



INFLUENCE OF DIETARY CHITOSAN SUPPLEMENTATION ON OVARIAN DEVELOPMENT AND REPRODUCTIVE PERFORMANCE OF NEW ZEALAND WHITE RABBIT DOES

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Abstract

This study aimed to determine the effect of dietary chitosan supplementation on the productive and reproductive performance of New Zealand White (NZW) rabbits. Forty healthy weaned female rabbits were randomly distributed into four experimental groups (10 females per group) and fed *ad libitum* for six months. A basal diet without chitosan supplementation was used as a control. The other three experimental groups were fed a basal diet plus 0.2, 0.4, or 0.6 g chitosan/kg diet. After eight weeks, three females from each group were sacrificed for morphological observation of ovarian tissues. The remaining animals were used for reproductive studies by a maximum of three parities. Morphological observation of ovaries demonstrated that females fed a diet containing 0.2 g/kg chitosan had increased ovarian diameter and elevated number of mature follicles compared with the control and the other experimental groups. Receptivity, conception rate, and kindling interval were significantly ($P < 0.05$) higher in females fed diets containing 0.2 and 0.4 g/kg chitosan compared to the other groups. Moreover, there was a trend toward a higher average milk yield throughout the lactating period in females fed a diet containing 0.2 g/kg chitosan ($P = 0.904$). Diets containing up to 0.4 g/kg chitosan were positively correlated with increased receptivity rate and several parities. However, diets containing up to 0.4 g/kg chitosan were negatively correlated with decreased receptivity time, kindling interval, litter size at weaning, mortality rate at weaning, bunny weight at weaning, and milk yield. The present study's findings indicate that diets containing 0.2 g/kg chitosan improved reproductive efficiency in female NZW rabbits.

Key words: rabbit females, chitosan, ovaries, productive, reproductive, performance

Rabbits could contribute significantly to solving the problem of meat shortage. Rabbits have several characteristics that would make them particularly suitable as meat-producing animals, especially when compared with other herbivorous animals. The meat of rabbits has a low cholesterol level, a high protein/energy ratio, and is relatively rich in essential fatty acids (Lukefahr and Cheeke, 1991; Kamal et al., 2022; Mohamed et al., 2022; Nasr et al., 2022).

Nutritionists and physiologists are under pressure from modern agricultural practices and a competitive market to find novel approaches to improve rabbits' performance. Feed additives, including antibiotics, probiotics, prebiotics, and immune modulators, might be suitable for promoting immunological responses, improving feed efficiency, and increasing weight gain (Swiatkiewicz et al., 2015; Ho et al., 2020; Okawa et al., 2021). Previous studies have demonstrated that using chitosan as a dietary supplement in animal feed has beneficial effects on the digestive system, including absorbing accumulated toxins, relieving chronic constipation, and accelerating

the healing of gastrointestinal ulcers. Chitosan reduces cholesterol absorption from food (Suthongsa et al., 2017; Wan et al., 2018; Huang et al., 2021).

Moreover, De Paiva et al. (2016) reported the utility of chitosan as a rumen modulator in the place of ionophores in the diets of dairy cows, with chitosan having beneficial effects on rumen fermentation resulting in improved milk yield in lactating dairy cows. Further, Del Valle et al. (2017) demonstrated that chitosan improves animal performance and nutrient utilization efficiency and increases long-chain fatty acid concentration, indicating the potential of chitosan as a feed additive in dairy cow diets without fat supplementation. Wan et al. (2018) reported that offspring growth was accelerated by adding chitosan oligosaccharide to the sow diet during late gestation and lactation. They posited that supplementation with chitosan during late gestation and lactation has beneficial antioxidant and anti-inflammatory effects in sows resulting in improved colostrum and milk composition. Ho et al. (2020) posited chitosan oligosaccharides as feed additives can improve pig production, milk pro-

duction, and general health in pregnant and lactating gilts and piglets.

