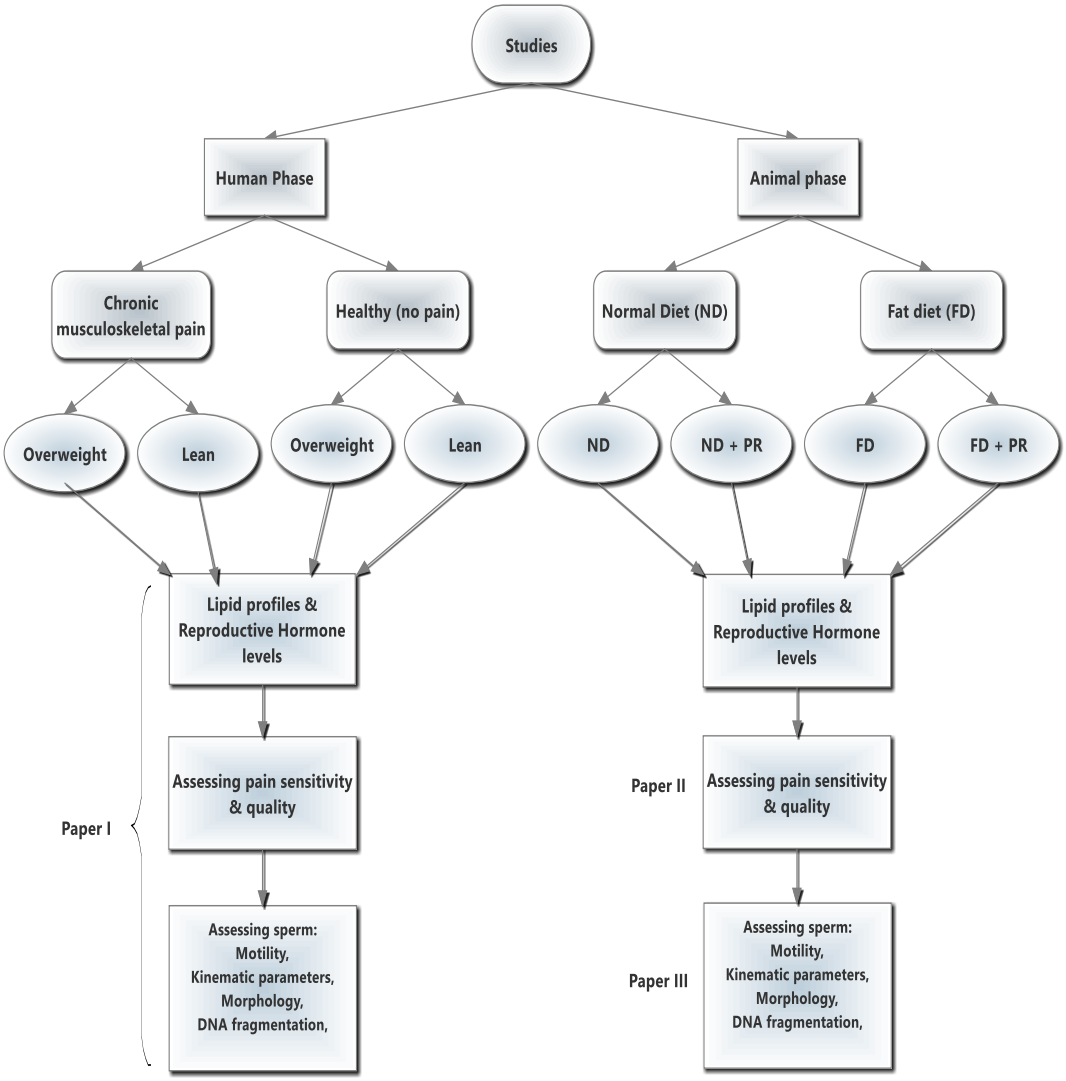

Figure 2. Illustration of the overall PhD study including the human and animal phases. (ND: Normal diet, FD: high fat diet, PR: probiotic.

MATERIALS & METHODS

1.6. PAPER I (HUMAN STUDY): EFFECTS OF CHRONIC MUSCULOSKELETAL PAIN ON FERTILITY POTENTIAL IN LEAN AND OVERWEIGHT MALE PATIENTS

In this study, a questionnaire survey was designed to recruit two groups of age matched overweight and lean male patients suffering from chronic pain. Reproductive hormone levels and sperm quality including motility, morphology and sperm DNA fragmentation index were assessed as biomarkers of male fertility potential for all patients. This assisted in finding a possible association between obesity; chronic pain and fertility in the study population.

