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Aims and Scope

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Effect of Partial Dietary Substitution of Prickly Pear (*Opuntia ficus indica* L.) Seeds Meal on Growth Performance and Carcass Characteristics of Broiler Chicken

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Keywords: *prickly pear, seeds meal, weight carcass, growth performance, broiler chicken.*

Abstract. *The objective of the present study was to investigate the effect of partial feed substitution with OFI meal on the growth performances and carcass characteristics of broiler chickens. One hundred and fifty 1-day-old broiler chicks (Arbor acres) were allocated into 5 groups with 3 replicates of 10 birds per group. The experimental groups received the diets substituted with 0 (control), 10% (B), 20% (C), 30% (D) and 40% (E) prickly pear seeds (PPS) meal of OFI during a 6-week duration divided into 4 dietary periods (starter diet, 0–12 days; grower diet, 13–33 days; finisher diet, 34–39 days; withdrawal diet, 40–45 days). At the end of the experiment, 5 birds from each sub-group were randomly selected and slaughtered, and the yield of the carcass segments was calculated. The growth performance of the broiler chickens seemed significantly affected ($P < 0.001$) by the PPS substitution level. The daily weight gain of the birds was exponential in the experimental groups. The results obtained indicate clearly that weight gain in the chickens fed treatments containing 10% PPS was significantly greater than in the chickens fed different treatments (20%, 30% and 40% PPS). Minimum feed intakes were observed in the birds with the diet substituted with 40% PPS. Feeding the birds with the substituted 10% PPS resulted in a significant increase ($P < 0.001$) in the weight of carcass and intestinal part at 45 days of age, when compared with the control group and the treated group. The results obtained under the conditions of this experiment showed that the partial dietary substitution of 10% PPS from Algeria can improve the growth performance and carcass yield in broiler chickens. Therefore, it is important to valorize the PPS by-product which would decrease the import invoice of animal feed, especially in broiler chickens.*

Introduction

Cacti are the most characteristic plants of the arid and semi-arid regions. *Opuntia ficus-indica* (OFI) L., commonly called prickly pear or nopal cactus, belongs to the dicotyledonous angiosper *Cactaceae* family, which includes about 1500 species of cactus. Also, cacti are known for their ability to thrive under environments recognized as stressful for most plant species, and are widely used to prevent soil erosion and to combat desertification (Scheinvar, 1995; Le Houerou, 2000). OFI has been exploited as a cheap and alternative source of food suitable not only for humans but also for animals. In addition, Cacti have been cultivated as ornamental crops to delimit lands (Estrada-Luna et al., 2008). Two parts of OFI are used for food, namely cladodes and prickly pears. Cladodes are consumed in Mexico as salads (Medina et al., 2007) whereas fruits are widely eaten fresh, dried or preserved (Medina et al., 2007). OFI fruits are fleshy and elongated berries, varying in shape, size and color and have a consistent number of hard seeds (Piga, 2004).

Opuntia gender is native of the Americas and has spread in many parts of the world including North Africa, the Mediterranean basin and the Middle East (El Mostafa et al., 2014). OFI is rich in phenolic compounds among other phytochemicals, and is widely used in folk medicine (Andreu et al., 2018). It is traditionally used for its antioxidant (Pês et al., 2016), anti-microbial (El Mostafa et al., 2014), anti-fungal (Ennouri et al., 2014), anti-inflammatory (Benayad et al., 2014), hypoglycaemic (Newman and al., 2016), or diuretic effects (Ammar et al., 2012). It helps preventing adipocyte hypertrophy and hepatic steatosis (Rodíguez-Rodríguez et al., 2015). The important biological activities of OFI are attributed to its different bioactive compounds such as gallic acid, catechin quercetin or resveratrol (Belviranli et

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al. 2019). Seeds of OFI often are considered as waste material during fruit processing but can be used to extract oil, due to its fatty acid composition.

In Algeria, poultry farming is an important sector of livestock production and contributes significantly to food security. However, feed costs affect negatively chicken production profitability. Therefore, the exploration of new feed resources and their effective use should be encouraged. In this country, OFI is a significant portion of the agricultural economy (150 000 hectares) (Boumaliet al., 2022), with huge production of fruits. According to the Direction of Agricultural Services, a large part of OFI is found in the northeast of Algeria and generates a huge quantity of residues. OFI by-products thus could be used in poultry feeding. To our knowledge, there are few studies on the effects of OFI seed meal on poultry performance under a different food matrix. Thus, the objective of the present study was to investigate the effect of partial feed substitution with OFI meal on the growth performances and carcass characteristics of broiler chickens.

Materials and Methods

The experimental protocol was approved by the Scientific Faculty Council of the University of El-Tarf (Algeria) and the authors followed the regulations applied in University of Liege (Belgium).

Study area

This study was conducted from August to September 2018 in Beni-Mazline locality, Western Algeria (36°27'N, 7°25'E). The annual winter rainfall in the region ranges between 400 and 500 mm. The mean temperature and humidity during the experimental period reached 28°C (min 22.1°C and max 36.8°C) and 56%, respectively.

Meal production and chemical analysis

Prickly pear fruitswere collected in March through April 2018 in El-Bourdj (Souk-Ahras province, Northern Algeria) (36° 17'N, 7° 57'E). The dry by-product of OFI was obtained in a traditional mill immediately after the pressing operation of OFI fruits. The mixture is passed through a sieve with mesh for the separation of the seeds. After then, the seeds are washed, dried in the open air and sent to extract oil. The by-product was manually treated in order to reduce their size using a hammer, and then crushed using a traditional grinder. The final product was dark brown with a particle size of between 0.1 and 2 mm.

The chemical analysis was carried out according to the procedures of AOAC (2000). Dry matter content was determined from a test sample of 5 g in an oven at 105°C for 24 h (AFNOR, 1982). Crude protein (CP) was determined by the Kjeldahl method (AOAC, 955.04) (N x 6.25), and ether extract (EE) by the Soxhlet method (AOAC, 920.39). Crude fiber (CF) was determined by the Weende method (AOAC, 978.10). Finally, atomic absorption spectrometry was used to assay minerals, calcium (Ca), phosphorus (Ph), potassium (K), sodium (Na), magnesium (Mg).

Animals and dietary treatments

One hundred and fifty 1-day-old broiler chicks (*Arbor acres*) were obtained from a commercial hatchery. The birds were randomly allocated into 5 groups with 3 replicates of 10 birds per group and housed in pens of identical size (2 x 2 m) on a deep litter system with straw as floor. The birds from the control group (A) were fed, *ad libitum,* balanced commercial broiler diets for a 6-week duration divided into 4 dietary periods (starter diet, 0–12 days; grower diet, 13–33 days; finisher diet, 34–39 days; withdrawal diet, 40–45 days). The experimental groups received the same diets substituted with 10% (B), 20% (C), 30% (D) and 40% (E) of prickly pear seeds (PPS) meal of OFI. The chemical composition of the OFI prickly pear seed meal is shown in Table 1. The ingredients and the composition of the diets used in the experiment are presented in Table 2.

Birds were vaccinated against Gumboro (IBA-VAC®) and Newcastle (BIO-VAC® B1) diseases according to laboratory recommendations. In order to prevent coccidiosis, the chicks were treated by anticoccidial at 15 and 28 days for 3 days (HIPRAVIAR®). They were reared for 6 weeks, maintaining all hygienic measures in a well-ventilated and temperature-controlled poultry house. Chicks were managed according to the guidelines suggested by Cobb Broiler Commercial Management Guide (*Arbos Acres* plus).

Growth performances and carcass characteristics

All birds were weighed individually at the same time after their arrival from the hatchery to the experimental farm and every 7 days until slaughter. Feed intakes (FI) per pen, individual body weight gain (WG) and feed conversion ratio (FCR) were calculated per period. Daily weight gain (DWG; g/d) was calculated as weekly body weight gain/7. Overall FCR was also calculated from total FI/overall WG. Mortality rate was recorded daily for each pen and calculated as a number of dead birds / total number of birds x 100.

At the end of the experiment, 5 birds from each sub-group were randomly selected and slaughtered. The slaughtered animals were plucked, eviscerated and weighed to calculate carcass weight including skin. The carcass was then dissected to characterize carcass segments. Carcass yield was calculated according to the ratio: eviscerated weight / live weight.

Statistical Analysis

The SAS® software (version 9.4, Institute Inc, Cary, NC, USA) was used for statistical analysis. The effects of diet, age and sex on feed intake, body weight gain and feed conversion ratio were evaluated by a mixed model. Statistical analysis was performed using the *t* test to compare between different groups. For repeated measurements on the same experimental unit, a similar model was used but including the effect of a compound symmetry covariance structure. Orthogonal polynomials were performed to determine

Table 1. Formulas (kg/100 kg feed) of the phases of starter (1–12 days), grower (13–33 days), finisher (34–39 days), and withdrawal (40–45 days) feed distributed to broiler chickens.

	Start-up phase (1–12 days)	Growth phase (13–33 days)	Finishing phase (34–39 days)	Withdrawal phase (40– 45days)
Ingredient composition				
Corn (%)	62.5	68	67.62	70
Oilcake of soy (%)	30	28	24	24
Milling issue (%)	3.5	1.3	5.38	4
Dicalcium phosphorus (%)	1.17	1	1	1
Limestone (%)	0.83	0.7	1	1
CMV DC (%)	1 [a]	1 [a]	-	-
CMV DL (%)	-	-	1	-
Antistress (%)	1	-	-	-
Analysis composition				
DM (%)	90.33	91.33	91.67	92
Crude protein (%)	17.9	29.9	20.4	19.1
Crude fiber (%)	3.8	3.5	3	2.9
Ash (% DM)	6.33	7	5	7
Raw ash (% Ash)	5.3	7	5.1	5.5
Insoluble ash (% Ash)	23.37	21.92	21.78	22.79
NDF (% DM)	43.39	34.35	40.45	35.63
ADF (% DM)	35.8	7.21	3.79	16.16
Ether extract (% DM)	22	16.67	19	13.33
Calcium (g/kg DM)	7.7	9.4	7.6	10.1
Phosphorus (g/kg DM)	6.1	7	5.6	7.1
Potassium (g/kg DM)	7.8	14.4	9.5	8.7
Sodium (g/kg DM)	2.1	2.8	0.9	0.1
Magnesium (g/kg DM)	2.2	2.4	1.7	1.8
Calculate composition				
Nitrogen (%)	2.86	4.78	3.26	3.06
EM (Kcal/kg)	2696	2774	2562	2057

DM: Dry matter; NDF: Number of fiber bags needed; ADF: Acid detergent fiber

the linear and quadratic effects of an increasing level of PPO in the diets. The data were expressed as mean ± SE, and $P < 0.05$ was considered significant.

Results

The chemical and mineral composition of *Opuntia ficus-indica* L. powder flour prickly pear seeds are presented in Table 2. Metabolizable energy (ME, 2593 Kcal/kg), number of fiber bags fed (NDF, 35.9%), and acid detergent fiber (ADF, 30.5%) were calculated on dry matter basis. The prickly pear seeds (PPS) meal is composed of 95% of dry matter, 2% of ash, 9.8% of crude protein, 47.7% of crude fiber and 19% of ether extract. This powder also contained significant amounts of minerals (Ca, K, Mg, and Na) which have a high content of calcium (4.2 g/kg DM) and potassium (3 g/kg DM), while phosphorus, magnesium, and sodium occupy the second place with 2.4 g/kg, 0.3 g/kg, and 1.5 g/kg of dry matter, respectively.

Initial and final body weights, average daily gain, mean daily consumption and consumption index during the different level treatments are shown in Table 3. The mortality rate of broilers did not exceed 1% during the period of the experiment in the treated and control groups. The growth performance of the broiler chickens seems to be significantly affected ($P < 0.001$) by the PPS substitution level. The daily weight gain of the birds was exponential in the experimental groups. The birds substituted with 10% PPS had a higher body weight (2939 g) than other groups substituted with 20%, 30% and 40% PPS (2622 g, 2527 g and 1745 g, respectively) during the experimental phases.

The average daily weight gain (from day 1 to 45) was higher for the control group than in chickens substituted with graded levels of 20%, 30% and 40%

Table 2. Chemical composition of prickly pear (*Opuntia ficus indica* L.) growing in Algeria

Composition	Proportion
Chemical composition	
DM (%)	95
Crude protein (%)	9.8
Crude fiber(%)	47.7
Ash (% DM)	2
Raw ash (% ash)	4
Insoluble ash (% ash)	23.9
NDF (% DM)	35.9
ADF (% DM)	30.5
EE (% DM)	19
ME (Kcal/kg)	2119
Mimeral composition	
Calcium (g/kg DM)	4.2
Phosphorus (g/kg DM)	2.4
Potassium (g/kg DM)	3
Sodium (g/kg DM)	0.3
Magnesium (g/kg DM)	1.5
Nitrogen (%)	1.6

DM: Dry matter; NDF: Number of fiber bags needed; ADF: Acid detergent fiber; EE: Ether extract; ME: Metabolizable energy.

Table 3. Effect of graded levels of dietary substitution of prickly pear (*Opuntia ficus indica* L.) on the performance of broiler chickens

	Substitution rate PPS (%)						
	0%	10%	20%	30%	40%	SEM	*P* value
Weight gain (g)							
1 day	44 [a]	45 [a]	45 [a]	45 [a]	45 [a]	19.54	0.02
Start-up phase (1–12 days)	400[a]	395 [a]	383 [a]	340 [b]	300 [c]	19.54	0.02
Growth phase (13–33 days)	1900 [a]	1908 [a]	1800 [b]	1593 [c]	1312 [d]	19.98	< 0.01
Finishing phase (34–39 days)	2502 [b]	2600 [a]	2200 [c]	2000 [d]	1500[e]	20.07	< 0.01
Withdrawal phase (40–45 days)	2825 [b]	2939 [a]	2622 [c]	2527[d]	1745[e]	20.18	< 0.01
Daily weight gain (g/days/subject)							
Start-up phase (1–12 days)	33 [a]	32 [a]	30 [a]	27 [b]	25 [b]	-	< 0.01
Growth phase (13–33 days)	79 [a]	82 [a]	76 [a]	60 [b]	51[c]	-	< 0.01
Finishing phase (34–39 days)	77 [b]	87 [a]	70 [c]	69 [c]	40[d]	-	< 0.01
Withdrawal phase (40–45 days)	36 [b]	41 [c]	51 [b]	64 [a]	27 [e]	-	< 0.01
Intake feed (g/day/subject)							
Start-up phase (1–12 days)	397 [b]	414 [a]	381 [c]	347 [d]	329 [e]	4.63	< 0.01
Growth phase (13–33 days)	2723 [a]	2676 [b]	2557 [c]	2059 [d]	1712 [e]	4.70	< 0.01
Finishing phase (34–39 days)	984 [a]	969 [b]	934 [c]	785 [d]	589 [e]	4.72	< 0.01
Withdrawal phase (40–45 days)	1051 [a]	1057 [a]	1021 [b]	799 [c]	648 [d]	4.73	< 0.01
Feed conversion ratio							
Start-up phase (1–12 days)	1.23 [a]	1.23 [a]	1.22 [a]	1.2 [a]	1.16 [b]	0.04	< 0.01
Growth phase (13–33 days)	1.69 [a]	1.65 [a]	1.68 [a]	1.58 [b]	1.58 [b]	0.04	< 0.01
Finishing phase (34–39 days)	1.88 [b]	1.75 [c]	1.96 [a]	1.73 [c]	1.93 [a]	0.04	< 0.01
Withdrawal phase (40–45 days)	2.13 [a]	1.89 [c]	1.91 [b]	1.64 [d]	1.94 [b]	0.04	< 0.01

[a,b,c,d,e] A significant difference in parameters of growth performances between the control group (0% PPS) and the treated groups (10%, 20%, 30% and 40% PPS) is indicated by letters ($P < 0.05$). SEM = Standard error of the mean

PPS.The results obtained indicate clearly that weight gain in the chickens fed treatments containing 10% PPS had a significantly greater weight gain than the chickens fed different treatments (20%, 30% and 40% PPS). Minimum feed intakes were observed in the birds with the diet substituted with 40% PPS, while the highest was noted in the chickens treated with 10% PPS. There is a significant difference between the control and the chickens fed different treatments ($P < 0.05$). Feed conversion rate (FCR) was low in birds supplemented with 30% PPS during the growth, finishing and withdrawal phases (1.58, 1.73 and 1.64, respectively) compared with other treatment groups.

The means of the weight of the carcass and the intestinal part for dietary treatments are shown in Table 4. Feeding birds with the substituted 10% PPS resulted in a significant increase ($P < 0.001$) in the weight of the carcass and the intestinal part at 45 days

Table 4. Effect of graded levels of dietary substitution of prickly pear (*Opuntia ficus indica* L.) on carcass part characteristics of broiler chickens

Items	Substitution rate PPS (%)						
	0%	10%	20%	30%	40%	SEM	*P* value
Carcass weight							
G	2182[b]	2271[a]	1959[c]	1912[d]	1263[e]	9.86	< 0.0001
%	73.27	73.51	73.17	74.48	71.31		
Head							
G	73[a]	75[a]	66[b]	57[c]	44[d]	0.88	< 0.0001
%	2.4	2.4	2.5	2.2	2.5		
Paws							
G	92[b]	103[a]	95[b]	78[c]	71[d]	1.87	< 0.0001
%	3.1	3.3	3.5	3	4		
Heart							
G	19[b]	21[a]	17[c]	13[d]	13[d]	0.46	< 0.0001
%	0.6	0.7	0.6	0.5	0.7		
Liver weight							
G	58[c]	63[b]	56[c]	66[a]	47[d]	1.1	< 0.0001
%	1.9	2	2.1	2.5	2.6		
Gizzard weight							
G	55[b]	62[a]	57[b]	63[a]	43[c]	1.07	< 0.0001
%	1.8	1.9	2.1	2.4	2.4		
Proventriculus							
G	92[b]	103[a]	95[b]	78[c]	71[d]	1.87	< 0.0001
%	3.1	3.3	3.5	3	4		
Intestine							
G	124[b]	132[a]	127[b]	117[c]	96[d]	1.35	< 0.0001
%	4.2	4.3	4.7	4.6	5.4		
Thighs							
G	712[a]	639[c]	659[b]	568[d]	366[e]	11.46	< 0.0001
%	32.63	28.13	33.63	29.70	28.87		
Wishbone							
G	520[c]	545[a]	530[b]	412[d]	284[e]	9.65	< 0.0001
%	23.83	23.99	27.05	21.54	22.48		
Wings							
G	293[b]	311[a]	282[c]	221[d]	183[e]	8.08	< 0.0001
%	13.42	13.69	14.39	11.55	14.48		

[a,b,c,d,e] A significant difference in carcass part characteristics between the control group (0% PPS) and the treated groups (10%, 20%, 30% and 40% PPS) is indicated by letters ($P < 0.05$). SEM = Standard error of the mean

of age, when compared with the control group and the treated group (20%, 30% and 40% PPS).

Discussion

Several studies have reported that cactus pear is used as feed to supplement livestock diets, due to its main constituents, namely water (80–95%), followed by carbohydrates (3–7%), fiber (1–2%), and protein (0.5–1%) (Ginestra, et al., 2009; Bouzoubaâ et al., 2016; Todaro et al., 2020). In the present study, the chemical analysis of a PPS cake revealed that moisture and dry matter content were 8.1% and 91.9 g/kg, respectively. The DM result is in agreement with the results previously described (Mokoboki & Sebola, 2017); however, Coşkuner and Tekin (2003) reported 71.5 g/kg of DM. According to the results of this study, the PPS contains 9.8 g/kg of crude proteins. Similar results (9.6%) have been described by Reda et al. (2020). Another investigation has demonstrated that a variety of *Opuntia*, which exhibited crude protein of 9.2%, is enough to enhance microbial growth (Mcitaka, 2008). Likewise, the study conducted by López-Cervantes et al. (2011) showed that the crude protein content of cactus pear cladodes flour was 7.24%. Meanwhile, Albergamo et al. (2022) have observed that *O. ficus-indica* seed had a higher protein content (17.34%). Our results also showed that the crude ash value was higher than those reported in previous investigation (Sawaya et al., 1983; Coşkuner & Tekin, 2003; Stintzing & Carle, 2005). These differences may be due to genetic factors, prickly pear varieties, cultivar or growth conditions, as well as geographical variations of prickly pear plants (De Wit et al., 2018). Moreover, variations in values between studies can be due to the stage of cactus maturity.

It is known that prickly pear is considered as a good source of minerals, namely potassium, magnesium, calcium and sodium (Chiteva & Wairagu, 2013; Ghazi et al., 2015; Albuquerque et al., 2016; El-Beltagi et al., 2019), although the site of culture and the physiological state of the vegetal tissue can influence the mineral content of OFI (Pastorelli et al., 2022). Our results showed different values of macro and microelements (Ca, Mg, K, and Na) and microelements (Fe, Mn, Cu, and Zn) in a PPS cake. The calcium content (4.2 g/kg) in PPS was similar to that obtained by Ghazi et al. (2015). However, Al-Juhaimi and Özcan (2012) have reported a low value of Ca and K ranging from 0.26 g/kg to 0.67 g/kg and 0.34 g/kg to 0.67 g/kg, respectively. In another investigation, Alsaad et al. (2019) have reported that the phosphorus content of a PPS cake was similar to those measured in our study. On the other hand, the phosphorus content was high in a PPS cake (2.4 g/kg) compared with those reported by Al-Juhaimi and Özcan (2012) and Alves et al. (2017a), which varies between 1.17 g/kg and 1.87 g/kg; and 1.92 to 4.56 g/kg, respectively. However, Ghazi et al. (2015) have reported a high level of phosphorus (1417.59 mg / 100 g). Regarding the magnesium content, the value was similar (1.5 g/kg) to the results reported by Alsaad et al. (2019), and was high compared with the results reported by Al-Juhaimi and Özcan (2012) (0.123 and 0.21 g/kg). These differences may be attributed necessarily to the harvesting season, soil properties, maturity degree or fruit processing, as well as to the equipment used (Benattia et al., 2019). Also, the variations in mineral content could be due to the age of the cladode (Alves et al., 2017b).