Previous studies have used nanoparticles containing chitosan in animal reproduction experiments. Hassanein et al. (2021) reported the fabrication of GnRH-loaded chitosan nanoparticles (GnRH-ChNPs) and demonstrated the conventional intramuscular GnRH dose used for artificial insemination in rabbits could be halved without affecting fertility. Bhat et al. (2019) found that chitosan nanoparticles conjugated to Eurycomanone enhanced gonadal growth with higher gonadosomatic index values. Eurycomanone was found to act on genes necessary for steroid synthesis and metabolism in gonads and on FSH β and LH β in brain tissues, thereby increasing testosterone levels and reproductive success. Further, recent studies have highlighted the potential use of chitosan as a feed additive to improve farm animals' productivity and reproductive performance (Suthongsa et al., 2017; Hernandez-Patlan et al., 2018). Because of the reported impacts of chitosan on many of reproductive traits in several animal species, the present study hypothesized that its use in rabbit does' feed may have positive effects on the ovarian development and reproduction in New Zealand White (NZW) rabbit does.

Material and methods

The present study was carried out at a Private Rabbitry Farm in El Kassassin, and the Laboratory of Animal Production Department, Faculty of Agriculture, Suez Canal University, Ismailia Governorate, Egypt, and executed from October 2020 to April 2021.

Animals and husbandry

Forty NZW rabbits does were used in the present study. Animals were healthy and free of external parasites and skin diseases. Animals were approximately five weeks of age with an average body weight of 671 $\pm$ 44 g. Animals were individually housed in galvanized wired cages (50 $\times$ 50 $\times$ 40 cm) and provided with feed and water *ad libitum*. All animals were maintained continuously under consistent housing and environmental conditions with a 16 h light/8 h dark cycle during the experimental period. Feed was provided in pellet form and comprised yellow corn, soybean meal, corn gluten, minerals, a vitamin premix, and molasses. The calculated chemical composition of the diet was 17.5% crude protein, 2.8% fat, 10% crude fiber, and 2600 kcal digestible energy/kg diet (NRC, 1991). Feed ingredients and chemical compositions of experimental diets are presented in Table 1. Chitosan was supplied by Chitosan Egypt (the Egyptian Company for Chitosan manufacturing, Dokki, Giza, Egypt).

Experimental design

Animals were individually weighed and randomly divided into four equal experimental groups (10 fe-

males per group). The control group was fed only on a basal diet. The other three experimental groups were fed a basal diet supplemented with 0.2, 0.4, or 0.6 g chitosan/kg. The experiment was extended to three parities.

Table 1. Ingredients and calculated chemical composition of the basal diet

Ingredients	(%)
Alfalfa hay	26.5
Barley	17.0
Yellow corn	15.0
Soybean meal (44%)	16.0
Wheat bran	20.0
Alfalfa straw	3.0
Limestone	1.65
Vitamins and minerals premix*	0.30
NaCl	0.30
DL-methionine	0.1
Anti-coccidia	0.05
Anti-toxin	0.1
Total	100.0
Chemical composition (on DM basis)	
crude protein	17.5
fat	2.8
digestible energy (kcal/kg)	2600
crude fiber	10.0
calcium	0.93
total phosphorus	0.62
lysine	0.84
methionine	0.65
methionine + cysteine	0.63

*Supplied per 1 kg diet: 6000 IU vit. A; 900 IU vit. D₃; 40 mg vit. E; 2.0 mg vit. K₃; 2.0 mg vit. B₁; 4.0 mg vit. B₂; 2.0 mg vit. B₆; 0.010 mg vit. B₁₂; 5.0 mg vit. PP; 10.0 mg vit. B₅; 0.05 mg B₉; 3.0 mg B₉; 250 mg choline; 50.0 mg Fe; 50.0 mg Zn; 8.5 mg Mn; 5.0 mg Cu; 0.20 mg I, and 0.00 mg Se.

Histological studies

At eight weeks into the experimental period, three females from each group were randomly chosen and sacrificed for morphological observation of ovarian tissues. Both ovaries from each female were immediately removed after sacrifice, fixed in 10% formalin solution, dehydrated in an ascending series of alcohol concentrations, and embedded in paraffin wax. Samples were sectioned and stained with hematoxylin and eosin (Bancroft and Stevens, 1990). Sections were examined under light microscopy.

Sexual receptivity and conception rate

Doe receptivity was measured using two methods: the proportion of mated females among all females provided mate according to Kishk et al. (2006), and the time (sec) to successful mating after the introduction of the buck according to Khalil et al. (2015).