This study was done at the Orthopedics department, Aalborg University Hospital, and Fertility Clinic in Dronninglund, Denmark from June 2014 to December 2015, following approval by the Scientific Ethics Committee of the Northern Jutland Region, Denmark (approval reference no. N-20140025).

For this study, 20 chronic pain patients and 20 pain-free healthy matched controls were recruited. The participants in each of these groups were select-recruited to create two equal subgroups of normal weight and overweight subjects.
The following inclusion and exclusion criteria were used (as indicated in the ethical approval protocol):

1.6.1. INCLUSION CRITERIA:

- ✓ Patients (men) within the age range of 18-46 years, attending the orthopedic outpatient clinic located at the Aalborg University Hospital, suffering from low back pain or knee pain.
- ✓ Pain more than 3 months.
- ✓ Patients can be on a daily use of Paracetamol 1 gram x 4, NSAID x -3-4 or combined. Patients will be included regardless of their BMI but will be put in their respective BMI subgroup after the required measurements have been taken.
- ✓ Regarding low back pain the LBPRS > 9, Knee pain the KOOS ≤75 (0-100, i.e. 100 = no problems)

- ✓ Healthy men in the age 18-46 years (matched with enrolled patients)

1.6.2. EXCLUSION CRITERIA:

Cases of:

- ✓ Malignancy
- ✓ Ongoing infection
- ✓ Drug addiction including cannabis, opioids or other psycho drugs
- ✓ Previous musculoskeletal, neurologic or mental illnesses
- ✓ Lack of ability to cooperate
- ✓ Patients with a pain rate of below 3 will be excluded
- ✓ Medication
- ✓ Patients taking Morphine drugs.

1.6.3. DESIGN AND METHODS:

The study was consisted of age matched groups of men 18-46 years old. These groups were defined as the following:
Group I: Overweight patients with chronic pain (OP)
Group II: Overweight patients without chronic pain (OC)
Group III: Lean patient with chronic pain (LP)
Group IV: Lean patient without chronic pain (LC)

1.6.4. DESCRIPTION OF TESTS

For each participant, height, and weight were measured and body mass index (BMI) was calculated. Demographics information, such as gender, age, ethnicity, level of education, history of chronic knee pain, pain medication and other information which might be possible confounders including caffeine intake, alcohol intake, smoking status (never, past, current, unknown), reproductive history, reproductive surgery and abstinence time were collected.

Blood samples were collected and analyzed at the Clinical Biochemistry Department at Aalborg University Hospital by trained professionals based on the hospital routine protocols. A volume of 9 ml

of blood was collected from each patient and assessed for lipid profiles (triglyceride, total cholesterol, low/very low density lipoprotein (LDL/VLDL)), high density lipoprotein (HDL), and hormone levels including: testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH). These procedures performed for healthy olunteers (as controls) like patient. After the tests, the remaining blood was discarded.

Afterwards, the participants were tested for their pain in terms of intensity (magnitude) and quality. Intensity was rated on a visual analogue scale (VAS 0-10) for ongoing pain, and pressure pain threshold was measured by application of a handheld pressure algometer (Somedic, Sweden) to identify muscle sensitivity. A validated Danish version of short form McGill Pain Questionnaire (SF-MPQ-2) was given to evaluate the quality of pain based on pain related word descriptors. In addition, distribution of pain was mapped by drawing on body charts included in this questionnaire. More information on these tests is given below.

1.6.5. ASSESSMENT OF PAIN CHARACTERISTICS:

1.6.5.1 Ongoing pain intensity (on VAS0-10):

The participants were asked to rate their pain intensity at rest condition at the time of experimental visit on a visual analogue scale by placing a horizontal mark on a 10 cm VAS where 0 means no pain and 10 is the most imaginable pain.

1.6.5.2 Pain quality (on SF-MPQ-2):

The SF-MPQ-2 includes four subscales (constant pain (6 items), intermittent pain (6 items), neuropathic pain (6 items) and affective descriptors (4 items)) for a total of 22 items asking about pain symptoms over the past week. The response to each item was scored on an 11-point numeric rating scale (0 = none; 10 = worst possible). A total score was calculated by summing the scores for each item. The SF-MPQ-2 has a high internal consistency and construct validity with the

Multidimensional Pain Inventory (MPI) severity scale in people with different pain conditions.

1.6.5.3 Pain drawings (on body charts)

To map the area of pain distribution, body charts were given and patients were asked to draw on the charts wherever in the body they sense pain.

