Research Article

Microencapsulated Symbiotic and Phytosteroids to Enhance Reproductive pattern in Male Albino Rats

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Abstract | Obesity affects metabolic hormones and consequently the male fertility. *spirulina platensis* (SP) and *Bifidobacterium longum* (BL) were known as health potentiators. The aim of this study was to produce microcapsules to act as functional feed additives to enhance general health and to study its effect on the reproductive performance of male albino rat. Total flavonoids, total phenolics, DPPH, HPLC and antimicrobial test against different types of bacteria were done for SP extract. BL has been microencapsulated in association with SP. Twentiy eight. adult male rats were divided into 4 equal groups. G1 received high fat diet (HFD), G2 received HFD and microencapsulated BL, G3 was supplemented by HFD plus encapsulated SP and BL. While G4 received basic diet as a control. After 2 months, animals were sacrificed. Results showed that SP had antimicrobial effect against pathogenic bacteria while, it kept probiotic bacteria alive. The animals fed on HFD showed elevated lipid profile, leptin, oxidative markers and low level of testosterone and the epididymal Spermatozoa had low sperm viability and higher abnormalities than control group. Feeding the microcapsules (G2,G3) improved the animals' health condition. Animals in G2 showed good spermatozoa profile while, those fed SP (G3) showed low sperm count and motility. Histopathological examination declared testicular degeneration in G1 and G3 and a high spermatogenesis in G2 compared to control group. It was concluded that spirulina may act as health potentiator but also, may negatively affect the male reproductive performance. Special care should be paid for the dose and frequency of administration of any polyphenol or health potentioators.

Keywords | *Spirulina platensis*, *Bifidbacterium longum,* Spermatozoa profile, Male fertility

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INTRODUCTION

Unhealthy high fat diets (HFD) contribute to the development of obesity and affect the metabolic and hormonal profile which control male fertility leading to lower testosterone level, erectile dysfunction, and disturbed spermatogenesis, changes in sperm concentration, motility and morphology (Glenn et al., 2019). *Spirulina platensis* (SP) strengthen the body, improves immunity and controls many of chronic diseases such as obesity, diabetes, rheumatism, hypertension (Karkos et al., 2011). It is categorized as prebiotic that enhances the growth of beneficial bacteria in the gut and act as adsorbents to other types of pathogens (Yousefi et al., 2019). Spirulina is rich in polyphenols. Earlier studies reported improved fertility of female fish fed on 8% spirulina in diet (James et al., 2006)

others mentioned that the treated semen with spirulina showed better sperm motility and fertilizing ability in swine (Granaci, 2007). SP is present in different forms in the market and gaining popularity as functional food (feed) supplements at nutritional and therapeutic levels (Karkos et al., 2011). Probiotics are viable microorganisms that are beneficial to the host when administered in appropriate quantities. Many commercial products containing *L. acidophillus*, *L. reuteri*, *L. rhamnosus* and *Bifidobacterium* used as functional food (El-Kholy et al., 2016).

Microencapsulation is a rapidly expanding technology in which every particle of the compound is surrounded or coated with a continuous film of polymeric material (Suganya and Anuradha, 2017). The encapsulation of bioactive agents can prevent the reaction between nutrients and reduce the astringency of the added bioactive compounds in the functional foods. It also improves their bioavailability by protecting them against oxidation and processing conditions for better delivery and to control their release (Mozafari et al., 2008). The objective of this study was to formulate microcapsules containing probiotic *Bifibacterium longum* (BL) and prebiotic (SP) to be used as functional feed additives used as health promoters and study the effect on male reproductive performance in albino rat model.

MATERIALS AND METHODS

Extraction of *spirulina platensis*

The blue-green alga, *Spirulina platensis* was provided by food industry and nutrition research institute, National Research Center, Cairo, Egypt. One gram of sample was mixed with 100 mL of methanol, ethanol or acetone and homogenized using the Ultra-Turrax homogenizer. The homogenates were kept at 4 °C for 12 h and then centrifuged at 10,000 rpm for 20 min. The supernatants were filtered through Whatman No. 1 filter paper (Whatman International Ltd., England) and the solvents were evaporated and stored at -20 °C until analysis (Duke et al., 2003). For testing the antimicrobial activity the extracts were mixed with Dimethyl sulfoxide (DMSO) and adjusted to a final concentration of 50 mg/ml.

Estimation of total phenolic and flavonoid content

Estimation of total phenolics, total flavonoids, antioxidant and polyphenol profile (HPLC) were done for the methanolic extract.

The total phenolic and flavonoid content was determined using gallic acid and rutin as standered (Kamboj et al., 2015; Ainsworth and Gillepsie, 2007).

Determination of radical DPPH scavenging activity

Free radical scavenging capacity was determined using the stable 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH$^{\bullet}$) compared to ascorbic acid as slandered (Mot et al., 2011).

Polyphenol profile by HPLC

Polyphenols HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using the Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (85% A) and 15-16 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 35 °C (Tian et al., 2004).