The incorporation of PPS in feed diet causes a very low mortality rate in the experimental groups. Nevertheless, the mortality rate remains acceptable as reported in livestock management. Ragab (2007) have recorded a mortality rate of 3.3% in quails fed diets containing 15% PPP shells. However, Moula et al. (2019) have reported a mortality rate of 10% in broilers fed with 10% OFI cladodes in the diet. On other hand, several authors have reported that the incorporation of the by-product of OFI processing (husks, oilcake and zest) did not affect mortality in the experimental groups of chickens and rabbits (Bakr et al., 2017; Badr et al., 2019; Cherif et al., 2022). This difference in the mortality rates may be due to broiler strain genetics, individual fragility and stress factors as transportation.

In livestock production, nutrition represents an important factor to increase animal performance and, consequently, improve carcass traits and meat yield to be marketed. In arid and semi-arid regions, especially in Algeria, irregular availability of animal feed is the major problem faced by an animal production system. Many studies have been undertaken to develop alternatives in animal diet to reduce the import invoices, focusing on the potential use of natural feed additives. It is known that cactus is a plant that grows in adverse conditions, with little rainfall and poor soils (FAO, 2001). Despite the low nutritional quality of cactus forage, it remains interesting to investigate the OFI effect on the animal growth performance with other sources of protein or energy (Flores-Hernandez et al., 2019). The present experiment was to study the effects of a substitute of the PPS cake in the diet on the growth performances in chicken broilers at 42 days. These results are in agreement with results obtained recently, where the incorporation of OFI processing by-products into broiler feed at rates of 10% and 20% improves zootechnical performance (Cherif et al., 2022). According to the results of this study, the body weight gain average of chickens was significantly affected by a dietary 10% PPS substitution, *i.e.* by an average day gain of 59 g. This is consistent with the findings of Belghiti et al. (2021), who discovered that incorporation of the prickly pear cactus OFI fruit into the chicken broilers diet improved feed intake and weight gain. Moreover, Badret al. (2019) have concluded that feeding commercial chicken diets containing 15% prickly pear peel (PPP) substituted with corn grain resulted in a better daily gain and feed conversion ratio, as compared with other groups. Other studies have reported that the administration of PPP

(15% to 60%) to quail showed a positive effect on the growth performance and could successfully replace yel-low corn in diet (Ali, 2001; El-Nagmy et al., 2001). On the other hand, Moula et al. (2019) have reported that chickens receiving OFI cladodes powder had numerically higher body weight and average daily gain than the controls, although not statistically significant. In another study, Ruiz-Feria et al. (1998) have reported that cactus prickly pear did not affect the feed intake when included in the diets versus the control group. Our results are in agreement with those reported by Gebremariam et al. (2006), where the body weight gain of growing lambs increased significantly with an increase of the cactus intake level. Nevertheless, Morshedy et al. (2020) have noted that the supplementation of 10 g of pear cactus seeds oil reduced the lamb weaning body weight compared with the control. Furthermore, Zedan et al. (2015) have reported that inclusion of prickly pear cladodes significantly increased the dressing percent-age and the carcass weight of growing rabbits. Meanwhile, other researchers have noted no significant difference among the treatment groups in lambs (Venkatesh et al., 2014; Ajith et al., 2017; Shashikumar 2017). The beneficial properties of *Opuntia* spp. are related also to their chemical content, such as polyphenols and flavonols that play an antioxidant capacity. However, the content of phenolic compounds in OFI is affected by several variables, such as maturity stage, harvest season, environmental conditions and species. OFI of the PPS cake at different levels (10%, 20%, 30% and 40%) revealed a decrease significant difference, with a significantly low FCR in broiler chicken compared with the control group. This result is similar with that reported by Badr et al. (2019) and Pascoal et al. (2020). However, some research has noticed that male broilers fed diets containing 30% PPP had the wrong FC value (El-Nagmy et al., 2001; Ragab, 2012). Moreover, other studies have reported that FI was not affected when replacing yellow corn by pear pick in quail (El-Nagmy et al., 2001) or fish diet (El-Kholy, 1999). A growth performance increase in broiler chickens could be due to the stimulation of chaperone activity by a cactus product, thus reducing the deleterious effects caused by stress. In the literature, heat shock proteins, also known as molecular chaperones, consist of conserved molecules found in all organisms, because under stress conditions, such as heat, proteins tend to denature (Sung et al., 2011). It is noted that these controversial results might be attributed to high energy density and digestibility nature of cactus fruit meal (Tsega et al., 2016). As noted by Hoffman et al. (1993), the cactus cladodes in diet are very palatable to rabbits, due to the high sugar and low acid content (pH = 5.3–7.1). Likewise, it has been indicated that broiler chickens consume enough feed to satisfy their energy requirement (Giachetto et al., 2003). For this reason, high feed intakes were exhibited in the diets which contained higher levels of PPS compared with the control group.

In our results, we also found that the substitution rate of PPS resulted in significant differences in the weight of different carcass parts in broiler slaughter compared with the control group. Interestingly, these findings included a significant ($P< 0.0001$) increase in liver, gizzard, proventriculus and intestines weight. The same observation was noted for liver and gizzard weights in previous studies (Moula et al., 2019; Cherif et al., 2022). Because cactus contains secondary compounds (Bouaouich et al., 2023), it is highly likely that the degradation of the prickly pear seeds meal of OFI during digestion was high, resulting in high weight carcass. PPS oil is rich in unsaturated fatty acids such as linoleic acid and oleic acid. Among the saturated fatty acids, the most important are palmitic acid and stearic acid (Labuschagne & Hugo, 2010). It is known that the liver is the main site of lipid production, whereas the abdomen is the main site of fat storage. Badr et al. (2019) have reported that the carcass meat composition was significantly influenced ($P< 0.05$) with higher protein and fat percentage in chicks fed different levels of PPP. Besides, the weight intestines raised could be explained by an increased epithelial cell turnover due to feeding of microbial. Note that absorption and digestion capacity are essential to animal development that involves high villi and mature enterocytes (Cera et al., 1988). It has been noted that OFI intake is a good source of soluble fiber as pectin; insoluble fibers are cellulose and lignin (Bisson et al., 2010). As discussed in one review paper (Sacranie et al., 2012), the insoluble fiber diet has been shown to increase gizzard development. Likewise, Sacranie et al. (2012) have reported that the addition of hulls, rich in fiber, increased gizzard weight and content, but it had no effect on the ability of the birds to handle intermittent feeding.

Conclusion

The results obtained under the conditions of this experiment showed that the partial dietary substitution of 10% PPS from Algeria can improve the growth performance and the carcass yield in broiler chickens. The by-product prickly pear (*Opuntia ficus indica* L.) seeds are also a good source of energy and minerals; and they are rich in fibers and proteins. Therefore, it is important to valorize the PPS by-product which would decrease the import invoice of animal feed, especially in broiler chickens. Also, other approaches are necessary, such as the histology of intestines, to understand deeper the impact of PPS by-product on the intestine villi and the enterocytes.

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Competing interests

The authors declare that they have no potential conflict of interest.

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True Vaginal Prolapse Complicated with Uterine Horn Intussusception and Urethral Obstruction in a Postpartum Bitch

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Keywords: *vaginal prolapse, intussusception, anuria, bitch.*

Abstract. *The current report presents a clinical case of a true vaginal prolapse complicated with unicornual uterine intussusception and anuria caused by urethral obstruction due to compression in a postpartum bitch. Transabdominal ultrasonographic examination showed no presence of retained fetuses into the birth canal, but an extremely enlarged urinary bladder. After repositioning of the protruded vaginal wall, laparotomy was performed, invagination of the right uterine horn into the uterus was observed, placed back to normal position, and spontaneous urination occurred. Ovariohysterectomy and cervicopexy to the abdominal wall for prevention of recurrence were done. The bitch had full recovery at one month follow-up. True vaginal prolapse in a bitch could be complicated with urethral obstruction due to compression, which may secondarily cause life-threatening azotemia. Reposition of the prolapsed vaginal tissue in the bitch is recommended to be followed by laparotomy in order to correct intussusception of the uterine horns and the body if presented.*

Introduction

Vaginal wall protrusion from the vulva in the bitch is occasionally due to the presence of a vaginal fold prolapse or hyperplasia as a result of edematous swelling of tissues during proestrus and estrus, influenced by increased levels of estrogens (Johnston, 2001). A very rare condition in the female dog is a true vaginal prolapse, which occurs around the time of parturition, influenced by decreasing progesterone, increasing estrogens and relaxin (Antonov & Karadaev, 2019). It is usually observed in bitches with constipation, dystocia or forced separation during breeding (Purswell, 2000; Alan, 2007; Ober, 2016).

During an early puerperal period in small animals, several uterine complications can occur (Johnston, 2001). A rarely described canine uterine disorder is intussusception of the uterine body or horns, and until now fewer than 10 cases of the condition have been reported in the scientific literature (Gorham & Spink, 1975; Izquierdo & Cueto, 2013; Pinto, 2015; Silva, 2019; da Silva, 2020; de Oliveira Néia, 2021; Antonov, 2022).

Several complications of a vaginal prolapse might occur: extensive edema, hemorrhage, necrosis and automutilation (Feldman & Nelson, 2004; Sontas, 2010), rectal prolapse (Ober, 2016), herniation of the colon (Yesilkaya, 2020) or retroflexion of the bladder (Alan, 2007; Canatan, 2015; Acar, 2017; Özgenç, 2017; Yesilkaya, 2020) into the prolapsed vaginal tissue, which may lead to partial or total urethral occlusion, leading to dysuria or anuria (Schaefers-Okkens, 2001; Sontas, 2010).

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In the present report, the treatment of a very rare true vaginal prolapse complicated with unicornual uterine herniation and urethral obstruction due to compression in a postpartum bitch is described.

Case description

A two-year-old, intact female Pincher dog, weighing 4.9 kg, was presented to the small animal clinic of the University Veterinary Hospital of the Faculty of Veterinary Medicine, Trakia University, in Stara Zagora. The owner reported that the bitch gave birth to one dead puppy 12 hours ago without any signs of dystocia, and the vaginal prolapse occurred during the subsequent night, but without exact information when and how it happened.

Physical examination of the animal showed presence of lethargia, dehydratation, tachypnea, tachycardia and anuria. The visible mucosae were red colored. The prolapsed vaginal wall was reddish, edematous, with dried and slight necrotic mucosa (Fig. 1). The external urethral orifice could not be visualized in the prolapsed tissue. The mammary gland was edematous and with presence of lactation.

Transabdominal ultrasonography showed no presence of retained fetuses into the birth canal, but an extremely enlarged urinary bladder. Complete blood cell counts and biochemical laboratory analysis showed no changes in the parameters.

Manual reposition of the prolapsed vaginal wall was done after subcutaneous premedication using 0.04 mg/kg of atropine sulfate (Atropinum sulfuricum; Sopharma; Bulgaria), followed by intravenous administration of 5 mg/kg of propofol (Propofol 1%, Fresenius, Germany). Perivulvar and perianal skin was cleaned and disinfected using Tinctura jodi 5% (Vetprom, Radomir, Bulgaria). In order to reduce tissue edema, the prolapsed vaginal tissue

was bandaged with sterile gauze, irrigated with 0.1% Rivanoli solution (Fig. 2), and then replaced by digital manipulation. During the manual reposition into the cranial vaginal lumen, a presence of soft tissue was palpated in the forward direction, so a decision for laparotomy was made.

Inhalation anaesthesia was performed after endotracheal intubation of the bitch with isoflurane (TerrellTM; Minrad Inc.; USA). Aseptic preparation of the ventral abdominal wall was made, a midline laparotomy was performed, and abdominal exploration revealed an extremely enlarged urinary bladder and full herniation of the pregnant right uterine horn into the uterine body (Fig. 3). There were no signs of tissue congestion or adherence of the invaginated uterine wall. Gentle retraction of the right uterine horn was done in the cranial direction in order to place it back to the normal position (Fig. 4), followed by spontaneous urination. Finally, ovariohysterectomy followed by cervicopexy to the ventral abdominal wall were performed to prevent recurrence with USP 2/0 polyglycolic acid (Marlin; Catgut GmbH; Markneukirchen). The abdominal cavity was closed with a cross-stitch pattern using the same suture material, and the skin was sutured with simple interrupted non-absorbable sutures USP 2/0 (Vitalon; Dr Hammer & Co. GmbH; Hamburg). Ringer's solution was administered intravenously at a dose of 20 mL/kg/h only on the day of operation. Post-operative therapy included oral application of antibiotic – 25 mg/kg of amoxicillin-clavulanic acid (Synulox RTU; Zoetis; USA), for 7 days. Lactation of the mammary gland was ceased by daily oral treatment with 5 mg/kg of cabergoline (Dostinex; Pfizer; Italiy), for 10 days. Skin sutures were removed after 12 days. Follow-up examination at one month showed that the bitch had full recovery and no complications.

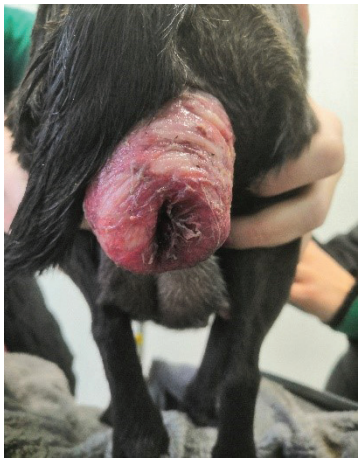

Fig. 1. Caudal view of the prolapsed vaginal tissue of the patient

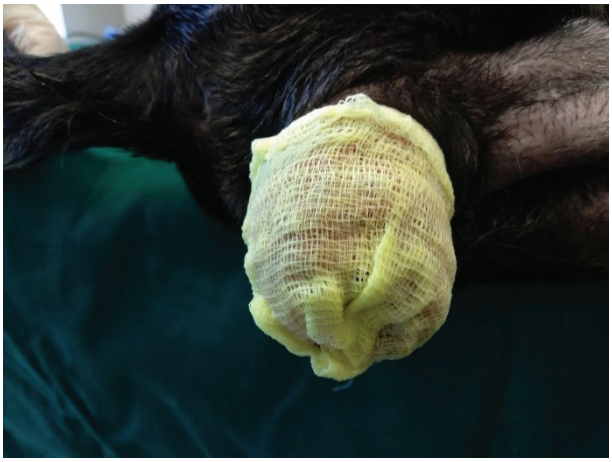

Fig. 2. Prolapsed vaginal tissue bandaged with sterile gauze, irrigated with 0.1% Rivanoli solution

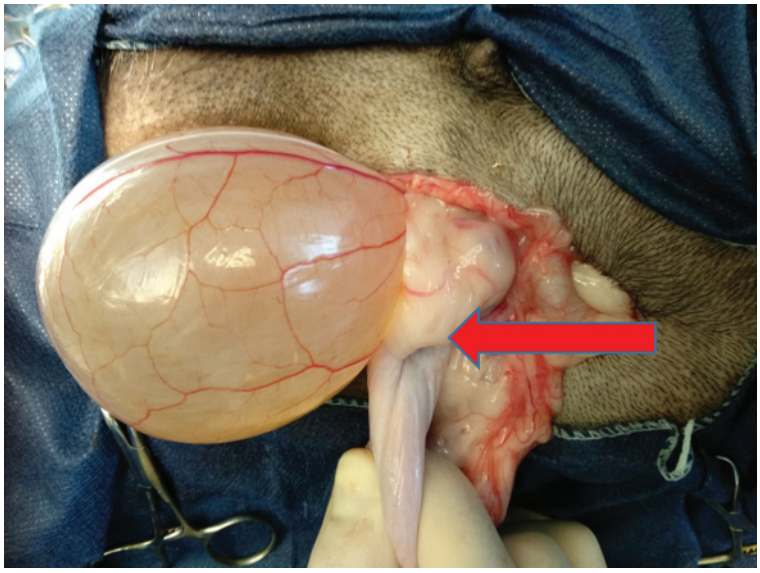

Fig. 3. Intraoperative appearance of the pregnant uterine horn intussusceptum of the patient (red arrow)

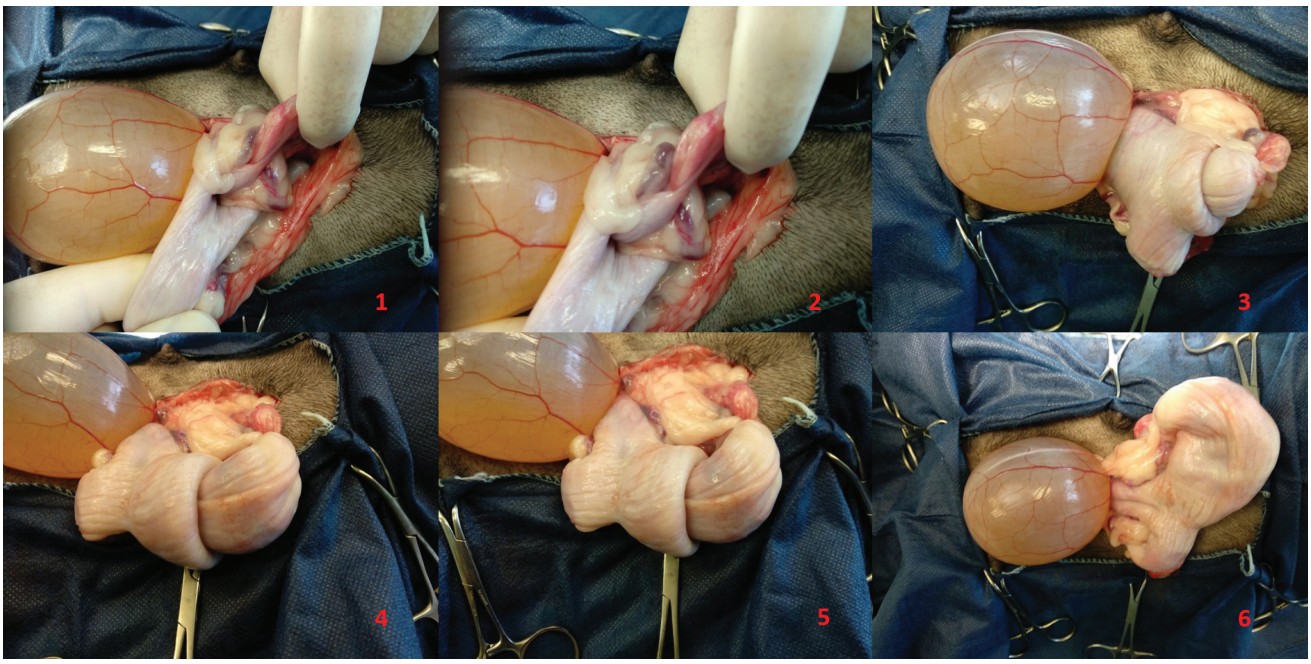

Fig. 4. Reposition of the right uterine horn during operation (consequent steps)

Discussion

A true vaginal prolapse in the bitch is a very rare condition, usually occurring during or shortly after parturition (Schaefers-Okkens, 2001; Ober, 2016; Antonov & Karadaev, 2019), which is often complicated with herniation of other abdominal organs (Wikes, 1986). In the present case, the pregnant uterine horn was invaginated into the uterine body and the cranial part of the vagina, which then caused anuria due to obstruction of the urethra as a result of external compression. Unfortunately, the presence of uterine horn intussusception was not detected during the ultrasound examination, even if it could be successfully used as a diagnostic method (Antonov, 2022), and it was recognized during the abdominal operation, as it usually happens (Gorham & Spink, 1975; Izquierdo & Cueto, 2013; Pinto, 2015; Silva, 2019; da Silva, 2020; de Oliveira Néia, 2021). In our opinion, the reason for this could be the extremely enlarged bladder, which almost filled the abdominal cavity.

Usually, the urethral obstruction leads to acute renal failure and post-renal azotemia, causing fast deterioration of the animal's general health condition (Niles & Williams, 1999). Establishing a patent urinary tract is an emergency, so early operative treatment of a vaginal prolapse is recommended (Canatan, 2015). In the described case, the vaginal prolapse and uterine horn intussusception were corrected within the first 12 hours after occurrence, so there were no signs of even initial kidney failure.

Conclusion

In order to reduce additional complications, reposition of the prolapsed vaginal tissue in the bitch should be done as soon as diagnosed, followed by laparotomy in order to correct possible abnormalities of the abdominal organs. Additionally, ovariohysterectomy and cervicopexy should be performed to minimize the risk of potential recurrence.

