



Original article

Effects of dietary plant polyphenols and seaweed extract mixture on male-rabbit semen: Quality traits and antioxidant markers



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ABSTRACT

Feed additive consisting of polysaccharides from brown seaweeds plus phenolic acid, hydroxycinnamic acids, tannins, and flavonoids from plant extracts, was used as rabbit food supplement. Main aim of the study was to determine the effect of natural mix of marine and freshwater seaweed polyphenols on selected reproductive characteristics of male rabbits during the 90-days experiment. Natural mix was incorporated in feed-pellets for rabbits in two different concentrations – 0.3% (T1 group) and 0.6% (T2 group), compared with a control group (C group). In experimental groups a significant increase of concentration of calcium during first 30 days of supplementation was found. An increase of alanine aminotransferase, glutathione peroxidase and ferric reducing ability of plasma and a decrease in aspartate aminotransferase after 90 days were recorded in the same groups. Except for that we noticed decrease of semen distance of curved line and velocity of curved line after 30 days though only while being supplemented with 0.6% proportion of seaweed polyphenols in feed mixture. Based on the results it can be stated that the natural mix in the tested levels do not show adverse effect on male rabbit reproductive parameters, and an improvement of antioxidant status was observed. The feed additives can have a very important effect on growth, health and development of animals in general as it supplies the with the much-needed minerals, nutritional substances and antioxidants, on which we focused in our study. © 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Generally, oxidative stress occurs in the organism whenever there is elevated level of free radicals over body antioxidant defense system. Oxidative stress cause alteration in body homeostasis condition leading to production of free radical species over internal antioxidants and leading to an inhibition of normal

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physiological activities (Rahal et al., 2014). Oxidative stress is categorized as drug-dependent oxidative stress, metabolic oxidative stress and environmental oxidative stress (oxidative stress occurring as a result of changes or critical environmental conditions such as nutrition, drought, and salinity) based on the sources of generation but in any case could be defined as metabolic process of imbalance between pro-oxidants and antioxidants favoring abundance and overbearing effects of the pro-oxidants. It causes disruption of redox signals, biochemical pathways and control leading to molecular damage in the body systems. Oxidative stress has been reported widely as the mechanism behind many pathological developments and diseases conditions including reproductive inefficiency, cardiovascular diseases, aging, alcohol-related diseases, adult respiratory distress, atherosclerosis, and inborn error of metabolism (Sikiru et al., 2018).

In the last decade, many studies have been focused on natural substances that can affect health of animals and challenge the new animal welfare prospective (Yeung et al., 2018). The present

study based on the claim that the dietary supplementation of antioxidants is a proven tool of reducing oxidative stress associated with reproductive performance and the need to discover different sources of antioxidants capable of improving reproductive activities is in high demand. Plant extracts represent a valid strategy to support a sustainable animal production (Rossi et al., 2020; Casamassima et al., 2017).

Brown seaweeds are considered an excellent source of vitamins, minerals and omega-3 fatty acids (Descamps et al., 2006). Moreover, it also contains sulfur polysaccharides, phlorotannin, catechins, carotenoids, tocopherols and diterpenes characterized by antimicrobial, antioxidant, anti-inflammatory and immunomodulatory activities (Maghin et al., 2014). These properties make these compounds interesting in livestock production for improvement of animal health and welfare (Vizzari et al., 2019a; Makkar et al., 2015). Previous studies reported that tannins, a heterogeneous group of polyphenols, showed antibacterial and antioxidant activities as recently reviewed by Huang et al. (2018). It is hypothesized that the combination of several bioactive compounds will act synergistically on the animal antioxidant status and reproductive parameters. The effects of this natural mixture on male reproductive parameters have not been reported in literature, thus its effect on the reproductive potential of male rabbits was assessed to determine its possible positive effects on reproduction. It is generally suggested that natural substances can have positive effect on health status, production and reproductive capabilities but there are still many concerns related to adequate dosage. The aim of the present study is to determine the effects of dietary natural mixture supplementation in two doses to male rabbits for 90 days on selected reproductive traits (mainly motility parameters), seminal plasma biochemical profile and antioxidant markers.

2. Materials and methods

2.1. Animals and experimental design

All the experimental procedures and management of animals were conducted in accordance with European Community guidelines no. 86/609/EEC regarding the protection of animals for experimental purpose. The choice of rabbit is due to being a good model for assessing the effects of toxic agents on semen quality, fertility and developmental toxicity. Rabbit semen is collected, evaluated and the fertility is tested under controlled conditions through artificial insemination (Foote and Carney, 2000), thereby providing a direct comparison with human semen analysis. Whole experiment lasted for 90 days and animals – adult males ($n = 24$) of New Zealand rabbits were provided by National Agricultural and Food Centre, Nitra (Slovak Republic). During the trial, the animals were placed in separate cages that were equipped with feeder and automatic watering system. Environmental conditions in the rabbitry was 16 h of light and 8 h of dark per day (maximal intensity being 80 lx), air temperature between 20 and 24 °C and 65% humidity. All male rabbits were divided into 3 homogeneous groups (aged 18.5 ± 1.5 months; 4.90 ± 0.87 kg body weight). Rabbits were fed a control diet (C; $n = 8$) or T1 ($n = 8$) and T2 ($n = 8$) diets, which were supplemented with 0.3% and 0.6% of feed additive consisting of polysaccharides from brown seaweeds (*Laminaria Digitate* and *Hyperborea*, ratio 1:1) plus phenolic acid, hydroxycinnamic acids, tannins, and flavonoids from plant extracts. The diets included no anticoccidials, antibiotics or other medications. The supplement was included in the basal mashed diet. All the experimental diets were pelleted. The two dosages of the natural extract were chosen after an *in vitro* evaluation of the minimal inhibitory concentration (MIC) against *Clostridium* spp., *Staphylococcus* spp., and *Escherichia coli* spp. (Tosi, personal communication), and based on previous