The conception rate was determined in rabbit does at 14–15 days after successful mating by abdominal palpation. The conception rate was calculated according to the methods of Ewuola et al. (2014) using the following equation:

$$\text{Conception rate} = \frac{\text{number of pregnant does}}{\text{number of mated does}} \times 100$$

Twelve healthy bucks of the same breed were used for natural mating (three bucks for each treatment). Does were transferred to buck cages for natural mating and monitored until the completion of natural mating.

Gestation periods and kindling intervals

The gestation period (days) was recorded in each doe from successful mating to parturition over three parities. Kindling interval (days) was recorded as the time between two consecutive parturitions in each doe over three parities.

Productive performance and mortality rates

The weekly milk yield for each doe was recorded on one day per week at 8:00 am as the difference in rabbit kitten weight before and after suckling. Rabbit bunnies were separated for 14 h before suckling during the four weeks of the lactating period, as described by Lukefahr et al. (1983).

Litter size (total, alive and dead) was recorded at birth and weaning (30 days of age). Litter weights were recorded at birth and at weaning. The average bunny weight was measured over a range of time points (birth and weaning). Animals were observed daily for mortality during the experimental period. The total mortality rate was calculated as the number of deaths of rabbits during the experimental period divided by the number of rabbits at the experiment beginning.

Data analysis

The experimental period covered three consecutive parities. Mean values for each measured parameter were calculated from means of three parities. Data were analyzed using the General Linear Model calculation in SPSS version 26.0 (SPSS, 2019). Differences between means were compared using Duncan's multiple range test (Duncan, 1955). Conception rates were compared using the chi-square test. Correlation coefficients were estimated between some traits. One-way analysis of variance (ANOVA) was used to compare all means using the following model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where:

Y_{ij} = the observation on the j^{th} individual from the i^{th} treatments,

μ = the overall mean,

T_i = the fixed effect of the i^{th} treatments (control, chitosan treatments),

e_{ij} = the random error associated with the individual ij .

Results

Histology analysis of ovarian tissues

Histological examinations of ovaries after treatment for eight weeks are shown in Figure 1. Morphological observation of ovarian tissues revealed that females fed a diet containing 0.2 g/kg chitosan had increased ovarian diameter and a higher number of mature follicles than the control and other treatment groups.

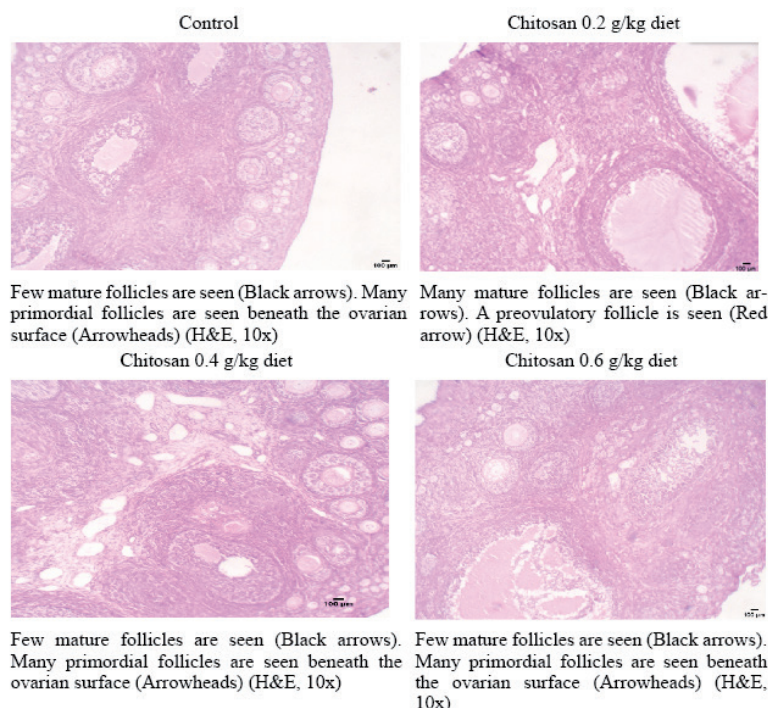


Figure 1. Histological evolution of ovaries of growing rabbits as affected by different chitosan treatments

