Research Article

Protective Effects of Encapsulated *Bacillus subtilis* on Oxidative Stress, Spermatogenesis, and Fertility Outcomes in Experimental Cholestasis

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ABSTRACT

Objectives: Probiotics such as *Bacillus subtilis* exhibit antioxidant and anti-inflammatory activities. However, their viability may be reduced in the gastrointestinal tract (GIT) due to acidity and enzymatic degradation. Encapsulation can improve probiotic survival and bioactivity. Probiotics have also been implicated in mitigating oxidative stress–induced reproductive toxicity. This study evaluated the effects of encapsulated *B. subtilis* on oxidative balance, sperm quality, and testicular histology in an experimental model of liver cholestasis.

Methods: Forty-eight male Wistar rats were randomly assigned to four groups: control, probiotic, free capsule, and encapsulated probiotic. Encapsulated *B. subtilis* (3 × 109 CFU/day) was administered orally for one week prior to bile duct ligation (BDL) and for three weeks afterward. At the end of the experiment, rats were euthanized, and blood and testes were collected for biochemical, hormonal, and histological analyses.

Results: BDL markedly reduced sperm concentration and viability while increasing morphological abnormalities. Encapsulated *B. subtilis* significantly improved sperm parameters compared to free probiotics. Testicular oxidative stress, evidenced by increased protein carbonyls and total oxidant status with decreased reduced glutathione, was attenuated by encapsulated probiotics. Histological analysis revealed disrupted testicular architecture and decreased Johnson's scores after BDL, whereas encapsulated *B. subtilis* restored seminiferous tubule integrity and spermatogenesis..

Conclusion: Encapsulated *B. subtilis* enhanced probiotic efficacy, improving sperm quality, antioxidant status, and testicular structure in cholestatic rats. These findings suggest a protective role for encapsulated probiotics in male infertility associated with oxidative stress and liver disease.

Keywords: Probiotics; Bacillus subtilis; Encapsulation; Cholestasis; Male infertility

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Introduction

Liver plays an important role in sex hormones regulation and spermatogenesis; therefore, liver disorders such as Liver fibrosis, caused by the imbalance between synthesis and degradation of extracellular matrix (ECM) can adversely affect male productive system leading to conditions such as atrophy of testis, reduction of testosterone levels, reduced libido and compromised fertility (1). Although liver has regenerative abilities, if not treated properly and in time, fibrosis can lead to irreversible damage to the male reproductive system function (2).

There are several models that can be induced to study liver fibrosis such as chemical models, induced by agents like carbon tetrachloride, thioacetamide, and dimethylnitrosamine, and surgical models such as bile duct ligation (BDL) which replicate physiological conditions associated with portal hypertension and liver cirrhosis in humans (3). BDL is particularly useful to study fibrosis in combination with cholestatic damage, wherein fibrotic change is caused by liver and circulatory overload of toxic bile acids due to bile duct obstruction. These bile acids are toxic to a number of organs, including those protected by barriers like the blood-brain and blood-testis barriers (4).

The blood-testis barrier protects the male reproductive organs, although xenobiotics such as heavy metals are able to breach this barrier, leading to reproductive disorders, including testicular necrosis, edema, hemorrhage, and decreased sperm quality (5). Although it is known that these toxic compounds exert negative influences on reproductive health, their mechanisms remain unclear. Studies reveal that the overproduction of reactive oxygen species (ROS) is capable of injuring cellular antioxidant defenses, increasing bile acid cytotoxicit (6). Ommati et al. referred to the association of cholestasis with reproductive abnormalities such as testicular injury, endocrine disturbances, and defective sperm qualit. Hydrophobic bile acids, being surfactants, possess the ability to solubilize lipids and proteins of the plasma membrane, disrupting cellular integrity (5, 7).

Available assisted reproductive technologies (ART), such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), are implemented in the treatment of infertility but may be limited in patients with high seminal ROS levels, necessitating alternative treatments (8). Probiotics are a recent development with promise in improving sperm quality in animal and human experiments (9). The World Health Organization (WHO) says that probiotics—live microorganisms conferring host health by maintaining gut microbiota—exert a positive effect on diseases like gastrointestinal diseases, inflammation, and allergies (10).

