Effects of Olive Leaf Extract and *Nannochloropsis oculata* Supplementation on Growth Performance, Oxidative Stress, and Lymphocytes Transformation in Pregnant Ewes

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Abstract

During pregnancy, ewes may encounter various stressors that can have significant effects on their well-being and the development of their offspring. These stressors include physiological, environmental, and psychological factors. This study aimed to investigate the effects of OLE supplementation (2% and 4%) and *Nannochloropsis oculata* (3%) on pregnant ewes' immune functions. This study was primarily focused on evaluating the lymphocytes transformation test (LTT) (as a measure of immune response), serum lipid peroxidation and superoxide dismutase levels (as indicators of oxidative stress). The study was conducted on healthy pregnant ewes in their last trimester of gestation. The ewes were divided into different groups, including a control group and groups supplemented with OLE and *N. oculata*. Performance parameters such as body weight gain and feed conversion ratio (FCR) were measured. Blood samples were collected to assess lymphocytes transformation, lipid peroxidation, and superoxide dismutase levels. The results showed that supplementation with OLE and *N. oculata* improved the FCR in pregnant ewes, indicating enhanced feed conversion efficiency. Additionally, the supplements significantly increased lymphocytes transformation and reduced lipid peroxidation levels, indicating improved immune response and reduced oxidative stress. In conclusion, this study explored the effects of OLE and *N. oculata* supplementation on pregnant ewes' immune functions. The findings suggest that these supplements can improve immune response and reduce oxidative stress in pregnant ewes, potentially benefiting both the ewes and their offspring.

Keywords: Antioxidant, Nannochloropsis oculata, Pregnant ewes, Immunostimulant, Olive leaf extract

Introduction:

During pregnancy, ewes may encounter various stressors that can have significant effects on their well-being and the development of their offspring. Physiological stressors include heat stress, which can directly affect fetal growth. In heat-stressed pregnant ewes, fetal body temperature rises along with maternal temperature, leading to fetal hyperthermia that may impact fetal development beyond placental insufficiency ***(Karimi et al., 2015; Tao et al., 2012)***. Environmental stressors, such as sub-optimal nutrition and housing conditions, can also impact pregnant ewes. Maternal under-nutrition in the last third of pregnancy has been consistently shown to impair lamb birth weight, vigor, and subsequent performance ***(Rooke et al., 2015)***. Psychological stressors, including fear and anxiety, can also affect pregnant ewes. Pregnant mice, rats, and ewes have been shown to exhibit reduced fear and anxiety behavior compared to non-pregnant animals in various stressful situations ***(Glynn et al., 2008)***.

Dietary immunomodulatory supplements can minimize the effects of stressors on the health of pregnant ewes by improving their immune response and reducing inflammation. Several studies have investigated the effects of different dietary supplements on pregnant ewes. Phytosterols extracted from microalgae have been found to influence cell proliferation and cytokine release during inflammation ***(Ciliberti et al., 2017)***. By modulating immune responses, phytosterols can help regulate inflammation and improve immune function in pregnant ewes. Dietary supplementation with eicosapentaenoic acid (EPA) and arachidonic acid (AA), both polyunsaturated fatty acids (PUFAs), has been shown to have immunomodulatory effects on the immune system ***(Roy et al., 2007)***. These PUFAs can help regulate intestinal inflammation and modulate gene expression related to inflammation. By incorporating these PUFAs into the diet, pregnant ewes can potentially improve their immune response and reduce inflammation.

Nannochloropsis oculata is a species of microalgae belonging to the class Eustigmatophyceae. It is a small yellow-green heterokont alga that has gained attention for its potential applications in various fields, including biodiesel production, aquaculture, and biotechnology ***(Olofsson et al., 2012; Sarker et al., 2018; Wang et al., 2009)***. Many studies have highlighted the high nutritional value of *N. oculata*, including its high concentrations of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) that proof the potential use of N. oculata as a dietary supplement in animals to improve the nutritional value of foods ***(Al-Hoqani et al., 2016; Camacho et al., 2019; Volkman et al., 1993)***. In addition, *N. oculata* has been studied for its potential immunostimulatory effects in animals. ***El-Hawy et al. (2022)*** investigated the effect of *N. oculata* supplementation on the growth performance and immunological status of rams.