Table 2. Effects of dietary chitosan oligosaccharides supplementation on reproductive performance in NZW rabbit does (Mean $\pm$ S.E)

Traits	Treatments (g chitosan/kg diet)				P-value
	Control	0.2	0.4	0.6	
Female receptivity (sec) ¹	22.8 $\pm$ 6.01 a	5.0 $\pm$ 0.70 b	6.6 $\pm$ 1.12 b	10.4 $\pm$ 0.92 b	0.004
Female receptivity (%) ²	65.0 $\pm$ 5.00 b	90.0 $\pm$ 5.75 a	93.3 $\pm$ 6.66 a	70.0 $\pm$ 5.77 b	0.020
Conception rate (%)	68.3 $\pm$ 4.41 b	88.3 $\pm$ 6.00 a	90.0 $\pm$ 5.77 a	71.7 $\pm$ 1.66 b	0.025
Gestation period (days)	31.5 $\pm$ 0.31	31.3 $\pm$ 0.37	31.1 $\pm$ 0.45	31.8 $\pm$ 0.37	0.622
Kindling interval (days)	43.4 $\pm$ 1.40 a	38.8 $\pm$ 0.37 b	38.8 $\pm$ 0.66 b	42.8 $\pm$ 0.97 a	0.003
Number of parities	3.0 $\pm$ 0	3.2 $\pm$ 0.20	3.2 $\pm$ 0.20	3.0 $\pm$ 0.00	0.999

a, b, c, d – means within the same row with different letters are significantly different ($P \leq 0.05$).

¹Receptivity time (sec) was determined using a stopwatch to determine the time of introducing doe to the buck until successful mating.

²Receptivity percent was calculated as a percentage of the number of mated females from all females provided to mating.

Reproductive parameters

Table 2 shows the effects of dietary supplementation with chitosan on reproductive performance in NZW rabbit does, including female sexual receptivity, conception rate, gestation period, kindling interval, and several parities. ANOVA demonstrated significant effects of chitosan supplementation on all reproductive traits except the gestation period. Females fed diets containing varying amounts of chitosan had significantly lower receptivity times than those in the control groups ($P < 0.05$). Further, receptivity, conception rate, and kindling intervals improved significantly in females fed diets containing 0.2 and 0.4 g/kg chitosan compared with the control and 0.6 g/kg chitosan groups ($P < 0.05$).

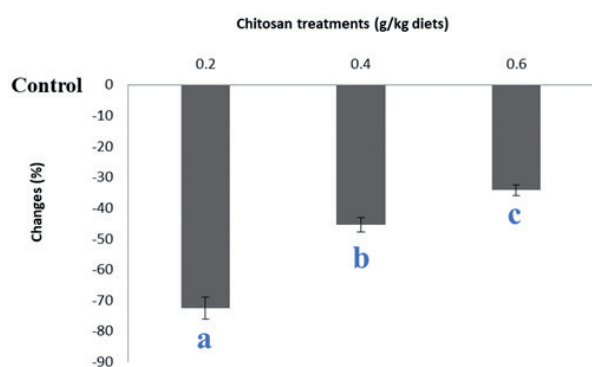


Figure 2. Changes (%) in time of receptivity of does (sec) between control and chitosan treatment groups

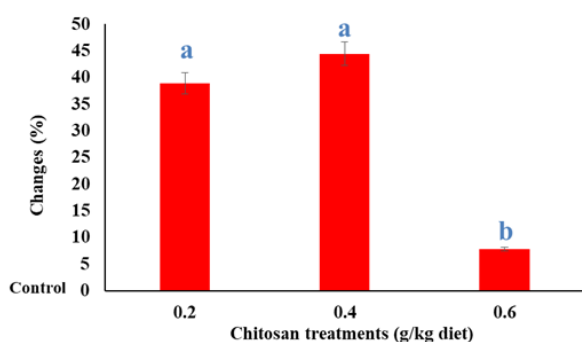


Figure 3. Changes (%) in receptivity rate of does between control and chitosan treatment groups