Bacillus subtilis (B. subtilis), which is present in a wide range of foods as a probiotic, is anti-inflammatory and can modulate immune responses by downregulating inflammatory cytokines. *B. subtilis* strains downregulate the expression of inducible nitric oxide synthase and cyclooxygenase-2, nitric oxide, and prostaglandin E2, and inhibit IL-1β and IL-6 levels, influencing inflammatory signaling pathways such as nuclear factor-κB, MAPK, and AP-. *B. subtilis* possesses anti-oxidant activities as reported by some research; hence, this ability may have some effects on oxidative-induced testicular tissue and sperm morphology and concentration damage.

Encapsulation of probiotics with alginate, arabic gum, and chitosan enhances their stability, viability, and gastrointestinal (GI) delivery. Alginate provides encapsulation efficiency, arabic gum supports structural integrity and controlled release, and chitosan facilitates mucosal adhesion and provides further antimicrobial protection for improved probiotic survival and gut health (11, 12). Alginate is able to dissolve in intestinal alkaline pH and also act as a substrate for gut microbiome; therefore, alginate is able to be degraded and dissolved in the intestine of the host, leading to probiotic release. Therefore, encapsulation with the aforementioned chemicals not only improves GIT viability but also facilitates intestinal release (13, 14).

The effect of alginate-arabic gum and chitosan-coated encapsulated *B. subtilis* on male reproductive system toxicity caused by bile duct ligation has not been explored. Therefore, this study aims to assess the effects of *B. subtilis* encapsulated with alginate-arabic gum with chitosan coating on testicular toxicity, with focus on biochemical, spermatological, histological and the impact on fertility in male Wistar rats following bile duct ligation.

Material and methods

Study's ethics

This study has the approval of Kashan University of Medical sciences in Kashan, Iran ethics committee under the code IR.KAUMS.AEC.1401.010. During the treatment period, rats were euthanized through cardiac puncture under anesthesia, which is in accordance with the guidelines set by the ethics committee for animal research at the same institution.

Preparation of Probiotic and Encapsulated Probiotic

Bacillus subtilis lyophilized powder was kindly provided by Zist Darman Mahan Co. (Tehran, Iran), and species identification was confirmed through standard morphological, cultural, physiological, and biochemical tests. Bacterial enumeration was performed using the pour-plate method on MRS agar after anaerobic incubation at 37°C for 48 h.

The preparation and characterization of alginate/Arabic gum–chitosan (APC) encapsulated *B. subtilis* were previously described in detail in our earlier work (15). In the present study, the same APC microcapsules were used.

Animals

Forty-eight Albino male Wistar rats aged 6–8 weeks (180–200 g) were maintained in standardized conditions in a controlled animal facility: temperature (25 ± 0.5°C), humidity (60%), and 12-hour light/dark cycle (08:00–20:00 light on). The animals were maintained in a clean environment with free access to food and water throughout the study.

Study Design and Treatment

The rats were selected at random and allocated into six treatment groups (n=8/group):

1. Normal Control (NC): Healthy rats without treatment.
2. Sham-Operated (SHC): Had laparotomy but without bile duct ligation (BDL).
3. BDL Control: Had BDL but without treatment.
4. BDL + Non-Encapsulated *B. subtilis*: Treated with 3×10^9 CFU of probiotic daily
5. BDL + free alginate-arabic gum with chitosan coating: Administered with 0.1 g/day of placebo microcapsules.
6. BDL + Encapsulated *B. subtilis*: Treated with 3×10^9 CFU of probiotic-loaded microcapsules daily.

Treatment was initiated one week prior to BDL surgery and continued for three weeks post-surgery.

Confirmation of BDL in Rats

Successful BDL was confirmed by jaundice (yellow discoloration of skin and urine) within 48–72 h post-surgery. On the fourth day, rats were placed in metabolic cages to obtain urine, and bilirubin levels were measured with test strips. Increased levels of bilirubin confirmed BDL induction and made rats suitable for inclusion in the study (16).

Sample Preparation

Rats were sedated and weighed after treatment in a CO_2 chamber. Blood was collected by cardiac puncture, followed by transection of the aorta to ensure euthanasia. Serum was separated by centrifugation (3000 ×g, 10 min to avoid hemolysis) and stored at −80°C for hormonal determination (17). The right epididymis was removed for sperm analysis, while the right testis was fixed in 10% formalin for histological examination. The left testis was snap-frozen at −80°C for oxidative stress determination.