Olive leaf extract (OLE) has been traditionally used as a herbal supplement and has been reported to associate with various health benefits such as immune system support, antioxidant activity, and anti-inflammatory effects ***(Liu et al., 2017a)***. These beneficial properties of OLE have been attributed to its bioactive compounds such as phenolic acids, phenolic alcohols, flavonoids, and secoiridoids. OLE has showed many immunomodulatory effects through inducing apoptosis, promotes monocyte/macrophage differentiation, and enhances immune responses in cancer cells and animal models ***(Samet et al., 2014)***. Moreover, OLE could help to protect against the oxidative stress biomarker, inflammation, and improved the growth performance of animals ***(Şahin and Bilgin, 2018)***.

There is a significant gap in research concerning the effects of Olive leaf extract (OLE) and *Nannochloropsis oculata* on sheep, with a particular focus on pregnant ewes. Limited studies have explored the potential impacts of these supplements on this specific population. Therefore, the objective of this study was to investigate the effects of OLE supplementation (2% and 4%) and *Nannochloropsis oculata* (3%) supplementation on pregnant ewes' immune functions. This investigation primarily involved evaluating the lymphocytes transformation test (LTT) as a means to assess immune response. Furthermore, we aimed to explore the influence of these supplements on serum lipid peroxidation and superoxide dismutase levels as indicators of oxidative stress. By addressing this research gap, we can gain valuable insights into the potential benefits and mechanisms of action of OLE and *Nannochloropsis oculata* in enhancing immune functions during pregnancy in ewes.

Material and methods:

Olive leaf extract (OLE)

Olive leaf extract was obtained from the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt. It was tested by HPLC in our laboratory to contain 7% oleuropein ***(Fayez et al., 2023)***.

Nannochloropsis microalga

Nannochloropsis oculate (NNO-1 UTEX Culture LB 2164) was obtained from the Algal Biotechnology Unit, Biological and Agricultural Research Division, National Research Centre, Dokki, Giza, Egypt. Gas chromatography mass was used at the complex laboratories of the National Research Centre in Dokki, Giza, Egypt, to analyze the chemical composition of *N. oculate*. The Chemical composition (g/100 g) of

N. oculata was detected by ***Aboulthana et al. (2018)***.

Experimental design

The experiment was conducted on 24 apparently healthy pregnant ewes aged between 2-3 years and weighed between 45-55 Kg in their last trimester of gestation period. The experiment was maintained for one month from August to September 2021, according to the ethical guidelines for the use of animals in laboratory experiments of the Faculty of Veterinary Medicine, Suez Canal University, Egypt. The animals were kept in a semi-closed pen, 12 m^2/ 6 lambs (2 m^2/lamb) for each pen, with walls between the groups at the sheepfold of a private farm next to the El-Salam canal in Sahl El Teena, East El-Qantara region, North Sinia, Egypt. Water was provided throughout the course of the trial by troughs, which served as the source of water, a cover was available for sun protection. The animals were fed a basal diet consisted of; wheat straw (35%), wheat bean (15%), Egyptian clover (25%), broken horse beans (9%) and yellow maize (15%). This diet contained; dry matter (71.23%), crude protein (12.95%), total digestible nutrient (65.85%), neutral detergent fiber (44.87%), acid detergent fiber (44.87%), ether extract (1.48%), calcium (0.69%), and phosphorus (0.43%) on DM basis. The health condition of the experimental animals was evaluated at the beginning by parasitological analysis of their feces samples ***(Soulsby, 1982)***, and the animals were examined clinically, according to ***Kelly (1984)***. After two weeks of acclimatization, they were divided into Group 1 (Control or C): Ewes were fed the basal diet, Group 2 (N 3%): Ewes were given the basal diet supplemented with 3% *N. oculate*, Group 3 (OLE 2%): Ewes were given the basal diet supplemented with 2% OLE, and Group 4 (OLE 4%): Ewes were given the basal diet supplemented with 4% of OLE. The experimental animals had full access to food and water. The experimental protocol was approved by the Institutional Research Ethics Committee at the Faculty of Veterinary Medicine, Suez Canal University (approval number 202089).

Performance parameters

Ewes' body weight (Kg) was recorded at the beginning and the end of experiment. Body weight gain (Kg) was calculated by subtracting the initial body weight (initial Bwt/Kg) from the final body weight (final Bwt/Kg), Feed intake was recorded daily, and the feed conversion ratio (FCR) was calculated to assess the lambs' performance according to ***Mohammady et al. (2013)***.

