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SHORT CUMMUNICATION
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Effect of Probiotic Supplementation on Spermatozoa Quality in Male Wistar Rats Fed a High-Fat Diet

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ABSTRACT

A high-fat diet is known to negatively impact reproductive efficiency due to the accumulation of free radicals in testicular tissue, leading to damage in the testes, Sertoli, and Leydig cells. This disruption affects the hypothalamus-pituitary-gonadal axis, which plays a critical role in spermatogenesis. Reducing saturated fat and total energy intake, along with probiotic supplementation, has been recommended to lower triglyceride levels in both humans and animals. This study aimed to evaluate the effectiveness of Lactobacillus sp. and Bacillus subtilis as probiotics in Wistar rats (Rattus norvegicus) subjected to a high-cholesterol diet. A total of 25 rats were divided into five treatment groups, with body weights ranging from 150–250g. Sperm morphology was assessed by analyzing the structural characteristics of 100 randomly selected spermatozoa stained with 0.05% eosin-Y. The sperm count analysis categorized sperm forms into normal and abnormal categories, including primary and secondary abnormalities, as well as sperm agglutination. A decline in motility was observed in the K+ group, which was given a high-fat diet, with values of 53.33±7.64 compared to the K- group with values of 83±1.75. Furthermore, the highest abnormality rates were recorded in the K+ group (11.67±4.93) compared to the K- group (4.67±1.15). However, after probiotic intervention, the KP3 group, which received a combination of Lactobacillus sp. and Bacillus subtilis, demonstrated the highest motility and the lowest abnormalities, indicating its potential in mitigating the adverse effects of a high-fat diet.

Keywords: Probiotics, Spermatozoa, High-fat diet.

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INTRODUCTION

One of the major contributors to global mortality is coronary heart disease (CHD). Coronary heart disease (CHD) is primarily driven by factors such as hypercholesterolemia, atherosclerosis, poor lifestyle habits, and the consumption of high-fat foods, all of which may worsen the progression of the disease (Smith & Brown, 2023). Recent studies indicate that a high-fat diet, obesity, and abnormalities in lipid metabolism contribute substantially to elevated LDL levels in the bloodstream, thereby worsening cardiovascular diseases (Jiang et al., 2022).

Triglycerides are the primary lipids found in food. Elevated triglyceride levels in the blood can increase very low-density lipoprotein (VLDL) concentration, raising the risk of arterial plaque formation and, consequently, atherosclerosis. Factors such as excessive fat and energy intake, obesity, and sedentary lifestyles contribute to high triglyceride levels. Lowering triglyceride levels is crucial for reducing the risk of atherosclerosis, which can be achieved through pharmacological interventions (medications) or non-pharmacological methods such as dietary regulation (Sadishkumar & Jeevaratnam, 2016). Changes in dietary habits, particularly reducing intake of saturated fat and

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cholesterol, play a vital role in restoring metabolic balance. Hypercholesterolemia affects multiple organ systems, including digestion, reproduction, immunity, and cardiovascular function. One significant consequence of an uncontrolled high-fat diet is a decline in reproductive efficiency.

A high-fat diet can impair reproductive efficiency due to the excessive accumulation of free radicals in testicular tissue. This oxidative stress leads to damage in the testes, Sertoli cells, and Leydig cells, disrupting the hypothalamus-pituitary-gonadal axis, which governs spermatogenesis (Nevin & Rajamohan, 2008). Studies have shown that a high-cholesterol diet can increase body weight while significantly reducing the weight of the testes, epididymis, seminal vesicles, ventral prostate, and vas deferens. Cholesterol imbalance adversely affects sperm function by interfering with spermatozoa maturation. Dietary interventions, including reducing saturated fat and total energy intake and incorporating probiotics, are highly recommended to lower triglyceride levels in both humans and animals (Mishra et al., 2015).