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Reproductive Performance and Progesterone Profile in Dairy Sheep after GnRH Administration on Day 4 Post Artificial Insemination

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Keywords: *dairy sheep, reproductive performance, progesterone, GnRH*

Abstract. *The current study aimed to determine the reproductive performance and progesterone profile in East Friesian sheep after GnRH administration on day 4 post artificial insemination (AI). The experiment was carried out with 67 sheep subjected to estrus synchronization and artificial insemination. The animals were divided in two groups: I (control group, n = 30) and II (GnRH group, n = 37), treated by GnRH on day 4 after AI. Rams were introduced on day 12 and removed on day 20 after AI. Ultrasound pregnancy check was on days 20 and 60 after AI, and sheep were separated on the basis of their reproductive status (RS). Blood progesterone (P4) concentrations were measured by ELFA on days 4, 12 and 20 after AI, and a likehood for early embryonic mortality was estimated. The percentages of non-pregnant and pregnant sheep in both groups on day 20 were close; however, there were significantly (P < 0.05) more pregnant sheep in the GnRH group on day 60 compared with day 20. The investigated factors affected the P4 concentrations independently of each other. Analysis of the main effects of GnRH and RS showed a significant (P < 0.005) influence on the progesterone profile. The day after AI had a strong positive effect on the P4 concentration, with significant (P < 0.001) differences between mean P4 values measured during the different days. The estimated likehoods of early embryonic mortality (EEM) for both groups was 25.4%. In conclusion, GnRH injection on day 4 after artificial insemination and the introduction of ram on day 12 did not have a direct effect on the pregnancy rate, but led to improvement of the reproductive performance at the flock level. Gonadotropin releasing hormone treatment, reproductive status and day after AI affected the progesterone concentrations in East Friesian sheep irrespective of each other, and had a significant (P < 0.005) effect on the hormonal profile. The treatment by GnRH on day 4 after AI tended to reduce early embryo mortality, but future investigations are needed to clarify this effect.*

Introduction

The main goal in dairy sheep farming is breeding sheep with a high milk yield and fertility resulting in production of more milk (Pollott & Gootwine, 2004; Tzanidakis et al., 2017). An achievement of good results requires optimal reproductive performance, an introduction of artificial insemination and different hormonal interventions (Valergakis et al., 2010; Gibbons et al., 2019; Hameed et al., 2021; Didarkhah & Vatandoost, 2022). In this aspect, most protocols for estrus synchronization include a combination of intravaginal progesterone releasing devices with equine chorionic gonadotropin (eCG) (Ataman et al., 2013; Hashem et al., 2015; Fernandez et al., 2018, 2019; Hajibemani et al., 2022). Although they provide an acceptable conception rate, the reproductive performance at a farm level is limited by embryonic and fetal losses (Diskin & Morris, 2008; Chundekkad et al., 2020). An early gestational period includes several events such as attachment of conceptus, implantation, placentation and initiation of fetal and placental growth, which are critical for maintenance of pregnancy (Bairagi et al., 2018; Reynolds et al., 2019). The embryonic mortality is an important factor leading to low reproductive efficiency and significant economic losses (Dixon et al., 2007; Moraes et al., 2009; Rickard et al., 2017). In sheep, the cases registered up to 18–20 days after fertilization are defined as early embryonic mortality, and from day 20 to day 45 as late embryonic loss (Thatcher et al., 2001; Dixon et al., 2007). Most of the losses in sheep are related to embryonic mortality, as 30% to 40% of fertilized ova are lost during the pre-implantation period and 70% to 80% of losses are during the first 3 weeks post-mating (Wilkins, 1997; Michels et al., 1998). The majority of embryonic losses occur during pre- and peri-implantation between days 12 and 17 of gestation (Rickard et al., 2017). Detection of early embryonic mortality is very difficult because of a lack of reliable diagnostic methods. Moreover, sheep with early embryo loss until gestation days 11–12 return to estrus between days 15 and 19 after mating (Edey, 1967).

A measurement of progesterone concentration on day 12 and transrectal ultrasound examination between days 10 and 17 after AI have been used for

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pregnancy diagnosis and retrospective determination of early embryonic mortality in sheep (Vinoles et al., 2012; Rickard et al., 2017), but still there are no detected accurate diagnosis methods.

A few studies report that administration of gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG) after AI of synchronized sheep lead to a decrease of embryonic loss and increased pregnancy rate (Cam & Kuran, 2004; Hashem et al., 2015; Farrag et al., 2017; Zonturlu et al., 2018; Nikbakht et al., 2022). However, other authors have determined no effect on the pregnancy rate after AI and recommended future investigations to explore the effect of gonadotropin treatments according to day post mating, season and breed of sheep (Fukuy et al., 2001; Fernandez et al., 2018, 2019). A single gonadotropin injection between days 4 and 12 after mating has been shown to improve the activity of the corpus luteum (CL) or the formation of accessory corpora lutea, and to increase angiogenic factors in pregnant sheep (Beck et al., 1996; Coleson et al., 2015). As a result, an increase in the P4 concentration has beneficial effects on early embryonic development and placentation, and decreased pregnancy loss (Cam & Kuran, 2003; Khan et al., 2007; Hashem et al., 2015). In contrast, Fernandez et al. (2018) have observed no increase in the concentration of serum P4 in GnRH generated accessory corpora lutea in sheep and a lack of effect on pregnancy results. Most of the studies in the last years (Hashem et al., 2015; Farrag et al., 2017; Nikbakht et al., 2022) have reported a positive influence of the treatment with luteotrophic agents after mating on the pregnancy maintenance, but the information evaluating the effect of GnRH injection during the early luteal phase is controversial and still studied (Hajibemani et al., 2022).

The current study aimed to determine the reproductive performance and the progesterone profile in East Friesian sheep after GnRH administration on day 4 post artificial insemination. In addition, a hypothesis for determination of the early embryonic mortality on the basis of progesterone concentrations measured on day 12 after AI was tested.

Materials and methods

Animals

The experiment was carried out with 67 sheep from East Friesian breed at the end of lactation, reared in dairy sheep farms, located at *latitude* 42° 28' 59.99" N and *longitude* 26° 01' 0.01" E. Animals were aged 2.5 ± 0.5 years, body weight was 67.5 ± 2.5 kg, and the housing technology was in group boxes. The animals' feeding (total mixed ration included hay, concentrate containing mineral supplements and corn silage) was in agreemnt with the requirements for dairy breed, age and stage of lactation, and water intake was *ad libitum*. The experiment was performed during the breeding season (from September throughout October). All procedures were in accordance with the minimum requirements for protection and welfare of experimental animals and requirements for use, rearing and/or their delivery included in Bulgarian legislation (Ordinance No 20/1.11.2012).

Estrus synchronization, artificial insemination and ram mating

All sheep were subjected to estrus synchronization by intravaginal sponges containing 30 mg of flurogeston acetate (Syncro-part® 30, Ceva Sante Animale, France) for 12 days and an intramuscular injection of 500 UI eCG (Folligon®, MSD Animal Health, USA) on day of a sponge removal. Artificial insemination with fresh-diluted semen was performed from 52 to 58 h after the sponge removal. Fresh-diluted semen of 0.2 mL with 300 x 10^6 motile sperms was introduced deep cervically by an experienced operator. The animals were divided in two groups: I (control group, n = 30) and II (GnRH group, n = 37). Group I was not treated and Group II received 50 µg of gonadorelin as diacetate (Ovarelin®, Ceva Sante Animale, France) on day 4 after AI. Rams with proven fertility in a ratio male/female 1/6 were introduced in each group on day 12 and removed on day 20 after AI.

Ultrasound examination and reproductive performance registration

Ultrasound pregnancy check was conducted on days 20 and 60 by ultrasound equipment SonoScape S2 Vet and multifrequency (7–12 MHz) linear transducer (SonoScape Medical Corporation. Shenzhen, China). On day 20, all sheep were subjected firstly to a transabdominal ultrasonography. In case of negative pregnancy diagnose, a transrectal ultrasonography was performed. On the basis of ultrasound results, the sheep were recoded non-pregnant and pregnant. A positive pregnancy diagnose on day 20 was given in visualization of an echogenic embryo, located in the anechogenic uterine lumen. On day 60, a fetus with visible cardiac activity and placentomas were observed. Pregnant animals with an ultrasound picture different from those observed on days 20 and 60 were recorded as the sheep *conceived after mating by ram (SCAMR)*. The reproductive status (non-pregnant and pregnant sheep) based on the data on day 20 and (non-pregnant, SCAMR and pregnant sheep) based on the data on day 60 and a total value of pregnant and non-pregnant animals in both groups were calculated and presented in percentages.

Progesterone assay

Blood samples for P4 assay in all the sheep were collected on days 4, 12 and 20 after artificial insemination. After the sample collection, the blood plasma was separated by centrifugation (3000 g for 15 min) and stored in a sterile tube at –20°C until analysis. Progesterone concentrations were measured by automated quantitative-enzyme-linked fluorescent immunoassay (ELFA, VIDAS, ImmunoDiagnostic Assay System, bioMerieux, France) and VIDAS®

Progesterone kit imprecision within and between runs of 5.7–3.8% and 6.2–3.8%, respectively. The progesterone concentrations according to group, reproductive status, and days after AI were calculated and compared.

Statistical analysis of the progesterone concentration in all animals in both groups on day 12 showed significantly ($P < 0.003$) higher P4 concentrations in pregnant sheep compared with non-pregnant sheep. For exclusion of a possible influence of the number of embryos and gonadotropin treatment, the lowest progesterone concentrations for animals in the control and the GnRH group on day 12 determined as pregnant by ultrasound on day 20 were accepted as indicative for pregnancy on day 12 after AI. The assumption was that the ewes recorded as non-pregnant by ultrasound on day 20 but with progesterone concentrations above the indicative for pregnancy on day 12 underwent embryo loss. The subgroups of pregnant, non-pregnant sheep and animals with EEM from each group were formed after the pregnancy check. The embryonic mortality was calculated as a percentage of animals determined with embryo loss compared with all sheep in each group.

Statistical analysis

Values of non-pregnant and pregnant animals between different groups and total values were compared by the non-parametric method for comparison of proportions with small samples. The effects of GnRH treatment, reproductive status of animals and day after AI on the P4 concentrations were determined by ANOVA based on the Wilks-lambda test. The progesterone values (mean ± standard deviation) of non-pregnant animals, sheep *conceived after mating by ram and* pregnant animals according to group and day after AI were compared by ANOVA and the post-hoc Tukey-test. Differences were considered significant at $P < 0.05$ level.

Results

The percentages of non-pregnant sheep in both groups on day 20 were close (Table 1). On the same day, the percentage of pregnant sheep in the GnRH group tended to be higher than in the control group, but there was no statistical difference ($P > 0.05$). Six sheep (37.5%) from the non-pregnant animals in group I and 10 (62.5%) from these in group II on day 20 were recorded as pregnant during the second ultrasound examination for pregnancy. The percentages of non-pregnant and pregnant animals in both groups on day 60 also did not differ significantly. However, there were significantly ($P < 0.05$) more pregnant sheep in the GnRH group on day 60 compared with day 20. The total value of pregnant animals in both groups was higher than the recorded one of non-pregnant sheep ($P < 0.05$).

Factorial analysis showed that the investigated factors affected the P4 concentrations independently of each other (*GnRH x RS, observed power* = 0.26, $P = 0.65$). The progesterone profiles of non-pregnant sheep in the control and the GnRH treated group did not differ considerably from day 4 to day 20 after artificial insemination (Fig. 1). In contrast, additional analysis of the main effects of GnRH and the reproductive status showed a significant influence on the progesterone profile (*Wilks-lmbda GnRH* = 0.80, $P = 0.0045$; and *RS* = 0.94, $P = 0.0000$) (Fig. 2AB). The mean progesterone concentrations of the non-pregnant sheep in both groups on day 12 were higher ($P < 0.05$), compared with P4 concentrations measured on day 4 (Table 2). After that, they started to decrease rapidly as on day 20 they were close to the concentrations of P4 registered on day 4, and significantly ($P < 0.05$) lower than those on day 12.

A similar enhancement in P4 between days 4 and 12 and a drop on day 20 after AI were observed in the sheep conceived after mating by ram. However, the mean concentration of P4 in SCAMR on day 20 was higher ($P < 0.05$) in the GnRH compared with the control group. It should be noted that regardless of a lower progesterone concentration in the GnRH group, compared with the control group on day 4 (3.03 ± 0.88 ng/mL vs 3.82 ± 1.95 ng/mL), the concentration of P4 on day 12 was relatively

Table 1. Non-pregnant and pregnant sheep in control and GnRH groups according to time of the ultrasound examination

Day after AI	Groups			
	Non-pregnant		Pregnant	
	Control n = 30	GnRH n = 37	Control n = 30	GnRH n = 37
	% (n)	% (n)	% (n)	% (n)
20	53.3 (16)	43.2 (16)	46.7 (14)	56.8 (21)[1]
60	30.8 (10)	16.2 (6)	69.2 (20)	83.8 (31)[2]
Total	23.9 (16/67)[a]		76.1 (51/67)[b]	

AI, artificial insemination; GnRH, gonadotropin-releasing hormone
Percentages within a column marked with different numbers differ at $P < 0.05$.
Percentages within a row marked with different superscripts differ at $P < 0.05$.

higher in the GnRH group (7.81 ± 2.44 ng/mL vs 6.50 ± 2.11 ng/mL), as on day 20, it remained higher than in the control group (3.20 ± 0.99 ng/mL vs 1.97 ± 0.88 ng/mL; P<0.05).

In the pregnant animals from the different groups, a strong positive effect of the day after AI on the concentration of P4 (Fig. 1) was determined with significant ($P < 0.05$) differences between the mean values of P4 measured during the different days (Table 2). These data supported the previous result about a more powerful effect on the reproductive status, in comparison with the effect of GnRH treatment on the progesterone concentration (*observed power 1 vs 0.74, respectively*). On day 4 after AI, the P4 level was relatively lower in group II compared with group I, but on days 12 and 20, it tended to be higher in GnRH treated sheep, compared with non-treated sheep.

A retrospective analysis of the progesterone concentration measured on day 12 after AI showed a likehood of an early embryonic mortality of 33.3% and 24.3% in the control and the GnRH treated sheep, respectively, but a significant difference was not registered ($P = 0.19$). A total value of 25.4% for both groups was estimated (Table 3).

Table 2 Progesterone concentration (Mean ± SD, ng/mL) of sheep in control and GnRH groups according to day after AI and reproductive status based on ultrasound results

Day after AI	Control group (n = 30)			GnRH group (n = 37)		
	Non-pregnant (n = 10)	SCAMR (n = 6)	Pregnant (n = 14)	Non-pregnant (n = 6)	SCAMR (n = 10)	Pregnant (n = 21)
4	2.45 ± 1.01^{a1}	3.82 ± 1.95^{ab1}	3.74 ± 0.73^{b1}	2.56 ± 0.79^{a1}	3.03 ± 0.88^{bc1}	3.45 ± 0.98^{b1}
12	5.82 ± 1.65^{a2}	6.43 ± 2.52^{ab1}	7.53 ± 1.09^{bc2}	6.50 ± 2.11^{ab2}	7.81 ± 2.44^{bc2}	8.38 ± 0.91^{c2}
20	1.91 ± 0.95^{a1}	1.97 ± 0.88^{a2}	9.47 ± 1.96^{c3}	1.93 ± 0.43^{a1}	3.20 ± 0.99^{b1}	10.26 ± 1.69^{c3}

AI, artificial insemination; GnRH, gonadotropin-releasing hormone; *SCAMR,* sheep *conceived after mating by ram*
Mean values within a column marked with different numbers differ at $P < 0.05$.
Mean values within a row marked with different superscripts differ at $P < 0.05$.

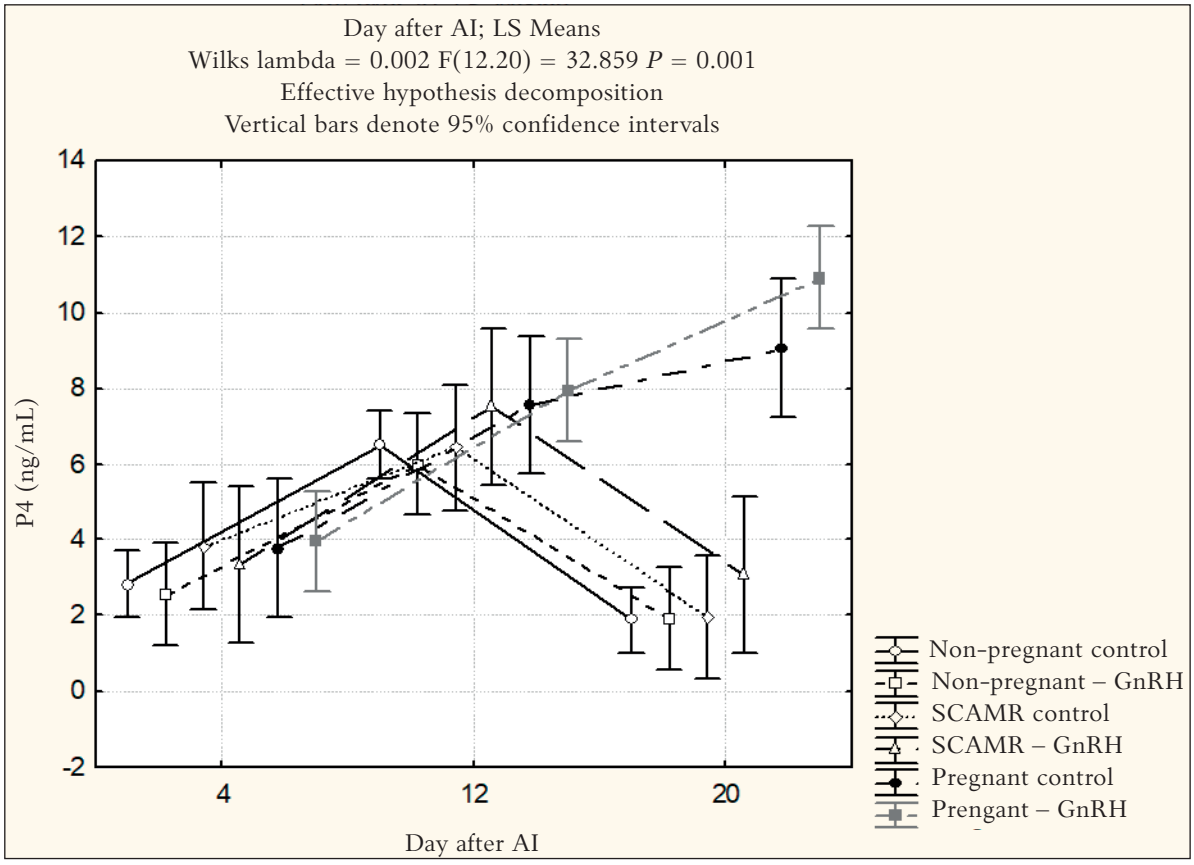

Fig. 1. Effect of day after artificial insemination on progesterone (P4) concentrations during the different days (ANOVA one way effects)
AI, artificial insemination; GnRH, gonadotropin-releasing hormone; *SCAMR,* sheep *conceived after mating by ram*

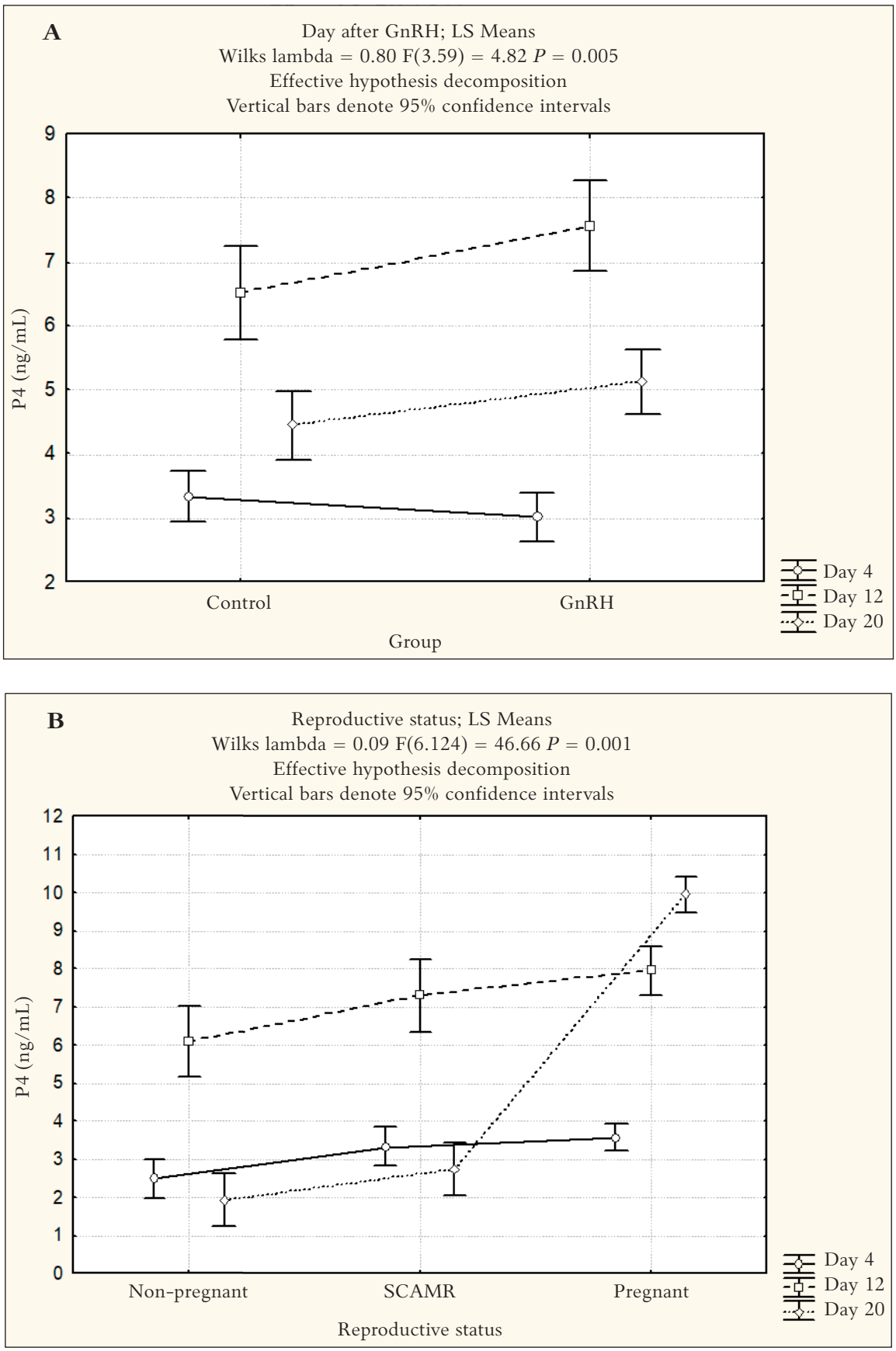

Fig. 2. Effect of GnRH (A) and reproductive status (B) on progesterone concentrations during the different days (ANOVA main effects)
AI, artificial insemination; GnRH, gonadotropin-releasing hormone; *SCAMR*, sheep *conceived after mating by ram*

Table 3. Early embryonic mortality (EEM) in the different groups based on minimal progesterone (P4) concentrations for pregnant animals measured on day 12 after artificial insemination

	Indicative P4 concentrations for pregnancy	
	(*P4 > 5.08 ng/mL*)	(*P4 > 6.60 ng/mL*)
	Control group (n = 30)	GnRH group (n = 37)
	% (n)	% (n)
EEM	33.3 (10)	24.3 (9)
Total	25.4 (17)	

Discussion

Administration of GnRH agonists has been used on the day of mating or days 1, 2, 4, 7 and 12 after that and showed to improve the reproductive performance in sheep (Cam & Kurran, 2004; Mirzaei et al., 2014; Hashem et al., 2015; Fernandez et al., 2019; Didarkhah & Vatandoost, 2022; Nikbakht et al., 2022). The obtained results are rather various and the information about the effect of GnRH treatment during the first several days after AI on the progesterone production, pregnancy rate and early embryonic mortality in dairy sheep is limited and sometimes controversial.