Blood samples

At the end of the experimental period (1 month), 5 mL of blood samples (n = 6/group) were collected from the jugular vein of ewes in the different groups. Each sample per animal was placed into 3 different tubes: lithium heparin tubes for lymphocytes transformation test (LTT) and plain tubes for sera separation. The separated sera were used for plasma MDA and SOD measurement.

Lymphocytes culture and transformation test (LTT)

Lithium heparinized blood samples collected from lambs were transferred to the laboratory in ice bags immediately. Lymphocytes transformation assay was performed on separated PBMCs from buffy coat using Ficoll-Hypaque according to ***Boyum (1968)*** and as mentioned *in vitro* study.

MDA and SOD

The plasma MDA and SOD in separated sera of ewes were assessed using kinetic enzymatic assay (BioVision, USA). The plasma MDA was measured using a colorimetric kit (Abcam, United Kingdom) and the protocols as described ***(Armstrong and Browne, 1994; Yagi, 1998).*** SOD plasma levels were measured using colorimetric kit (BioVision, USA) according to ***Koivunen and Krogsrud (2006)***

Statistical analysis

Data of the present study were analyzed using One-way Analysis of Variance (one way-ANOVA) ***(Placha et al., 2014)*** considering ($P < 0.05$) and procedures were done according to ***Snedecor and Cochran (1989)*** for testing the significance among the studied groups. Means separation and pairwise comparisons were done by Duncan's Multiple Range test according to ***Duncan (1955)***. Statistical analyses were conducted by SPSS for windows (SPSS version 20) and Statistical Analysis System (SAS Institute 2003) software.

Results:

Sheep performance

Regarding the influence of OLE supplementation in the ewes' diet on the performance, ewes given 2% and 4% dietary OLE and 3% dietary *N. oculata* showed improvement in the growth performance measures where there was a significant ($P<0.05$) decrease in FCR compared to the control group. However, there was non-significant change in the body weight gain and the final Bwt (Table 1).

Lymphocytes transformation test (LTT)

The supplementation of ewes' diet with OLE at 2% and 4% and *N. oculata* at 3% significantly ($P< 0.05$) enhanced the level of lymphocytes transformation compared to the control group. OLE 4% group significantly ($P< 0.05$) elevated the level of lymphocytes transformation higher than the *N. oculata* at 3% concentration group (Table 1).

MDA and SOD

The addition of OLE at 2%, 4%, and *N. oculata* at 3% concentrations to pregnant ewes' diet significantly ($P<0.05$) decreased the level of MDA than control group. All treated groups OLE 2%, 4% and N 3% significantly ($P<0.05$) enhanced the levels of SOD compared to the control group. OLE group supplemented with 4% concentration showed a significant effect in reducing the level of MDA and improving the level of SOD compared to all other treated groups (Table 1).

Table 1: *The effect of supplementation with N. oculata and OLE on growth performance, lymphocytes transformation test, and oxidative stress biomarkers in pregnant ewes.*

Groups / Parameters	Control	N 3%	OLE 2%	OLE 4%	Sig.
Initial Bwt (kg)	50 ±1.44	50.33 ±0.93	50.33 ±0.73	51±0.76	P=0.913
Final Bwt (kg)	62 ±1	64 ±1.25	64±1.32	64 ±0.58	P=0.479
Bwt gain (kg)	12 ±0.76	13.67 ±0.44	13.67±0.67	13 ±0.29	P=0.162
FCR	15.29 ±0.92[a]	13.02±0.43 [b]	12.45 ±0.66 [b]	12.8 ±0.29[b]	P=0.018
LTT (µg/mL)	1.08±0.04[c]	2.7 ±0.15 [b]	2.86 ±0.1[ab]	3.22 ±0.17 [a]	P=0.000
MDA (µM/mL)	1.72 ±0.05[a]	1.34±0.08 [b]	1.24±0.07 [b]	1.03±0.02[c]	P=0.001
SOD (µg/mL)	735.5 ±240.7[c]	837 ±156.59[b]	852.75 ±148.69[b]	916.25±153.34[a]	P=0.000

Data was expressed as mean ±SE. different superscript (a, b, c) between columns considered significant at (P<0.05). Body weight (Bwt), feed conversion ratio (FCR), Lymphocytes Transformation test (LTT), serum lipid peroxidation or Malondialdehyde (MDA) and superoxide dismutase (SOD)

Discussion:

In the current study the results regarding dietary supplementation of *Nannochloropsis oculata* at 3 % concentration and OLE at 2% and 4% concentrations in pregnant ewes' diet had non-significant differences on body weight gain and final body weight; however, enhanced the feed conversion ratio. Previous reports have shown that nutrient restriction during pregnancy can lead to intrauterine growth retardation (IUGR) in offspring. However, realimentation and supplementation with specific nutrients have been found to prevent IUGR and improve growth performance ***(Wu et al., 2006)***. In this context, the dietary supplementation of *N. oculata* and olive leaf extract may have contributed to improved feed conversion efficiency in pregnant ewes, as indicated by the enhanced feed conversion ratio.