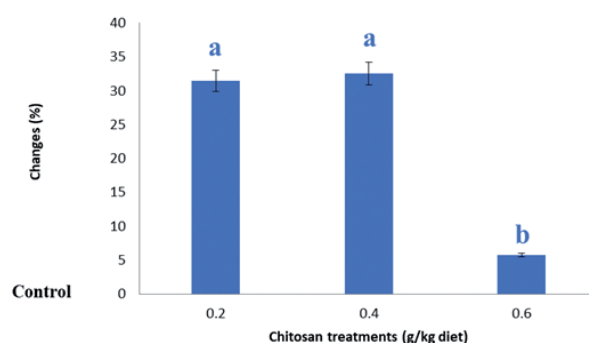


Figure 4. Changes (%) in conception rate of does between control and chitosan treatment groups

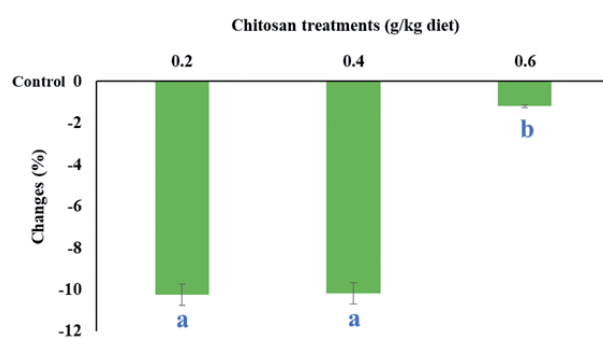


Figure 5. Changes (%) of kindling interval between control and chitosan treatment groups

The effects of diets supplemented with chitosan on receptivity, conception rate, and kindling interval are shown in Figures 2–5. Diets containing 0.2 g/kg, 0.4 g/kg and 0.6 g/kg chitosan decreased receptivity time (sec) by 72.57%, 45.43%, and 34.26%, respectively, increased receptivity by 44.44%, 38.89%, and 7.78%, respectively, improved conception rate by 32.54%, 31.43%, and 5.71%, respectively, and decreased the kindling interval by 10.24%, 10.19%, and 1.19%, respectively, compared to the control group. Further, receptivity (proportion and time), conception rate, and kindling intervals were significantly ($P \leq 0.05$) higher in does fed chitosan at levels of 0.2 and 0.4 g/kg chitosan compared to the diet containing 0.6 g/kg chitosan ($P < 0.05$) and the control group.

Table 3. Effects of dietary chitosan oligosaccharides supplementation on productive performance of NZW rabbits (Mean $\pm$ S.E.)

Traits	Treatments (g chitosan/kg diet)				P-value
	Control	0.2	0.4	0.6	
Average litter size at birth/doe (No)	8.4 $\pm$ 0.87	7.6 $\pm$ 0.40	7.7 $\pm$ 0.40	8.2 $\pm$ 0.58	0.701
Average litter size at weaning/doe (No)	7.2 $\pm$ 0.37	7.2 $\pm$ 0.20	7.0 $\pm$ 0.31	6.8 $\pm$ 0.58	0.892
Average bunny weight at birth/doe (g)	48.9 $\pm$ 2.39	42.5 $\pm$ 0.90	42.4 $\pm$ 2.14	45.9 $\pm$ 3.17	0.180
Average bunny weight at weaning/doe (g)	388.8 $\pm$ 33.15	398.9 $\pm$ 13.05	350.1 $\pm$ 15.89	395.5 $\pm$ 18.88	0.384
Average litter weight at birth/doe (g)	411.4 $\pm$ 47.61	322.0 $\pm$ 12.90	320.0 $\pm$ 14.93	373.4 $\pm$ 30.82	0.134
Average litter weight at weaning/doe (g)	2838.4 $\pm$ 356.53	2878.0 $\pm$ 158.68	2465.0 $\pm$ 194.10	2714.0 $\pm$ 306.00	0.639
Average milk yield (g/day/doe) during lactating period	132.8 $\pm$ 3.06	133.7 $\pm$ 3.29	126.8 $\pm$ 3.99	128.9 $\pm$ 13.81	0.904
Total mortality rate of bunny (%)	2.0 $\pm$ 0.95	1.6 $\pm$ 0.40	1.8 $\pm$ 0.80	2.4 $\pm$ 0.25	0.849
Mortality rate of adult rabbits (%)	0.00	0.00	0.00	0.00	1.000