The information about close percentages of non-pregnant and pregnant sheep in both groups on day 20 after AI was indicative of an insignificant direct effect of the gonadotropin treatment on the pregnancy rate. It was in agreement with the results of other studies using GnRH administration on day 4 (Fernandez et al., 2019) or days 2 and 5 post AI (Nikbakht et al., 2022). However, the registration of significantly higher ($P < 0.05$) cumulative pregnancy in the GnRH group on day 60 was an indicator of an indirect positive influence of the GnRH treatment on the pregnancy results. It was supported by the fact that more animals from group II recorded as non-pregnant on day 20 were returned to estrus before day 20 after AI and were conceived *after mating by ram*. A possible reason for this finding could be a rapid increase of the P4 level after the GnRH treatment resulting in the attainment of the P4 level required to lysis of corpus luteum and earlier resumption of regular estrus activity in the treated sheep. In ruminants, oxytocin, progesterone, and estradiol regulate the uterine secretion of prostaglandin F2a that causes luteolysis (Silva et al., 1991; Goff, 2004). Regardless of a lower percent of sheep conceived after mating by ram in group I, the significant ($P < 0.05$) difference between the total value of pregnant and non-pregnant animals indicated that a combination of GnRH treatment on day 4 after AI and an introduction of fertile rams on day 12 may improve the reproductive performance on the flock level. Zonturlu et al. (2018) have also reported an increase of the pregnancy rate (up to 96.6%) after GnRH treatment on days 0 and 9 post-mating and an introduction of ram into the flock.

The information about the progesterone profile of the sheep treated by GnRH after mating or artificial insemination is contradictory. Hashem et al. (2015) injected 4.2 µg of buserelin on days 0, 7 and 0+7 post-mating in non-lactating Rahman ewes. The greatest overall mean P4 concentration was observed for GnRH 7 and GnRH 0+7 groups, followed by GnRH 0 and the control group. An investigation in Lake-Ghashghaei ewes during the spring season revealed a significantly higher serum progesterone concentration in all groups receiving GnRH (25µg as diacetate) on days 1, 2, 5, 7 and 12, compared with the control group (Nikbakht et al., 2022). However, Ayaseh et al. (2020) have reported that an injection of hCG or 4.2 µg of buserelin on days 0 and 5 after did not increase serum P4 concentrations in Karakul ewes during the non-breeding season.

The present study showed a similarity in the progesterone profiles of sheep with the same reproductive status in the control and the gonadotropin treated group. This result does not exclude fully a beneficial effect of GnRH treatment on the luteal function, followed by an increase in endogenic progesterone production. It was in agreement with the analysis of the main effect of the GnRH on the P4 concentration. The rapid increase of the P4 concentration in *SCAMR between days 4 and 12* after AI, in spite of a lower baseline of P4 on day 4, compared with the control group, could be accepted as evidence for enhanced luteal activity in the GnRH treated sheep. It is known that many factors (age, breed, body condition and lactating status of sheep, season, level of feeding, etc.) affect the progesterone concentration in the blood circulation of ruminants (Nawito et al., 2015; Kamil, 2019; Garcia-Baccino et al., 2022). Moreover, an adequate response of the animals to gonadotropin treatment depends on the type of a GnRH agonist and a dose (Mirzaei et al., 2011; Picard-Hagen et al., 2015). Mirzaei et al. (2011) have treated cross-bred fat tailed ewes with a low (4.2 µg) dose and a high (8.4 µg) dose of buserelin on days 5 and 19 after ram introduction, respectively. On day 12 after treatment, the high dose GnRH resulted in significantly lower plasma P4 concentrations ($P < 0.05$), compared with the treatments by the low dose GnRH and without GnRH. All aforementioned may explain the discrepancy of the current results with the data of previous authors for different P4 profiles

in non-treated and GnRH treated sheep. Probably the response of the East Friesian sheep to the used GnRH treatment was lesser compared with other sheep breeds. Additional detailed investigations with a large number of animals may clarify the question about a dose of the used GnRH agonist and the time for treatment after AI that can provide a significantly higher increase in P4 of East Friesian sheep at the end of lactation.

The reproductive status had a significant effect on the progesterone profiles of the animals with a close pattern in non-pregnant and SCAMR between days 4 and 20 after AI. A similar elevation of P4 until day 12 and a decrease until day 18 were established by Garcia et al. (2022) in non-pregnant sheep. Although the factorial analysis did not show a simultaneous influence of all factors, there was a tendency ($P = 0.057$) for P4 concentration to increase in SCAMR and pregnant animals after GnRH administration. The abovementioned subgroups had relatively lower baseline P4 levels, compared with the same non-treated subgroups on day 4, but their progesterone levels were relatively higher on days 12 and 20. The same effect on the mean plasma progesterone concentration was observed by Farrag et al. (2017) in adult Barki ewes.

The pregnancy affected the progesterone profile expressing in a gradual rise between days 4 and 12 and a rapid increase until day 20 after AI. This result was in unison with the data reported by Ganaie et al. (2009) in pregnant Corriedale ewes. They determined a significant increase in the mean plasma progesterone concentration from day 0–6 to day 16–30, while the progesterone level dropped to less than 1.0 ng/mL in the case of ewes that returned to estrus. The significant differences ($P < 0.05$) between the progesterone concentrations of non-pregnant and pregnant sheep in groups I and II on day 20 indicated that the measurement of P4 can be used for pregnancy detection, but only in a lack of ram introduction after artificial insemination. Otherwise, there is a risk of a large number of false negative diagnoses, as a result of re-conception by ram of non-pregnant but returning to estrus sheep. It was confirmed by the registration of pregnant animals on day 60 coming from those recorded as non-pregnant on day 20 after AI.

According to Vinoles et al. (2012), the pattern and concentrations of progesterone were affected by the pregnancy as the values were higher on day 12 in pregnant than non-pregnant ewes from all groups ($P = 0.01$). The P4 concentrations decreased in non-pregnant ewes between days 12 and 17, the expected time of return to estrus. The same effect was estimated in our study, and on this base, the likehood of early embryonic mortality in different groups was calculated. Regardless of the insignificant differences between EEM in gonadotropin treated and non-treated animals, the obtained value in the GnRH group was 9% lesser, compared with the controls. Related to this, the significantly ($P < 0.05$) higher mean P4 concentration in treated than non-treated SCAMR on day 20 supported indirectly the hypothesis for a positive influence of the gonadotropin administration on the early embryonic mortality, conditioned by stimulation of the P4 production from the corpus luteum or formation of accessory corpora (Cam & Kuran, 2004). Progesterone predominantly exerts an indirect effect on the conceptus via the endometrium to regulate blastocyst growth and conceptus elongation (Spencer, 2013; Coleson et al., 2015). The absence of a sufficiently developed conceptus to signal maternal pregnancy recognition results in the genes implicated in uterine receptivity being "turned off" as luteolysis ensues, P4 concentrations decline, and the animal returns to estrus for another opportunity to mate (Spencer, 2013). The aforementioned information was in accordance with the obtained result for more sheep returning to estrus after GnRH treatment and determined as pregnant during the second ultrasound examination. An effective prevention of the embryonic death in ewes has been achieved by the application of GnRH or FGA on days 4 and 12 after mating (Ataman et al., 2013). The total value of EEM in our study was close to the registered embryo loss of 27% between days 10 and 17 and 30 ± 13% between ovulation and pre-implantation in Merino ewes (Vinoles et al., 2012; Rickard et al., 2017). On the other hand, the absence of a statistical difference in EEM between non-treated and GnRH treated sheep implies an influence of additional factors different than progesterone on the embryo loss in the earliest gestational phase. Hoskins et al. (2021) suggest that while progesterone may accelerate conceptus development, its role in the mechanisms of implantation and pregnancy is complex and requires further research to investigate its therapeutic properties in livestock reproduction.

Conclusion

Administration of GnRH on day 4 after artificial insemination and an introduction of ram on day 12 do not have a direct effect on the pregnancy rate, but lead to improvement of the reproductive performance on the flock level by an increase of the number of sheep conceived after mating by ram. Gonadotropin releasing hormone treatment, reproductive status and day after AI affected the progesterone concentration in East Friesian sheep irrespective of each other, and had a significant (*Wilks-lambda tests, P* < 0.005) effect on the hormonal profile. The treatment by GnRH on day 4 after AI tended to reduce early embryo mortality, but future investigations are needed to clarify this effect.

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Pigeon Paramyxovirus-1 Infection and the Public Health Importance: A Review Article

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Keywords: *Newcastle disease or paramyxovirus, clinical picture, pigeon, human, vaccination*

Abstract. *Pigeons are highly susceptible to Newcastle disease virus (NDV) infection, which causes economic losses in terms of increased mortalities, immunosuppression, vaccination costs, and probable trade restrictions. NDV belongs to the avian paramyxovirus serotype 1 (APMV-1). The antigenic variant of APMV-1 is termed as pigeon paramyxovirus type 1 (PPMV-1), which is classified in the genus Avulavirus of the subfamily Paramyxovirinae and family Paramyxoviridae. Infections of pigeons with PPMV-1 have been detected since the 1930s, and the virus is still circulating in many countries until now. Domestic, feral, and racing pigeons, doves, and exotic birds are susceptible to PPMV-1 infection. The virus is rapidly spreading between birds through the horizontal route. Infected pigeons may show circling, ataxia, torticollis, head and neck tremors and twisting, leg and wing paralysis, greenish diarrhea, respiratory signs, and polyuria. Some pigeons could be infected with PPMV-1 without apparent signs, and they act as reservoirs for other domestic or free-living birds. The diagnosis of suspected PPMV-1 cases is based mainly on the isolation and identification of the virus, serological detection of specific antibodies, and molecular characterization of the virus. Adoption of strict biosecurity measures as well as vaccination using traditional live or inactivated NDV or even the specific PPMV-1 vaccines are the gold standard methods for preventing pigeons from such infection. Therefore, this review article was designed to focus on PPMV-1 infection regarding the virus characteristics, epidemiology, diagnosis, human infection, and control.*

Introduction

Newcastle disease (ND) is an acute, highly contagious, and endemic viral disease of poultry worldwide (Abdisa & Tagesu, 2017; Suarez et al., 2020). Respiratory, gastrointestinal, and neurological manifestations are the main clinical pictures of ND infections. The disease is associated with significant global economic losses in the poultry industry, including high morbidity and mortality rates, immunosuppression, increasing the costs of vaccinations and control, and probable trade restrictions (Alexander, 2001; Ganar et al., 2014). The World Organization for Animal Health included ND in the list (A) of notifiable diseases (OIE, 2012). It has been reported that about 236 free-living avian species could be infected either naturally or experimentally with ND (Kaleta & Baldauf, 1988).

Avian paramyxoviruses have been divided into 12 different serotypes based on hemagglutination inhibition (HI) and neuraminidase inhibition assays (Dimitrov et al., 2016). Newcastle disease virus (NDV) belongs to avian paramyxovirus serotype 1 (APMV-1) in the family *Paramyxoviridae* and the genus *Avulavirus* (Cox & Plemper, 2017). The virus is a single-stranded, negative-sense, and non-segmented RNA genome that contains 6 genes in the sequence of 3′-NP-P-M-F-HN-L-5′ (Gogoi et al., 2017; Dimitrov et al., 2019). APMV-1 is circulating as an enzootic infection in many continents, such as Europe, Africa, Asia, and America (Miller et al., 2010). Infection with APMV-1 has shown variation in pathogenicity, from asymptomatic to lethal disease, due to the significant differences in strains virulence (Heiden et al., 2014). Three major pathotypes of NDV have been recognized in poultry based on the degree of the pathogenicity indices. They include the mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI). Apathogenic NDV strains are non-virulent and show enterotropism, while lentogenic strains are of low virulence and produce mild respiratory manifestations (Alexander, 1997). Mesogenic strains of NDV are of moderate pathogenicity and infect mainly the respiratory tract, causing death in birds under 8 weeks of age (Beard & Hanson, 1984), while highly virulent velogenic strains (viscerotropic and neurotropic) induce systemic infections and a high mortality rate (Susta et al., 2011; Miller & Koch, 2013).

Pigeons could be divided into 3 categories: meat-type pigeons, homing pigeons, and fancy pigeons. Recently, the pigeon industry has shown rapid development, but the disease situation caused by viral affections is not helping and is not hopeful. Owing to the migratory nature of pigeons, difficulties in vaccinations, and their existence in live bird markets and backyard houses, they are regarded as a major threat for NDV transmission to domestic chickens. APMV-1 infects almost all avian species, including pigeons. Therefore, pigeons are regarded as a natural host of APMV-1 and play an important role in the

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virus ecology (Pestka et al., 2014). ND in pigeons is called paramyxovirosis and is caused by an antigenic "pigeon variant" of the virus (pigeon paramyxovirus type 1, PPMV-1). PPMV-1 is an antigenic variant of APMV-1, which is mainly associated with infection of pigeons (Pestka et al., 2014). Since the genotype VI of NDV is frequently detected in pigeons, this strain is commonly named PPMV-1 (Alexander et al., 1985b; Tian et al., 2020; Xie et al., 2020). Genotypes VI, XX, and XXI of NDV are sometimes assigned as PPMV-1, which is regarded as the most genetically diverse group among the virus strains (Dimitrov et al., 2016). Despite the fact that PPMV-1 is responsible for severe disease conditions in domestic pigeons (Alexander, 2009), it occasionally spreads to a wide variety of other species, including feral pigeons, doves, and exotic birds (Alexander, 2000). Nowadays, PPMV-1 has been reported as an enzootic infection of feral, racing, and fancy pigeons (Alexander, 2011). Young pigeons are more susceptible to PPMV-1 and show neurological disorders with high morbidity and mortality rates (Chang et al., 2021; Badr et al., 2022). The central nervous system, respiratory system, alimentary tract, and kidney are usually affected by PPMV-1. Routine diagnosis of PPMV-1 is based on primary isolation in specific pathogen-free (SPF) embryonated chicken or pigeon eggs or on tissue culture (Gough et al., 1988), followed by a hemagglutination (HA) assay. Confirmation of infection should be carried out using conventional serological tests such as HI test or molecular-based techniques (OIE, 2021).

Infection of humans with APMV-1 is usually rare and may cause an asymptomatic condition or a mild disease, especially in immune-competent people (Capua & Alexander, 2004). The direct contact of humans with diseased pigeons may lead to conjunctivitis or, rarely, to flu-like symptoms (Zehetbauer et al., 1971; Schemera et al., 1987). In severe cases, infected people may show long-term vision damage (Beard & Hanson, 1984; Lamb & Parks, 2007). The disease usually shows self-limiting symptoms, which develop within 24 hours of exposure to PPMV-1 and resolve within a week (Swayne & King, 2003). Though the zoonotic potential of PPMV-1 is low, veterinary authorities urge people not to touch diseased or dead pigeons.

Despite vaccination, PPMV-1 is still enzootic in pigeons in some countries (Alexander, 2001). The gold standards for the prevention of PPMV-1 infection in pigeons are the administration of vaccines (Vindevogel & Duchatel, 1985; Zhao et al., 2010; Soliman et al., 2019), along with the adoption of effective biosecurity measures.

From the abovementioned, this review article was designed to focus on PPMV-1 infection in regards to the virus characteristics, epidemiology, diagnosis, human infection, and control.

The virus

According to the International Committee on Taxonomy of Viruses, *Orthoavulavirus* 1 (NDV) belongs to the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Avulavirinae*, and genus *Orthoavulaviruses* (ICTV, 2020). It is an enveloped virus with single-stranded RNA that is linear, pleomorphic (mostly spherical), non-segmented, and has a negative polarity (Amarasinghe et al., 2019). The genome of NDV is around 15–19 kilobases in length and encodes 6 structural proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), and RNA large polymerase (L) protein in the following order of 30-NP-P-M-F-HN-L-5 (Czegledi et al., 2006). Besides, the gene P encodes 2 additional non-structural (V and W) proteins by RNA editing (Steward et al., 1993; Karsunke et al., 2019). Both F and HN glycoprotein spike projections on the virus envelope are important for infection, fusion, and propagation of most paramyxoviruses in the host cell (Lamb & Parks, 2007). Moreover, the F protein is regarded as a key factor in NDV pathogenicity (Panda et al., 2004). Other internal proteins are involved in some functions, including transcription and replication of the virus (Dimitrov et al., 2016; Gogoi et al., 2017).

Although APMV-1 has a single serotype, the phylogenetic characterization of F gene sequences reveals the presence of 2 distinct classes (class I and class II) (Diel et al., 2012). Both classes are consequently subdivided into many different genotypes and clades. Class I of the virus consists of a single genotype, and it is mostly isolated from natural reservoirs of free-living wild and domestic waterfowl without pathogenicity to chickens (Kim et al., 2007; Chen et al., 2021; Lu et al., 2021). Class I virus strains are characterized by a 15,198-nt genomic RNA. On the other hand, class II can be separated into 21 genotypes (I to XXI, no XV) and represents the major cause of the disease outbreaks all over the world (Diel et al., 2012; Courtney et al., 2013; Dimitrov et al., 2019). Class II viruses show variable pathogenicity (both avirulent and virulent strains), as genotypes V, VI, VII, VIII, and XI–XVIII are the most pathogenic ones (Snoeck et al., 2013). Two new genotypes of XX and XXI and some strains of genotype VI have been designated as variant NDV strains, particularly in Columbiformes (Dimitrov et al., 2019). The strains of PPMV-1 are clustered into genotypes VI and XXI (Aldous et al., 2004). Genotype I–IV viruses are early lineage with a genome size of 15 186 nt, while genotype V–VIII viruses are recent lineage with a genome of 15 192 nt (Ujvari et al., 2006). Moreover, class II genotype VI is further divided into VIa–VIg. Moreover, Aldous et al. (2003) defined 6 lineages (class I viruses were lineage 6) with many sub-lineages. PPMV-1 viruses are placed in class II and lineage 4b or VIb, depending

on the used nomenclature. NDV has a specific amino acid sequence at the fusion protein gene cleavage site ["multiple basic amino acids (positions 113–116) at the C-terminus of the F2 protein and phenylalanine at position 117 at the end of the F1 protein"] (OIE, 2021). At the cleavage location of the F0 precursor, the most virulent strains of NDV has a sequence of "112R/K-R-Q-R/K-R*F117", in comparison with avirulent strains that have "112G/E-K/R-Q-G/E-R*L117", which is considered as a major determinant of the virus virulence (Collins et al., 1993). Therefore, the virulence of NDV strains could be determined through the F protein cleavage site amino acid sequence analysis (Le et al., 1988), as well as through the ability of separation of specific cellular proteases to cleave the F protein pathotypes (Ogasawara et al., 1992). The World Organization for Animal Health has defined virulent NDV as APMV-1 with at least 3 multiple basic amino acid residues (arginine or lysine) at the C-terminus of the F2 protein (between residues 113 and 116) and phenylalanine at residue 117, which is the N-terminus of the F1 protein (Alexander, 2008, 2011). It has been documented that lentogenic or avirulent NDV strains have fewer basic amino acids in the F protein cleavage site than either mesogenic or velogenic isolates, which have similar cleavage site sequences (Glickman et al., 1988).

Epidemiology

Distribution

Despite the fact that infections of pigeons with PPMV-1 have been detected since the 1930s (Doyle, 1933), the 3rd NDV panzootic started during the late 1970s (Yang et al., 1999) and is present in many countries until now. Around 1979, PPMV-1 was also reported in the Middle East (Kaleta et al., 1985a), and then the virus strains were detected in some European countries (Alexander et al., 1985a, b). In 1984, feedstuff was contaminated by feral pigeons in the docks in Liverpool, United Kingdom, resulting in 19 NDV outbreaks due to PPMV-1 in chickens (Alexander et al., 1985a). Moreover, outbreaks of PPMV-1 in commercial and backyard poultry have been reported in Brazil (Zanetti et al., 2001; Souza et al., 2018; Thomazelli et al., 2021; Pereira et al., 2022), Poland (Smietanka & Minta, 2011), Ireland (O'Reilly et al., 1994), Germany (Werner et al., 1999), Switzerland (Alexander et al., 1985b; Annaheim et al., 2022), Slovenia (Krapez et al., 2010), Macedonia (Dodovski et al., 2013, 2017), Japan (Mase & Kanehira, 2015), China (Liu et al., 2006; Wang et al., 2015; Qiu et al., 2017; Wei et al., 2018; He et al., 2020; Tian et al., 2020; Xie et al., 2020; Zhan et al., 2022), South Africa (Pienaar & Cilliers, 1987; Abolnik et al., 2004, 2008), Nigeria (Snoeck et al., 2013), Iran (Mayahi et al., 2017; Rezaei Far et al., 2017), and Egypt (Mohamed et al., 1980; Ahmed & Sabri, 1989; Shakal, 1989; Abou Hashem, 1993; Ibrahim et al., 2005; Soliman et al., 2016; Mansour et al., 2017).