Antimicrobial assay of spirulina platensis extract

The microorganisms used in antimicrobial assays were supplied by Department of Dairy Microbiology Laboratory at National Research Center. The bacterial species taken for the study were (*Escherichia coli* 0157: H7 ATCC *6933, Bacillus cereus* ATCC 33018, *Staphylococcus aureus* ATCC 20231, *Yersinia enterocolitica* ATCC 27729 and *Salmonella typhimurium* ATCC 14028. *Listeria monocytogenes*V7 strain serotype 1 (milk isolate) was obtained from the Department of Food Science, University of Wisconsin, Madison, USA. *Lactobacillus plantarum* ATCC 8014, *Lactobacillus rhamnosus* ATCC 7469, *Leuconostoc mesenteroides* ATCC 8293, and *Bifidobacterium longum* ATCC 15697). Each strain was activated in Tryptone soy broth or MRS broth by incubation at 37 ºC for 24 h. The antimicrobial activity of algal extracts (using methanol, ethanol and acetone) were determined by the agar well diffusion method with some modification. One ml of the activated culture indicator strain (10^5 cells/ml) was inoculated into 20 ml of Mueller-Hinton agar (Becton Dickinson, USA) and poured in Petri dishes. After solidification of the agar, wells of 5 mm in diameter were cut from the agar with a sterile borer and 50µL of algal extracts were poured in each well. The zone diameter of wells cut in Mueller-Hinton agar was 5.0 mm and the diameter of inhibition zone (DIZ) of negative control for each bacterium was also 5.0 mm. The plates were incubated at 37°C for 24 h. Zones of inhibition were measured at the end of the incubation period (El-Sayed et al., 2017).

Microencapsulation procedure by freeze drying method

The culture of *Bifidobacterium longum* was sub-cultured in

MRS broth supplemented with 2 gm/l sodium propionate and 3 gm/l lithium chloride and incubated for 48h at 37 ºC. The BL cells were harvested and washed by centrifugation at 5000 rpm for 15 min at 4 ºC. The cell suspension (25 g) was mixed with a 100 ml of wall material sodium alginate solution (3.0% w/ v) (Fayed et al., 2018; El-Shenawy et al., 2016). After that, 3% of spirulina powder was added to amount of the coated BL and stirred using magnetic stirrer. The mixture was kept for 24 h at -8ºC then subjected to Freeze dryer for 8h. The obtained microcapsules or freeze-dried powder was stored at 4ºC. The same encapsulation process was done for the remaining part of bacterial suspension coated by alginate only without adding SP. The two types of microcapsules were stored at 4 ˚C until be used (El-Sayed et al., 2021).

Identification of microencapsulation efficiency (ME)

One gm of the microcapsules was dissolved in 9 ml of sterile tri sodium citrate solution 2% (w/v) and stirred to release the bacterial cells, and then serial dilution with physiological saline was done. Cultivation on plates containing MRS agar supplemented with 2gm/l sodium propionate and 3gm/l lithium chloride, anaerobically incubated at 37ºC for 48h (Mahmoud et al., 2020). The microencapsulation efficiency (ME) was determined using the following equation:

$$ME = Log_{10}N / Log_{10}No \times 100$$

N = the number of the *B. longum* loaded inside the microcapsules; No= the number of the free *B. longum* added to the sodium alginate mixture during the process of microencapsulation.

Diet formulation

Two types of diets were prepared for animal feeding, normal fat diet and high fat diet (Table 1).

Table 1: Ingredients of control and treatment diets.

Ingredients	Control diet %	High fat-high cholesterol diet %
Casein	10	10
Cellulose	10	0
Corn oil	10	0
Bile Salt	4	0.25
Cow fat	0	20
Cholesterol	0	2
Salt mixture	0	4
Vitamin mixture	1	1
L-Cystine	0.018	0.018
Choline chloride	0.025	0.025
Corn starch	64.957	63.545

Biological experiment

Animal experiment was designed and done under the regulation of ethical committee of NRC (ethical approval NO. 20170), 28 Sprague- dawely adult male rats were divided into 4 groups (G1, G2, G3, G4) 7 rats each. G1 received high fat diet (HFD), G2 received HFD and encapsulated BL (10^8 CFU/ml), G3 was supplemented by HFD plus encapsulated BL (10^8 CFU/ml) coated with SP (370 mg/kg bwt). G4 received basic diet only and considered as control group. After 8 successive weeks of treatment, animals were fasted over night and sacrificed. Heparinized blood samples were collected for analysis of plasma chemistry. Fecal samples were collected for microbiological investigation. Both testis were collected, weighed, kept in 10% formol-saline and sent for histopathological examination. Also, Epididymal semen was collected for microscopic examination.

Plasma chemistry

Plasma level of testosterone, cholesterol, high density and low density lipoproteins (HDL and LDL), triglycerides, leptin hormone, oxidative markers (MDA and Catalse), Gama glutamyl transaminase (GGT), urea and creatinine, alkaline phosphatase, albumin, zinc and phosphorus were performed using commercial reagent kits supplied by Roche Diagnostics (Laval, QC, Canada).