Probiotics are live microbial food supplements that provide health benefits by balancing intestinal microbiota. Unlike prebiotics, which are indigestible food components that stimulate the growth of beneficial gut bacteria, probiotics directly influence gut health. Combining probiotics and prebiotics, known as synbiotics, supports the growth and survival of beneficial gut bacteria. In this context, probiotic strains such as *L. delbrueckii*, *L. casei*, and *B. animalis* have been shown to lower serum cholesterol levels and improve liver structure in obese BALB/c mice (Bubnov et al., 2017). *Lactobacillus casei* is classified as a probiotic due to its ability to enhance digestive function by producing lactic acid, which suppresses the growth of harmful bacteria in the gut. Given this background, an in-depth investigation is necessary to assess the efficacy of Lactobacillus sp. and Bacillus subtilis as fat-degrading probiotics.

Recent research has revealed that probiotic supplementation can reduce the negative impact of a high-fat diet (HFD) on male reproductive health. Liu et al. (2024) show *Lactiplantibacillus plantarum* 1008 has been shown to improve testicular function and spermatogenesis in male mice with HFD-induced obesity by modulating gut microbiota. The combination of probiotics and metformin administration enhanced sperm quality in obese male mice. This therapeutic approach reduced inflammation and oxidative stress, while increasing androgen production, which in turn improved spermatogenic function in the testes (Liu et al., 2025). Studies conducted on humans, horses, and dogs with varying treatment durations (ranging from 3 weeks to 6 months) have demonstrated that probiotic administration can influence the success or failure of improvements in semen quality parameters (Helli et al., 2022; Mahiddine et al., 2023; Cooke et al., 2024). Yanai & Endo (2021) state that in adult individuals, long-term probiotic administration must consider the aging process and the potential for additional side effects that may arise with advancing age, so that prolonged probiotic treatment may be applicable to mice, with treatment starting during puberty to evaluate its effects from the early stages of development. The primary objective of this research is to evaluate the effectiveness of Lactobacillus sp. and Bacillus subtilis as probiotics administered to Wistar rats (Rattus norvegicus) with high cholesterol levels on quality sperm in rat.

MATERIALS & METHODS

Ethical Approval

This experimental laboratory research was conducted in accordance with ethical guidelines for the use of animals in research. Ethical approval for this study was obtained from the Ethics Committee of Animal Hospital Hasanuddin University, with Ethical Clearance Number 0035/KKEH/RSHUH/EC/2023. All experimental procedures involving animals adhered to institutional and international ethical standards, ensuring the humane treatment and welfare of the animals throughout the study.

Experimental Animals and Parameters Studied

This study used 25 male Wistar rats (Rattus norvegicus) obtained from local breeders in Makassar, South Sulawesi. The rats weighed between 150 and 250g. They were housed in plastic containers covered with wire mesh and maintained in controlled conditions. Prior to the experiment, the rats were acclimatized for seven days, during which they were provided with food and water *ad libitum*. The animals were divided into five experimental groups are K- (Negative Control): Standard diet without high-fat feeding, K+ (Positive Control): High-fat diet without probiotic supplementation, KP1: High-fat diet with Bacillus subtilis supplementation, KP2: High-fat diet with Lactobacillus sp. supplementation. and KP3: High-fat diet with a combination of Bacillus subtilis and Lactobacillus sp.

The high-fat diet was administered once daily for 30 days. After this period, the treatment groups (KP1, KP2, and KP3) received probiotic supplementation for an additional 30 days, while the K- and K+ groups continued with their respective diets. The probiotics were dissolved in distilled water, containing 4.72×10^{8} CFU/mL of Bacillus subtilis or Lactobacillus sp. (Chen et al., 2013; Negi et al., 2024). Sperm samples were collected from the testicles of euthanized rats. Euthanasia was performed by administering a high dose of ketamine and xylazine, followed by necropsy. The testicular organs were extracted, and spermatozoa were collected from the cauda epididymis. For motility assessment, a wet mount was prepared from the sperm suspension diluted in 0.9% NaCl solution. Sperm motility was evaluated under a light microscope. Sperm morphology analysis was assessed by staining sperm samples with 0.05% eosin-Y. A total of 100 sperm cells per sample were examined for normal and abnormal morphology, including primary and secondary abnormalities and sperm agglutination.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was set at $P<0.05$.