experimental experiences on rabbit species (Rossi et al., 2020; Vizzari et al., 2019a). Rabbits were fed *ad libitum* and the ingredients and the chemical composition of the diets are reported in Table 1 in dried form. All analyses on experimental diets were performed in accordance with the methods of the Association of Analytical Chemists (AOAC, 2000). The dietary supplement was analyzed for the identification and quantification of natural compounds with the HPLC-DAD (Russo et al., 2017), and the polyphenols profile composition is reported in Table 2.

2.2. Semen sampling and analyses

The semen samples were collected on day 0, 30, 60 and 90 of the experiment with the help of artificial vagina. The obtained semen samples were diluted with physiological solution in the ratio of 1:5. After processing, the samples were stored in the cultivator at the temperature 37 °C and were analyzed immediately. Each of such prepared samples were evaluated using a Computer Assisted Semen Analyzer (CASA) system – Sperm Vision (Minitub, Tiefenbach, Germany) equipped with a microscope (Olympus BX 51, Japan) to assess the spermatozoa motility (Massányi et al., 2008). Each sample was placed into Makler Counting Chamber (depth 10 µm, Sefi-Medical Instruments, Germany). Using the rabbit specific set up, the following parameters were evaluated – spermatozoa concentration (CONC, 10⁶/mL), total motile spermatozoa (%), motility > 5 µm/s, progressive motile spermatozoa (%), motility > 20 µm/s, DAP (distance of average path, µm), VAP (velocity of average path, µm/s), DCL (distance of curved line, µm), DSL (distance of straight line, µm), VCL (velocity of curved line, µm/s), VSL (velocity of straight line, µm/s), STR (straightness, VSL:VAP ratio), LIN (linearity, VSL:VCL ratio), WOB (wobble, VAP:VCL ratio), ALH (amplitude of lateral head displacement, µm/s) and BCF (beat cross frequency, Hz).

2.3. Semen plasma analysis

One aliquot from all collected semen in each sampling time was centrifuged for 20 min at 3000 rpm to obtain semen plasma and the following parameters were determined: magnesium (Mg), calcium (Ca), phosphorous (P), total protein, urea, cholesterol, triacylglycerols (TAG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and selected antioxidant markers and additional parameters, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), ferric ion reducing antioxidant power (FRAP), albumins and uric acid. Urea, uric acid, albumins, Ca, P, Mg, AST, ALT, cholesterol and TAG were measured using commercial kits DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) on the Randox RX Monza analyzer (Crumlin, United Kingdom) (Kovacic et al., 2017). SOD activity was assessed using the Randox RANSOD commercial kit (Randox Laboratories, Crumlin, Great Britain) employing xanthine and xanthine oxidase (XO) to generate superoxide radicals, which will react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. SOD activity was subsequently measured by the inhibition degree of the reaction at 505 nm using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.). The results are expressed as U/mg protein. Glutathione peroxidase (GPx) activity was evaluated using the Randox RANSEL commercial kit (Randox Laboratories), applying the method of Paglia and Valentine (1967). GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase (Gr) and NADPH the oxidized glutathione is subsequently converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease of absorbance was measured using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.) at 340 nm. GPx activity is expressed as U/mg protein. Protein concentration was

Table 1
Ingredients and chemical composition of experimental diets (g/kg).

Ingredients	Experimental diet [§]		
	C	T1	T2
Maize	282	279	276
Alfalfa hay	305	305	305
Sunflower meal	135	135	135
Palm seed oil	8	8	8
Soybean oil	7	7	7
Wheat	80	80	80
Cane molasses	20	20	20
Carob bean meal	90	90	90
Oat	53	53	53
Calcium carbonate	7	7	7
Sodium Chloride	3	3	3
Dicalcium phosphate	2	2	2
Methionine (99%)	2.5	2.5	2.5
Lysine (78.5%)	1.6	1.6	1.6
Choline (75%)	1.4	1.4	1.4
Vitamin and mineral premix*	2.5	2.5	2.5
Natural extract supplement	0	3	6
Chemical composition, [†]			
Crude protein	184	183.6	183.5
Ether extract	35.7	35.5	35.5
Crude fibre	187	186.8	187
Ash	86	85.7	85.8
Nitrogen free extract	507	507.1	506.9
NDF	302.1	301.5	301.7
ADF	195.8	195.4	195.3
ADL	39.9	39.5	39.5

[§] C: control group; T1: group supplemented with 0.3% of natural extracts; T2: group supplemented with 0.6% of natural extracts.