Microbiological evaluation of fecal samples

At the end of the experiment, rat fecal samples were collected for microbiological evaluation by soaking in tubes containing 9 ml of serial diluted solution of sterile saline. *B. longum* were counted on MRS agar supplemented with 2gm/l sodium propionate and 3gm/l lithium chloride and the plates incubated at 37 ˚C for 72h under anaerobic condition. Total bacterial counts were aerobically incubated using nutrient agar at 25˚C for 48h. Coliform groups were detected using Violet Red bile Agar and the plates incubated at 35 ºC for 24 h. Mold and yeast counts were determined using acidified potato dextrose agar and the plates incubated at 25 ºC for 3 days (Fayed et al., 2019).

Evaluation of epididymal spermatozoa motility

The fluid obtained from the cauda epididymis with a pipette was diluted to 2 mL with Tris buffer solution 0.3 M Tris (hydroxymethyl) aminomethane, 0.027 M glucose, 0.1 M citric acid. A warmed glass slide was placed on phase-contrast microscope, and an aliquot of this solution was placed on the slide and percent motility was evaluated visually at a magnification of 400 times. Motility estimations were performed from 3 different fields in each sample. The mean of the 3 estimations was used as the final motility score. Samples for motility evaluation were kept at 37˚C (Sonmez et al., 2007).

Spermatozoa count

The epididymal spermatozoa concentration was determined with a hemocytometer using a modified method described by Türk et al. (2007). The right epididymis was finely minced by anatomical scissors in 1 mL of isotonic saline in a Petri dish. It was completely squashed by a tweezers for 2 min, and then allowed to incubate at room temperature for 4 h to provide the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue-fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid containing all epididymal spermatozoa was drawn into the capillary tube up to 0.5 lines of the pipette designed for counting red blood cells. The solution containing 0.595 M sodium bicarbonate, 1% formalin and 0.025% eosin was pulled into the bulb up to 101 lines of the pipette. The contents of the pipette were mixed by holding the ends of the pipette between the thumb and the index finger and shaking it vigorously in 100 back-and-forth 30 cm movements. The bulb of the pipette contains a small glass bead that makes thorough mixing possible. Sufficient solution was then blown from the pipette to ensure that the diluents containing no sperm were flushed from the capillary. This gave a dilution rate of 1:200 in this solution. Approximately 10 µL of the diluted sperm suspension was transferred to both counting chambers of an Improved Neubauer (Deep 1/10 mm, LABART, Darmstadt, Germany) and allowed to stand for 5 min. The sperm cells in both chambers were counted with the help of a light microscope at 200× magnification.

Spermatozoa abnormality

To determine the percentage of morphologically abnormal spermatozoa in the cauda epididymis, the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate) were prepared, then viewed under a light microscope at 400x magnification. Two-hundred spermatozoa were examined on each slide. The head, tail and total abnormality rates of spermatozoa were expressed as percent. Also the live sperms were white (unstained) while, dead sperms acquired the stain. 100 sperms per rat were counted and viability percent (%) was calculated (Turk et al., 2008).

Histopathological examination of testis

Testes specimens were harvested from different groups then fixed in 10 % neutral buffered formalin for 48 hr, routinely processed and stained with Haematoxylin and Eosin (Suvarna et al., 2018). Spermatogenesis was measured on histologic sections using modified Johnsen's score. Categorized from 1 (no seminiferous epithelium) to 10 (full spermatogenesis) for each seminiferous tubule cross-section (El-Makawy et al., 2019). The germinal epithelium of at least thirty randomly chosen seminiferous tubules in each group under magnification power x400 was assessed for each testis and the mean Johnsen's score per rat was calculated.

Statistical analysis

Results were statistically computed using SPSS 11 program one way ANOVA test.

RESULTS AND DISCUSSION

Chemical analysis of the methanolic extract showed that SP is rich in Flavonoids (Figure 1) (3.56) compared to gallic acid (2.68) and Rutin (2.65) as standard. The phenolic compounds were (36.3) compared to gallic acid (36.8) and rutin (40) as standard expressed as mg per gram dry weight (Figure 2). The free radical scavenging activity (Figure 3) showed that Sp has high ability to inhibit the free radicals (77.2%) compared to ascorbic acid (94.6%) as standard.

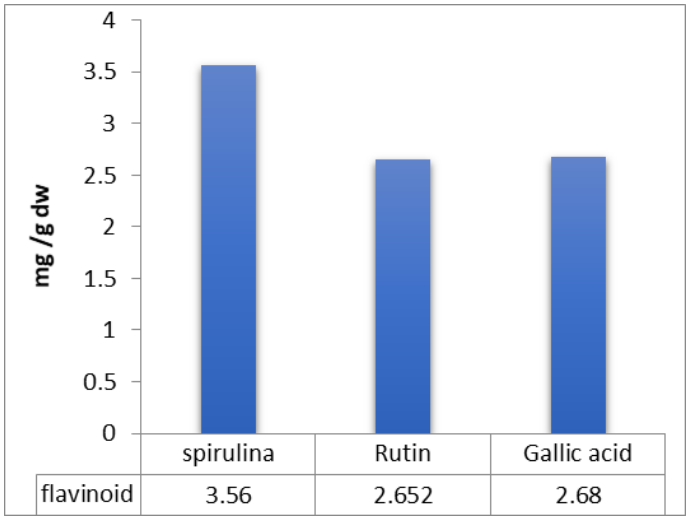

Figure 1: Total flavonoids in *spirulina platensis* extract.