1.6.5.4 Assessment of evoked pain by mechanical stimulation

Pressure algometry has been used in clinical and experimental settings for testing of tissue sensitivity to mechanical pressure (Bovim, 1992; Arendt-Nielsen, 1997).
Pressure pain thresholds (PPTs) were measured by a handheld pressure algometer (Somedic, Sweden) in predefined points around the knee (in total 5 points) and one reference point on upper arm of non-dominant hand. Subjects were instructed that the PPT is 'the point at which the pressure sensation just becomes painful'. Each measure was repeated three times with a stimulus interval of 1 min. Averages of the three repetitions were used for analysis. The pressure was delivered at a constant rate (30 kPa/s) by the investigator while the algometer tip (1 cm^2 probe area) applied perpendicularly at the stimulation site of the target tissue. A window display on the pressure algometer aids in the application of pressure at a constant rate.
This test was performed for all participants in both healthy control and patient groups.

1.6.6. BIOLOGICAL SAMPLE (SPERM) COLLECTION:

Semen samples were collected by masturbation and allowed to liquefy for 30-45 minutes at room temperature. Following liquefaction, the volume was measured using a graduated pipette; samples were then divided into three parts in small sampling tubes and used for the following assessments:

1.6.6.1 Concentration, Motility, and kinematic parameters:

A "Leja chamber slide" (20 μm deep) (Leja Products B.V., Nieuw Vennep, Netherlands) was filled with five micro-liters of the liquefied sperm suspension and assessed at a total magnification of 100X using a Nikon E50i microscope equipped with a phase contrast condenser and a Basler sca780 (Basler, Germany) camera. The motility and concentration module of the Sperm Class Analyzer (SCA®, Ver. 5.4, Barcelona, Spain) computer aided sperm analysis (CASA) system was used to analyze the concentration and detailed kinematic parameters of the spermatozoa.

The detailed motion parameters provided by the SCA were used to categorize the sperm into different velocity and progression groups according to the current WHO reference values [109].
The reference and cut-off values used by the SCA® for the categorization of human and animal spermatozoa have been illustrated in the papers included in the appendix section.

1.6.6.2 Morphology

Air-dried sperm smears were stained using "Spermblue" (Microptic S/L, Barcelona, Spain) per the manufacturer's instructions assessed using the morphology module of the SCA system at 1000X magnification.

1.6.6.3 DNA fragmentation (Halosperm kit)

The DNA fragmentation of the samples was performed using the Halosperm kit (Halotech, Madrid, Spain) according to the manufacturer's instructions.
In general, unfixed sperm cells are immersed in an agarose micro gel on a slide, incubated in an acid unwinding solution that transforms DNA breaks into single-stranded DNA, followed by immersion in a lysing solution to remove protamines. After staining, the spermatozoa without fragmented DNA show stained nucleoids with big halos of spreading of DNA loops, whereas those with fragmented DNA appear with a small or no halo. The halo sizes and fragmentation index were assessed using the "SCA® DNA fragmentation" module.

1.7. PAPER II AND III (INTERVENTIONAL ANIMAL STUDIES)

Paper I, demonstrated an association between obesity, chronic pain, and fertility in the defined sub-populations of the human patients (Obese and lean patients with or without chronic musculoskeletal pain).

In parallel, animal studies were designed and performed to examine if probiotic based diet programs can be used as a novel weight management strategy to reduce nociception and improve the sperm quality in diet induced obesity (DIO) mice models.

Paper II and III were based on animal models to reduce the confounding factors due to the feasibility and possibility of using a restricted and controlled diet while also providing the possibility of some required invasive assessments not possible in human studies. Furthermore, the animal studies were required as a proof of concept, efficacy, and safety, in order to be able to acquire the required approvals to initiate human trials in collaboration with the probiotic producing company (Bifodan A/S, Hundested, Denmark)

1.8. PAPER II: "POTENTIAL NOCICEPTIVE REGULATORY EFFECT OF PROBIOTIC LACTOBACILLUS RHAMNOSUS PB01 (DSM 14870) ON MECHANICAL SENSITIVITY IN DIET-INDUCED OBESITY MODEL" [91].

1.9. PAPER III: "LACTOBACILLUS RHAMNOSUS PB01 (DSM 14870) SUPPLEMENTATION AFFECTS MARKERS OF SPERM KINEMATIC PARAMETERS IN A DIET-INDUCED OBESITY MODEL"

1.9.1. ANIMALS IN PAPER II, III:

A total of twenty-four, 6 weeks-old male C57BL/6NTac mice were acquired from Taconic (Ejby, Denmark). The mice were housed at the Aalborg University Hospital animal facility (Aalborg, Denmark) and maintained in accordance with Danish Animal Protection Act (12/09/2015).
The mice (one mice per cage), were allowed to acclimatize in an environmentally controlled room (22°C and 60% relative humidity) under a 12-hour light/12-hour dark schedule for two weeks.
All mice were weighed every two weeks throughout the experiment. Furthermore, the mice were allowed a 2-week period of adaptation before the commencement of the experiment receiving food and water ad libitum. All the animal experiments in this study were performed under approval by the Danish Animal Experiments Inspectorate (AEI), Denmark (2014-15-0201-00026) and according to the guidelines of "the ethical committee for research on laboratory animals" of Aalborg University.