* Supplied per kg diet: 13,500 I.U. vitamin A (trans-retinyl acetate); 800 I.U. vitamin D3 (cholecalciferol); 35 mg vitamin E (α -tocopherol min 91%), 35 mg copper (cupric sulphate pentahydrate), 150 mg aminoside sulphate;

[†] Analyses determined in triplicate.

Table 2
Polyphenols content (mg/kg DW) in the natural extract supplement.

Phyto-derivate family name	Compound	Amount in natural mix*
Phenolic acid	Dihydroxybenzoic acid	≤LOD
	Syringic acid	1059.79 ± 62.82
Hydroxycinnamic acids	Neochlorogenic acid	7979.23 ± 468.11
	Chlorogenic acid	21.45 ± 3.65
	Trans sinapic acid	105.54 ± 8.09
	Rosmarinic acid	126.54 ± 8.67
Tannins	Ellagic acid	2440.88 ± 148.29
Flavonoids	Rutin	272.37 ± 20.82
	Myricetin	53.88 ± 5.68
	Kaempferol	≤LOD

* Limit of detection; values expressed as means (n = 4) ± standard deviation.

assessed using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and the semi-automated clinical chemistry photometric analyzer Microlab 300 (Merck, Darmstadt, Germany). The measurement is based on the Biuret method, according to which copper sulfate reacts with proteins to form a violet blue color complex in alkaline solution, and the intensity of the color is directly proportional to the protein concentration when measured at 540 nm (Tvrdá et al., 2016). Analysis for FRAP was performed according to method proposed by Benzie and Strain (1996). This test determines the total antioxidant power, based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. The FRAP reagent contains 10 mmol/L TPTZ (2, 4,6- tripyridyl- s- triazine) solution in 40 mmol/L HCl (Centralchem, Bratislava, Slovak Republic) plus 5 mL of 20 mmol/L FeCl₃ (Centralchem, Bratislava, Slovak Republic) and 50 mL 0.3 mol/L acetate buffer (pH = 3.6;

Centralchem, Bratislava, Slovak Republic). Aliquots of 100 μ L sample were mixed with 3 mL FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm for 4 min using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) (Tvrdá et al., 2015).

2.4. Statistical analysis

Statistical program GraphPad Prism (version 3.02 for Windows; GraphPad Software, La Jolla California USA) was used for the elaboration of experimental data. Values were compared using Tukey's Multiple Comparison Test, and significance levels of differences between groups was set to P < 0.05.

3. Results

3.1. Feed additive analysis

The major bioactive compounds of the natural extracts' mixture were identified and quantified by HPLC-DAD as shown in Table 1. Four main phyto-derivate families were identified, such as phenolic acid, with syringic acid as the most represented; hydroxycinnamic acids group, with neochlorogenic acid as the most abundant; tannins class, with ellagic acid as the most present; and flavonoids group, with rutin as the most represented.

3.2. Semen quality traits

The effect of dietary natural mixture on semen quality traits are reported in Table 3. The concentration of spermatozoa did not significantly change among experimental groups after 3 months of dietary treatment. Also the spermatozoa motility parameters (motility and progressive motility) did not significantly change between groups. After one month of dietary supplementation a decrease of all distance parameters (DAP, DCL, DSL) was observed in T2 group. On the other hand, we have seen increase of DAP and DSL in T1 group (Table 4). One month of dietary supplementation with natural mixture had a decreasing effect on distance spermatozoa parameters in T2 group compared to C group. The alteration of DCL between C and T2 groups was statistically significant (Table 4). Two months after the application of the additive, there was a decreasing trend of the DCL parameter in both experimental groups (Table 4). At the end of the experimental period (90d) an increasing trend in all monitored distance parameters was observed in both T1 and T2 groups (Table 4). In both cases a decrease in VAP, VCL and VSL in both experimental groups – T1 and T2 was detected while decreasing trend in velocity parameters was seemingly higher in group which was supplemented by the additive in higher concentration (0.6%) (Table 4). The effect was stronger in the animal fed with higher level of additive and the difference between C and T2 group is statistically significant. After 2 months of dietary supplementation a positive enhancement of all velocity parameters in T1 group was found. In T2 group an improvement in VAP and VSL values was observed but the value of VCL was decreased.

In Table 5, additional parameters of rabbit semen are reported. After one and three months of application, increasing trend of STR was found in the T2 group. After 2 months of application a decrease of STR in T1 group and decrease in T2 group was noticed. After one and three months, a statistically significant increase in linearity (LIN) was found; higher increase was in the group with higher concentration of additive, that being the T2 group. After 2 months, there was a slight increase of LIN in T1 group and slight decrease in T2 group (Table 5). Two months of supplementation completely opposite results to control – increase

Table 3
Quality traits of rabbit semen.