Olive leaf extract has been recognized for its potential health benefits, including its antioxidant, anti-inflammatory, and immunomodulatory properties. These properties may have contributed to the improved feed conversion ratio observed in the pregnant ewes. The bioactive compounds present in olive leaf extract, such as phenolic compounds, have been associated with various health benefits, including improved glycemia, lipidemia, and cardiovascular health ***(Brown et al., 2015)***. These effects may have indirectly influenced the feed conversion efficiency of the pregnant ewes.

Nannochloropsis oculata, a species of microalgae, has been studied for its potential as a dietary supplement in animals. It has been shown to have high nutritional value, including high concentrations of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA). PUFAs are known to have immunomodulatory effects and can influence various physiological processes, including inflammation and immune response. The inclusion of *N. oculata* in the diet of pregnant ewes may have contributed to the observed improvements in feed conversion ratio, potentially through its immunomodulatory effects ***(Van Vooren et al., 2012)***.

Our findings showed that supplementation of *N. oculata* at 3% concentration and OLE at 2% and 4% concentrations significantly enhanced the transformation of lymphocytes in comparison with the supplemented group. As previously mentioned, *N. oculata* contains high nutritional value, including high concentrations of PUFAs which can influence various physiological processes, including inflammation and immune response. Therefore, inclusion of *N. oculata* in the diet of pregnant ewes may have contributed to the observed enhancement in lymphocytes transformation. The bioactive compounds present in *N. oculata*, such as PUFAs, may have influenced the immune system of the pregnant ewes, leading to improved lymphocytes transformation ***(El-Hawy et al., 2022)***. These immunomodulatory effects could potentially enhance the immune response and overall health of the pregnant ewes, which may have contributed to the observed improvements in feed conversion ratio ***(Salem et al., 2022)***.

On the other hand, dietary supplementation of olive leaf extract in pregnant ewes' diet might enhance lymphocytes transformation due to its potential immunomodulatory properties. Olive leaf extract is rich in phenolic compounds and flavonoids, such as Ole, hydroxytyrosol, and luteolin 7-glucoside, which have been associated with various health benefits, including immunomodulation ***(Muzzalupo et al., 2020)***. These bioactive compounds in olive leaf extract may interact with immune cells, including lymphocytes, and modulate their function and response. Previous studies have demonstrated the antimicrobial activity of olive leaf extract, indicating its potential to influence immune responses ***(Liu et al., 2017b)***. The presence of antimicrobial compounds in the extract may stimulate the immune system and enhance lymphocytes transformation as part of the immune defense against pathogens.

Dietary supplementation of *N. oculata* at 3 % concentration in pregnant ewes' diet in the present study significantly decreased MDA and increased SOD levels. *N. oculata* has been shown to have high nutritional value including EPA which are known to have antioxidant effects and can help reduce oxidative stress ***(Unuofin and Lebelo, 2020)***. The inclusion of *N. oculata* in the diet of pregnant ewes may have contributed to the observed decrease in MDA levels. MDA is a marker of lipid peroxidation and oxidative stress, and a decrease indicated a reduction in oxidative damage ***(Kinalski et al., 2000)***. The antioxidant properties of *N. oculata*, possibly attributed to its high PUFA content, may have contributed to the decrease in MDA levels. Moreover, *N. oculata* supplementation may have led to an increase in SOD levels. SOD is an important antioxidant enzyme that helps neutralize superoxide radicals and protect cells from oxidative damage ***(Fereidouni et al., 2022)***. The antioxidant compounds present in *N. oculata*, such as PUFAs and other bioactive compounds, may have stimulated the production or activity of SOD, leading to increased SOD levels in pregnant ewes. This increase in SOD levels indicates an enhanced antioxidant defense system, which can help counteract oxidative stress and maintain cellular health.