1.9.2. EXPERIMENTAL DESIGN:

Phase I (Week 1-4): Following adaptation, the mice were randomly assigned to two groups, receiving either a standard or a high fat diet (60%) (D12492, Research Diets Inc., USA). The mice on standard diet were used as the normal weight (NW) model while the group on high-fat diet resulted in the diet-induced obesity (DIO) model.

Phase II (Week 5-8): Each of the DIO and NW groups were then divided into two subgroups randomly, and maintained on their previous diet. One DIO subgroup and one NW subgroup were randomly selected as the test group, receiving a single daily dose of *Lactobacillus Rhamnosus* diluted in normal saline while the other two subgroups received only normal saline with no probiotic supplement (control group) by intra-gastric administration (gavage), for four weeks.
The above procedure resulted in the following four groups: ND (Normal Diet), NDPR (Normal Diet + Probiotic supplement), FD (Fat diet) and FDPR (Fat diet + Probiotic supplement).

1.9.3. PRESSURE PAIN SENSITIVITY:

Sensitivity to mechanical stimulation by electronic von Frey was performed every second week (3 times on each mice) by placing the mice in an acrylic box (15 × 15 cm) with a metal mesh floor (5 × 5mm square openings) preventing extra movement while providing enough room to show reaction.
This test evaluated how soon paw withdrawal due to mechanical pressure occurred. The more sensitive the paw, the faster the withdrawal, which would be reflected on lower pressure amount.

Before preforming the "pressure pain threshold" test the mice were weighed using a digital scale.
A steadily increasing pressure was applied to the plantar surface of the mouse's hind-paw by a filament (0.1–10 μL pipette tip) loaded on to the sensor of the Von Frey device, until withdrawal occurred. The amount of pressure (g) at the time of hind-paw retraction was recorded by the Von Frey device. Averages of three consecutive readings (with 3-minute intervals) from the same hind paw were recorded for further statistical analysis.

1.9.4. BIOLOGICAL SAMPLE (SPERM AND BLOOD) COLLECTION, AND ELISA ASSAYS:

Blood samples from the facial vein of conscious mice were collected at baseline and end of weeks 4 and 8. Centrifugation (500 g for 10 min at 4C) was used to obtain blood serum which was stored at −20C until being used to assess blood lipid profiles (HDL, LDL/VLDL, total cholesterol, and cholesterol) using a commercially available ELISA assay kit (ab65390, ABCAM, UK) based on the manufacturer's directions.
The serum testosterone, FSH and LH levels were assessed using the total antioxidant capacity kit (ab65329, Abcam, United Kingdom), testosterone ELISA kit (ab108666, Abcam, United Kingdom), FSH ELISA kit (MBS703380, MyBioSource, U.S.A) and LH ELISA kit (MBS041300, MyBioSource, U.S.A), respectively (according to the manufacturers protocols).

1.9.5. PREPARATION OF SPERM SUSPENSIONS:

Sperm collection and preparation of suspension were carried out using a modified procedure. Briefly, the caudal portion of the epididymis was placed in 30-mm petri dishes containing 2ml DMEM (Dulbecco's Modified Eagles Medium), cut open longitudinally and washed (by pipetting with the DMEM medium) to flush out all spermatozoa. The epididymis was then removed and the sperm supernatant fluid could incubate for 20 to 30 min at 37°C before being used for the analysis of sperm parameters.

1.9.6. SPERM ANALYSIS:

Sperm samples were assessed using the Sperm Class Analyzer (SCA®, version 5.4.0.0, Microptic S.L., Barcelona) for sperm motility and kinematic parameters similar to the procedure mentioned above.

RESULTS

1.10. PAPER I: EFFECTS OF CHRONIC MUSCULOSKELETAL PAIN ON FERTILITY POTENTIAL IN LEAN AND OVERWEIGHT MALE PATIENTS

1.10.1. PAIN INTENSITY AND QUALITY:

The minimum and maximum pain intensity levels within last 3 months on the VAS. scale was respectively reported to be 5.15 ± 2.21 cm and 7.78 ± 1.97 cm for the overweigh patients, and 4.7 ± 1.97 cm and 8.01 ± 1.77 cm for the lean chronic pain patients.
Throbbing, shooting, boring, sharp, hot, taut, exhausting, grueling, blinding and nauseating were the maximum (30%) reported descriptors of pain via chronic pain patients.
LP and OP showed a decreasing tendency compared to LC and OC; however, no statistically significant difference was detected in any other measured spots among the sub-groups (Paper I, Table2). The anatomic sites for pressure pain threshold evaluations over the muscles are demonstrated in Paper I (figure1).