RESULTS & DISCUSSION

The results of this study indicate that sperm motility and morphology were significantly affected by the high-fat diet and probiotic treatment. The negative control group (K-) exhibited the highest sperm motility, while the positive control group (K+) showed the lowest motility, confirming the detrimental effects of a high-fat diet. Among the treatment groups, KP3, which received a combination of Bacillus subtilis and Lactobacillus sp., demonstrated the highest motility recovery, suggesting a synergistic effect between the two probiotics.

Table 1 presents the average spermatozoa motility results. The statistical differences in mean spermatozoa motility among treatment groups were analyzed using statistical tests. The ANOVA test results on day 30 indicated no significant ($P>0.05$) differences, and the Tukey test confirmed no significant differences among the treatment groups. In contrast, spermatozoa motility on day 60 showed that the mean motility in the K- group was significantly different from that in the K+ and KP1 groups but not significantly different from that in the KP2 and KP3 groups. Meanwhile, the K+ group did not exhibit significant differences compared to any of the treatment groups (KP1, KP2, and KP3) (Table 1).

Table 1: Results of motility (%) of rat spermatozoa on day 30 and 60 of experiment

Treatment	Day 3	Day 60
K-	82.5±3.53aA	83±1.73aB
K+	55.0±7.07a	53.33±7.64b
KP1	57.5±3.53a	61.67±7.64b
KP2	57.5±10.6aA	65±5.00bB
KP3	57.0±7.07aA	67.67±5.77bB

Values (mean±SD) bearing different small letters in a column and capital letters in row differ significantly ($P<0.05$).

The average calculation of spermatozoa abnormalities is presented in Table 2. The statistical differences in mean spermatozoa abnormalities among treatment groups were analyzed using statistical tests. The results of the ANOVA and Tukey tests showed no significant differences among the treatment groups on both day 30 and day 60 (Table 2). Sperm abnormalities were most pronounced in the K+ group, with a higher percentage of morphological defects compared to the other groups. The administration of probiotics, particularly in the KP3 group, significantly reduced the incidence of abnormalities, further supporting the protective role of probiotics in sperm quality (Mishra et al., 2015; Wang et al., 2017).

Table 2: Results of abnormalities (%) of rat spermatozoa on day 30 and 60 of experiment

Treatment	Day 3	Day 60
K-	6.0±2.82a	6.0±1.00b
K+	10.0±8.48a	11.67±4.93b
KP1	10.0±5.66a	6.67±0.58b
KP2	9.0±1.41a	6.33±3.21b
KP3	9.0±1.41a	4.67±1.15b

Values (mean±SD) bearing different small letters in a column differ significantly ($P<0.05$).

Prepare a Panel of Photos of Sperm Abnormalities of Each Treatment so that Readers Could Understand/see these Sperm Abnormalities

The image above illustrates spermatozoa abnormalities observed under a microscope at 400x magnification. Fig. 1 illustrates the observed sperm abnormalities, such as detached heads, broken and folded tails, shortened tails, tails folded over the heads, and bent tails. These morphological defects can be categorized into primary and secondary abnormalities (Paoli et al., 2020).