Sperm Concentration

Epididymal sperm suspension was prepared according to WHO standards in PBS (pH 7.4), incubated at room temperature for 5 min, and diluted (10 µL epididymal sperm suspension + 990 µL distilled water). Sperm heads were counted from four hemocytometer chambers under light microscope (Nikon, Japan), and concentration was expressed as million sperm/mL (18).

Sperm Viability

Viability was assessed using an eosin-nigrosin staining kit (DDK Co., Italy). 5 µL sperm suspension was mixed with 5 µL eosin and 10 µL nigrosin. Two hundred sperm were counted microscopically per sample, and the viability percentage (percentage of unstained sperm) was determined (18).

Sperm Morphology

Sperm abnormalities were evaluated by staining 10 µL of sperm suspension with Papanicolaou stain. Two hundred sperm per slide were evaluated as normal or abnormal based on deficiencies in head, neck, or tail, and the abnormality rate was expressed as a percentage (18).

Biochemical Analysis

Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels were determined using commercially available ELISA kits (Zell Bio, Germany) according to the manufacturer's manual guide.

Tissue Homogenization

Testicular tissue (100 mg) was homogenized in liquid nitrogen and lysed in buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.2% Triton X-100, and protease inhibitor). After incubation (20 min, 4°C), samples were centrifuged (14,000 ×g, 20 min) and supernatants utilized for protein quantitation (Bradford assay) and oxidative stress analysis (19) .

Determination of oxidative/ anti-oxidative status in rat testis

Presence of PCO was measured spectrophotometrically by the color developed during reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH). Intensity of color was measured at 360 nm wavelength (20). Total oxidant status (TOS) was calculated by oxidation of ferrous ions to ferric ions, which reacted with xylenol orange to develop colored complex in acidic medium. This reaction is observed in the presence of various oxidants, and the developed intensity of the colour was read at 560 nm (21). The amount of reduced glutathione (GSH) in homogenized testicular tissue was quantified using 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), which is a thiol compound that reacts with GSH to form a yellow colour (22). Nitric oxide (NO) levels were assayed by Griess method in which nitrate in the sample is converted to nitrite by nitrate reductase. The nitrite forms an azo complex with Griess reagents that is measurable at 540 nm (23).

Histological Examinations

The appropriate testis and epididymis were fixed in 10% formalin (24–48 h), paraffin embedded, and

sectioned (5 µm). After H&E staining, the blinded pathologist scored 10 tubules for each rat according to Johnsen's scoring system (24):

Score 10: Uniform spermatogenesis with abundant sperm. Score 9: Numerous sperm but disorganized epithelium, Score 8: Few sperm present, Score 7: No sperm; numerous spermatids, Score 6: Few spermatids, Score 5: Only spermatocytes, Score 4: Few spermatocytes, Score 3: Spermatogonia only, Score 2: Sertoli cells only, Score 1: Absence of germ cells.

Statistical Analysis

Values are expressed as mean ± SD. The groups were compared by one-way ANOVA with Dunnett's post-hoc test (GraphPad Prism v10); non-parametric data were compared by Kruskal-Wallis test. A *p*-value <0.05 was considered statistically significant.

Results

Dosage of probiotic

After performing serial dilution and cultivation of *B. subtilis* for 48 hours in De Man–Rogosa–Sharpe (MRS) agar, the dosage of probiotic was evaluated to be $3\times 10^{10}\times 10^{10}$ CFU/gr. After determining the dosage of *B. subtilis*, microcapsules with the dosage of $3\times 10^{9} \times 10^{9}$ CFU/gr were prepared and used for treating rats.

Effect of encapsulated *B. subtilis* on sex hormone levels

As shown in the figure 1 BDL surgery significantly reduced FSH ($P \leq 0.05$), LH ($P \leq 0.001$) and testosterone ($P \leq 0.01$) levels compared to the Sham control group (SHC). Even though the levels of these three hormones increased in the free probiotic treatment group, the change was not significant. The levels of sex hormones in free capsule treatment group showed no difference compared to the BDL group. However, treatment with encapsulated *B. subitlis* increased the levels of FSH ($P \leq 0.05$), LH ($P \leq 0.05$) and testosterone ($P \leq 0.05$) significantly compared to the BDL control group. Data are presented in figure 1.