Susceptibility

All ages of pigeons are susceptible to PPMV-1 infection with high morbidity and mortality rates (Kim et al., 2008; Qiu et al., 2017; Tian et al., 2020). Not only domestic pigeons are susceptible to PPMV-1, but also feral pigeons may carry the virus as asymptomatic carriers (Teske et al., 2013; Mase & Kanehira, 2015). Feral or wild pigeons have shed PPMV-1 during foraging in rural areas and posed a potential threat to the spread of the disease to free-range chickens (He et al., 2018). The PPMV-1 isolate that belongs to sub-genotype XXI.2 has been reported in collared doves in Italy (Bonfante et al., 2012) and in Eurasian collared doves in Iran (Esmaeelzadeh-Dizaji et al., 2022). PPMV-1 has also been isolated from wild North American doves (Kim et al., 2008). The study of Śmietanka et al. (2014) has shown that PPMV-1 strain was highly virulent to pigeons, followed by chickens and turkeys, but quails and geese showed the highest level of innate resistance to the used strain. Nevertheless, the passing of PPMV-1 in chickens has resulted in increased ICPI. There is no evidence of PPMV-1 infections in wild non-Columbiformes. However, the virus is occasionally isolated from migratory birds (Alexander et al., 2012).

Transmission

PPMV-1 could be excreted in the nasal, buccal, and ocular secretions and droppings of the infected pigeons. The virus is transmitted horizontally among birds either by inhalation or ingestion (Kaleta et al., 1992). Direct contact between healthy and diseased birds helps in the rapid spread of infection. The rapid transmission of PPMV-1 among Columbiforms may also be possible during competition flights (racing pigeons), exhibitions (show pigeons), live bird markets (meet pigeons), or intensive trade (Aldous et al., 2014; Sabra et al., 2017).

Clinical picture

The incubation period of PPMV-1 infection is 7–14 days. The clinical picture of infection varies according to the virulence of the infective strain, the immune status of the host, and the presence of other infections (Alexander & Senne, 2008). Pigeons show clinical signs similar to neurotropic NDV-infected chickens. Affected pigeons may display moderate to severe depression, circling, ataxia, torticollis, head and neck tremors and twisting, leg and wing paralysis, greenish diarrhea, and respiratory manifestations (Vindevogel & Marlier, 2006; Wang et al., 2015; Badr et al., 2022). Viscerotropic strains of PPMV-1 exhibit specific affinity for the kidneys; thus, the first observed sign is polyuria, and neural symptoms appear only in individual birds (Pestka et al., 2014). However, an asymptomatic course of infection may occur (Alexander et al., 1984a). Infection with PPMV-1 may cause a mortality rate that ranges from 10% to

70%, as does the morbidity, which ranges from 30% to 80% in the infected pigeons (Hutchison, 1984; Mansour et al., 2017; Qiu et al., 2017). Concurrent infections with other bacterial or parasitic infections increase the mortality rate. Survived pigeons may shed the virus and, therefore, represent a source of infection (Alexander et al., 1984b). The post-mortem lesions of dead pigeons with PPMV-1 have revealed soft or friable tissues of the brain and/or hemorrhages, severe hemorrhagic enteritis, petechial hemorrhage in the gizzard, and congested liver (Dodovski et al., 2017; Badr et al., 2022). It has been documented that PPMV-1 in chickens is of intermediate virulence (a mesogenic virus) (Collins et al., 1994; Hüppi et al., 2020), and the infection may vary from subclinical to a marked drop in egg production (Guo et al., 2014). Though PPMV-1 strains sometimes cause mild disease, multiple passages in chickens may result in increasing virus virulence in the form of signs, mortality, and neuro-invasiveness (Kommers et al., 2001, 2003; Dortmans et al., 2011). Moreover, many NDV outbreaks in chickens have been attributed to PPMV-1 infection (Alexander et al., 1984b; Werner et al., 1999). Several point mutations are sufficient to increase the pathogenicity of PPMV-1 in chickens (Meulemans et al., 2002; Dortmans et al., 2009). The histopathological examination of the examined brains of PPMV-1-infected pigeons has revealed the presence of non-suppurative encephalitis, necrosis, and microgliosis (Wakamatsu et al., 2006; Pereira et al., 2022). Diffuse inflammation of the respiratory and intestinal tract tissues has also been detected (Aldous & Alexander, 2001).

Human infection

Infection of humans with APMV-1 is rarely detected, and it is not considered a life-threatening condition so far. However, sporadic infections have been reported in patients with occupational exposure to commercially infected poultry flocks (Capua & Alexander, 2004). Most of the APMV-1 human cases have occurred through direct contact with infected birds; mostly, workers in poultry farms, laboratories, and processing plants are at a high risk of getting infections. The clinical signs are usually transitory mild conjunctivitis, and most of the patients resolve without medical or clinical interference (Steele & Beran, 1981). Frequent symptoms of eye disorders (Lippmann, 1952; Capua & Alexander, 2004), unilateral or bilateral eye redness, eyelid edema, conjunctivitis, sub-conjunctival hemorrhage, and acute keratoconjunctivitis have been reported in a case with concurrent NDV and human adenovirus infection (Prajna et al., 2021). Nevertheless, lethal pneumonic studies of APMV-1 have been found in immune-compromised persons following blood stem cell/allogeneic bone marrow transplantations (Goebel et al., 2007; Kuiken et al., 2018). Another child case caused by APMV-1 has shown fatal encephalitis after hematopoietic stem-cell transplantation (Winter et al., 2021). The strain of APMV-1 isolated from a pneumonic patient was also of pigeon origin (Goebel et al., 2007). Kuiken et al. (2017) have found that PPMV-1 from a fatal human case induced pneumonia in experimentally infected Cynomolgus macaques. Moreover, APMV-1-associated pneumonia and death have been detected in a 64-year-old person following contact with a live pigeon (Abbo et al., 2007). Following extensive laboratory investigation of the previous case, both APMV-1 and *Acinetobacter baumannii* (ABA) have been detected. Recently, a similar case was reported in 2020 in China, where a 64-year-old man presented with severe acute respiratory distress syndrome and sepsis, followed by death within a few days (Zou et al., 2022). The patient had close contact with pigeons before illness, and PPMV antibodies were detected in his blood within 20 days of illness. Besides, the metagenomic sequencing revealed the presence of ABA and APMV-1 genotype VI.2.1.1.2.2 in the broncho-alveolar lavage fluid, and the virus nucleic acid was found in the pigeon feathers. This study emphasizes the cross-species transmission of PPMV-1 between infected pigeons and humans.

Diagnosis and differential diagnosis

The primary isolation of PPMV-1 could be achieved via inoculation of SPF embryonated chicken or pigeons eggs. The embryos die between the 2nd and 8th day of inoculation and show curling and sometimes dwarfing with head and body hemorrhages as well as presence of urates even mixed with allantoic fluids. The HA of allantoic fluid may confirm the presence of PPMV-1. Tissue culture, including chicken embryo fibroblasts, chicken primary neuronal cells, pigeon embryo fibroblasts, and pigeon primary neuronal cells have been used for primary isolation of PPMV-1 (Guo et al., 2018; Zhan et al., 2020). Serological tests, particularly the HI assay, have been used for detection of antibodies in the serum of PPMV-1 infected pigeons (Ibrahim et al., 2005).

The pathogenicity of the virus could be determined by assessing the MDT in 10-day-old SPF embryonated chicken or pigeon eggs and the ICPI in 1-day-old SPF chicks based on standard procedures (OIE, 2012). Virulent NDV strains are those with MDT less than 60 hours and ICPI values of 0.7 or more (defined as the mean observed score per bird as 0 if normal, 1 if diseased, or 2 if dead over the 8-day period) (Dortmans et al., 2009, 2010). The IVPI of PPMV-1 could be determined after inoculation of freshly infective allantoic fluids into a 6-week-old pigeon, as each bird should be examined daily for a period of 10 days and scored for assessment of the virus virulence (mesogenic, velogenic, etc.).

The molecular identification of PPMV-1 has been comprehensively carried out using polymerase chain

reaction (Aldous et al., 2004, 2014; Wei et al., 2018; Esmaeelzadeh-Dizaji et al., 2022). Virulent PPMV-1 strains have a specific amino acid sequence at the F protein gene cleavage site (amino acids positions 113–116 at the C-terminus of the F2 protein and phenylalanine at position 117 at the end of the F1 protein) (Dimitrov et al., 2017; OIE, 2021).

Signs of PPMV-1 in pigeons are similar to pigeon herpes virus infection, sodium chloride poisoning, and overdose of ronidazole or vitamin B1 deficiency. Therefore, laboratory tests are essential for an accurate diagnosis (Hamouda et al., 2017).

Control

There is no specific treatment for PPMV-1 infection because infected birds usually die within 72 hours; however, some recovered birds may survive with supportive treatment. Control of PMV-1 infection can be achieved via the adoption of strict biosecurity measures and a proper vaccination regimen (Miller & Koch, 2013). Vaccination of pigeons could reduce the losses and the hazards of the spread of infection to other pigeons and/or other bird species. Pigeons could be protected against infection using chicken NDV-live or -inactivated oil-emulsion vaccines (Viaene et al., 1984). Ibrahim et al. (2005) have found a close immunogenic relationship among PPMV-1 and NDV vaccine strains, which may answer the question of why we use NDV vaccines for controlling PPMV-1 infection in pigeons. Immunization of commercial or racing pigeons with inactivated APMV-1 vaccines is also important (Alexander et al., 1985b). Lentogenic strains of NDV showed some virulence for pigeons and might be excreted 3–7 days post inoculation; thus, inactivated vaccines are preferred for racing pigeons (Vindevogel et al., 1982). Viaene et al. (1983, 1984) reported that LaSota and oil-inactivated NDV vaccines provided protection for pigeons against PMV-1 infection for up to 6 months. However, it has been reported that the Hitchner B1 live NDV vaccine could not protect pigeons against the virus as it could not propagate in pigeon tissues and thus could not induce suitable immunity (Kaleta et al., 1985b). Alexander et al. (1986) demonstrated that vaccination of pigeons against PPMV-1 was not analogous to vaccination against NDV in chickens due to the differences in the hosts or the antigenic variations between pigeons and classical PPMV-1 strains, including the vaccinal viruses.

Homologous vaccine is necessary for a complete protection of pigeons against the disease (Fritzsch et al., 1984; Eskelund, 1986; Stone, 1989; Amer, 2008). Vaccinated pigeons with a homologous oil emulsion vaccine against PPMV-1 infection were more highly protected than pigeons vaccinated with commercial live NDV vaccines for a year (Alexander and Parsons, 1984; Amer et al., 2013). Inactivated NDV vaccines provoked a relatively high antibody titer, which was sufficient to protect pigeons against PPMV-1, while the Hitchner B1 live vaccine did not (Polten et al., 1985; Kosters et al., 1986). Duchatel and Vindevogel (1986) found that inoculation of 0.2 mL of inactivated aqueous-suspension vaccines prepared from the LaSota strain of NDV gave a high resistance to a severe challenge with PPMV-1. Box et al. (1985) suggested using double doses of an inactivated oil-emulsion vaccine 4 weeks apart to provide a good protection for pigeons. Inactivated vaccines from the local PPMV-1 strain were developed, and the results revealed a protection rate of 80% with a high level of immune response that lasted for 5 months (El-Zanaty et al., 1992; Hassan, 1997). Wawizkiewicz et al. (1991) demonstrated that the oil emulsion PPMV-1 vaccine gave a higher HI antibody response on the 3rd week after the 2nd vaccination dose. Besides, Amer et al. (2013) have reported that the PPMV-1 vaccine gave 100% protection for vaccinated pigeons against the homologous virus compared with only 10% protection in non-vaccinated birds. A combined inactivated vaccine containing oil adjuvanted local strains of PPMV-1 and *Salmonella typhimurium* is safe, potent, and provides a full protection in terms of increasing the HI antibody titers and reducing mortality post-challenge (Khedr et al., 2016). These findings suggest that sub-strains may exist in the PPMV-1, which necessitates the continuous update of the vaccine master seeds with new field isolates to maintain high protection level (Soliman et al., 2019). Subcutaneous inoculation of pigeons with an aluminum hydroxyl-based formula of the PPMV-1 YA/14 vaccine provides a higher humoral immune response with a lower shedding level than the oil-based vaccine (Soliman et al., 2019).

Conclusion

PPMV-1 infection is widespread; therefore, prevention of the pigeon's infection looks significant and needs thorough investigation. Besides, the mechanisms by which APMV-1 causes severe human infection should be more thoroughly explored. Pigeons may not show clear signs of PPMV-1 infection or deaths, but they can catch the virus and induce specific antibodies. Despite vaccination, PPMV-1 is still enzootic in pigeons in certain countries. Vaccination of pigeons with the NDV vaccine or a specific PPMV-1 vaccine gains attention to avoid the virus shedding to other birds. The use of a homologous vaccine is the most suitable solution for the control of such infection. Personnel should wear protective clothes and equipment when handling and processing potentially infected poultry. The surveillance studies of PPM-1 are necessary to prepare health authorities better.

Conflict of Interests

The author declare that there is no conflict of interests.

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Effect of Feeding Common (*Agaricus bisporus*) and Oyster (*Pleurotus ostreatus*) Mushrooms on Performance, Intestinal Microbiology and Morphology of Female Japanese Quails (*Coturnix coturnix japonica*)

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Abstract. *This research was performed to study the performance, intestinal microbiology and morphology of Japanese quails supplemented with two types of edible mushrooms including common (Agaricus bisporus) and oyster (Pleurotus ostreatus) powders. A total of 420 twenty-one day old female quail chicks were randomly allocated to seven experimental treatments. Each treatment consisted of 3 replicates of 20 birds. The birds within the control group were given the basal diet for the respective growth stage. The other six groups were fed experimental diets based on the basal diets containing 0.5%, 1%, and 2% of dried common or oyster mushroom powders. Birds were given free access to feed and water during the 84 days of the experimental period. During the experiment, performance characteristics were measured. Count of coli-form bacteria in the gut and intestinal morphological characteristics were studied at the age of 84 days. Egg weight, feed intake, egg mass and feed conversion ratio in 84 days were not significantly influenced by the supplementation of mushrooms. Bifidobacteria and Lactobacilli populations were significantly increased ($P < 0.05$) by 2% of mushrooms compared with the control. Total counts (Aerobes) and Escherichia coli were significantly decreased ($P < 0.05$) by 2% of mushrooms compared with the control. Crypt depth and papillae height in parts of 10%, 50% and 70% of the gut were positively influenced by the supplementation of mushroom ($P < 0.05$). Therefore, it seems that mushrooms could increase useful microflora and prove helpful in the fight against pathogenic organisms colonizing in the quail chicks gut.*

Introduction

Due to banning the use of antibiotics in animal nutrition, some additives such as herbal materials, probiotics, prebiotics and organic acids can be used instead of antibiotics in poultry diets (Fouladi et al., 2018). Recently, natural materials such as medicinal plants, mushrooms and herbs have been investigated. Wang et al. (1998) reported antimicrobial activities, immune enhancement and stress reduction in farm animals given natural medicinal products from fungi and herbs. Asadi Dizaji et al. (2014) reported that 2% of mushrooms (*Agaricus. bisporus*) in the diet positively affect performance parameters and some internal organs of quails. Mushrooms have long been appreciated as an important source of bioactive compounds of medicinal value (Breene, 1990). Use of 2% of mushrooms (*Pleurotus. ostreatus*) in the diet positively affects blood biochemical characteristics of quails (Asadi Dizaji et al., 2017). Some fungi have been used for centuries to combat disease outbreaks in many parts of the world and are still used in ethnoveterinary medicine in Asian and Mediterranean countries (Chang & Buswell, 1996). Mushrooms may have a wide range of activities (Guo et al., 2003). For particular interest, extracts derived from various mushrooms are known to confer health-promoting benefits, due to a multitude of compounds with antioxidant, antibacterial, immune-enhancing, and stress reduction properties on farm animals (Dalloul & Lillehoj, 2006; Dalloul et al., 2006). Guo et al. (2004a) reported that the population of *bifidobacteria* and *lactobacilli* were significantly increased with the addition of a shiitake mushroom extract (*Lentinusedodes*). It was found that the immunologically active components in medical mushrooms and plants may include polysaccharides, glycosides, alkaloids, volatile oils, and organic acids (Yang & Feng, 1998). Anti-microbial activity including anti-bacterial, anti-parasitic, anti-fungal and anti-viral agents is a widespread therapeutic effect reported in mushrooms (Wasser & Weis 1999; Kettering et al., 2005).

Intestinal microflora play an important role in the health status of host animals. Intestinal microflora constitute a dynamic ecosystem that is essential to the health of the chicken. In general, intestinal bacteria may be divided into species that exert either harmful or beneficial effects on host health (Macfarlane & Cummings, 1991). Therefore, a common approach to maintain host health is to increase the number of desirable bacteria in order to inhibit colonization of invading pathogens (Rolfe, 1991). The composition and activity of intestinal macrobiotics can be altered by diet composition and dietary manipulations such as

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the use of feed additives and antibiotics (Coates et al., 1981; Jensen, 1993). Guo et al. (2004b) investigated several mushrooms and herb polysaccharides, as alternatives for an antibiotic, on growth performance of broilers, and found *Lentinula edodes* to be a significant growth promoter in broilers. Similarly, Willis et al. (2007) noted enhanced beneficial *bifidobacteria* production from a mushroom extract (*Lentinulaedodes*) given to broiler chickens. Mahfuz et al. (2020) also stated that supplementing *Agaricus bisporus* and *Pleurotus ostreatus* in broiler diets led to an increase *bifidobacteria* and *lactobacilli* in the caecum and the ileum.Therefore, the aim of this study was to investigate the effects of supplementation of different levels of dried powder of common and oyster mushrooms on the performance, intestinal morphology and microflora composition in female Japanese quails.

Materials and Methods

Birds and experimental design

A total of 420 twenty-one day old female quail chicks were randomly allocated to seven experimental treatments. Each treatment consisted of 3 replicates of 20 birds. The birds within the control group were given the basal diet for the respective growth stage. The other six groups were fed experimental diets based on the basal diets containing 0.5%, 1%, and 2% of dried common (*Agaricus. bisporus*) or oyster (*Pleurotus. ostreatus*) mushroom powders. Birds were given free access to feed and water during the 84 days of the experimental period. Each replicate was housed in separate stainless floor pens under controlled temperature and light conditions. Each pen was 100 × 100 cm. The lighting cycle was 23 h/day maintained at all growth times. The diets were formulated to meet the nutrient requirements of poultry as recommended by the National Research Council (NRC, 1994). Table 1 presents the ingredients and the composition of the basal diets fed in a mash form.

Preparation of mushroom diet

Fresh fruiting bodies of mushrooms were obtained from mushroom producers. The mushroom powders were obtained from oven dried mushrooms. The whole mushrooms were dried out at 60°C for 12 h

Table 1. Composition of experimental diets of female Japanese quails with or without mushroom powder (%)

Ingredients	Control	0.5% *Agaricus bisporus*	1% *Agaricus bisporus*	2% *Agaricus bisporus*	0.5% *Pleurotus ostreatus*	1% *Pleurotus ostreatus*	2% *Pleurotus ostreatus*
Yellow corn	53.31	52.00	52.00	52.08	52.00	52.00	52.18
Soybean meal	39.69	39.00	38.75	39.50	39.00	38.78	39.60
Corn gluten meal	3.07	4.20	4.00	2.50	4.20	4.00	2.50
Vegetable oil	1.00	1.38	1.33	0.98	1.38	1.30	0. 80
Oyster mushroom	-	0.50	1.00	2.00	-	-	-
Commom mushroom	-	-	-	-	0.50	1.00	2.00
Oyster shell	1.22	1.22	1.22	1.22	1.22	1.22	1.22
Di calcium phosphate	0.77	0.77	0.77	0.77	0.77	0.77	0.77
L-lysine	0.06	0.06	0.06	0.06	0.06	0.06	0.06
DL-methionine	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Mineral-vitamin premix*	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sodium chloride	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Calculated analysis							
ME (Kcal/Kg)	2900	2900	2900	2900	2900	2900	2900
CP (%)	24.00	24.00	24.00	24.00	24.00	24.00	24.00
Calcium (%)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Phosphor (%)	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Sodium (%)	0.11	0.11	0.11	0.11	0.11	0.11	0.11
Lysine (%)	1.30	1.30	1.30	1.30	1.30	1.30	1.30
Methionine + Cycteine (%)	0.89	0.89	0.89	0.89	0.89	0.89	0.89

*Supplemented for kg of the diets: Vit. A, 12000 IU; D3, 2000 IU; E, 20 mg; K3, 3 mg; B2, 7 mg; B3, 12 mg; B5, 3 mg; B12, 0.03 mg; Biotin, 0.1 mg; Choline chloride, 300 mg; Mn, 130 mg; Fe, 70 mg; Zn, 60 mg; Cu,12 mg; I,1 mg;Se, 0.2 mg, and adequate antioxidant.

and were added to the experimental diets of chicks after carefully grinding. After drying, fruiting bodies were milled to a powder approximately less than 1 mm in particle size using a cyclotec grinder (Tecator, Hoganas, Sweden). The chemical composition of *Pleurotuso streatus* and *Agaricus Bisporus* powders was determined by the standard AOAC (Association of Official Analytical Chemists) methods outlined by Conniff (1995), as shown in Table 2.

Data Collection

At day 84 of age, the feed consumption and egg weight of each pen were used to calculate the feed conversion ratio (FCR) and some laying performance values (egg production percentage and egg mass).