On the other side, our findings demonstrated that dietary supplementation of OLE in pregnant ewes' diet significantly decreased MDA levels and increased SOD levels. These results can be attributed to the antioxidant properties of OLE. OLE is rich in various phenolic compounds which have been associated with potent antioxidant effects ***(Wu et al., 2006)***. These compounds can scavenge free radicals and reduce oxidative stress, leading to a decrease in MDA levels, which is a marker of lipid peroxidation and oxidative damage ***(Erişir et al., 2020)***. The bioactive compounds in olive leaf extract may stimulate the production or activity of SOD, leading to increased SOD levels in pregnant ewes. This increase in SOD levels indicates an enhanced antioxidant defense system, which can help counteract oxidative stress and maintain cellular health ***(Pang et al., 2021)***.

Conclusion:

In conclusion, this study investigated that OLE and *N. oculata* supplementation improved the feed conversion ratio (FCR) without significant changes in body weight gain and final body weight. Furthermore, the supplementation enhanced lymphocytes transformation and reduced oxidative stress, as evidenced by decreased malondialdehyde (MDA) levels and increased superoxide dismutase (SOD) activity. These findings suggest the potential of OLE and *N. oculata* as immunomodulatory supplements to enhance immune functions during pregnancy in ewes. Further research is needed to explore the underlying mechanisms and long-term effects of these supplements.

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تأثير إستخدام مستخلص ورق الزيتون و طحلب النانوكلوروبسيس أوكيولاتا مكملات غذائية على أداء النمو، التوتر التأكسدي وتحول الخلايا الليمفاوية في النعاج الحوامل

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المستخلص العربى

خلال فترة الحمل، قد تتعرض النعاج لعوامل مجهدة مختلفة يمكن أن تؤثر بشكل كبير على صحتها وتطور أفرادها الصغار. تشمل هذه العوامل العوامل الفسيولوجية والبيئية والنفسية. لذا تهدف هذه الدراسة إلى التحقق من التأثير المناعي لطحلب النانوكلوروبسيس اوكيولاتا ومستخلص ورق الزيتون على النعاج الحوامل. الدراسة الحقلية أُجريت على 24 من النعاج الحوامل يتراوح أعمارها ما بين 2-3 عاماً و تزن ما بين 45-55 كجم. قُسمت تلك الحيوانات الى 4 مجموعات (6 نعاج لكل مجموعة)

كالتالى المجموعة الأولى: المجموعة الضابطة و تلقت نظاما غذائيا يفى بإحتياجات النعاج الحوامل طول فترة التجربة، المجموعة الثانية : تلقت النظام الغذائى الأساسى مضافاً عليه الطلحب نانوكلوربسيس أوكيولاتا بنسبة 3% لكل كجم علف، المجموعة الثالثة: تلقت النظام الغذائى الأساسى مضافاً عليه مستخلص أوراق الزيتون بنسبة 2% لكل كجم علف، المجموعة الثالثة: تلقت النظام الغذائى الأساسى مضافاً عليه مستخلص أوراق الزيتون بنسبة 4% لكل كجم علف. أمتدت لشهر كامل تم فيه قياس وزن الجسم مع ابتداء التجربة ومقارنته بوزن جسم الحيوانات مع انتهاء التجربة. كذلك تم سحب عينة الدم من الحيوانات مع انتهاء مدة التجربتين وكان قياس كميات العلف المستهلكة يتم أسبوعيًا.ثم بعد ذلك تم إجراء تقييمات في المختبر لمدى تكاثر الخلايا الليمفاوية، وللمؤشرات الحيوية للإجهاد التأكسدى. أظهرت النتائج أنه لم يكن هناك فرق معنوى للمعالجات على وزن الحيوانات مقارنة بالمجموعة المراقبة و لكن أظهرت المجموعات المتعالجة بأنخفاض فى معدلات تحويل الغذاء مقارنة بالمجموعة المراقبة كما أظهرت هذه المجموعات ازدياد معنوي فى الخلايا الليمفاوية، انخفاض معنوى فى مستوى المالونديالدهيدات، و ارتفاع فى مستوى فوق أكسيد الدسميتاز مقارنة بالمجموعة المراقبة. في الختام، استكشفت هذه الدراسة تأثير الأضافة الغذائية لكل من ورق الزيتون و طحلب النانوكلوربسيس اوكيولاتا مكملات غذائية على وظائف المناعة لدى الأغنام الحوامل. تشير النتائج إلى أن هذه المكملات يمكن أن تحسن استجابة المناعة وتقلل من التوتر التأكسدي لدى الأغنام الحوامل، مما قد يكون له فوائد على الأغنام ومولوديها.