1.10.2. SEMEN ANALYSIS:

1.10.2.1 Concentration, motility, and sperm kinematic parameters

The percentage of progressively motile sperm was significantly lower ($P \leq 0.05$) in LP group compared to LC group; however, LP group demonstrated a higher percentage of non-progressively motile sperm compared to the LC group. A same trend in OP group was seen compared to the matched healthy controls, however it remained insignificant. (Paper I, Table 3).
Moreover, the sperm motion kinematics was significantly affected by chronic pain. For more information refer to Paper I (Table 4).

1.10.2.2 Morphology and DNA fragmentation

LC and OC groups demonstrated lower, but not significant trend, in DNA fragmentation compared to LP and OP groups. Entirely, there was no difference in morphology and DNA fragmentation in lean and overweight chronic pain patients compared with the matched healthy controls (Paper I, Table 3).

1.10.2.3 Blood serum lipid profile and reproductive hormones:

There was no significance difference in plasma testosterone levels in lean and overweight chronic pain patients compared with the matched healthy controls and the levels remained within the reference ranges for the hormone (10.3 - 27.4 nmol/L) ("Testosteron (R-nr. 515) - Statens Serum Institut," 2015). FSH and LH levels were found to be lower in LP and OP groups compared to math healthy groups, however insignificant. The OP and LP group also demonstrated higher cholesterol levels compared to OC; however, that was also insignificant (Paper I, Table 5).

1.11. PAPER II AND III:

PAPER II: EFFECT OF LACTOBACILLUS RHAMNOSUS PB01 ON DIFFERENT ASPECTS OF OBESITY, PAIN, AND FERTILITY POTENTIAL IN ANIMAL TRAIL:

PAPER III: LACTOBACILLUS RHAMNOSUS PB01 SUPPLEMENTATION EFFECTS ON BODY WEIGHT AND TESTICULAR WEIGHT:

In week 4, groups on a high fat diet, had gained significantly higher weight in comparison to the group on normal diet ($P<0.01$) and maintained a rising trend after week 4. The probiotic supplemented groups upheld a steady weight without any significant change until the end of the study at week 8 (Paper II, Figure 1).
FD group demonstrated the significant rising trend in gaining weight in week 8 ($P<0.002$).
However, The NDPR and FDPR groups maintained a stable weight after week 4, following the start of probiotic supplementation (Paper II, Figure 1).
In regards to testicular weigh, normal diet groups with or without *L. rhamnosus* did not demonstrate any significant difference while probiotic supplemented DIO group showed significantly higher testicular weight compared to DIO group without probiotics (0.127 ± 0.01 gr and 0.117 ± 0.01 gr, respectively).

1.11.1. *L. RHAMNOSUS* PB01 SUPPLEMENTATION EFFECTS ON PRESSURE PAIN THRESHOLD:

After 4 weeks of the probiotic supplementation, both normal diet and diet–induced obese mice demonstrated remarkable changes in the pain pressure threshold (PPT) values (Paper II Figure 1).

PPT ranges were between 15 and 17.7 g in the baseline. During the first 2 weeks of the study, a slight increase was observed in the PPT values with no significant difference between the normal or fat diet groups; however, this difference became clearly significant at week 4 ($P<0.01$) (Paper II Figure1).

In week 8, all groups showed a significant ($P<0.01$) difference in the PPT results.
Pain pressure thresholds in the NDPR, FDPR, ND and FD groups, were (22.17, 16, 12 and 7.83) grams respectively (Paper II Figure1). As a remarkable point, NDPR demonstrated the highest PPT results.

1.11.2. *L. RHAMNOSUS* PB01 SUPPLEMENTATION EFFECTS ON LIPID PROFILES, REPRODUCTIVE HORMONES AND TOTAL ANTIOXIDANT CAPACITY:

Paper II, Table 1 presents the blood serum lipid profiles (serum total cholesterol, LDL-cholesterol, HDL-cholesterol), reproductive hormone levels (LH, FSH and testosterone) and total antioxidant capacity of the mice at following 8 weeks on the respective diet with or without probiotic supplementation.