Table 4. Correlation coefficients among some productive and reproductive traits in NZW rabbit does

	RR	RT	NP	KI	LSW	MRW	BWW	MY
Ch Tr	0.915	-0.823	0.866	-0.866	-0.866	-0.721	-0.750	-0.799
RR	1	-0.982	0.994	-0.994	-0.590	-0.939	-0.419	-0.488
RT		1	-0.997	0.997	0.428	0.987	0.241	0.315
NP			1	-0.999**	-0.500	-0.971	-0.319	-0.391
KI				1	0.500	0.971	0.319	0.391
LSW					1	0.277	0.980	0.993
MRW						1	0.082	0.159
BWW							1	0.997*

ChTr – Chitosan treatment, RR – Receptivity rate, RT – Receptivity time, NP – Number of parities, KI – Kindling interval, LSW – Litter size at weaning, MRW – Mortality rate at weaning, BWW – Bunny weight at weaning, MY – Milk yield, *($P \leq 0.05$), **($P \leq 0.01$).

Productive parameters

Diets containing chitosan affect product performance, including litter size, weight at birth, weight at weaning, bunny weight at birth, bunny weight at weaning, milk yield during the lactating period, and bunny mortality and adult mortality (Table 3).

No statistically significant differences in productive parameters in NZW rabbits were observed between the control and treated groups. Further, no statistically significant differences in total mortality rates in bunny or adult does were observed between treatments. However, a trend was toward a higher average milk yield throughout the lactating period in females fed a diet containing 0.2 g/kg chitosan.

Correlation coefficients among productive and reproductive traits in NZW rabbits

Table 4 presents the correlation coefficients among productive and reproductive traits in NZW rabbits. We observed a significant positive correlation between bunny weight at weaning and milk yield and a negative correlation between the number of parities and kindling interval.

Moreover, diets containing up to 0.4 g/kg chitosan were positively correlated with receptivity (proportion and time) and the number of parities. However, the diet containing up to 0.4 g/kg chitosan was negatively correlated with receptivity time, kindling interval, litter size at weaning, the mortality rate at weaning, kitten weight at weaning, and milk yield.

Discussion

The results of the present study demonstrated that diets containing up to 0.4 g/kg chitosan improved most reproductive traits in NZW rabbit females, including accelerated ovarian development, increased doe receptivity and conception rate, and decreased kindling interval compared with the control females.

These improvements in reproductive performance indicate chitosan improves sexual hormone levels and genital organ growth in NZW does. Diets containing up to 0.4 g/kg chitosan were positively correlated with doe receptivity and conception rate but negatively correlated with doe receptivity time and kindling interval. These results corroborate the findings of Irawan et al. (2020), who reported that chitosan increased ovarian follicle diameter, increased the number of endometrial arterioles, and increased endometrial thickness in Wistar rats. Further, Wan et al. (2016 and 2018) demonstrated that dietary supplementation with chitosan improved reproductive performance in sows. Huang et al. (2021) found that chitosan promotes the proliferation of ovarian germ stem cells and modulates ovarian function by improving the ovarian microenvironment in mice. Moreover, Kohiruimaki et al. (2013) reported intrauterine administration of a chitosan solution in postpartum anestrous cows positively affected reproductive performance. Okawa et al. (2021) posited that intrauterine infusion of chitosan solution during the early postpartum period accelerates

uterine recovery from endometritis and may be a suitable alternative to PGF₂ α administration. Alfari et al., (2017) reported the successful use of PGF₂ α with chitosan for infertility treatment in cows suffering from the repeated estrous or cystic ovaries. Osama et al. (2017) reported average receptivity and conception rates of 54.80% and 69.39%, respectively, in NZW rabbit does under normal managerial conditions.

Accordingly, our results indicate that diets containing up to 0.4 g/kg chitosan improve receptivity and conception rates in NZW rabbits. A 15% increased conception rate was observed in sows treated with chitosan compared to a control group (Ho et al., 2020). Moreover, Hassanein et al. (2021) reported the successful fabrication of GnRH-loaded chitosan nanoparticles and demonstrated the conventional intramuscular GnRH dose used for artificial insemination in rabbits could be halved without affecting fertility.