Effect of encapsulated *B. subtilis* on sperm quality

Our results exhibit that sperm concentration and viability reduced significantly and morphological abnormalities increased significantly in BDL control group compared to SHC group ($P \leq 0.001$, $P \leq 0.0001$ and $P \leq 0.0001$ respectively). The changes on sperm quality factors in free probiotic and free capsules' treatment group were not significant. However, treatment with encapsulated *B. subtilis* significantly increased sperm concentration ($P \leq 0.01$) and viability ($P \leq 0.01$) and reduced sperm morphological abnormalities ($P \leq 0.0001$). Data are presented in figure 2.

Effect of encapsulated *B. subtilis* on oxidative status

Rats subjected to bile duct ligation (BDL) demonstrated significantly elevated levels of protein carbonyl (PCO; $P \leq 0.001$) and total oxidant status (TOS; $P \leq 0.05$), along with reduced glutathione (GSH) levels ($P \leq 0.05$), compared to the Sham control group. Administration of free *B. subtilis* significantly decreased PCO and TOS levels ($P \leq 0.05$) and moderately increased GSH levels relative to the BDL control group. Treatment with the empty capsule resulted in non-significant reductions in GSH, TOS, and PCO. In contrast, rats treated with *B. subtilis* encapsulated in alginate-arabic gum with a chitosan coating exhibited a pronounced reduction in PCO ($P \leq 0.0001$) and TOS ($P \leq 0.01$), as well as a significant increase in GSH ($P \leq 0.05$). When comparing free and encapsulated probiotic treatments, the encapsulated form resulted in numerically lower TOS and higher GSH levels than the free probiotic, though these differences were not statistically significant. However, PCO levels were significantly lower in the encapsulated probiotic group

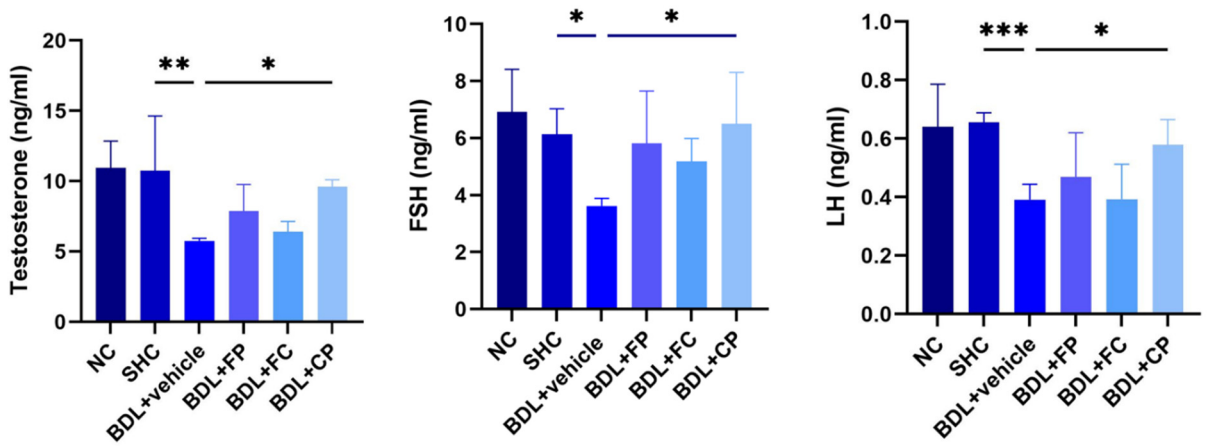

Figure 1. Effects of free and encapsulated probiotics on serum testosterone, FSH, and LH levels in Bile Duct-Ligated rats. Abbreviation of groups are as followed: NC: normal control, Sham: Sham control, BDL+vehicle: bile duct surgery group, BDL+FP: BDL rats treated with free probiotic, BDL+FC: BDL rats treated with free capsule and BDL+CP: BDL rats treated with encapsulated probiotics. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ compared to BDL+vehicle group.