1.11.3. *L. RHAMNOSUS* PB01 SUPPLEMENTATION EFFECTS ON SPERM MOTILITY (PROGRESSION):

No significant difference was seen in the percentage of sperm categorized by progressive motility, between the groups on normal diet regardless of probiotic supplementation.
However, a significantly lower percentage of immotile sperm and a significantly higher percentage of non-progressively and progressively motile sperm were observed in the normal diet group (ND) compared to the fat diet group (FD) (Paper III Figure 2)**.**
A significantly higher percentage of non-progressively ($P\leq0.05$) and progressively motile ($P\leq0.01$) sperm and a significantly lower percentage of immotile ($P\leq0.01$) sperm were also seen in the probiotic supplemented fat-diet group (FDPR) compared to the fat diet group (FD) (Paper III Figure 2).

1.11.4. *L. RHAMNOSUS* PB01 SUPPLEMENTATION EFFECTS ON SPERM KINEMATIC PARAMETERS:

The FD group demonstrated lower values in all the kinematic parameters when compared to the ND group (Paper III Figure 3).
The probiotic supplemented high fat diet and normal diet groups (NDPR and FDPR) demonstrated higher values of VCL, VAP, VSL, BCF and ALH compared to the groups without probiotic supplementation in week 8 (ND and FD); however, the deference remained insignificant. A significant difference in VSL ($P<0.01$), VAP ($P<0.05$) and VCL ($P<0.05$) was seen between the FD and FDPR group (Paper III Figure3)**.** A comparison of the kinematic parameters of sperm in the DIO and NW groups, with and without probiotic supplementation after 4 weeks has been illustrated in (Paper III Figure 3).

DISCUSSION

1.12. HUMAN STUDY:

The main purpose of the human study (Paper I) was to investigate if patients suffering from obesity, musculoskeletal chronic pain, or a combination of the two, demonstrate different pain sensitivity to mechanical pressure (pressure pain threshold), reproductive hormone levels and sperm quality in comparison with age-matched healthy controls. It was also investigated, whether chronic pain in lean or overweight subjects is related to the possible change in sperm quality. The novelty of this study resides in investigating the influence of chronic pain, as a factor by itself, or in combination with obesity, on male fertility potential.

To the best of the author's knowledge, this is the first investigation on biomarkers of male fertility including sperm motility and detailed kinematic parameters, sperm morphology, DNA fragmentation and reproductive hormone levels in lean and overweight chronic pain patients compared to age-matched healthy controls.

1.12.1. ASSOCIATION BETWEEN OBESITY, CHRONIC PAIN, AND SPERM QUALITY:

Study of lean and overweight male chronic pain patients compared to age-matched healthy controls demonstrated that chronic pain patients have a generally lower sperm quality in terms of sperm motility, compared with healthy matched controls. This difference in the sperm quality was observed as significantly lower percentage of progressively motile sperm in the lean chronic pain group compared to the lean control group while the overweight chronic pain group demonstrated significantly lower VCL, STR and WOB compared to the overweight control group.
Our findings in musculoskeletal pain confirm the results from a meta-analysis by Fu et al., in 2014 [110] on male fertility indicating that chronic pelvic pain syndrome (CPPS) significantly reduces sperm concentration and sperm kinematic parameters.

Both lean and overweight chronic pain patients also demonstrated decreased testosterone levels compared with the healthy matched controls; however, insignificant. A possible explanation for this change could be that chronic pain can induce a stress response through persistent stimulation of the nociceptors and this recurrent stressor on the hypothalamic–pituitary–adrenal (HPA= axis and Gonadotropin-releasing hormone (GRH) axis, may lead to decreased testosterone plasma levels [110–113].Testosterone is intricately involved in endogenous opioid activity [114,115]; therefore, adequate levels of testosterone could produce pain control in males while decreased plasma testosterone levels could result in raised muscle sensitivity reflected on lower (PPT) values, allowing for testosterone to play a role in pain modulation [112,116,117].

The lower testosterone levels in LP and OP groups could explain the tendency to decreased PPT values, which is in line with the previously conducted studies demonstrating decreased PPT values in chronic-pain patients [117–121].

Testosterone also has an important role in spermatogenesis; therefore, an alteration in the testosterone secretion could result in the dysfunction of the production and maturation process of the spermatozoa, consequently affecting the sperm concentration and kinematic parameters. Accordingly, our study demonstrated significant differences in the sperm kinematic parameters, but no difference in morphology and DNA fragmentation between LP and LC groups. No significant correlation was demonstrated between chronic pain, hormonal profiles and the PPT values. The LH and testosterone hormone levels were not significantly different in the overweight test and control groups but the tendency similar to what was seen in the "lean groups" can still be found. LH levels was higher in obese musculoskeletal chronic pain patients and FSH and testosterone levels was lower in obese musculoskeletal chronic pain patients.