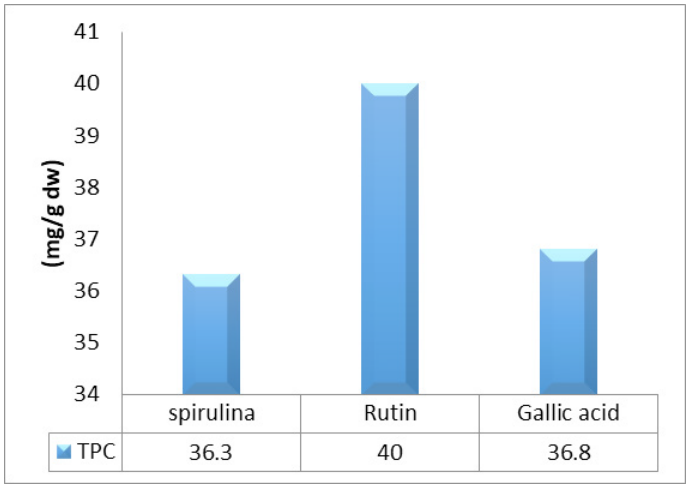

Figure 2: Total phenolic compounds of *spirulina platensis* extract.

Screening the polyphenol profile of the SP methanolic extract by HPLC for the presence of bioactive compounds

(Table 2) showed that SP is rich in phenoilc compounds and flavonoids with higher concentration for Gallic acid, Kaempferol, Querectin, Ferulic acid, Naringenin, Coffeic acid, Methyl gallate and cinnamic acid, respectively.

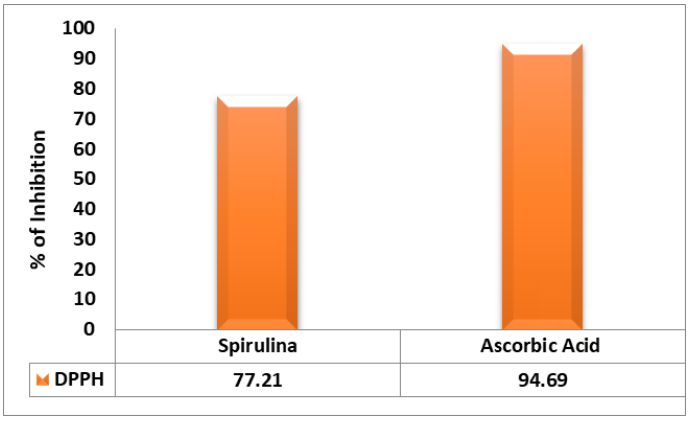

Figure 3: Free radical Scavenging activity (DPPH) of *spirulina platensis* extract.

Table 2: Polyphenol profile of *spirulina platensis*.

The polyphenol compound	Spirulina platensis Conc. (µg/mL)	Standard Conc. (µg/ml)
Gallic acid	9.40	8.4
Chlorogenic acid	ND	14
Catechin	ND	33.75
Methyl gallate	0.10	5.1
Coffeic acid	0.15	9
Syringic acid	ND	8.6
Pyro catechol	ND	14.6
Rutin	ND	30.5
Ellagic acid	ND	17.15
Coumaric acid	ND	6.6
Vanillin	ND	6.45
Ferulic acid	0.26	6.2
Naringenin	0.21	7.5
Daidzein	ND	10
Querectin	0.32	6.4
Cinnamic acid	0.07	2.9
Kaempferol	0.34	6
Hesperetin	ND	6.6

Conc., concentration; ND, not detected.

Gallic acid was reported as strong antioxidant. It induced body mass loss through reduction of plasma lipids, visceral fat, and insulin resistance (Rather and Saravanan, 2013; Totani et al., 2011).

Earlier studies reported that Ferulic acid present in SP is treated with ferulic acid esterase present in BL and release ferulic acid esterase which has antioxidant and antimicrobial activity (Szwajgier and Dmouska, 2010).

Most of research work proved that consuming food rich in polyphenols is good for general health condition and disease. In some circumstances consuming polyphenols may have unfavorable effect on reproductive health depending on the phenolic acids content, dose and duration of consumption (Ly et al., 2015).

Table 3: Antibacterial activity of *Spirulina platensis* extracts against some bacterial strains.