There is a lack of information regarding the potential of adding chitosan to rabbits' diets. Consequently, it is proposed that chitosan supplementation could improve the reproductive attributes in female rabbits, which was evidenced by ovarian histological exploration. This response might be accredited by the antioxidant capacity of chitosan. Chitosan can sustain an amino acid or two hydroxyl groups, which can respond with oxidative stress, thus producing high scavenging capacity (Abd El-Hack et al., 2020).

However, in the present study, we observed no statistically significant differences in any parameters of productive performance in NZW rabbits between the control and treatment groups. These results corroborate the findings of Ho et al. (2020), that observed no significant difference in litter size or overall individual birth weight with maternal chitosan supplementation compared to the control group in swine. Furthermore, Duan et al. (2020) reported that dietary chitosan did not affect litter size, piglet survival, average initial litter weight, or individual weight at birth compared to the control group of swine. In spite of improving the litter sizes at weaning and at birth, there were insignificant effects of chitosan dietary inclusion. This is previously explained by Sun et al. (2007), who clarified that the high molecular weight of chitosan had lower antioxidant capacity contra some superoxide and hydroxyl radicals. Thus, further investigations are desired to explore the underlying mechanisms for these responses especially at the molecular pathways. In addition, exploring modified dietary chitosan to improve fertility outcomes is still unexplored. Our future works will consider these topics for discovering more about the multifunction of chitosan in animals.

Regarding offspring performance, Wan et al. (2016 and 2018) reported dietary supplementation with chitosan in sows improved offspring growth performance during sucking by increasing the content of IgA, IgG, and IgM in colostrum and milk. This effect of chitosan supplementation during late gestation and lactation may be attributable to the antioxidant and anti-inflammatory

effects of chitosan in sows. Further, Cheng et al. (2015) and Ho et al. (2020) reported increased litter sizes of 5% and 18.5%, respectively, in sows treated with chitosan compared to controls. The offspring growth performance observed in the present study is in keeping with the results of these previous studies.

Also, ANOVA demonstrated no statistically significant differences in the present study's total mortality rates between bunny and adult's females among experimental groups. However, there was a trend toward greater viability during the growth period in rabbits fed diets containing 0.2 g/kg or 0.4 g/kg chitosan compared to the control and other treatment groups. These findings agree with Ho et al. (2020). They demonstrated that dietary supplementation with chitosan did not affect the number of piglets born alive or the total number of dead piglets compared to the control group. Further, Duan et al. (2020) reported that dietary chitosan did not affect the total number of piglets born, the number of piglets alive at birth, initial litter weight, or individual weight at birth compared with controls.

Mortality rates of bunnies were insignificantly lower in the chitosan treatment groups compared to the control group in the present study.

Non-significantly, the antioxidant effects of chitosan were confirmed in broiler chicken (Jang et al., 2004; Je et al., 2006). Further, adding chitosan at 100–200 mg/kg to the diets of adult farm animals can improve and support many reproductive traits, such as pregnancy and its outcomes, milk production, and egg production in dairy cows, swine, and laying hens (Wan et al., 2018; Hamady and Farroh, 2020).

However, Wan et al. (2018) reported that dietary chitosan increased the IgM content of colostrum in sows. The immune system of piglets has been shown to continue development after birth, including the development of humoral and innate immunity and inflammatory responses (Mair et al., 2014; Shokryazdan et al., 2017). A previous study reported piglets weaned on a diet containing chitosan augmented immune function by increasing serum IgG, IgA, IgM, and interleukin concentrations (Sun et al., 2009). Overall, an improvement in reproductive features was found by producing more viable rabbits per doe using 0.2 or 0.4 g/kg diet feeding chitosan. This response was confirmed by studying the histological architecture in rabbits' ovaries. However, beside these positive effects of feeding chitosan, additional research is necessary to shed more light on this subject.

Conclusion

The present study showed that adding chitosan to female rabbits' diet significantly improved most productive and reproductive traits compared to a basal diet. Further, the diet containing 0.2 g/kg chitosan resulted in greater reproductive efficiency in NZW rabbits than those with high chitosan concentrations. We suggest that more examination of the molecular mechanisms underlying the action of chitosan molecules is required to develop inno-

vative dietary tactics to sustain the reproductive potential in rabbits.

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