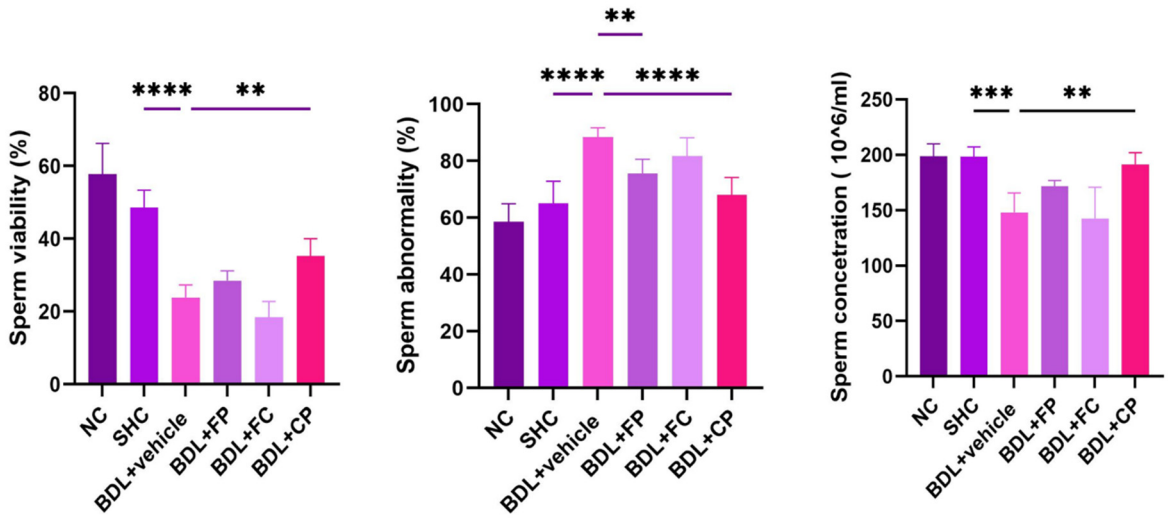

Figure 2. Impact of free and encapsulated probiotic treatment on sperm concentration, viability, and morphological abnormalities in Bile Duct-Ligated rats. Abbreviation of groups are as followed: NC: normal control, Sham: Sham control, BDL+vehicle: bile duct surgery group, BDL+FP: BDL rats treated with free probiotic, BDL+FC: BDL rats treated with free capsule and BDL+CP: BDL rats treated with encapsulated probiotics. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ compared to BDL+vehicle group.

compared to the free probiotic group. No significant differences were observed in nitric oxide (NO) levels across the experimental groups. Data are presented in figure 3.

Effect of encapsulated *B. subtilis* on histological parameters

Our results indicate that in the healthy control and sham groups, all seminiferous tubules appeared normal. Complete spermatogenesis and mature spermatozoa were observed in some tubules. Additionally, a significant number of spermatozoa were visible in the epididymal tubules of these two groups. In the BDL group, the seminiferous tubules exhibited a reduction in complete spermatogenesis and mature spermatozoa compared to the control and sham groups, with some tubules also showing decreased cellular density. Furthermore, the number of spermatozoa in the epididymal tubules of the BDL group was notably lower than in the healthy control and sham groups. A similar reduction was observed in the treatment groups as well. The picture of testicular tissue is shown in figure 4 with X100 magnification.

Also, testicular tissue was assessed by a professional pathologist using the Johnson's scoring method. As the results indicate, the Johnson's score reduced significantly ($P \leq 0.01$) in the BDL control group than SHC group. treatment with free probiotic and free capsules did not lead to any significant change; however, treatment with encapsulated *B. subitlis* alleviated BDL induced testicular injury which was concluded by increased Johnson's score ($P \leq 0.01$) (Table 1).

Discussion

In the present study, obstruction of common bile duct was employed to induce cholestatic liver dysfunction and damage in male Wistar rats to assess the effects of treatment with *B. subtilis* encapsulated with alginate-arabic gum coated with chitosan in alleviating testicular damage and reproductive issues linked with bile duct obstruction.