Bacterial strains	Methanol extract	Acetone extract	Ethanol extract
	Diameter of inhibition zone (mm)		
Listeria monocytogenes	9	8	7
Bacillus cereus	11	10	8
Staphylococcus aureus	10	9	8
Escherichia coli 0157: H7	12	10	10
Yersinia enterocolitica	8	6	6
Salmonella typhimurium	13	11	11
Pseudomonas aeruginosa	11	9	9
Lactobacillus plantarum	Nil	Nil	Nil
Lactobacillus rhamnosus	Nil	Nil	Nil
Leuconostoc mesenteroides	Nil	Nil	Nil
Bifidobacterium longum	Nil	Nil	Nil

Concerning the antimicrobial activity of SP, results (Table 3) showed that the greatest inhibition zone of *SP* extracts (50 mg/ml) was against *B. cereus* and *S. aureus* Gram positive bacteria and *S. typhimurium*, *E. coli*, *P. aeruginosa*, *L. monocytogenes* and *Y. enterocolitica* gram negative bacteria respectively. SP extract did not show inhibition zone against lactic acid strains and Bifidobacterium. So that, *Bifidobacterium longum* (BL) has been chosen in this study to be given to animals as symbiotic bacteria with *spirulina platensis* (*SP*) and prepared as microcapsules. The evaluation of the microencapsulation efficiency of both of them recorded 93.4%. Previous studies reported that Freez-drier yield great encapsulation efficiency (Heidebach et al., 2010; Semyonov et al., 2010). Antimicrobial activity of spirulina could be attributed to the presence of bioactive compounds like cyclic peptides, γ-linolenic acid, sulfoquinovosyl, diacylglycerols alkaloids and lipopolysaccharides (Katircioglu et al., 2006). Previous studies reported *Candida albicans*, Gram positive then Gram negative bacteria as the most susceptible microorganisms to the antimicrobial activity of SP respectively. This may be attributed to the different permeability and structure of the bacterial cell membrane (Abu-Shanab et al., 2005). Probiotics should be resistant to antibiotics to survive in the gastrointestinal tract the presence of resistant genes to some antimicrobial agents was reported in many lactic acid and befidobacteria species (Sánchez et al., 2013).

Microbiological evaluation of rat fecal samples (Table 4) after feeding the microcapsules showed significant

elevation of the probiotic bacteria (BL) in G2 and G3 on the expense of natural inhabitant coliform bacteria, mold and yeast compared to control groups G1 and G4. Spirulina as prebiotics provide binding sites for pathogenic bacteria, flushing them out of the digestive tract. Also, it contains Fructooligo saccharides which selectively promote the growth of beneficial bacteria by acting as a food source for host-beneficial bacteria, which competitively excludes pathogenic bacteria (Yousefi et al., 2019).

Table 4: Fecal microbial colony count of rats after feeding the microcapsules.

Treatment	Total bacterial count	Coliform counts	*B. Longum* counts	Mold and yeast counts
	(Log CFU/g)			
G1	7.89	4.34	4.47	5.83
G2	9.46	2.95	8.84	4.70
G3	9.07	3.04	8.97	3.90
G4	6.39	4.89	3.51	4.59

Analysis of plasma (Table 5) showed significant elevation of cholesterol, LDL and triglycerides in G1 (fed on the HFD). Feeding the microcapsules in G2 and G3 took these values back towards normal compared to G4 (the control group fed on normal diet). SP is rich source of bound phenolic acids like ferulic acid (Itagaki et al., 2009). *Befidobacterium longum* was reported to secret ferulic acid esterase. The esterified ferulic acid lowers plasma cholesterol level. This leads us to consider *Befidobacterium longum* as an ideal candidate bacteria used to delay the symptoms of metabolic syndrome (Rose et al., 2015).

The current results showed that the prepared microcapsules were safe on health and did not affect on liver or kidney function. *Spirulina platensis* previously mentioned to contains C-phycocyanin which exhibits antidiabetic, antioxidant, hypolipidemic, anticancer, antiviral, immunomodulatory, hepatoprotective, renoprotective, neuroprotective, and antigenotoxic properties (Rose et al., 2015).

The current study showed marked decrease in testosterone level in G1 that fed on HFD. It is well known that diabetic dyslipidemia or metabolic syndrome is associated with low androgen level due to hormonal disruption (Kapoor et al., 2006). Also, the elevated lipid peroxides in tissues negatively affect gonads function and sperm morphology causing deformities (Hala et al., 2012). Probiotics controls the release of such free radicals and its cytotoxic hazards this was clear in our results where animals in G2, fed on BL plus HFD, showed better values of the hormone and semen picture. Conversely, the third group of animals (G3), fed on the microcapsules of SP plus BL beside the HFD, showed down regulation of the hormone compared to control group G4. This may be referred to the presence of phytosterols (B-sitosterol and stigmasterol) in SP which lowers the intestinal absorption of cholesterol and consequently lowers the synthesis of testosterone (Basha et al., 2008). Earlier study showed that when rats fed phytosterol for 22 days showed reduced testosterone level by 33% (Rose et al., 2015). Also, the current results showed that leptin level was positively correlated with body fat mass and negatively correlated with testosterone level. It is well known that leptin, as a metabolic hormone, has a role in GnRH and testosterone production at different reproductive stages (Mervat et al., 2010). It is produced by adipose tissue and sends information to brain about the body's energy resources to regulate a number of physiological processes, including reproduction. The new scientific data confirms the tight relation between fertility and the body state of energy via metabolic hormones like leptin and ghrelin (Farooqi and O'Rahilly, 2009).