Items	Diet*			P-value [†]
	C	T1	T2	
Concentration (10 ⁶ .ml ⁻¹)				
0 d (basal)	0.64 ± 0.28	0.64 ± 0.60	0.61 ± 0.49	(-)
30 d	0.65 ± 0.18	0.70 ± 0.35	0.87 ± 0.39	(-)
60 d	0.64 ± 0.25	0.54 ± 0.31	0.59 ± 0.46	(-)
90 d	0.86 ± 0.29	0.49 ± 0.10	0.61 ± 0.21	(-)
Motility (%)				
0 d (basal)	84.76 ± 2.69	85.84 ± 9.27	77.70 ± 12.74	(-)
30 d	85.09 ± 7.53	87.21 ± 8.25	89.38 ± 8.02	(-)
60 d	82.84 ± 4.51	87.63 ± 4.57	77.33 ± 12.71	(-)
90 d	92.10 ± 4.21	88.67 ± 5.26	85.68 ± 7.96	(-)
Progressive motility (%)				
0 d (basal)	74.51 ± 4.95	69.94 ± 18.98	62.42 ± 14.26	(-)
30 d	74.28 ± 12.60	79.07 ± 13.89	81.28 ± 11.37	(-)
60 d	70.89 ± 4.40	77.16 ± 4.91	64.39 ± 18.44	(-)
90 d	84.61 ± 4.61	82.04 ± 6.59	76.02 ± 14.47	(-)

* C = control group; T1 = group supplemented with 0.3% of natural extract; T2 = group supplemented with 0.6% of natural extract.

† Within the same row, means with different letters differ significantly (P < 0.05). Results are expressed as means ± SD.

Table 4
Distance and velocity parameters of rabbit semen.

Items [§]	Diet*			P-value [†]
	C	T1	T2	
DAP (μm)				
0 d (basal)	31.73 ± 6.96	26.87 ± 7.89	26.30 ± 5.43	(-)
30 d	33.15 ± 6.69	33.96 ± 3.42	25.99 ± 4.65	(-)
60 d	29.40 ± 4.89	29.07 ± 4.01	24.84 ± 6.58	(-)
90 d	28.51 ± 2.94	34.71 ± 2.35	28.82 ± 7.73	(-)
DCL (μm)				
0 d (basal)	57.82 ± 12.12	52.86 ± 18.39	52.10 ± 12.74	(-)
30 d	62.76 ± 10.64 ^A	61.07 ± 5.13	47.23 ± 6.64 ^B	(-); (+)
60 d	58.09 ± 7.49	56.74 ± 7.34	51.56 ± 7.74	(-)
90 d	54.39 ± 3.90	62.67 ± 9.13	54.37 ± 5.14	(-)
DSL (μm)				
0 d (basal)	25.45 ± 6.41	21.28 ± 7.71	20.55 ± 4.22	(-)
30 d	24.51 ± 5.90	25.66 ± 3.86	20.61 ± 4.45	(-)
60 d	21.50 ± 6.56	20.63 ± 2.18	18.81 ± 4.30	(-)
90 d	21.22 ± 3.17	27.01 ± 4.84	23.77 ± 7.34	(-)
VAP (μm.s ⁻¹)				
0 d (basal)	73.91 ± 15.99	61.29 ± 15.86	60.19 ± 11.82	(-)
30 d	77.55 ± 16.03	79.05 ± 8.32	60.68 ± 11.06	(-)
60 d	68.09 ± 12.23	66.15 ± 7.30	57.03 ± 15.93	(-)
90 d	66.67 ± 7.25	80.71 ± 5.85	65.51 ± 18.69	(-)
VCL (μm.s ⁻¹)				
0 d (basal)	134.20 ± 27.64	119.30 ± 36.92	118.00 ± 27.40	(-)
30 d	146.20 ± 25.83 ^A	141.60 ± 12.42	109.50 ± 15.36 ^B	(-); (+)
60 d	133.80 ± 18.68	128.20 ± 13.88	117.20 ± 19.86	(-)
90 d	126.50 ± 8.55	144.80 ± 19.01	122.70 ± 14.45	(-)
VSL (μm.s ⁻¹)				
0 d (basal)	59.56 ± 14.99	48.71 ± 15.80	47.22 ± 9.20	(-)
30 d	57.61 ± 14.07	59.96 ± 9.03	48.31 ± 10.68	(-)
60 d	50.16 ± 15.87	47.28 ± 3.29	43.44 ± 10.58	(-)
90 d	49.99 ± 7.50	63.26 ± 12.36	54.25 ± 17.68	(-)

* C = control group; T1 = group supplemented with 0.3% of natural extract; T2 = group supplemented with 0.6% of natural extract; † Within the same row, means with different letters differ significantly (P < 0.05).

§ DAP – average distance; DCL – curvilinear distance; DSL – straight distance; VAP – average velocity; VCL – curvilinear velocity; VSL – straight velocity. Results are expressed as means ± SD.

in WOB were observed. After two and three months a positive effect of additive on WOB in T1 group and insignificant negative or no effect in T2 group (Table 5) was detected. Over the entire duration of the experiment a decrease in value of ALH was noticed. Decrease was in all groups, more significant for higher concentration of additive (Table 5). An increase in BCF was observed in both experimental groups 1, 2 and 3 months of feeding with the additive. After one and two months, the increase was higher in T2 group whereas after 3 months it was higher in T1 group (Table 5).

3.3. Semen plasma analysis

Table 6 presents results of selected biochemical markers in rabbit seminal plasma. Analysis of ALT shown an increasing trend, suggesting the lowest concentration of ALT in control group, whereas in T1 group the concentration was higher and the highest concentration of ALT was detected in T2 group. After one and two months of additive supplementation, we noticed the lowest concentration of ALT in T1 group (0.3% of additive) and highest in T2 group (0.6% of additive). After 3 months, decrease of ALT was

Table 5
Additional parameters of rabbit semen quality.