Our findings showed increased bilirubin levels in the urine of rats on the third day following surgery to ligate the bile duct (BDL). Our finding is consistent with that noted in earlier studies by Hajian et al (24). The pathologic processes after BDL show time-dependent evolution, where bile acid composition, gene expression, and immune cell infiltration change over days to weeks following surgery. For optimal assessment of hepatic fibrosis, we recommend that rats must be assessed between 21 and 28 days after BDL.

Table 1. Assessment of testicular spermatogenesis using Johnson's Score in different rat groups. Data are presented as Mean ± SD

NC	SHC	BDL+ vehicle	BDL+FP	BDL+FC	BDL+CP
6.75±0.5	6.5±0.5 **	3 ± 1.22	3.25 ± 0.95	3 ± 0.81	5.25 ± 0.95 **

Abbreviation of groups are as followed: NC: normal control, Sham: Sham control, BDL+vehicle: bile duct surgery group, BDL+FP: BDL rats treated with free probiotic, BDL+FC: BDL rats treated with free capsule and BDL+CP: BDL rats treated with encapsulated probiotics. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ compared to BDL+vehicle group.

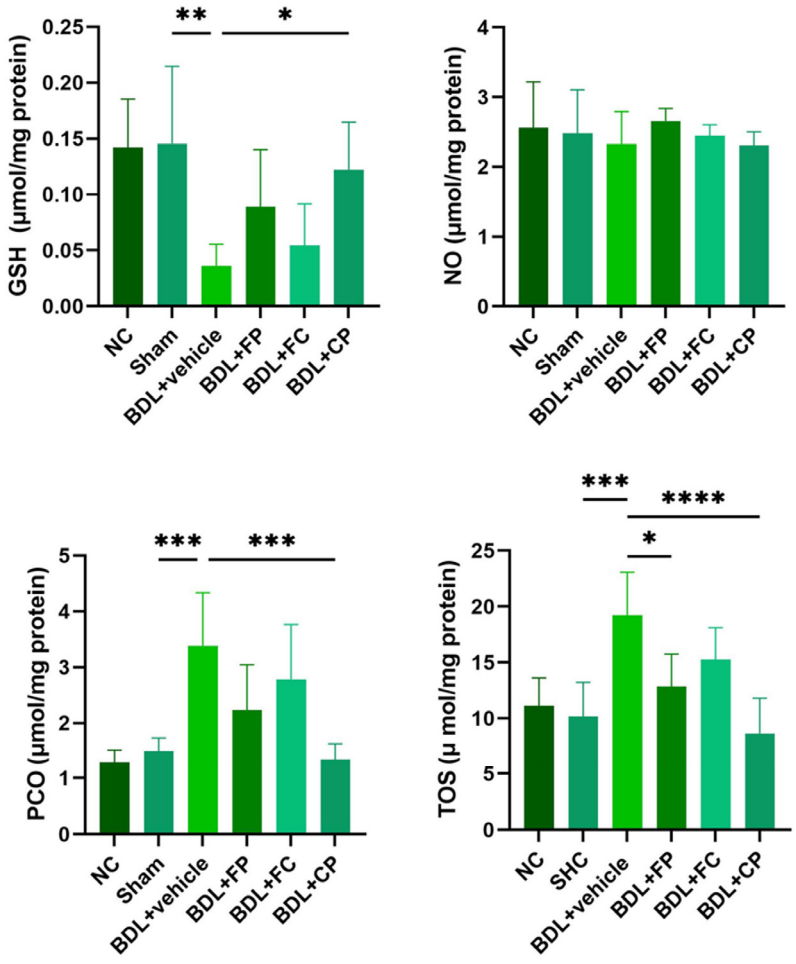

Figure 3. Antioxidant and oxidant parameters in BDL rats treated with free or encapsulated probiotics. Abbreviation of groups are as followed: NC: normal control, Sham: Sham control, BDL+vehicle: bile duct surgery group, BDL+FP: BDL rats treated with free probiotic, BDL+FC: BDL rats treated with free capsule and BDL+CP: BDL rats treated with encapsulated probiotics. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ compared to BDL+vehicle group.