Table 5: Plasma chemistry of male rats treated with microcapsules.

Parameter/group	G1	G2	G3	G4
Cholesterol (mg/dl)	127.80 ± 4.3^{a}	79.89 ± 5.26^{b}	65.71 ± 2.01^{bc}	63.36 ± 2.49^{c}
HDL (mg/dl)	12.13 ± 0.41^{a}	24.10 ± 2.5^{b}	25.43 ± 0.25^{b}	16.18 ± 3.43^{ab}
LDL (mg/dl)	112.5 ± 3.90^{a}	75.40 ± 3.30^{b}	45.77 ± 8.50^{b}	30.24 ± 2.30^{c}
Triglyceride (mg/dl)	155.63 ±10.48^{a}	143.68 ± 5.76^{a}	119.82 ± 6.65^{b}	76.16 ± 3.87 ^{c}
Testosterone (pg/ml)	120.2± 2,62^{a}	137.4± 7.79^{ac}	114.3± 8.9^{b}	143.9± 9.45 ^{c}
Leptin (ng/ml)	1.81± 0.20 ^{a}	1.43 ± 0.45 ^{ab}	1.98 ± 0.08^{b}	0.66 ± 0.07 ^{c}
MDA (mMol/L)	27.25 ± 2.6^{a}	12.64 ± 0.44 ^{b}	6.20 ± 0.54 ^{c}	12.75 ±0.85 ^{b}
Catalase (IU/ L)	291.3 ± 29.6^{a}	344.0 ± 18.1^{a}	469.17 ±48.61^{b}	254.5 ± 7.6 ^{a}
GGT(U/L)	8.69 ± 1.25 ^{a}	7.36 ± 0.49 ^{a}	7.17 ± 1.21 ^{a}	10.64 ± 0.67 ^{a}
Alk ph (IU/L)	12.39 ± 1.47^{a}	15.66 ± 1.19 ^{a}	19.45 ± 2.23 ^{a}	13.65 ± 0.54 ^{a}
Urea (mg/dl)	35.73± 1.64 ^{a}	47.6± 2.21^{b}	40.48 ± 3.39^{ab}	25.39 ± 1.87^{c}
Creatinine (mg/dl)	0.86 ± 0.10^{a}	0.81± 0.09 ^{a}	1.08± 0.13^{a}	0.91± 0.16^{a}
Albumin (g/dl)	3.15 ± 0.21^{a}	2.75 ± 0.32^{a}	3.66 ± 0.38^{a}	3.06 ± 0.20^{a}
Zinc (ug/dl)	148.70 ± 7.0^{a}	163.2 ± 16.06^{a}	222.8 ± 16.59^{b}	179.4 ± 17.42^{a}
Phosphorus (mg/dl)	10.81 ±0.97^{a}	11.19 ±1.17^{a}	12.58 ± 0.77^{a}	10.05 ± 0.73^{a}

Different superscripts within rows (a, b, c) means significant differences P≤ 0.05

Evaluation of the oxidative status of animals showed higher levels of MDA in animal Fed on HFD (G1) compared to control group (G4). Animals in G2 and G3 fed on the microcapsules showed lower level of MDA and higher level of Catalase indicating better oxidative status compared to control groups G1 and G4. It is well known that diets high in fats increase fat-mediated oxidative stress in tissues and decrease antioxidant capacity (Coudray, 2009). Recently, antioxidant properties of probiotics and yeast showed protective effect on reproductive system. *Befidobacterium longum* releases esterified ferulic acid from the dietary fibers which is strong antioxidant (Mikelsaar et al., 2008; Tovar-Ramı´rez et al., 2010; Szwajgier et al., 2010).

Concerning Albumin, GGT, Alkaline phosphatase, Urea and creatinine, which reflect the liver and kidney function, showed nearly non-significant changes after feeding the microcapsules indicating its bio safety. Zinc showed higher levels in plasma of G3 fed on the microcapsules of Sp compared to control groups earlier studies showed that spirulina is rich in trace elements and vitamins (Nilay et al., 2017).

Results in Table 6 showed that animals in G1 had the highest body weight and lowest gonad weight compared to other groups but did not reach statistical significance. Rats supplemented with microcapsules and HFD showed equal or less body weight than control normal animals. Previous studies showed the same results in rats supplemented with high fat diet and probiotic bacteria (Chen et al., 2013).