At day 84 of age, two birds from each pen picked out randomly were killed by cervical dislocation in a germ-free isolation chamber sterilized by ultraviolet radiation (Fallah et al., 2016). The caecum was then removed from each bird, and the fresh excreta of the caecum were gently squeezed and carefully collected in sterilized 25-mL tubes, each tube containing pooled excreta for 5 birds (per pen). Three grams of fresh caecal contents were diluted with 10 mL of distilled water and vortexed before pH 6 and viscosity 7 were measured.

One gram of a wet sample was diluted with 10 mL of sterilized distilled water, of which 1 mL was transferred into 9 mL of sterilized distilled water. The samples were serially diluted from 10^{-1} to 10^{-7}. One-tenth milliliter of each diluted sample was plated on the appropriate medium for enumeration of microbial populations. Bacterial counts were performed using the appropriate dilution and plate culture techniques under aerobic or anaerobic conditions according to Barnes and Impey (1970). The results were expressed as colony forming unit's $\log_{10}$ per gram of fresh sample. The bacterial groups and species determined included total aerobes (nutrient blood agar), *Lactobacilli* (citromalic acid-enriched MRS agar), *Escherichia coli* (MacConkey agar), and *bifidobacteria* (*bifidobacterium* agar composed of tomato juice, 400 mL; dissoluble amylum, 0.5 g; peptone, 15 g; yeast extract, 2 g; glucose, 20 g; sodium chloride, 5 g; Tween-80, 1 mL, 5% cysteine, 0.5 mL; liver extract, 80 mL; agar powder, 20 g; and distilled water, 520 mL; pH = 7.0, at 37°C for 72 h).

Collection of intestinal tissue samples

At day 84 of age, two birds per replicate were randomly chosen, based on the average weight of the group and slaughtered, and the digestive tract was carefully excised. After removing the intestinal contents, approximately 5 cm lengths on 10%, 50% and 90% of the jejunum (the mid point of the jejunum) were removed for gut morphological measurements. The intestinal samples were immersed in formalin, before fixation in Bouin's solution and paraffin embedding. The samples were then transferred into 70% ethanol after 24 h.

Histology of the jejunum

Histological examinations were carried out according to the method of Iji et al. (2001).

Statistical analysis

Data were statistically analyzed using the general linear model (GLM) procedure of SAS (2005). The test of significance for the differences between the means of each classification was done by the Duncan multiple range test (Duncan, 1955).

Results and Discussion

Performance parameters

Egg weight, feed intake, egg mass and feed conversion ratio at 84 days of age were not significantly influenced by the supplementation of mushrooms at day 84 (Table 3). The results of the current study were in line with the findings of Cho et al. (2010) who conducted an experiment with a 5% to 15% fermented spent mushroom substrate and found no effects on egg production, egg weight, egg mass, and feed conversion ratio. Uuganbayar et al. (2005) reported a decrease in egg weight and egg mass when layers were fed a 0.5% green tea supplemented diet.

Increased egg production was also reported by the supplementation of herbs (Awadein et al., 2010). However, Park et al. (2010) found a linear increase in egg weight and egg mass. Data from previous studies summarized by Windisch et al. (2008) suggested that the effects of phytogenic products on production performance of poultry vary widely with respect to botanical origin, processing procedure, composition, as well as animal species, animal age, and environmental hygiene.

Table 2. Proximate analysis of common and oyster mushroom powders

Component	Oyster mushroom *(Pleurotus ostreatus)*	Common mushroom *(Agaricus bisporus)*
ME (Kcal/kg)	1898	1843
Moisture (%)	7.01	15.11
Ash (%)	6.55	12.18
Ether extract (%)	2.3	2.5
Crude protein (%)	21.86	23.21
NFE (%)	62.28	47

Morphometric analysis of the jejunum

Crypt depth and villus height of female quails given the common (*Agaricus bisporus*) and oyster (*Pleurotus ostreatus*) mushrooms at the level of 2% were significantly higher than from the quails in the control group (Table 4). In other trial in broilers, Giannenas et al. (2010) reported that the use of *Agaricus bisporus* mushroom did not produce any significant effect on villus height and crypt depth. But other trials in turkey poults showed a villi height increased by *Agaricus bisporus* mushroom supplementation in all the intestinal section; however, the use of mushroom did not have any effect on crypt depth (Giannenas et al., 2011). An addition of probiotics in broiler diets caused an increase in villus height in the ileum (Nava et al., 2001).

Intestinal microflora composition

The effect of various levels of mushrooms on total counts (aerobes), *Bifidobacteria*, *Lactobacilli* and *Escherichia coli* populations is shown in Table 5.

Total counts of microflora (aerobes) and *Escherichia coli* populations from female quails given the mushroom common (*Agaricus bisporus*) and oyster (*Pleurotus ostreatus*) 2% diet were significantly lower than the respective population values for quails in the control group. *Bifidobacterial* populations from female quails given the 2% common (*Agaricus bisporus*) and oyster (*Pleurotus ostreatus*) mushroom diets (treatments 4 and 6) were significantly higher than the respective population values for quails in the control group (treatment 1); and for treatments 3 (1% *Agaricus bisporus*), 5 and 6 (0.5% and 1% *Pleurotus ostreatus*) were also significantly varied ($P < 0.05$), but not in treatment 2 (0.5% *Agaricus bisporus*).

Bifidobacteria are major species components of the chicken gut microflora (Mead, 1987) that may quantitatively and qualitatively influence the intesti-nal microflora. The results of the current study were in line with the findings of Mahfuz et al. (2020) who stated that supplementing *Agaricus bisporus* and *Pleurotus ostreatus* in broiler diets led to an increase in *bifidobacteria* and *lactobacilli* in the intestine.

Lactobacilli populations from female quails given the 2% common (*Agaricus bisporus*) and

Table 3. Effects of mushrooms on egg weight, feed consumption, egg production, egg mass and feed conversion ratio of female Japanese quails (day 84)

Treatments	Egg weight (g)	Egg production percentage	Feed consumption (g/day/per bird)	Egg mass (g)	Feed conversion ratio
Control	10.75	76.70	27.13	8.24	3.29
Agaricus bisporus 0.5%	10.43	76.14	27.00	7.94	3.41
Agaricus bisporus 1%	10.79	78.74	27.00	8.48	3.18
Agaricus bisporus 2%	10.87	76.40	27.07	8.28	3.26
Pleurotus ostreatus 0.5%	10.86	78.33	28.40	8.49	3.34
Pleurotus ostreatus 1%	10.88	68.33	26.78	8.30	3.23
Pleurotus ostreatus 2%	10.83	78.55	27.11	8.51	3.19
SEM	0.27	2.37	0.57	0.29	0.14
P value	0.9128	0. 1954	0.5338	0.8182	0.9008

Table 4. Effects of dietary supplementation of mushrooms on villus height and crypt depth in different parts of the jejunum at day 84 of age in female Japanese quails (μm)

Treatments	Crypt depth (10% jejunum)	Crypt depth (50% jejunum)	Crypt depth (90% jejunum)	Villus height (10% jejunum)	Villus height (50% jejunum)	Villus height (90% jejunum)
Control	64.30^{cd}	61.37^{c}	54.56^{d}	280.10^{c}	226.42^{b}	135.26^{d}
Agaricus bisporus 0.5%	65.31^{cd}	61.93^{c}	54.80^{d}	280.63^{bc}	223.87^{d}	137.07^{cd}
Agaricus bisporus 1%	64.50^{b}	63.16^{b}	55.51^{abc}	281.74^{ab}	225.60^{bc}	137.81^{cd}
Agaricus bisporus 2%	67.13^{ab}	63.93^{ab}	57.20^{b}	283.27^{a}	228.33^{a}	140.85^{ab}
Pleurotus ostreatus 0.5%	65.17^{d}	62.01^{c}	54.91^{d}	281.21^{bc}	224.50^{cd}	137.48^{cd}
Pleurotus ostreatus 1%	65.54^{bc}	64.14^{a}	56.41^{bc}	281.19^{bc}	225.67^{bc}	138.48^{bc}
Pleurotus ostreatus 2%	69.83^{a}	64.74^{a}	58.41^{a}	283.61^{a}	228.33^{a}	141.70^{a}
SEM	0.68	0.26	0.34	0.48	0.48	0.81
P value	0.0008	0.0001	0.0001	0.0010	0.0002	0.0009

Means with different superscripts in the same column represent significant difference at $P < 0.05$.

Table 5. Effects of dietary supplementation of mushrooms on intestinal microbiology of female Japanese quails at day 84 of age (log_{10} CFU/g)

Treatments	Total counts (Aerobes)	*Bifidobacteria*	*Lactobacilli*	*Escherichia coli*
Control	5.894^{a}	8.559^{cd}	7.552^{c}	6.790^{a}
Agaricus bisporus 0.5%	5.759^{abc}	8.527^{d}	7.707^{c}	6.670^{bc}
Agaricus bisporus 1%	5.647^{cd}	8.765^{bc}	7.930^{bc}	6.572^{d}
Agaricus bisporus 2%	5.500^{d}	9.217^{a}	8.492^{a}	6.140^{e}
Pleurotus ostreatus 0.5%	5.838^{ab}	8.772^{bc}	7.585^{c}	6.736^{ab}
Pleurotus ostreatus 1%	5.690^{bc}	8.939^{b}	7.887^{bc}	6.587^{cd}
Pleurotus ostreatus 2%	5.585^{cd}	9.155^{a}	8.293^{ab}	6.192^{e}
SEM	0.0535	0.0636	0.1268	0.0276
P value	0.0116	0.0006	0.0076	0.0001

Means with different superscripts in the same column represent a significant difference at $P < 0.05$.

oyster (*Pleurotus ostreatus*) mushroom diets were significantly higher than the respective population values for quails in the control group.

Quails given the 2% common (*Agaricus bisporus*) and oyster (*Pleurotus ostreatus*) mushroom diets had the lowest intestinal *Escherichia coli* population count when compared with population values for quails in the control group. Guo et al. (2004b) indicated that mushroom and herb polysaccharide extracts stimulated beneficial bacteria (*bifidobacteria* and *lactobacilli*) while reducing the number of harmful bacteria. Willis et al. (2009) reported an increase in *bifidobacterial* populations and a reduction in *Salmonella* from broilers given the mushroom extract. The mechanism by which mushrooms stimulate *bifidobacterial* growth and survival in the gut of chickens is not known. However, it is sufficient to assume that the shiitake mushroom extract, which is known to be rich in β-glucans (polysaccharides), has components that may either act as quality and specific nutritional factors or create a buffered physical chemical environment in which *bifidobacteria* can survive and multiply compared with the levels in control samples.

Conclusion

Our results clearly demonstrate that tested mushrooms have greater abilities to decrease the fecal *Escherichia coli* population count. Also, the results of this study showed that *Bifidobacterial* and *Lactobacilli* population counts increased by supplementing diets with mushrooms. Mushroom powders exhibit potential benefits because they seem to stimulate health-enhancing *bifidobacteria*, thereby competitively reducing the *Escherichia coli* population. Mushrooms also cause an increase in crypt depth and villus height in male and female quails. This study shows that mushrooms could be helpful in the fight against pathogenic organisms colonizing and increase villus height of quail chicks. In an overall conclusion, the mushrooms could be a beneficial supplement in Japanese quail diet.

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A One Health View over Environment Contaminants in Wild Otter Populations

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Abstract. *In the 20th century, wild otter populations declined across the world. This decline was associated with several factors, such as habitat destruction and hunting. Other underlying causes were anthropogenic contaminant loads in their food and water sources. Aquatic mammals such as otters are very susceptible to chemical contamination and, as top predators, can be considered sentinel species for the health of the ecosystem. This brief review aims to show the presence of environmental contaminants in different otter species and how they may have contributed to the population decline of these species. Although many studies have been carried out on the presence of these compounds in otters, in the future, it is important to continue monitoring these populations to understand their impacts under the One Heath concept.*

Introduction

In the 20th century, American and Eurasian otter species declined. This decline was associated with several factors, such as habitat destruction and hunting. Other causes were the high environmental contaminant load in food and water, heavy metals and persistent organic pollutants in particular (Nelson et al., 2015; Roos et al., 2001). Pollutants such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCB) are examples of lipophilic persistent organic pollutants (POPs). Although they have been banned in many countries for several decades, they are still found in high concentrations in biota (Agostini et al., 2020; Islam and Malik, 2018). These compounds can bioamplify within the food chain, which means that residues not only affect species at the base of the chain, but also spread further up the food chain, reaching maximum concentrations in top predators (Rivera et al., 2021) (Figure 1).

River and sea otters are exposed to pesticides through their diet. Yet, as top predators, they are also particularly prone to the accumulation of pesticide residues that biomagnify with trophic levels (Nelson et al., 2015). Deaths resulting from direct poisoning are rarely reported in these species. However, sublethal effects associated with some compounds have been reported, such as organ failure, diseases (e.g., tumors, cardiovascular diseases, chronic nephropathies, autoimmune diseases), behavioral and reproductive changes (Peterson and Schulte, 2016).

One Health is an approach that recognizes that the health of people is closely linked to the health of animals and the environment (CDC, 2022). It is a collaborative, multidisciplinary approach to understanding the ecology of emerging zoonotic diseases to perform risk assessment and develop response and control tactics otter populations (Mackenzie and Jeggo, 2019). Aquatic mammals such as otters are susceptible to chemical contamination (Murata et al., 2008). As top predators, they can be considered sentinel species for the health of the ecosystem, as they interact strongly with the biotic and abiotic components of their habitat (Peterson and Schulte, 2016). Studying the concentration of environmental contaminants in otters can provide researchers with information about temporal and

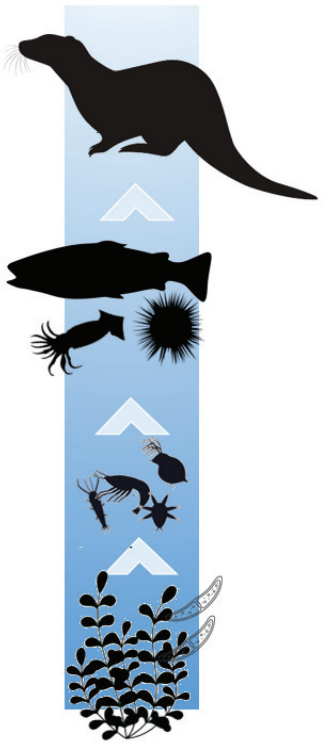

Fig. 1. Example of biomagnification of environmental contaminants moves further up in the food chain

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spatial changes in ecosystem contamination and contribute to the assessment of potential risks to human health due to dietary coincidences coupled with continuous exposure to different contaminated matrices (Rivera et al., 2021).

In this short review, we present just a few examples of some of the most significant works on environmental pesticides in some otter species, both aquatic and marine.

Otters and anthropogenic environmental contaminants

Otters are carnivorous mammals from the subfamily Lutrinae (Kruuk, 2006). Thirteen different species of otter can be found on every continent except Australia and Antarctica. These species are semiaquatic, aquatic or marine and their diets are based on fish and invertebrates (Kruuk, 2006). According to the IUCN Red List of Threatened Species, 11 out of 13 species are threatened (The IUCN Red List of Threatened Species, 2023).

Many substances have been studied in recent years in this species. In Table 1, we present some of the examples described in this review, the group and its impact on vertebrates (Harley et al., 2019; Hart et al., 2009; Kannan and Perrotta, 2008; Murata et al., 2008, 2008).

Environmental pollutants can be dispersed through the air, penetrate the soil, enter bodies of water, or be absorbed by plants and animals (Agostini et al., 2020; Baker et al., 2013; Chaturvedi et al., 2013). Their fate in the ecosystem depends on the physical and chemical properties of the compound, as well as environmental conditions. Their physical and chemical properties determine soil mobility, water solubility and volatility. Once released into the environment, these compounds can be broken down by exposure to sunlight (photolysis), exposure to other chemicals (oxidation and reduction), microbial activity (bacteria, fungi, and other microorganisms), plants or animals (metabolism), and exposure to water (hydrolysis) (Ames, 1992; Fry, 1995; Islam and Malik, 2018). Figure 2 shows the routes by which pesticides can enter the environment, their path through the food chain and how otters can be affected.

Sea otter

Table 2 presents some studies regarding environmental contaminants (pesticides and others) in sea otters (*Enhydra lutris kenyoni and Enhydra lutris nereis*). Table 3 shows some examples of heavy metals and metalloids in sea otters.

River otter

Table 4 presents some studies regarding environmental contaminants (pesticides and others) in otters (*Lutra lutra, L.canadensis, L. longicaudis*). Table 5 presents some examples of heavy metals and metalloids.

Implications about environment contaminants, otter population and One Health concept

Knowledge about the presence and concentration of environmental contaminants is essential in the One Health context. These studies acquire even more importance in species that do not share the same habitat as humans, but which, with greater or lesser proximity, suffer the effects of pollution irreparably. Otters, as semi-aquatic or marine animals, can be affected by contaminants in terrestrial and aquatic ecosystems, providing essential data on their health and sources of habitat contamination (Jessup et al., 2004).Therefore, they can be considered a good indicator of pollution, being a sentinel species (Harding, 1999; Jessup et al., 2004). However, several knowledge gaps remain in ecotoxicological studies on this species, as these animals are often ignored and neglected during epidemiological surveillance.

The presence of anthropogenic contaminants in the environment appears to be associated with the decline of some otter populations (Harding, 1999; Huang et al., 2018), particularly in areas where the use of components such as pesticides has been or continues to be very high (South America, India, North America) (Elliott et al., 2008; Halbrook et al., 1996). In this brief review, it is also possible to see that the otter with higher levels of contamination live in the areas that are more industrialized areas such as Europe or North America.

An example that illustrates the influence of environmental contaminants on the decline of otters is the case of PCB in Sweden. It was first suggested that PCBs were an important factor in the decline of the European otter in Sweden when a comparison was made between PCB content in the tissues of Swedish and Norwegian otters. Concentrations were highest in southern Sweden and lowest in northern Norway. These differences were correlated with differences in the status of the otter population (census taken 1975–1977). The census showed that Swedish otters in the north of the country were thriving, while otter populations in the south were declining. Sandegren et al. (1980) pointed out that the PCB concentration in these otters was greater than 50 mg/kg of lipids, a level that had already been associated with infertility in mink (Harley et al., 2019; Hart et al., 2009; Kannan and Perrotta, 2008; Murata et al., 2008, 2008; Roos et al., 2001).

Other factors such as habitat destruction, hunting for fur and climate change have also had a huge influence on the reduction of these populations. For example, Enhydra lutris populations in the Aleutian archipelago declined by 75% between 1965 and 2000 (Doroff et al., 2003), not only due pollution but also due to hunting.

Despite the confirmed presence of pesticides in otter tissues, some studies (Basu et al., 2007; Grove and Henny, 2008; Huang et al., 2018) show that these

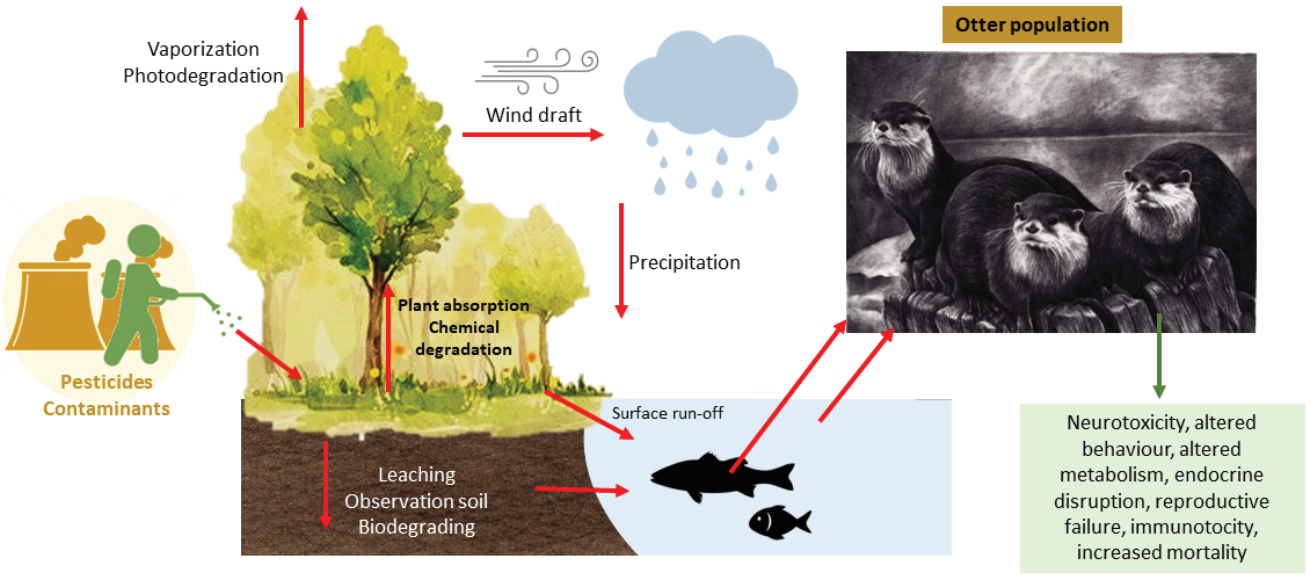

Fig. 2. Schematic representation of pesticide dispersion in the ecosystem and how they affect otter populations

Table 1. Examples of pollutant compounds and their impact in vertebrates

Compound	DL50	Characteristic	Effects in vertebrates	Ref.
Heavy metals and metalloids	Pb (50 µg/L), Cd (5 µg/L), Cr (50 µg/L), Zn (5000 µg/L), Cu (1500 µg/L), Co (10 µg/L), and Ni (100 µg/L)	Metallic chemical element with a relatively high density and toxic at low concentrations	Mutagenicity, teratogenicity, carcinogenicity; induced poor body conditions, reduced reproduction rate, immunosuppression	(Balali-Mood et al., 2021)
Perfluorooctanesulfonate (PFOS)	5.2 mg/L	Belongs to the synthetic chemicals perfluoroalkyl and polyfluoroalkyl with repellent properties	Infertility, cancer, preeclampsia, preterm labor, low birth weight gestational diabetes, thyroid and kidney disease, hypercholesterolemia	(Huang and Jaffé, 2019)
Dichlorodiphenyltrichloroethane (DDTs)	0.5 mg/m^3	It is an organochloride used as an insecticide.	Cancer, immunosuppression, teratogenic, toxic, seizures, liver disease	(Kabasenche and Skinner, 2014)
polybrominated diphenyl ethers (PBDEs)	0.5–5 g/kg	Synthetic chemicals used in plastics, textiles and electrical/electronic equipment to make them less flammable.	Cancer, teratogenic	(Palacio-Cortés et al., 2017)
Polycyclic aromatic hydrocarbons (PAHs)	0.2 mg/m^3	Class of organic compounds produced by incomplete combustion or high-pressure processes	Toxic, carcinogenic, pulmonary and gastrointestinal disease, infertility	(Boehm, 1964)
Organochlorine pesticides (OCs)	50 mg/kg	Synthetic chlorinated hydrocarbons used as insecticides	Toxic, effects in the nervous system, cancer, teratogenic, infertility, immunosuppression	(Mason and Macdonald, 1994)
Polychlorinated dibenzofurans (PCDFs)		Organic compounds with one or several of the hydrogens in the dibenzofuran structure replaced by chlorines used as pesticides	Severe skin lesions, altered liver function and lipid metabolism, weight loss, depression of the immune system, and endocrine alterations	(Muir et al., 1996)

Table 2. Papers that evaluated environmental contaminants in sea otters *(Enhydra lutris kenyoni* and *Enhydra lutris nereis)* regarding the number of animals, substance type, year, sample type analyzed, country, and compound concentration in the tissue. Results are in lipid weight (l.w.) or wet weight (w.w.).