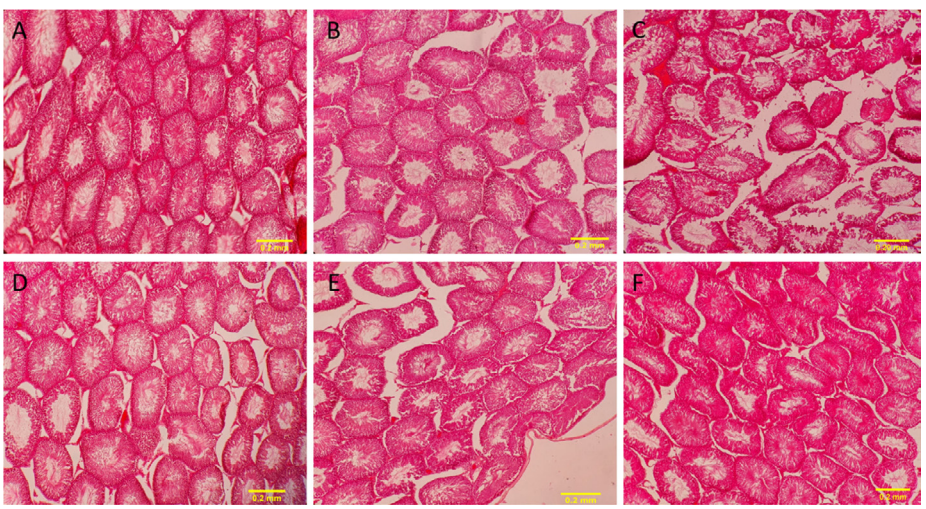

Figure 4. Histopathological effects of free and encapsulated *B. subtilis* on testicular tissue in BDL rats. Testicular tissue sections stained with H&E at 100X magnification with a 2mm scale are shown. (A) normal control (NC), (B) sham control (SC), (C) BDL control, (D) BDL+Free Probiotic group, (E) BDL+Free capsule group, and (F) BDL+Encapsulated probiotic group.

In the present work, we have investigated the impact of *B. subtilis* encapsulated in alginate-arabic gum with a chitosan coating on sperm parameters, testicular histology, oxidative condition, and sex hormone levels. The probiotic therapy was initiated one week before BDL and continued for three weeks post-surgery.

Our results indicated that although free *B. subtilis* affected sex hormone concentrations, the changes were not significant; on the other hand, treatment with encapsulated *B. subtilis* significantly increased sex hormone levels. Recent evidence suggests that probiotics are capable of modifying sex hormone levels like testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The mechanisms involved in these actions could be through alteration of gut microbiota, alteration of sex hormone metabolism and precursor pool, and hormonal regulation through the gut-brain axis. Probiotics could also directly influence stimulation of hormone secretion through increased Leydig cell function and prevention of testicular atrophy through the release of metabolites like acetate, propionate, and butyrate. Although no present study directly addresses action mechanism of *B. subtilis* in increasing sex hormones, several studies suggest that *B. subtilis* can alter gut microbiome, produce and release SCFAs that affects gut-brain-gonadal axes and indirectly increase GnRH and subsequently increase FSH, LH and then testosterone (25, 26). Therefore, the changes of sex hormone levels can be attributed to this. However, free probiotics effect was not significant while the encapsulated form's effect was; this can be attributed to low treatment dosage and short time period, and also low viability of *B. subtilis* cells during gastrointestinal transit leading to lower effectiveness. Thus, we can conclude that encapsulation of *B. subtilis* can increase its effectiveness towards increasing sex hormone levels in blood serum.