The previously proven negative effect of overweightness on sperm quality, which could overlap and cover the possible effect of musculoskeletal chronic pain, could be another explanation for why the differences of the parameters in the overweight groups are not significant.

The statistical analysis of the results in this study cannot conclude if musculoskeletal chronic pain has an influence on sperm quality in obese

patients. More research on a larger number of obese chronic pain patients (e.g. 45 patients in each group with power of above 80%) with further focus on differentiating the effect of obesity from chronic pain in overweight patients is required before a definite conclusion can be made.

1.13. INTERVENTIONAL ANIMAL STUDIES:

The interventional animal studies were designed to investigate the novel idea of using the previously proven "weight-gain control" [71] and "anti-inflammatory" [122] effect of probiotics (*Lactobacillus rhamnosus pb01)* supplementation to reverse the adverse effects of chronic pain on sperm quality in lean mice (Paper II).
Considering somewhat inconclusive results on the effect of chronic pain in overweight patients, the interventional animal studies could also provide an insight and allow for further evaluation on the relation among obesity, chronic pain, and sperm quality in a fully controlled setting. It was also hypothesized that the mentioned properties of probiotics may also result in lower pain sensitivity (Paper II, III).
These studies were based on animal models (mice) of obesity due to the feasibility and possibility of using a restricted and controlled diet and related ethical issues. Animal models also provided the possibility of some required invasive assessments, which were not possible in humans.

1.13.1. EFFECT OF PROBIOTICS ON BODY WEIGHT, LIPID PROFILE AND HORMONE LEVELS:

In the present study, we observed that feeding C57BL/6NTac mice a high fat diet for 4weeks produced significant increases in body weight (diet-induce obesity models). Oral Administration of *Lactobacillus rhamnosus* supplementation resulted in a significantly reduced body weight. Several previous studies have also reported the anti-obesity effects of some bacterial strains such as *Lactobacillus* spp. [75–77].

Our results demonstrated that the total cholesterol (TC) and low density lipoprotein (LDL) levels in serum were decreased and high density lipoprotein (HDL) level was increased in the FDPR group compared to

FD. The hypercholesterolemic effect of probiotics in both mice and human has also been reported ins several previous studies [123–130].

In contrast, a few studies have reported that probiotics have no influence on lipid profile changes [70,131–133]. These controversial results could be due to the differences in the used strains, and dissimilar delivery methods where the probiotic carrier (matrix) could have influenced the cholesterol-decreasing effect of probiotics [134].

Our results showed that the testosterone, LH and FSH levels in serum were increased in the FDPR group compared to FD group. Lower level of testosterone in the DIO groups in this study, could be a result of a possible degeneration of Leydig cells due to the detrimental effects of cholesterol-rich diets on the secretory capacity of Leydig and Sertoli cells.

Serum estrogen levels would raise as the result of the conversion of androgens to estradiol [135,136], which can be associated with the lower levels of LH and FSH in the DIO group.

Following the increased estradiol levels in obese models, the production and secretion of FSH and LH would be reduced resulting in reduced intra-testicular and circulating testosterone function and production [136,137]. The direct toxic effect of excessive estradiol on spermatogenesis has also been previously suggested [136,137].

1.13.2. EFFECT OF PROBIOTICS ON PRESSURE PAIN THRESHOLD:

Our results revealed that the pressure pain threshold rates were increased in the FDPR group compare to FD, which can show lower sensitivity in this group.
Several studies have confirmed that probiotics have powerful anti-inflammatory effects [86,88,90]. This anti-inflammatory effect could also be an explanation for the reduced pain sensitivity in the probiotic supplemented test groups observed in this study. Nevertheless, other unidentified mechanisms may have also been involved.

1.13.3. EFFECT OF PROBIOTICS ON SPERM MOTILITY AND SPERM KINEMATIC PARAMETERS:

The most important feature of the spermatozoon is sperm motility. It is known as a main indicator of sperm function [138].

Our results revealed significantly higher percentage of progressively motile sperm and significantly lower percentage of immotile (static) sperm in FDPR groups compared to (FD). This finding confirms the adverse effects of obesity and beneficial effects on sperm motility [46,139–143], while also supporting the results of previous studies assessing the effect of probiotics in rodents [104,144].

The diet induced obesity mice showed lower values in all kinematic parameters, which are also observed as the reduction in the level of progressive motility, possibly implying that spermatozoa are unable to migrate along the female reproductive tract efficiently to reach the site of fertilization *in-vivo* [143].