Species	Substance*	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Enhydra lutris kenyoni	PFOS, PFOSA, PFNA, PFOA, PFUnDA, PFDA	Liver	Alaska	1992–2007	0.9 to 8 ng/g w.w.	(Hart et al., 2009)
	CHLs, PCBs, DDTs, PBDEs	Liver	Alaska	1992–2010	PCBs 262,000 ng/g l.w., DDTs 8,800 ng/g l.w., PBDEs 4,600 ng/g l.w.	(Harley et al., 2019)
Enhydra lutris nereis	PCBs, DDTs, HCHs, chlordane, HCB	Liver, kidney, brain	California	1992–1996	PCBs 58±8700 and DDTs 280±5900 ng/g w.w.	(Nakata et al., 1998)
	OTs- mono- to tributyltin, -phenyltins, and -octyltins	Liver	California, Washington, Alaska, Russia	1992–2002	34 to 4100 ng/g w.w.	(Murata et al., 2008)
	POPs	Liver	California	2000–2005	DDTs- 635 ng/g l.w., PCBs 177 ng/g l.w., PBDEs 48.1 ng/g l.w.,	(Miller et al., 2007)
	PBDEs, PCBs	Liver	California	1992–2002	PBDEs 10–26,800 ng/g and PCBs 81–210,000 ng/g l.w.	(Kannan et al., 2007)
	PAHs	Liver	California	1992–2002	588–17 400 ng/g l.w.	(Kannan and Perrotta, 2008)
	OCs, PCB, butyltins	Liver	California	1992–1996	PCB 8,700 ng/g w.w., DDT 5,900 ng/g w.w.	(Kannan et al., 2004)
	POPs, COECs	Blood	Alaska, California	1997–1998	PCBs 705 ng/g l.w., DDTs 145 ng/g l.w., HCHs 145 ng/g l.w., CHL 135 ng/g l.w.	(Jessup et al., 2010)
	OCs, PCBs, including non–ortho PCBs, PCDDs, PCDFs	Liver	Alaska, California, Aleutian	1998–1992	Dichlorodiphenyltrichloroethane concentrations in California otters 850 mg/kg w.w., Aleutian otters 40 mg/kg w.w. and Alaska 1 mg/kg w.w. PCBs in Aleutian otters 310 mg/kg w.w., California otters 190 mg/kg w.w., and Alaska otters 8 mg/kg w.w.	(Bacon et al., 1999)
	PFOS, PFOA	Liver	California	1992–2002	PFOS < 1, PFOA < 5–147 ng/g w.w.	(Kannan et al., 2006b)

*(Perfluorooctanesulfonate (PFOS), perfluorooctanesulfonamide (PFOSA), perfluorooctanoate (PFNA), perfluorooctanoate (PFOA), perfluoroundecanoate (PFUnDA), perfluorooctanoate (PFDA), chlordane (CHLs), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDTs), polybrominated diphenyl ethers (PBDEs), hexachlorocyclohexanes (HCHs), Hexachlorobenzene (HCB), organotin compounds (OTs) - mono- to tributyltin, -phenyltins, and -octyltins, persistent organic pollutants (POPs), polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCs), and polychlorinated dibenzofurans (PCDFs).

Table 3. Papers that evaluated environmental contaminants in sea otters *(Enhydra lutris* and *Enhydra lutris nereis)* regarding the number of animals, substance type, year, sample type analyzed, country, and compound concentration in the tissue. Results are in dry weight (d.w.) or wet weight (w.w.).

Species	Substance*	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Enhydra lutris	As, Cd, Cu, Pb, THg, Se	Brain, kidney, gonad, liver, and stomach contents	Alaska	2021	As 0.05 to 3.01 mg/kg w.w., Cd 81.10 mg/kg .w., Cu 0.83 to 38.30 mg/kg w.w., Pb 0.01 to 6.47 mg/kg w.w., THg 0.01 to 2.26 mg/kg w.w., Se 0.70 to 7.83 mg/kg w.w.	(Brown et al., 2021)
	Hg	Fur	Russia	2003–2019	5.4 µg/g d.w.	(Ryazanov et al., 2023)
Enhydra lutris nereis	Cu, Zn, Cd, Hg, Pb	Liver	California	1992–2002	Zn 95.0–542 µg/g w.w., Cd and Hg > 15 µg/g w.w., Pb 0.019 to 1.06 µg/g w.w., Cu 5–50 µg/g w.w., Cd 0.5–25 µg/g w.w.	(Kannan et al., 2006a)
	Se, Al, Cu, Zn, Cd	Liver	California	1991 and 2002	Se > 3 ppm d.w., Al < 5.0 to 198 ppm d.w., Cu 15.3 to 191 ppm, d.w. Zn 00 to 258 ppm, d.w., Cd 6.03 –2.03 ppm w.w.	(Brancato et al., 2009)

*Arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), total mercury (THg), selenium (Se), mercury (Hg).

have been decreasing over time. This phenomenon can be associated with the fact that mitigation measures have been imposed to reduce these compounds in agriculture, veterinary and industry worldwide (OA US EPA, 2013; OP US EPA, 2013). Furthermore, as some species have an endangered conservation status (The Habitats Directive and Habitats Regulations, 2023), in some regions, otter populations have been recovering (Gutleb et al., 1998; Kruuk and Conroy, 1996). In 1998, the otter population in Netherlands became extinct. In 2002, a program for re-introducing otters was been implemented and the number increased to 360 otters in 2018 (Nature and Form, 2019)

The impact of these contaminants on the otter population is variable and depends on many factors, such as environmental conditions and concentrations of contaminants. Neurotoxicity, changes in behavior and metabolism, endocrine disruption, reproductive failure, immunotoxicity, neoplasia and mortality in extreme cases are some of the effects associated with these compounds (Köhler and Triebskorn, 2013). As some of these compounds will bioaccumulate in tissues in small quantities, the damage caused to animals can only be observed after a long period (years) (Ames, 1992; Köhler and Triebskorn, 2013). Some of these effects are subclinical, such as immunosuppression or decreased fertility, which are not easily detected without a prolonged study of the population. These effects have already been observed in humans and other animal populations with shorter lives (Brander et al., 2016; Chaturvedi et al., 2013; Damásio et al., 2010).

Conclusion

Environmental contaminants are a problem for wildlife and humans. These compounds are present in otter habitats and otters are susceptible to their presence. Years of overuse and abuse of these compounds are taking their toll on otters, directly and indirectly contributing to their decline.

In the future, it is important to develop One Health projects that include otters as sentinels of pollution, climate change and other anthropogenic threats. Within the scope of the One Health system, we can identify threat factors, understand their impact, establish measures to prevent or minimize their consequences and predict the emergence or re-emergence of diseases in different ecosystems, particularly aquatic ecosystems.

Table 4. Review of papers that evaluated environmental contaminates in river otters (*Lutra lutra, Lontra canadensis, Lontra longicaudis*) regarding the number of animals, substance type, year, sample type analyzed, country, and compound concentration in the tissue. Results are in lipid weight (l.w.) or wet weight (w.w.).

Species	Substance	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Lontra longicaudis	2,4-D, acetochlor, ametrine, atrazine, carbendazim, carbofuran, diazinon, dimethoate, emamectin, glyphosate, imazalil, λ-cyhalothrin, malathion, methomyl, metoxuron, molinate, parathion, picloram, pyraclostrobin, thiabendazole	Feaces	Mexico	2018–2019	Acetochlor 21.4, Ametrine 216.2, Diazinon 100.1, Emamectin 56.4, Glyphosate 373.2, Imazalil 3544.5, λ-cyhalothrin 75.4, Malathion 824.4, Methomyl 31.6, Molinate 64.1, Picloram 1280.7 μg kg^{-1} l.w.	(Rivera et al., 2021)
Lutra lutra	OCs	Muscle, liver, pericaudal fat	France	1987–1994	0 to 5.71 mg kg-1 l.w.	(Thome et al., 1995)
	PCB, DDE	Muscle	Sweden	1974–2004	DDE 1.4–970 mg s l.w., PBC 0.0–24 mg l.w.	(Roos et al., 2001)
	PCB	Muscle	Sweden	1970–2010	70 to 8 mg/ kg l.w.	(Roos et al., 2012)
	PCB, DDT	Muscle	Sweden	1968–1999	PCB 44±860 mg g^{-1} l.w., DDT 1.2 ± 27 mg g^{-1} l.w.	(Roos et al., 2001)
	PBDEs, PCBs, DDT	Liver	England and Wales	1995–2006	12–70 000 ng g^{-1} l.w.	(Pountney et al., 2015)
	OCs, PCBs	Tissue, faeces	Ireland	1991	p,p-DDE 1.61mg l.w., dieldrin 1.07mg l.w., PCBs 2.22nmg kg-1 l.w.	(O'Sullivan et al., 1993)
	Aldrin, chlordane, DDE, DDD, DDT, dieldrin, a-endosulfan, b-endosulfan, endrin, HCB, heptachlor, isodrin, methoxychlor, mirex, PCBs	Liver	Spain	2004–2006	PCB 3873–5426 ng g^{-1} l.w.	(Mateo et al., 2012)
	PCB, OCs	Faeces	England	1989–1991	Lindane 11.7 mg.kg^{-1}, 0.4 mg.kg^{-1} dieldrin, 22.3 mg.kg^{-1} p,p-D, 60.51 mg.kg^{-1} PBC	(Mason and Macdonald, 1994)
	PCB, OCs	Faeces	Scotland	1990–1991	9.4-19.5 mg kg^{-1} l.w.	(Mason et al., 1992)
	PCB, OCs	Liver	France	2004–2008	organochlorine pesticides 9.4 mg kg^{-1} l.w., PCBs 64.8 mg kg^{-1} l.w.	(Lemarchand et al., 2010)
	PCB, OCs	Faeces	France	2004–2005	2.72 mg kg^{-1} l.w. OC, 13.58 mg kg^{-1} l.w. PCBs	(Lemarchand et al., 2007)
	PCB, OCs	Liver	Scotland	1987–1992	PCB 14 ppm l.w.	(Kruuk, 2006)

Table 4 (cont.)

Species	Substance	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Lutra lutra	PCBs, OCPs	Liver	Italy	2014–2019	0.010 mg/kg to 4.99 mg/kg, OCPs and PCBs, IDs 1.59 mg/kg, 0.170 mg/kg, 1.09 mg/kg, and 2.64 mg/kg	(Esposito et al., 2020)
	OCs	Liver	France	2013–2014	1.96 mg/kg l.w.	(Alomar et al., 2016)
	PBDEs	Liver	England	1985–2005	BDE 47 ng/g w.w., BDE 153 ng/g w.w., BDE 100 ng/g w.w., PBDEs 3–718 ng/g w.w.	(Walker et al., 2013)
	PCB	Feaces, liver	Ireland	1984–1990	livers 0.18-1 23.2 mg kg^{-1} l.w., faeces 0.21–18.22 mg kg^{-1} l.w.	(Mason, 1989)
	Dieldrin, DDE, PCBs, lindane	Faeces	England	1981–1991	Lindane 0.39 ± 0.08 mg kg^{-1} l.w., DDE 1.61 4. 0.21 mg kg^{-1} l.w., PCBs 2,22 4. 0.54 mg kg^{-1} l.w., dieldrien 1,07 4. 0.16 mg kg -1 l.w.	(Mason and Macdonald, 1993a)**1993a**
	PCBs, DDE, DDT, lindane, Dieldrien	Liver, tissue	Denmark	1980–1990	Lindane 0.81 mg kg^{-1} lipid, Dieldrin 1.10 mg kg^{-1} l.w., DDE 2.88 mg kg^{-1} l.w., PCBs 16.76 mg kg^{-1} l.w.	(Mason and Maden, 1993)
	Perfluoroalkyl sulfonic acids (PFSAs),	Liver	Wales, England	2007–2008	PFAS 109 - 7652 µg/kg w.w.	(O'Rourke et al., 2022)
	DDE, PBCs	Liver	England, Wales	1992–2009	7660 µg kg^{-1} l.w., DDE 15.2 to 7868.6 µg kg^{-1} l.w.PCBs	(Kean et al., 2021)
	PCB	Muscle	Norfolk	1984	433 and 75 mg/kg l.w.	(Keymer et al. 1988)
	Lindane, dieldrin, DDE, PCBs	Faeces	Wales	1989–1992	Dieldren 1.19- 3.68 mg kg-I, DDE 2.12- 6.03 mg kg^{-1}, PCB 4.96- 7.99 mg kg-I l.w.	(Mason and Macdonald, 1993b)
	PCB	Faeces	England	1985– 1991	0.21 mg kg -1 l.w.	(Mason and Macdonald, 1993c)
	Dieldrin, DDE, PCBs, DDT	Muscle, Liver	Ireland	1984–90	Dieldrin 0.08-16.01 mg kg^{-1}, DDE 0.05–25.70 mg kg^{-1}, PCB 0.18–123.24 mg kg-I, DDT 0.07–44.47 mg kg^{-1} l.w.	(Mason and O'Sullivan, 1992)
	PCB	Faeces	Scotland	1992	19.5 mg kg^{-1} l.w.	(Mason et al., 1992)
	PCBs, HEOD, DDE	Liver	Scotland	1987–1992	DDE 0.12–2.81 ppm l.w., HEOD 0.08–0.28 ppm l.w.	(Kruuk and Conroy, 1996)
	Ocs, PCBs, PCDDs, PCDFs, PBDEs	Liver, testes	Oregon and Washington	2005–2007	PCB 540 ng/g w.w.,, PCDDs 172 to 2,783 pg/g w.w., PCDFs 1.50 to 2,719 pg/g w.w., PBDEs 0.82 to 436 ng/g w.w., DDT 0.71–2.20 ng/g w.w., Dieldrin 6.43–22.2 ng/g w.w., endrin 0.92–3.97 ng/g w.w, chlorpyrifos 0.62–1.50 ng/g w.w., aldrin 0.46-1.52 ng/g w.w.,	(Stansley et al., 2010)

Table 4 (cont.)

Species	Substance	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Lontra canadensis	PCB, HCB BHC, HE, Chlordane, Aldrin, Dieldrin, Endrin, DDT, DDE, DDD, Mirex, Pentachlorobenzene, Toxaphene, Methoxychlor	Liver, Fat tissue	Alberta	1980–81 to 1982–83	PCB 0.0130(0.014) ng/g w.w. chordane 0.001(0.001) ng/g w.w. DDE 0.002(0.001) ng/g w.w	(Somers et al., 1987)
	PBDEs	Feaces, blood	Vancouver Island	2009–2010	BDE-47 blood samples 0.37 lg/g l.w., BDE-206 0.18 lg/g v, BDE-47 0.16 lg/g l.w., scat samples, BDE-47 in blood 0.82 mg/kg l.w., scat 0.26 mg/kg l.w., BDEs blood 1.12 lg/g l.w., and scat 0.35 lg/g l.w.,	(Nelson et al., 2015)
	PCB, PBDEs, Ocs	Faeces, blood	Vancouver Island	2009–2010	PCB 0.713 (0.0903-11.1) mg/kg l.w., OCP 0.214 (0.0240–0.640) mg/kg l.w., PBDE 0.146 (0.0200–1.31) mg/kg l.w.	(Huang et al., 2018)
	Ocs, PBC	Liver	British Columbia	1995–1996	DDE 110 ng/g w.w., PCBs 120 ng/g w.w.,	(Harding et al. 1999)
	PHAH, OCPs, PBDEs	Feaces	British Columbia	2006–2009	PCB 6.0–38.2 mg/kg l.w., DDT 0.13[a] (0.04–0.93) mg/kg l.w., mirex 0.01[a] (0.002–0.26) l.w., PBDE 0.36A (0.04–2.7) mg/kg l.w.	(Guertin et al., 2010)
	Ocs, PCBs, PCDDs, PCDFs	Liver	Oregan, Washington	1994-1996	OCs 100 $\mu g kg^{-1}$ w.w., PCB < 1 $\mu g g^{-1}$ w.w.	(Grove and Henny, 2008)
	PCBs, Ocs, PCDDs, PCDFs, OCDD	Faeces	British Columbia	1991–1992	DDE 0.012.12 mg/kg l.w, HCB: 0.003–0.25 mg/kg l.w, OCDD 120–19,100 ng/kg l.w, PBC 49 – 12.3 mg/kg l.w, TCDD 1500 ng/kg l.w	(Elliott et al., 2008)
	PBDEs	Liver	Wisconsin	2009–2010	PBDE 0.5 to 72.9 ng/g l.w.	(Dornbos et al., 2015)
	OHCs, PCB, DDE	Liver	Illinois	2009–2011	dieldrin 14.4–534 ppb w.w., PCB: 30–3450 ppb w.w.	(Carpenter et al., 2014)
	PCB, OCP, PBDE	Cerebral cortex	Ontario and Nova Scotia	2002–2004	PCBs 70.9 12.1 ng/g l.w., OCPs 21.2 3.7 ng/g l.w., PBDEs were 3.2 0.6 ng/g l.w.	(Basu et al., 2007)
	Ocs, PCBs, PCDDs, PCDFs	Liver	Columbia	1994/95 and 1995/96	DDE 110–3056 ng/g l.w., PCBs 138 - 4615 l.w., 153 5769 ng/g l.w., and 180 7308 ng/g l.w., OCDD a – 1860 pg/g l.w.; b – 6923 pg/g l.w.	(Harding, 1999)
Lontra canadensis	DDT, PCBs, dieldrin, mirex	Muscle, hair, liver	Georgia	1979–80 and 1980 –81	0.08 to 91.90 ppm DDT, Mirex, dieldrin, and PCBs 0.16 to 75.40, 0.03 to 1.26, and 0.57 to 84.20 ppm.w.w.	(Clark et al., 1981)
	PCB, DDE,	Liver	New York	1982– 1984	PCB 114 µg/g l.w.,, DDE 5.14 µg/g, l.w.	(Foley et al., 1988)

Table 4 (cont.)

Species	Substance	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Aonyx capensis, Lutra maculicollis	DDE, dieldrin, PCBs	Faeces	South Africa	1990	Dieldrin 0.30–1.15 ng/g l.w., DDE 0.03-2.31 ng/g l.w., \PCBs 0.09–1.60 ng/g l.w.	(Mason and Rowe, 1992)

*(Organochlorinated pesticides (OCs), dibenzo-p-dioxins (PCDDs), Perfluorooctanesulfonate (PFOS), perfluorooctanesulfonamide (PFOSA), perfluorooctanoate (PFNA), perfluorooctanoate (PFOA), perfluoroundecanoate (PFUnDA), perfluorooctanoate (PFDA), chlordane (CHLs), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDTs), polybrominated diphenyl ethers (PBDEs), hexachlorocyclohexanes (HCHs), Hexachlorobenzene (HCB), organotin compounds (OTs) - mono- to tributyltin, -phenyltins, and -octyltins, persistent organic pollutants (POPs), polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCs), and polychlorinated dibenzofurans (PCDFs).

Table 5. Papers that evaluated environmental contaminants in sea otters *(Lutra lutra, Lontra canadensis, Lontra longicaudis)* regarding the number of animals, substance type, year, sample type analyzed, country, and compound concentration in the tissue. Results are in dry weight (d.w.) or wet weight (w.w.).