Items [§]	Diet*			P-value [†]
	C	T1	T2	
STR (VSL:VAP ratio)				
0 d (basal)	0.79 ± 0.05	0.78 ± 0.06	0.78 ± 0.09	(-)
30 d	0.73 ± 0.04	0.75 ± 0.05	0.79 ± 0.07	(-)
60 d	0.72 ± 0.12	0.72 ± 0.08	0.76 ± 0.05	(-)
90 d	0.75 ± 0.06	0.78 ± 0.13	0.82 ± 0.04	(-)
LIN (VSL:VCL ratio)				
0 d (basal)	0.44 ± 0.05	0.40 ± 0.01	0.40 ± 0.06	(-)
30 d	0.39 ± 0.04	0.42 ± 0.03	0.43 ± 0.07	(-)
60 d	0.37 ± 0.08	0.37 ± 0.04	0.36 ± 0.04	(-)
90 d	0.39 ± 0.04	0.44 ± 0.12	0.43 ± 0.10	(-)
WOB (VAP:VCL ratio)				
0 d (basal)	0.54 ± 0.03	0.52 ± 0.04	0.51 ± 0.04	(-)
30 d	0.52 ± 0.03	0.55 ± 0.01	0.55 ± 0.05	(-)
60 d	0.50 ± 0.02	0.51 ± 0.01	0.47 ± 0.06	(-)
90 d	0.52 ± 0.04	0.60 ± 0.07	0.52 ± 0.10	(-)
ALH (μm.s ⁻¹)				
0 d (basal)	4.44 ± 0.14	4.36 ± 0.60	3.97 ± 1.18	(-)
30 d	5.26 ± 0.71	5.13 ± 0.70	4.15 ± 1.36	(-)
60 d	4.63 ± 1.03	4.43 ± 0.80	3.89 ± 1.23	(-)
90 d	4.97 ± 0.53	4.39 ± 1.12	3.85 ± 0.46	(-)
BCF (Hz)				
0 d (basal)	33.86 ± 4.24	31.76 ± 5.77	32.91 ± 6.13	(-)
30 d	30.89 ± 3.65	31.75 ± 3.76	32.56 ± 4.82	(-)
60 d	31.24 ± 5.62	32.83 ± 3.50	33.91 ± 2.85	(-)
90 d	30.58 ± 2.53	35.88 ± 4.72	34.36 ± 2.43	(-)

* C = control group; T1 = group supplemented with 0.3% of natural extract; T2 = group supplemented with 0.6% of natural extract; † Within the same row, means with different letters differ significantly (P < 0.05).

§ STR -straightness of movement; LIN - linearity; WOB - wobble; ALH - amplitude of lateral head displacement; BCF - beat cross frequency. Results are expressed as means ± SD.

Table 6
Biochemical markers of rabbit seminal plasma.

Items [§]	Diet*			P-value [†]
	C	T1	T2	
ALT (μkat.l ⁻¹)				
0 d (basal)	0.40 ± 0.08	0.56 ± 0.15	0.57 ± 0.17	(-)
30 d	0.54 ± 0.24	0.43 ± 0.14	1.12 ± 0.71	(-)
60 d	0.61 ± 0.24	0.47 ± 0.14	1.52 ± 2.07	(-)
90 d	1.12 ± 0.45 ^A	0.48 ± 0.26 ^B	0.58 ± 0.17	(-); (+)
AST (μkat.l ⁻¹)				
0 d (basal)	2.67 ± 1.69	1.45 ± 1.44	2.38 ± 1.34	(-)
30 d	3.22 ± 1.36	3.37 ± 2.08	3.07 ± 1.47	(-)
60 d	3.20 ± 2.00	2.79 ± 2.20	2.77 ± 1.19	(-)
90 d	1.24 ± 1.79 ^A	3.27 ± 1.20	3.66 ± 1.05 ^B	(-); (+)
Cholesterol (mmol.l ⁻¹)				
0 d (basal)	1.14 ± 0.48	1.16 ± 0.32	1.46 ± 0.37	(-)
30 d	1.38 ± 0.18	1.32 ± 0.32	1.54 ± 0.64	(-)
60 d	1.40 ± 0.37	1.11 ± 0.33	1.40 ± 0.28	(-)
90 d	1.41 ± 0.38	1.13 ± 0.28	1.46 ± 0.24	(-)
Urea (mmol.l ⁻¹)				
0 d (basal)	10.62 ± 1.46	18.56 ± 10.32	10.18 ± 3.01	(-)
30 d	12.09 ± 2.95	10.76 ± 2.78	15.17 ± 7.85	(-)
60 d	11.24 ± 2.71	9.77 ± 2.57	8.99 ± 1.62	(-)
90 d	15.58 ± 3.60	24.54 ± 7.35	10.67 ± 2.08	(-)
Total proteins (g.l ⁻¹)				
0 d (basal)	18.65 ± 1.20	15.73 ± 1.71	16.82 ± 3.54	(-)
30 d	14.79 ± 1.41	17.81 ± 4.31	13.59 ± 2.19	(-)
60 d	14.70 ± 2.94	15.08 ± 1.82	15.67 ± 1.13	(-)
90 d	17.55 ± 2.53	15.94 ± 1.64	16.80 ± 1.56	(-)

* C = control group; T1 = group supplemented with 0.3% of natural extract; T2 = group supplemented with 0.6% of natural extract; † Within the same row, means with different letters differ significantly (P < 0.05).