The increased motion path parameters including linearity (Lin), straightness (STR) and beat cross frequency (BCF) and significantly ($P<0.05$) higher velocity parameters (VSL, VCL and VAP) in the probiotic supplemented DIO group compared to the respective control groups without probiotic supplementation suggest a general capability of probiotics in reversing the deleterious effects of DIO on sperm swimming speed.

The mechanisms behind this decline of motility in the obese mice may be related to the decrease in serum testosterone, because of increased estradiol levels alongside with the general adverse effects of increased body weight; while the weight reduction and hormone regulating effect of the probiotics could be the considered as the mechanism behind the better quality of the sperm in the probiotic supplemented groups. However, an exact mechanism is yet to be identified.

The detailed kinematic parameters of sperm assessed by CASA have been less focused in the literature. The motion parameters including linearity (Lin), straightness (STR) and beat cross frequency (BCF) were increased in both DIO and NW groups after 4 weeks of probiotic supplementation while ALH was marginally-significantly ($P=0.057$) higher in the FDPR compared to the FD group.

The sperm in the “rapid velocity” category demonstrated significantly higher average VSL, VCL, VAP and ALH values in the probiotic supplemented DIO groups. This increase in velocity parameters can be

interpreted as increased capability of sperm to reach the oocyte in the female reproductive tract and further emphasizes the positive effect of probiotic supplementation on the kinematic parameters of the sperm.

High-fat diet can provoke oxidative stress leading to sperm damage while probiotic supplementation may improve sperm quality to some extent by reducing the level of oxidative damage [144]. Other *Lactobacillus rhamnosus* strains (L. rhamnosus GG) have demonstrated similar capabilities including significant increase in seminiferous tubule cross-sectional profiles, increased Leydig cell numbers per testis and spermatogenesis in mice [145].

This is the first study reporting the capability of probiotics in reversing the adverse effects of DIO on sperm kinematic parameters. The mechanism of action behind this finding has not been yet investigated, but could be associated with the increasing level of testosterone during spermatogenesis and maturation, reducing the level of oxidative damage and inducing weight loss, which would also possibly reduce scrotal fat regulating of the testes' temperature.

In this relation, recent studies focusing on diet and exercise interventions in an obese mouse model [146] have also reported that the sperm function is correlated with the metabolic health of an individual. Therefore, the ability of probiotic supplementation in improving metabolic health through mechanisms such as the lowering of cholesterol to normal levels could be associated with improvements in molecular compositions such as reductions of oxidative stress and reduced DNA damage resulting in a better sperm motility [146].

Further investigation is still required to determine the exact mechanism of action for the protective effect of probiotics on the sperm kinematic parameters

CONCLUSION:

Paper I: The excessive impact of obesity itself on the sperm parameters renders it rather difficult to distinguish the distinct effect of musculoskeletal chronic pain on sperm quality in overweight human patients. However, musculoskeletal chronic pain in lean human patients altered the balance of reproductive hormone levels and demonstrated a significant negative effect on progressively motile sperm percentage.

Paper II and III: When assessing similar conditions in animal models, irrespectively of the underlying mechanism, *Lactobacillus rhamnosus* PB01 demonstrated a potential as an innovative approach for the management of weight and nociception. In addition, this probiotic could positively affect the male fertility potential, especially in obese groups.

Translation of the weight and nociception regulating effect of the probiotics to humans may potentially suggest a novel beneficial strategy in conditions where male infertility, obesity and pain co-exist.

Overall, due to the previously demonstrated excessive adverse effects of obesity on sperm characteristics, the results of the human phase of this study should be interpreted cautiously. A study with a larger sample size would be required to provide a conclusive conclusion on the different effects of obesity and chronic pain on sperm characteristics

1.14. PERSPECTIVES AND FUTURE STUDIES:

A study with a large number of samples in both male and female models from different strains and species is suggested to provide solid and reliable results and account for the possible overlapping of the effect of obesity and pain, variability in sperm kinematics and/or hormonal influence.

Another study on DIO models, focusing on the effect of the probiotic supplementation in relation to pain induced by injecting an algogenic substance or creating injury neuropathic models could provide more concrete confirmation of the nociceptive-regulatory effect of probiotics.

In addition, a diverse range of outcome measures and biomarkers, would provide better insight in underlying mechanisms.

Furthermore, metabolomics proteomics and gene expressions analysis should be conducted to identify the underlying mechanisms behind the weight and nociception regulation and its consequent effect on male fertility potential.

Human clinical trials, assessing the effect of probiotics on fertility potential, and inflammatory -pain are required. Translation of the results from this animal study to humans could lead to a novel beneficial strategy to manage co-existing cases of male infertility, obesity, and pain.

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APPENDIX: PAPER I - III

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