The current study (Table 7) showed poor viability and high abnormalities of sperm in G1 supplemented with HFD compared to other groups which had direct relation with elevated free radicals in the same group (Table 5). It is well known that sperm cell membrane is rich in polyunsaturated fatty acids and consequently, very susceptible to lipid peroxidation by free radicals. The increased sperm membrane permeability induces an interruption in respiratory chain and ATP production leading to decreased phosphorylation of axonemal proteins (Flaherty et al., 2006; Fernandes et al., 2016) These finding coincide with histological alterations and lesion score in the testes (Figure 4a, b) where moderate to severe testicular degeneration exhibited by small diameter of seminiferous tubules, degeneration of spermatogoneal cells and incomplete spermatogenic series were domenestrated. Additionally, examined sections exhibited marked vacuolation of spermatogenic cells and Leydig cells as well as interstitial oedema.

Previous literature reported that obesity in animal models showed alterations in metabolic profile and histological findings in gonads similar to those reported in human (Fernandes et al., 2016). Obese males have less number of Sertoli and Leydig cells (Miao et al., 2018), disruptions of seminiferous tubules (Demirci and Sahin., 2019) and reduction in sperm quality indices, such as concentration and motility (Demirci and Sahin., 2019; Crean and Senior 2019; Merino et al., 2019).

Examination of testes of rat in G4 (fed on basic diet) revealed the normal histological architecture of seminiferous tubules with complete spermatogenic series and sperm production (Figure 4h, i).

Table 6: Body and Gonads weight in male rats treated with microcapsules.

Treatment	Testicle 1 (gm)	Testicle 2 (gm)	Epididymis 1 (gm)	Epidydimis 2 (gm)	Body weight (gm)
G1	2.35 ± 0.21	2.33 ± 0.19	0.23 ± 0.02	0.22 ± 0.03	325.33 ± 10.84
G2	2.49 ± 0.21	2.45 ± 0.11	0.24 ± 0.04	0.23 ± 0.02	296.33 ± 8.84
G3	2.46 ± 0.24	2.42 ± 0.26	0.24 ± 0.01	0.22 ± 0.02	287.67 ± 17.65
G4	2.58 ± 0.10	2.47 ± 0.04	0.29 ± 0.01	0.22 ± 0.01	292.00 ± 5.77
F	0.08	0.12	0.79	0.14	1.81
P value	0.9697	0.9457	0.5356	0.9304	0.2336

Table 7: Epididymal Spermatozoa profile of male rats treated with microcapsules.

Treatment	Sperm conc (×10⁶/ml)	Motility (%)	Live sperm(%)	Dead sperm(%)	Abnormal morphology(%)
G1	440.00 ± 87.37	70.00 ± 5.00	89.67 ± 3.53	10.00 ± 3.61	40.00 ± 7.09
G2	476.67 ± 96.67	78.67 ± 6.67	94.67 ± 2.85	8.33 ± 2.85	25.00 ± 3.06
G3	390.00 ± 23.09	56.67 ± 4.41	90.67 ± 0.33	9.33 ± 0.33	30.33 ± 7.51
G4	433.00 ± 57.74	75.00 ± 5.77	91.00 ± 0.58	9.00 ± 0.58	35.33 ± 0.88
F	0.24	15.42	0.39	0.32	1.37
P value	0.8636	0.0011	0.7661	0.8136	0.3199

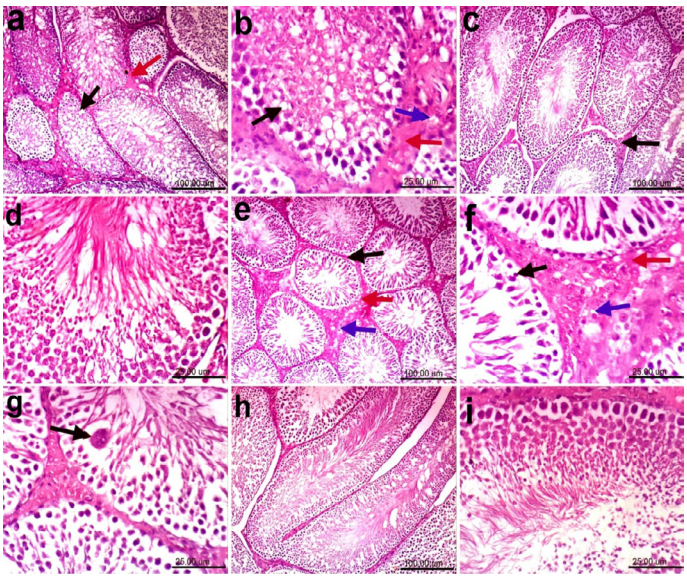

Figure 4: Representative photomicrographs of H & E stained sections of testes (scale bar, 100µm and 25 µm); a and b Fed on HFD; showing marked vacuolation of spermatogenic cells (black arrow) and Leydig cells (blue arrow) as well as interstitial oedema (red arrow). c and d Fed on HFD plus microcapsules of BL; showing normal seminiferous tubules with complete spermatogenic series and sperm production associated with slight intertubular oedema (black arrow). e, f and g Fed on HFD plus encapsulated SP and BL; e and f showing vacuolation of Leyding cells (blue arrow), marked vacuolation and necrosis of spermatogenic cells (black arrow) as well as interstitial oedema (red arrow); g) showing spermatid giant cell (black arrow). h and i Fed on basal diet (normal control) showing the normal histological architecture of seminiferous tubules with complete spermatogenic series and sperm production.