§ ALT - alanine aminotransferase; AST - aspartate aminotransferase. Results are expressed as means ± SD.

observed in both experimental groups in comparison with control group, whereas the difference between T1 and C groups was statistically significant (Table 6). After one and two months of feed additive application a decrease of AST concentration in T2 group was found, compared to control group. Similar decrease was spotted

in T1 group, but only after two months of supplementation. After 3 months, a significant increase of AST was detected – the difference between T2 and C group was statistically significant (Table 6). In comparison with control group a decreasing trend of cholesterol in T1 group (0.3% of additive) and gain trend in level of cholesterol

in T2 group (0.6% of additive) after 2 and 3 months was noticed. After one month of oral application an increase in both experimental groups in comparison with control group was detected (Table 6). In semen plasma an increase in concentration of urea in T1 group and decrease in T2 group after three months of application was detected. After first month a decrease of urea in T1 group and increase in T2 group was found, whereas after second month, decrease in both experimental groups related to control group was found (Table 6).

Changes in concentration of total proteins were occurred in all time periods. After 1 month an increase in T1 group and decrease of TP in the group T2 was found. After second month there was increase of concentration of TP in both experimental groups in comparison with control group. After last collection (3 months) a decrease of TP was noticed (Table 6). For mineral parameters concentration of magnesium, calcium and phosphorus were analyzed (Table 7). In control samples a decrease of Mg concentration in experimental group T1 and its increase in experimental group T2 were noticed. The differences between T1 and T2 groups were statistically significant. Lowest concentration of Mg after first month was in the control group, whereas this concentration was higher in T1 group and the highest in T2 group. After three months, decrease of concentration of Mg in both experimental groups in semen plasma was noticed. Concentrations of calcium were the lowest in control group after one and two months of dietary treatment, whereas after 3 months, lowest concentration of Ca was found in T2 group. The change in Ca concentration between control group and T1 group was statistically significant. Statistically significant results were also noticed after one month. Comparing three groups of male rabbit showed also changes in concentration of phosphorus. In comparison with control group decrease in concentration of phosphorus in T1 group and increase in T2 group was detected. Changes in concentration of phosphorus after one, two and three months had the same trend – increase in concentration of phosphorus in both experimental groups in comparison with control group (the increase was more notable in group T2). Concentrations of TAG shown a decrease of concentration in T1 group and increase in T2 group. After one, two and three months of experimental feeding a similar trend was observed – decrease of concentration in both experimental groups compared to control group. The decrease was more noticeable in T1 group (Table 7).

3.4. Antioxidant markers and additional parameters in semen plasma

In Table 8, data regarding the antioxidant markers and additional parameters in seminal plasma are reported. At the end of the dietary treatment (after 90 days), in the experimental group T2 an increase in activity of SOD was observed. This increase was also similar in T1 group after one and three months of dietary application, whereas a decrease in SOD activity in T1 group was detected. After three months, the increase was higher in T1 group and after 2 months it was in T2 group.

Activity of glutathione peroxidase also shown changes – at the beginning of the experiment a decrease of GPx in both experimental groups in comparison with control group was found; decrease was more substantial for higher feed additive concentration (T2). After one and three months, same trend GPx activity in comparison with control group was found – an increase in both experimental groups compared with control group. After second month of administration there was a slight decrease of GPx in T1 group and increase in T2. Differences in activity of GPx after 3 months between control group and T1 group as well as between T1 and T2 group were statistically significant ($P < 0.01$).

During the whole observation period, FRAP parameter shown us similar tendencies after two and three months of oral feeding. In comparison with control group an increase of FRAP in T1 group and decrease in T2 group was detected. The differences after 3 months between control and T1 group and between T1 and T2 groups were statistically significant. The levels of FRAP were generally lower in T2 group compared to T1 (except the results after 60 days) which can indicate that this concentration has positive effect.

Some differences in concentration of uric acid were also detected. After one month of application the highest concentration of uric acid was in T1 group and the lowest in T2 group. After 3 months of application of the additive showed similar trend. Concentration of albumins also shown changes, but these were not statistically significant. After one month of application we observed highest concentration in T2 group and lowest in control group. After 2 months we spotted increase in both T1 and T2 groups, being stronger in T2 group. Likewise, it was after 3 months where increase in both experimental groups was observed, but it was higher in T1 group.

Table 7
Mineral elements and triacylglycerols (TAG) in rabbit seminal plasma.