Biliary obstruction may induce oxidative stress by facilitating bile salt and other metabolite buildup. This one can disrupt cell structure, cell function and increase oxidative stress within the testicular tissue. Literature demonstrates that following 7 days of BDL, the markers of oxidative stress were elevated in testes, impairing testicular function, spermatogenesis and sperm quality. The result of this research shows that bile duct ligation (BDL) of rats leads to severe testicular oxidative injury, as evident from significant elevation of protein carbonyl (PCO) and total oxidant status (TOS), and reduction in glutathione (GSH) content with respect to sham-operated controls. These findings are in accordance with previous studies that cholestasis induces systemic oxidative stress via mechanisms involving bile acid accumulation and mitochondrial dysfunction (32). Of note, free *B. subtilis* administration decreased these markers of oxidation significantly by decreasing PCO and TOS and slightly increasing GSH, suggesting that BS possesses native antioxidant activity possibly mediated by direct free radical scavenging and perhaps through enhancement of host antioxidant defense mechanisms. Whereas the blank capsule group showed only non-significant changes in the oxidative parameters, the encapsulated form of BS—alginate-arabic gum with chitosan coating—evinced a meaningful and statistically significant improvement over both BDL and free BS treatments, particularly for PCO. This improved efficacy of encapsulated BS can be attributed to various factors like improved survival and directed delivery of live bacteria to the gut by virtue of the protective advantages of the encapsulation matrix, in addition to potential synergistic antioxidant activity by chitosan itself. Encapsulation may also assist in controlled release and prolonged probiotic action within the gastrointestinal tract, and thus overall antioxidant effect is increased. While encapsulated BS decreased TOS and increased GSH in numbers versus free BS, diminishment in PCO alone was significant statistically between these two groups. Interestingly, NO concentrations were not significantly different between groups, indicating that oxidative stress in this model may be mediated by mechanisms that do not involve NO-derived reactive species. These findings are consistent with evidence showing that *B. subtilis* on antioxidant defense mechanisms such as the Keap1/Nrf2 pathway, and that advanced delivery platforms like nanoencapsulation can supplement the antioxidant function of probiotics and their delivery systems (33). However, the study has the limitation of not mechanistically studying upstream regulatory pathways and tissue-specific responses, and lacking any available data on long-term reproductive or hormonal endpoints. Subsequent research should close these gaps by studying transcriptional regulators of the antioxidant response, performing histopathological analysis of testicular tissue, optimizing encapsulation formulations, and ascertaining clinical utility of reduction of oxidative stress in spermatogenesis and endocrine function. In total, this study recognizes the therapeutic ability of encapsulated *B. subtilis* to abate oxidative testicular damage induced by BDL and emphasizes the importance of new delivery approaches in providing maximum probiotic action in cholestatic models of injury (27-31).

Biliary obstruction leads to elevated serum bilirubin levels, which can adversely impact sperm quality and testicular function. Studies suggest that bilirubin disrupts the blood-testis barrier, exacerbates oxidative stress, and induces structural damage in testicular tissue, ultimately reducing sperm count and viability. Consistent with previous findings, our BDL control group exhibited a significant decline in sperm concentration, viability, and an increase in morphological abnormalities. As previously discussed, *B. subtilis* may counteract these effects by boosting testosterone levels, mitigating oxidative stress, and modulating the gut microbiome. These mechanisms likely contribute to improved sperm parameters. Our findings confirm that both free and

encapsulated *B. subtilis* significantly enhanced sperm count and viability, while also reducing morphological abnormalities. Although the encapsulated form demonstrated a stronger positive effect on sperm quality compared to the free probiotic, the differences between the two treatments were not statistically significant (30, 32, 33).

To identify histological features of testicular tissue, Johnson's score method was used by a pathologist. Similar to what hajian et al. already demonstrated, BDL can result in an opposite effect on testicular tissue and cause reduced spermatogenesis, seminiferous tubules diameter and area, spermatogenic epithelium area, ratio of spermatogenic epithelium area and Johnson score that are induced due to toxicity of biliary acids accumulated in blood stream (24) (34, 35).

In summary, based on the obtained results from our study, B. subtilis encapsulated with Alginate-arabic gum with chitosan coating has protective effects on testicular tissue of rats that underwent BDL surgery by reducing oxidative status and were able to enhance production and secretion of sex hormones such as FSH, LH and testosterone and sperm quality parameters. Additionally, as our results suggest, effectiveness of treating BDL-induced testicular damage with encapsulated *B. subtilis* might be higher than free *B. subtilis* treatment.

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During the writing of this article the author used ChatGPT artificial intelligence in order to paraphrase texts and check the grammar. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the article.

Author Contribution

MG: Methodology, Investigation. **SA:** Writing – review & editing, Methodology, Investigation. **MM:** review & editing, Supervision, Investigation, Conceptualization. **MS:** review & editing, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **AS:** Methodology, Investigation. **MA & MK:** Methodology, Investigation, Formal analysis. **EA:** Validation, Methodology. **HM:** Validation, Methodology.

Conflict of interest

The authors declare that there is no conflict of interest.

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