Rats administered the microcapsule BL in G2 showed high sperm concentration, motility and live sperm and the least number of dead and abnormal sperm morphology compared to other groups (Table 7 and Figure 4c, d). The histological findings of this group showed normal seminiferous tubules with complete spermatogenic series and sperm production associated with slight intertubular oedema. Earlier studies showed that the probiotic bacteria produce antioxidants that can dispose, scavenge and suppress the formation of free radicals and improve the sperm concentration, viability and motility (Chen et al., 2013).

The spermatozoa profile of animals in G3 which were given the SP (370mg/kg bwt) plus Bl capsules showed average concentration of sperm, and abnormal morphology while it showed marked decrease in sperm motility (Table 7). This group of animals showed severe histopathological alterations exhibited by small diameter seminiferous tubules, vacuolation of Leydig cells, marked vacuolation and necrosis of spermatogenic cells as well as interstitial oedema and formation of spermatid giant cell (Figure 4e, f). Also, there was significant decrease in histopathology Johnsen's score (Table 8) of the testes of G1 and G3 as compared to control group (P<0.005).

Table 8: Illustrated the histologic grading of spermatogenesis

Groups	Johnsen's score
G1	$6.60^{b} \pm 0.24$
G2	$9.60^{a} \pm 0.24$
G3	$6.40^{b} \pm 0.24$
G4	$10.00^{a} \pm 0.00$

Means with different superscripts (a, b, c) are significantly different at (P < 0.05). n=5. note the significant decrease in Johnsen's score of the testes of groups 1 and 3 as compared to control group (P<0.005).

Many literatures recommend the intake of food rich in polyphenols for good health. Actually this may be good for growing animal's bred for meat production. The case is different for breeding animals where the improper intake of phenolic compounds may affect the reproductive performance of the parents and their progeny (Amir et al., 2019). Spirulina is rich in gallic acid and other polyphenols. Earlier study mentioned that using low concentration of EGCG (Epigallocatechingallat) during in vitro fertilization increased fertilization rate than high concentration (Spinaci et al., 2008). Polyphenols were taken by the hypothalamus, testis, ovary, uterus, placenta and fetus (Demirci and Sahin, 2019; Miao et al., 2018; Merino et al., 2019). Some isoflavones are converted to a nonestrogenic metabolite (genistein and biochanin A) and others are converted to a potent estrogenic metabolite (formononetin and daidzein). Earlier study referred the reproductive disorders observed in sheep herds to such compounds (Reed, 2016). Also, high levels of polyphenol may massively scavenge reactive oxygen species (El-Desoky et al., 2017; Gessner et al., 2017). Whereas, moderate levels of reactive oxygen species are essential for completion of many biological functions in cells including gametes production and embryo growth (Spinaci et al., 2019; Arando et al., 2019) so that, when high polyphenol levels were added to semen/sperm in assisted reproductive technology (ARTs), showed hazardous effect on sperm membrane integrity, mitochondrial membrane potential, sperm capacitation, sperm motility, sperm-zona pellucida binding and thus in vitro fertilization outcomes (Menzel et al., 2007; Spinaci et al., 2018; Hashem et al., 2020).

Earlier studies showed that the testis of rats treated with Spirulina (500mg/kg bwt) revealed variable degrees of degenerative changes in the seminiferous tubules with malformed spermatozoa up to complete cellular destruction.

Treatment with higher dose of spirulina 1000 mg/kg bwt showed more obvious destructive alterations such as marked widening of the interstitial spaces, atrophied lumen and congested blood capillaries. Also, the seminiferous tubules revealed disorganized germinal epithelium with highly deformed spermatogenic cells, necrotic areas, and disappearance of almost all spermatozoa. The author referred these results to the presence of certain bioactive compounds causing disturbance in the reproductive hormones level (testosterone, FSH and LH) (Fathy and Ashraf, 2015). Other study reported that feeding spirulina at 10%–20% of feed amount is considered safe (Tantawi and Abozeid, 2020).

CONCLUSIONS AND RECOMMENDATIONS

Microencapsulation showed high efficiency to keep the viability and delivery of bacteria. The BL microcapsules showed positive effect on general health condition and in the same time improved the spermatozoa profile and testosterone level, SP has positive effect on health, lowered lipid profile and improved the oxidative state but unfortunately the sperm concentration and motility were negatively affected, Overall, SP is rich in polyphenols which exert its function according to chemical structure, level, cell sensitivity, and half-time of reactive oxygen species, and this might explain our unfortunate results in G3.

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NOVELTY STATEMENT

This was the first time to prepare microcapsules containing microalgae combined with probiotic bacteria to be used as functional food additive to correct the metabolic disorders of obese diabetic individuals.

AUTHOR'S CONTRIBUTION

All authors contributed equally in the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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