Items	Diet*			P-value†
	C	T1	T2	
Magnesium (mmol.l ⁻¹)				
0 d (basal)	3.73 ± 0.36	3.50 ± 0.75	3.79 ± 0.27	(-)
30 d	3.70 ± 0.40	3.33 ± 0.45 ^A	4.04 ± 0.16 ^B	(-);(+)
60 d	3.83 ± 0.48	3.92 ± 0.25	3.96 ± 0.18	(-)
90 d	4.01 ± 0.11	3.78 ± 0.55	3.95 ± 0.22	(-)
Calcium (mmol.l ⁻¹)				
0 d (basal)	1.67 ± 0.24 ^A	4.83 ± 2.17 ^B	3.27 ± 1.14	(-); (+)
30 d	1.80 ± 0.30 ^A	2.23 ± 0.34 ^B	5.01 ± 2.21 ^C	(-); (+)
60 d	2.21 ± 0.85	4.92 ± 2.89	2.47 ± 1.19	(-)
90 d	3.03 ± 1.46	4.28 ± 2.35	2.25 ± 0.77	(-)
Phosphorus (mmol.l ⁻¹)				
0 d (basal)	1.61 ± 1.98	1.27 ± 0.95	2.43 ± 2.41	(-)
30 d	1.45 ± 1.04	2.68 ± 2.62	3.40 ± 3.0	(-)
60 d	0.80 ± 0.60	2.12 ± 0.89	2.45 ± 1.79	(-)
90 d	1.92 ± 0.60	2.42 ± 1.29	3.28 ± 2.25	(-)
TAG (mmol.l ⁻¹)				
0 d (basal)	3.23 ± 1.59	3.26 ± 0.44	3.93 ± 1.16	(-)
30 d	2.65 ± 0.68	2.32 ± 0.80	2.49 ± 0.58	(-)
60 d	2.19 ± 1.02	1.45 ± 0.51	2.26 ± 0.55	(-)
90 d	2.05 ± 0.48	1.50 ± 0.40	2.05 ± 0.49	(-)

* C = control group; T1 = group supplemented with 0.3% of natural extract; T2 = group supplemented with 0.6% of natural extract.

† Within the same row, means with different letters differ significantly ($P < 0.05$). Results are expressed as means ± SD.

Table 8
Antioxidant markers and additional parameters of rabbit seminal rabbit.

Items [§]	Diet [*]			P-value [†]
	C	T1	T2	
SOD (U.mg ⁻¹ TP)				
0 d (basal)	0.42 ± 0.10	0.66 ± 0.58	0.35 ± 0.06	(-)
30 d	0.30 ± 0.19	0.44 ± 0.27	0.35 ± 0.15	(-)
60 d	0.38 ± 0.12	0.30 ± 0.10	0.42 ± 0.09	(-)
90 d	0.26 ± 0.11	0.43 ± 0.24	0.31 ± 0.09	(-)
GPx (U.g ⁻¹ TP)				
0 d (basal)	44.63 ± 15.37	36.33 ± 0.48	30.37 ± 0.60	(-)
30 d	23.28 ± 12.33	41.76 ± 28.01	35.79 ± 16.64	(-)
60 d	32.96 ± 12.30	32.75 ± 14.00	44.42 ± 15.62	(-)
90 d	26.60 ± 11.25 ^A	65.58 ± 19.31 ^B	30.53 ± 4.53 ^A	(-);(+)
FRAP (μmol Fe ²⁺ .g ⁻¹ TP)				
0 d (basal)	97.42 ± 48.96	142.90 ± 138.90	60.60 ± 12.10	(-)
30 d	75.59 ± 54.34	106.30 ± 64.98	69.75 ± 26.35	(-)
60 d	59.24 ± 15.33	53.31 ± 22.54	68.02 ± 28.52	(-)
90 d	45.46 ± 22.51 ^A	103.00 ± 36.10 ^B	47.11 ± 7.53 ^A	(-); (+)
Uric acid (μmol.g ⁻¹ TP)				
0 d (basal)	0.73 ± 0.29	0.51 ± 0.27	0.56 ± 0.36	(-)
30 d	0.49 ± 0.26	0.55 ± 0.20	0.40 ± 0.16	(-)
60 d	0.65 ± 0.14	0.27 ± 0.08	0.57 ± 0.31	(-)
90 d	0.41 ± 0.23	0.44 ± 0.05	0.41 ± 0.081	(-)
Albumins (g.g ⁻¹ TP)				
0 d (basal)	0.26 ± 0.10	0.82 ± 1.09	0.16 ± 0.08	(-)
30 d	0.23 ± 0.24	0.43 ± 0.27	0.46 ± 0.50	(-)
60 d	0.35 ± 0.35	0.38 ± 0.19	0.62 ± 0.44	(-)
90 d	0.12 ± 0.08	0.35 ± 0.25	0.16 ± 0.05	(-)

^{*} C = control group; T1 = group supplemented with 0.3% of natural extract; T2 = group supplemented with 0.6% of natural extract.

[†] Within the same row, means with different letters differ significantly ($P < 0.05$).

[§] SOD -superoxide dismutase; GPx - glutathione peroxidase; FRAP - ferric ion reducing antioxidant power. Results are interpreted as mean ± SD.

4. Discussion

In this study, we report that the natural feed mixture in the tested concentrations has no negative impact on monitored spermatozoa motility parameters. On the other hand, some indications of improved values of biochemical parameters and antioxidant markers in seminal plasma at the lower concentration suggest possible positive effect.