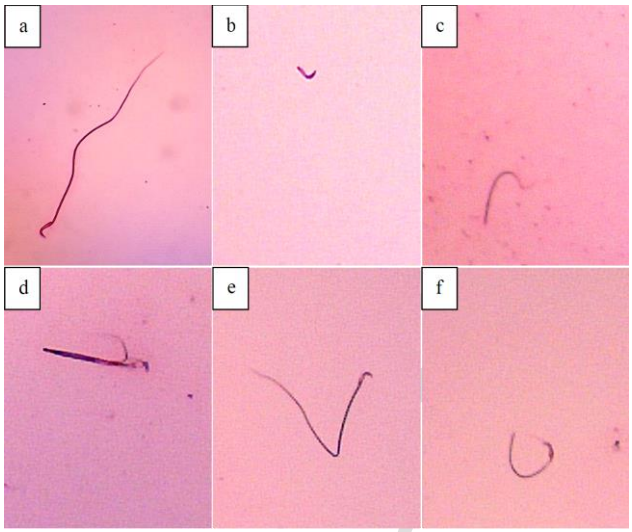

Fig. 1: Observation results of spermatozoa morphology: (a) normal sperm, (b) decapitated sperm (head only), (c) broken tail, (d) folded tail, (e) bent tail, (f) short tail.

Primary abnormalities, such as spermatozoa with coiled tails, originate during spermatogenesis within the testes and are often indicative of disruptions in the developmental process of sperm cells. These defects may result from increased reactive oxygen species (ROS) levels, which can adversely affect the plasma membrane integrity of spermatozoa. Secondary abnormalities, including decapitated spermatozoa, headless spermatozoa, and spermatozoa with broken tails, are typically acquired post-spermiation. Such defects are often associated with mishandling or environmental stressors affecting spermatozoa after they have exited the epididymis.

The research results show that from day 30 to day 60, there was an improvement in sperm motility with an increased percentage in the treatment groups. Meanwhile, the rate of abnormalities decreased, indicating an improvement in sperm quality after the administration of probiotics. The observed improvements in sperm quality in the probiotic-treated groups can be attributed to the antioxidant properties of probiotics, which mitigate oxidative stress induced by the high-fat diet. Previous studies have shown that probiotics enhance sperm viability by reducing lipid peroxidation and modulating the gut microbiota to improve metabolic health (Chen et al., 2014).

A high-fat diet is known to induce oxidative stress, which negatively affects sperm parameters, including motility, morphology, and viability (Nevin & Rajamohan, 2008). The significant reduction in sperm abnormalities observed in probiotic-treated groups indicates that probiotics may counteract these adverse effects by improving the antioxidant defense system. According to Mishra et al. (2015), probiotics can enhance antioxidant enzyme activity, reduce nitric oxide content, and lower

malondialdehyde levels, thereby preventing oxidative damage. Research indicates that probiotics can increase the proportion of normal sperm and modify sperm head shape, potentially enhancing sperm swimming capabilities (Sanchez-Rodriguez et al., 2024).

Furthermore, probiotics have been reported to improve lipid metabolism and modulate inflammatory responses, which may contribute to the observed improvements in sperm quality (Sadishkumar & Jeevaratnam, 2016). The role of probiotics in maintaining intestinal microbiota balance is crucial for reducing systemic inflammation, which has been linked to poor reproductive health outcomes (Seo et al., 2015).

Overall, these findings suggest that probiotics, particularly when used in combination, can effectively counteract the negative effects of a high-fat diet on sperm quality. Further research is warranted to explore the underlying molecular mechanisms and potential applications in reproductive health.

Conclusion

This study demonstrated that a high-fat diet negatively affects sperm motility and morphology in male Wistar rats. The administration of probiotics, particularly a combination of Bacillus subtilis and Lactobacillus sp., significantly improved sperm motility and reduced abnormalities. These findings highlight the potential of probiotics in mitigating the adverse effects of a high-fat diet on male reproductive health. The observed improvements in sperm quality are likely due to the antioxidant and anti-inflammatory properties of probiotics, which help counteract oxidative stress and maintain gut microbiota balance. Future research should focus on elucidating the molecular mechanisms underlying these protective effects and exploring their applications in human and veterinary medicine.

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