Investigating the effect of natural substances on reproductive potential is nowadays very actual topic. There are multiple studies aimed to find out the effect of natural substances on reproductive characteristics of males (Vizzari et al., 2019b; Halenár et al., 2017; Tvrdá et al., 2016). Diet has effect on secretory function of additional reproductive glands, which produce semen plasma – which was already confirmed in last century (Mann and Walton, 1953). Vizzari et al. (2019a) examined the effect of same plant-based extracts on reproductive parameters of rabbit does which were fed during gestation and lactation (65 days). They managed to find out that this extract does not have effect on number of born kits, neither on the birth live weight of the animals or their weaning weight. These results agree with our data because no effect of this natural extract on monitored reproductive parameters of male rabbits were observed, but an improvement of antioxidant status was recorded. Okab et al. (2013) reported that supplementation with 2% effect of seaweeds causes significant decrease of concentration, volume of ejaculate and ratio of live spermatozoa, as well as increase in total and progressive motility, distance parameters, velocity parameters and linearity of movement. In our experiment, after 1 month of dietary supplementation in T2 group, only statistically significant decreases were observed in DCL and VCL parameters. We did not manage to confirm statistically significant differences in concentration of spermatozoa in any of the experimental groups, and the length of the supplementation did not have any particular effect either. Reference values of fresh rabbit semen

stated by International rabbit reproduction group (2005) are – Progressive motility – 30–90%, pH 7.1, VCL 80–100%, VSL 30–50%, VAP 50–70%.

Likewise, Mourvaki et al. (2010) found no effect with use of dietary flaxseed on volume and spermatozoa concentration in rabbit. Motility is very important characteristic of ejaculate, as it is primary supposition for spermatozoa movement in female reproductive tract to the place of fertilization. Some research confirm that the existence of selected antioxidants positively correlates with motility of spermatozoa (Talevi et al., 2013). During the *in vivo* experimental period, no significant increase of motility in groups supplemented with two levels of natural extract contained seaweeds and polyphenols was confirmed. Cholesterol, TAG and total proteins have positive relation towards spermatozoa motility. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are both known as markers of increased oxidative stress in semen – which causes damage of membranes of spermatozoa (Aragwal et al., 2014; Asadpour, 2012; Bozkrut et al., 2011).

Changes in activity of hepatic enzymes (ALT and AST) are related to damage of spermatozoa membranes that can happen as effect of increased oxidative stress (Colenbrander et al., 1992). During our analysis, statistically significant decrease in concentration of ALT after 3 months of supplementation with the additive in 0.3% concentration and significant increase in concentration of AST after 3 months of supplementing with the additive in concentration of 0.6% were noticed. AST is generally negatively correlated with spermatozoa plasma membrane integrity (Umar et al., 2018). The increased activity of AST in experimental groups suggest, that the effect of analysed feed additive used in this study should affect the membrane integrity mainly in groups with the higher concentration of the supplement. Also a relatively wide range (deviation) of detected values is reported (Yousef et al., 2007; Viudes de Castro et al., 2015). There was no statistically sig-

nificant change in concentration of cholesterol, TAG and total proteins.

There is no universal system in existence that could provide us with information about actual antioxidant strength of certain antioxidant or complex of antioxidants and therefore it is difficult to evaluate antioxidant capacity of certain substance (Litescu et al., 2010). During their experiment authors found toxic effects of curcumin (decrease in motility of mice spermatozoa) with high concentrations – 100 µM, whereas it acted as a substance with protective and antioxidative functions in the same experiment when it was in concentration of 1–50 µM.

The activity of GPx after 3 months of supplementation was significantly higher in T1 (0.3%) group in comparison with control group; whereas in T2 group a significant decrease in activity of GPx in comparison with T1 group was observed. Perry et al. (2009) reported that SOD is used to neutralize superoxide radicals in ejaculate of rabbits, whereas in humans it is mostly GPx. In present study, no significant changes in activity of SOD were detected, but on the other hand after 90 days of supplementation a significant increase in activity of GPx in group T1 (0.3%) was noticed. Compared to our results, it has also been reported that surveying SOD activity in seminal plasma could be a useful tool for determining spermatozoa fertilization potential and could improve the diagnosis of male infertility (Shiva et al., 2011). In general, antioxidant dietary supplementation leads to improvement of the antioxidant markers profile in the seminal plasma, in particular when algae-based feed additive is supplemented in the diet (Murphy et al., 2017).

Nowadays, there are many studies describing a relation between mineral profile of semen plasma and quality of semen. It was already confirmed that increased concentrations of calcium, magnesium, sodium, potassium and zinc associate with increased fertility of males (Rodríguez et al., 2012). In our experiment there was no significant change in concentrations on Ca, Mg, P in any of the experimental groups and in none of the selected sampling time periods. Also, basic nitrogenous components of semen such as urea or uric acid were detected. Urea enters ejaculate from cardiovascular system (Setchell, 1991). Xu et al. (2010) reported that uric acid could serve as substrate for ROS and thus it protects important biomolecules against oxidative damage, they also proved that it is capable of stabilizing antioxidant activity of ascorbic acid in semen plasma and parameters of ejaculate, for example motility. During our experiment no significant changes in concentrations of uric acid in semen plasma were observed.

5. Conclusion

Based on the present results it can be stated that the natural mix in the tested concentration does not show negative effect on any of monitored reproductive parameters. Improved results of multiple profiles parameters and antioxidant markers in seminal plasma underlines the positive effect of dietary feed additive strategy to counteract the oxidative stress in intensively reared rabbit farms. The use of the supplement did not show any visible negative effects on the organism of rabbits. On the contrary, it enhanced the antioxidant capability of the seminal plasma, therefore the use of this feed supplement, especially in lower doses can be recommended.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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