Species	Substance*	Sample	Country/city	Year	Compound concentration in the tissue	Ref.
Lutra lutra	Pb, As, Cu, Cd, Hg	Liver	France	2004–2008	Pb 1.0, Cd 0.3 and As 0.1 mg kg^{-1} d.w., Hg 2.1 mg kg^{-1} w.w.,	(Lemarchand et al., 2010)
	Fe, Zn, Al, Mn, Cu, Pb, Cr , Cd	Faeces	South Korea	1994–1996	Cr 0.47–1.38 mg/Kg d.w., Zn 77.5–162.3 mg/Kg d.w, Cd 0.07–0.24 mg/Kg d.w, Cd 0.07–0.24 mg/Kg d.w, Pb 0.9–5.3 mg/Kg d.w, Cu 2.1–8.0 mg/Kg d.w, Fe 924–2451 mg/Kg d.w, Mn 39.5–98.1 mg/Kg d.w, Al 49.7–205.0 mg/Kg d.w	(Han et al., 2002)
	Hg, Cd, Pb, Ca, Zn	Liver	Ireland	1984–1990	Hg 5 mg kg^{-1} w.w, Cd10 mg kg^{-1} w.w., Ca 1 mg kg^{-1} w.w., Zn 100 mg kg-1 w.w.	(Mason and Sullivan, 1993)
	Cd, Pb	Liver	Austrian, Czech Republic, Hungary	1989–1994	Cd 4.6–5.4 μg g^{-1} d.w., Pb 3.5 μg g^{-1} d.w.,	(Gutleb et al., 1998)
	Hg, Cu, Zn, Pb, Cd	Liver	Hungary	2008	Hg 0–29.59 mg/Kg d.w., Cu 0–76.20 mg/Kg d.w., Zn 41.49–368.13 mg/Kg d.w., Pb 0–1.383 mg/Kg d.w.,, Cd 0-1.168 mg/Kg d.w.,	(Lanszki et al., 2009)
	Hg, Cu, Zn, Cd	Liver, kidney, fur	Finland	1986–2000	Hg 1.17–3.54 mg/Kg w.w., Cd 0.004–0.025 V, Cu o.44-7.76 mg/Kg w.w., Zn 19.50–19.91 mg/Kg w.w.,	(Hyvärinen et al., 2003)
	Cu, Zn, Cd, Pb, As	Faeces	Spain	1999–2003, 2006	Cu 21.1–133.0 ppm d.w., Cd 0.9–5.3 ppm d.w., Zn 113.4–672.5 ppm d.w., Pb 0.45-12-8 ppm d.w., As 0.83–84.5 ppm d.w.,	(Mateo et al. 2012)
Lontra longicaudis	Hg, Pb, Cd	Faces	Mexico	2008–2009	Hg0.02–0.17 mg kg^{-1} w.w., Pb 117.87 mg kg^{-1} w.w., Cd 9.14 mg kg^{-1} w.w.	(Ramos-Rosas et al., 2013)
Lontra canadensis	As, Cd, Cu, Co, Fe, Pb, Mg, Hg, Zn	Liver, kidney	California	2009 to 2016	As 0.14 μg/g w.w., Cd 0.2 μg/g w.w., Co 0.02 μg/g w.w., Cu 3.69 μg/g w.w., Fe 152.75 μg/g w.w., Pb 0.07 μg/g w.w., Mg 152.55 μg/g w.w., Hg 1.68 μg/g w.w., Zn 21.53 μg/g w.w	(Sanders et al., 2020)

*Arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), selenium (Se), mercury (Hg), cobalt (Co), zinc (Zn), iron (Fe), magnesium (Mg), calcium (Ca)

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Protein Profiles of Seminal Plasma and their Correlation with Semen Quality in Aceh Bulls (*Bos indicus*)

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Abstract. *The Indonesia Artificial Insemination Center wants the production of frozen semen from Aceh bulls as soon as possible to take advantage of the desired genetics. Genetic selection and breeding soundness examination are required to be accepted in the artificial insemination industry. Therefore, seminal plasma protein analysis and semen quality evaluation have prospective use in the selection of superior bulls. This study aims to characterize the protein profile of seminal plasma in Aceh bulls and to determine its correlation with semen quality. A total of four Aceh bulls (24–34 months) belonging to the Livestock Breeding and Forage Center of Indrapuri were selected randomly. Semen ejaculation was used for the evaluation of semen quality (semen volume, colour, consistency, and pH, mass motility, sperm motility, sperm viability, and sperm morphology). The remaining volume of semen was centrifuged to obtain seminal plasma. Total seminal plasma protein concentration (μg/μL) was calculated using the Bradford method, and then the seminal plasma protein was electrophoresed on SDS PAGE and visualized with Coomassie Blue. The results of protein visualization found 16 protein bands with different molecular weights, ranging from 11 to 180 kilo Dalton. In general, the protein band of 15.24 kilo Dalton was more prominent in Aceh bulls. In addition, the seminal plasma protein concentration showed a positive correlation with sperm motility, sperm viability, and sperm morphology. In conclusion, the seminal plasma protein of Aceh bulls is positively correlated with several semen quality variables and may be a useful as an additional parameter for determining semen quality or bulls fertility.*

Introduction

In Indonesia, there are several local beef cattle whose reproductive performance and production must be improved to be used sustainably for food security. One of the cattle whose performance needs to be improved is the Aceh cattle. Aceh cattle are one of the local beef cattle in Indonesia that have been genetically identified, and research is increasingly being carried out on reproductive efficiency to increase production (Abdullah et al. 2012; Sutarno et al. 2019; Panjaitan et al. 2021). Efforts to increase the reproductive efficiency of Aceh cattle have been carried out through several studies, ranging from follicle dynamics studies (Siregar et al. 2016; Armansyah et al. 2017), application of oestrous synchronization, and artificial insemination (AI) (Hafizuddin et al. 2012; Ramli et al. 2016). However, the reproductive efficiency of Aceh bulls has not been widely reported. Male reproductive efficiency continues to be developed through several studies finding a tool or method of male fertility assessment (Kaya and Memili 2016; Karunakaran and Devanathan 2017; Druart and de Graaf 2018; Hafizuddin et al. 2020). Male fertility is influenced by several factors, including physical condition, semen quality, and biochemical content of seminal plasma (Assumpção et al. 2005; Almadaly et al. 2016).

Semen characteristics, especially the sperm motility variable and sperm morphology, are the main criteria used in the assessment of semen quality. According to Rodrigues et al. (2013) and Boe-Hansen et al. (2015), if the seminal plasma as the microenvironment of spermatozoa cells is evaluated, it has a high potential for assessing semen quality or male fertility. This potential can explain how the characteristics of sperm are related to protein expression in the reproductive tract, both at maturation and during the transport of spermatozoa.

Several studies in mammals have reported the protein profile in seminal plasma and its effect on semen characteristics. Research in ram showed that seminal plasma protein was correlated with sperm motility (Rodrigues et al. 2013), and sperm capacitation (Carballada and Esponda 1998). Nonetheless, there have also been studies reporting negative effects of seminal plasma protein on semen quality (Iwamoto et al. 1995; Schöneck et al. 1996; La Falci et al. 2002). Thus, it is necessary to identify seminal plasma protein and its correlation with several semen quality variables as the main variable for assessing fertility in bulls.

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Materials and methods

Animals

This study used four Aceh bulls (24–34 months) who were randomly selected at the Livestock Breeding and Forage Centre of Indrapuri, Ministry of Agriculture, The Republic of Indonesia. Bulls have a good body condition score with criteria between 3 and 4 on a score scale of 5.

Semen collection

Semen was collected using an artificial vagina, previously dried and cleaned alongside the rubber and reservoir tubes in order to prevent contamination (Sutriana et al. 2022).

Semen quality examination

Semen macroscopic examination

Semen samples were obtained from each animal and analyzed as previously reported by Hafizuddin et al. (2021) unless stated in other references. After collection, the quality was macroscopically evaluated based on volume, colour, consistency, and pH.

Semen microscopic examination

Spermatozoa mass motility

A drop of 5 µL of raw semen was deposited on a pre-warmed glass slide (≈37°C), and the edge of the drop was observed at low magnification (10 × objective) on the thermally controlled stage of a phase contrast microscope. Observations at the edges of the drop provide for assessment of the rapid flogging of black waves and whirlpools on a grey background which is termed as the wave motion or mass sperm motility. This mass sperm motility was scored subjectively from 0 (no motion) to 5 (numerous rapid waves) on a scale with steps equal to 1 (David et al. 2015)

Spermatozoa motility

Spermatozoa motility was evaluated by dripping the sample on a glass slide, then one drop of physiological NaCl was added, followed by observation through a microscope with 40 x 10 magnification. The number of motile sperm was calculated based on the movement, categorized as fast progressive (A), slow progressive (B), circular (C) and vibration (D) (Syafruddin et al. 2020). The percentage was determined using the following formula:

$$\% \text{ Motility} = \frac{A}{A+B+C+D} \times 100\%.$$

Spermatozoa viability

The examination of viability was performed by introducing one drop of spermatozoa on a glass slide, followed by the addition of one staining eosin-nigrosin drop. A smear preparation was made and fixed on a spiritus lamp, then evaluated using a microscope of 40 x 10 magnification. The dead cells absorb a red pigmentation, while the live spermatozoa tend not to absorb any colour, leading to a white appearance. The spermatozoa were then counted and divided by the total visible and presented as a percentage value (Padrik et al. 2010).

$$\% \text{ Live} = \frac{\text{Total of live spermatozoa}}{\text{Total live and dead spermatozoa}} \times 100\%.$$

Spermatozoa morphology

This observation was performed by dripping spermatozoa and eosin-nigrosin on the object glass, fixed on a spiritus lamp, and observing in a microscope with 40 x 10 magnification. The morphological examination identified deformities that are categorized as primary (small/large head size, double head or double tail, and abnormal head shape) and secondary abnormalities (head rupture, tail breaking at the neck or middle, and folded tail) (Klimas et al. 2012). The minimum spermatozoa observed were 200 cells, and the calculations were conducted using the following formula:

$$\% \text{ Normal sperm morphology} = \frac{\text{Normal sperm morphology}}{\text{Normal sperm morphology} + \text{Abnormality}} \times 100\%.$$

Identification of seminal plasma proteins

SDS-PAGE of 4 biological replicates of seminal plasma (90 µg/per lane) was performed using self-cast 12.5% separating polyacrylamide gels according to the method of Laemmli (1970). After that, electrophoresis gels were stained overnight (0.05% CBB R-250, 50% methanol, 10% acetic acid) and destained with 5% methanol and 7% acetic acid. Seminal plasma protein concentration (µg/µL) was calculated using the Bradford method (Bradford 1976), and then the seminal plasma protein was electrophoresed on SDS PAGE and visualized with Coomassie blue.

Data analysis

Semen characteristics data and seminal plasma protein profiles were presented descriptively. Meanwhile, the correlation between seminal plasma protein and semen characteristics was determined using the Pearson test.

Results

Semen characteristics

The mean semen characteristics in Aceh bulls are summarized in Table 1.

Seminal plasma protein profile

The present data showed that the mean protein concentration in the semen of Aceh bulls was 1.83 µg/µL, and the range was 1.72–1.97 µg/µL. Furthermore, 16 protein bands with different molecular weights were found, ranging from 11 to 180 kDa. In general, the protein band of 15.24 kDa was more prominent in the Aceh bull. A typical SDS-PAGE profile of Aceh bull seminal plasma is shown in Figure 1.

Correlation of seminal plasma protein with semen quality

Based on the results of the Pearson test, the concentration of seminal plasma protein had a positive correlation with semen volume (r = 0.166), sperm motility (r = 0.877), sperm viability (r = 0.716) and sperm morphology (r = 0.646). Meanwhile, mass motility had a negative correlation (r = –0.877) (Table 2).

Table 1. Semen characteristics in Aceh bulls

Parameter	Unit	Bulls (n=4)	Range
Semen volume	mL	2.90 ± 0.53	2.5–3.5
Colour	-	Cream	-
Consistency	-	Moderate	-
Semen pH	-	6.50 ± 0.17	6.4–6.7
Mass motility	score	2.67 ± 0.58	2–3
Sperm motility	%	76.67 ± 2.89	75–80
Sperm viability	%	86.33 ± 4.65	82.50–91.50
Sperm morphology	%	91.50 ± 5.57	86.50–97.50

Table 2. The correlation coefficient (r) between seminal plasma protein with semen characteristics

Parameters	Correlation coefficient (r)				
	Semen volume	Mass motility	Sperm motility	Sperm viability	Sperm morphology
Seminal plasma protein	0.166	–0.877	0.877	0.716	0.646

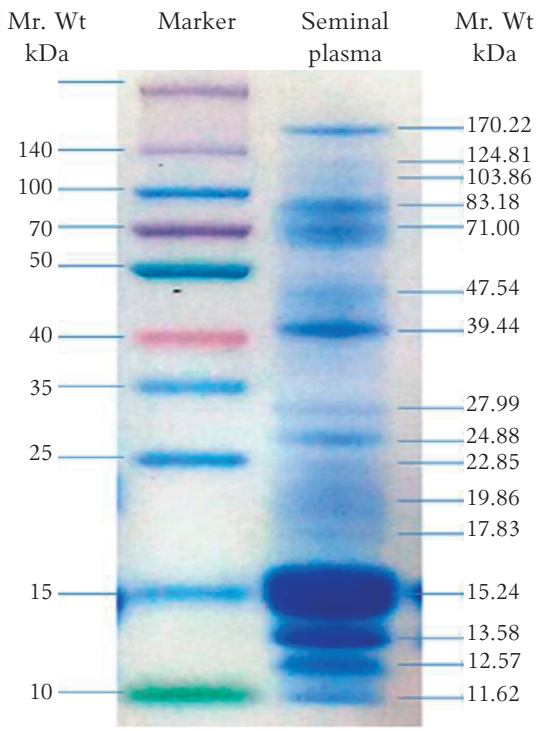

Fig. 1. SDS-PAGE of seminal plasma proteins in Aceh bulls
Mr. Wt = molecular weight; kDa = kilodalton

Discussion

Semen characteristics

The semen volume reported in this study was lower than that reported by Isnaini et al. (2019) and Vince et al. (2017). The mass motility was higher than that reported by Gopinathan et al. (2018), whereas the motility of sperm, viability of sperm, and morphology of sperm were similar to the results of other studies in bull (Rego et al. 2015; Vince et al. 2017). Based on the comparison of these data, the semen collection and evaluation in this study are still within the range of general bull semen characteristics.

Seminal plasma protein profile

The results of this study found low molecular weight protein bands similar to previous studies on bulls by Jobim et al. (2004). The study identified proteins in seminal plasma with low molecular weight (10–30 kDa). Our studies found proteins with small sizes of 11.62, 12.57, 13.58, 15.24, 17.83, 19.86, 22.85, 24.88, and 27.99 kDa. Previous studies on ram found that the concentration and number of protein bands with low molecular weight (11, 13, and 22.5 kDa) were found more in the highly fertile group than in the fertile and sub fertile groups (Almadaly et al. 2016).

The medium molecular weight protein in this study was similar to that found in buffalo bull seminal plasma. In that study, it was found that the protein of medium size was 45 and 55 kDa (Asadpour et al. 2007). Overall, the 16 protein bands found in this study were similar to the results of a recent proteomic study on seminal plasma (Soleilhavoup et al. 2014).

Low and medium molecular weight proteins such as BSP1, BSP3, BSP5, sperm-adhesin, albumin, TIMP, AK1, and PEBP1 have previously been reported in bulls that were significantly more in the highly fertile group (Kasimanickam et al. 2019)

Correlation of seminal plasma protein with semen quality

There was a positive relationship between seminal plasma protein concentration and several semen quality criteria observed in this study. Testing the spermatozoa motility variable is currently the most informative test, because the results of the study consistently show that spermatozoa motility is correlated with male fertility. In addition, the percentage of morphologically normal sperm is also a variable that is often used in the assessment of male fertility. These two variables are the main criteria used in the breeding soundness examination (BSE) (Chenoweth and McPherson 2016). Therefore, microscopic evaluation of sperm motility and morphology are likely to continue to be the two most important predictors of fertility in terms of utilization and fertility variation explained by these variables (Almadaly et al. 2016).

Several previous studies have reported the relationship between semen quality and seminal plasma protein, such as the relationship between seminal plasma and ram sperm motility (Rodrigues et al. 2013), and the relationship between seminal plasma proteins and the percentage of morphologically normal sperm in bull (Boe-Hansen et al. 2015).

Previous studies have been reported in bull by Killian et al. (1993), who stated that seminal plasma protein has positive features with bull male fertility.

Other studies that have found a positive correlation of seminal plasma protein with semen quality and male fertility have been reported in rams (Rodrigues et al. 2013; Almadaly et al. 2016), boars (Novak et al. 2010a), bulls (Karunakaran and Devanathan 2017), and stallions (Novak et al. 2010b). Based on these data, our study has similarities with previous studies. This can support the efforts to characterize seminal plasma proteins as biomarkers of semen quality and bull fertility.

Conclusion

The seminal plasma protein of Aceh bulls is positively correlated with several semen quality variables and may be useful as an additional parameter for determining semen quality or bull fertility. Characterization of deeper protein in seminal plasma is needed in the search for male fertility biomarkers.

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Conflict of interests

The authors declare that they have no conflict of interest.

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Detection of Genes Responsible for Antimicrobial Resistance in Bacteria Isolated from Bovine Mastitis Milk

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Abstract. *Bovine mastitis is considered a problem that is impossible to eradicate, due to the indiscriminate use of antibiotics, mainly β-lactams. The aim of this study was to detect antimicrobial resistance genes in bacteria isolated from bovine mastitis in Western Nicaragua. The antimicrobial resistance profile of 30 bacterial strains was evaluated using the phenotypic method for the antibiotics amoxicillin/clavulanic acid (AMC), ceftriaxone (CRO), gentamicin (CN), cephalexin (CL), vancomycin (VA) for gram-positive bacteria, while the antibiotics amoxicillin/clavulanic acid (AMC), trimethoprim sulfamethoxazole (SXT), ceftriaxone (CRO), gentamicin (CN), cephalexin (CL) in gram-negative bacteria. In addition, the evaluation of the genotypic method was carried out using the PCR (Polymerase Chain Reaction) technique for the detection of extended-spectrum β-lactamases (ESBL) in gram-negative bacteria and the mecA gene for bacteria of the Staphylococcus genus (MRS). It was possible to identify that the bacterial isolates presented a greater resistance to amoxicillin and oxacillin with 24/30 and 10/14, respectively. On the other hand, 100% of the bacterial strains showed sensitivity to the antibiotics gentamicin (CN) and sulfamethoxazole (SXT). In the present study, 2 antimicrobial resistance genes were detected in the gram-negative isolates related to ESBL, the blaSHV gene coding for the strains: Enterobacter, Serratia and E. coli, while the blaTEM gene was detected in the strains: Enterobacter and Serratia. The blaCTX gene in gram-negatives and the mecA gene in Staphylococcus were not detected.*

Introduction

The use of antibiotics in veterinary medicine constitutes one of the main therapeutic tools used for the prevention, treatment and control of infectious diseases of bacterial origin (Ibrahim et al., 2020). However, there are publications that indicate the existence of bacterial multiresistance, not only to antimicrobials of the same family, but also to drugs with different structures and mechanisms of action (Artemyeva et al., 2020; Ríos Padilla, 2021; Arbab et al., 2021; Saeed et al., 2022).

Mastitis is one of the most important diseases in dairy cattle. It is a multi-etiological pathology recognized worldwide for causing harmful effects, either for animal welfare or for dairy farming (Ruegg, 2017). It is considered a multifactorial problem that is impossible to eradicate. Its control depends on the application of comprehensive systems such as the reduction in the rate of new infections and the appropriate use of drugs commonly used in the livestock sector, generally β-lactam antibiotics (Jiménez Velásquez et al., 2020).

Bovine mastitis is caused by gram-negative bacteria, from the coliform group (*Escherichia coli*, *Enterobacter, Klebsiella*), *Pseudomonas* and *Serratia* (Das et al., 2017), gram-positive bacteria (*Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *Streptococcus agalactiae* and *Mycoplasmas* spp.), each of them expressing one or more types of antimicrobial resistance genes (Santiago et al., 2012; Liapi et al., 2021).

It is important to highlight that bovine mastitis has considerable effects on public health due to the transmission of zoonotic bacteria together with antibiotic resistance genes. These bacteria are present in animals and are exposed to antimicrobial pressure, thus developing strategies of survival through evolutionary adaptations (Allen and Stanton, 2014).

Enterobacteria acquire genes for β-lactamase enzymes that generate resistance to β-lactam antibiotics such as penicillins, 2nd, 3rd and 4th generation cephalosporins and monobactams (Lima et al., 2020). The detection of these genes is carried out with molecular techniques that determine extended-spectrum β-lactamases (ESBL) in gram-negative bacteria. For their part, methicillin-resistant *S. aureus* (MRSA) strains are resistant to all β-lactam antibiotics and resistance is mediated by the acquisition of *mecA*, which encodes a penicillin-binding protein (PBP) (Allen and Stanton, 2014).

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In Nicaragua, like in many countries in the region, antimicrobial susceptibility tests are not routinely performed as a diagnostic tool against any agent that causes bacterial diseases. The personnel in charge of certain livestock areas, such as cattle, use antibiotics as control and treatment measures for any clinical symptomatology, without prescriptions or veterinary consultations, contributing to an increase in antimicrobial resistance to most available treatments (Giono-Cerezo et al., 2020). In the country, resistance studies have been carried out in isolated enterobacteria from clinical samples in humans, with gram-negative bacteria found to be multidrug-resistant (92%; 50/54 isolates) by PCR (Dretler et al., 2020). However, the data relating to bacterial resistance to antibiotics in animals are scarce, especially in the case of bovine mastitis.

Antimicrobial resistance is a multisectoral problem because resistance genes are transmitted between food-producing animals and humans by direct exposure or through the food chain and the environment. This phenomenon between human health, veterinary and food production systems supports the need for health approaches (Quiñones Pérez, 2017). For this reason, the aim of this study is focused on the detection of antimicrobial resistance genes in bacteria isolated from the milk of cows with mastitis in bovines from Western Nicaragua.

Material and methods

A descriptive cross-sectional study was designed, reactivating 107 bacterial strains that had been isolated from the milk of cows with mastitis from 14 farms in Western Nicaragua from January to March 2021 and maintained in the strain collection of the Centro Veterinario de Diagnóstico e Investigación (CEVEDI). Farms were exclusively owned by small producers (less than 50 cattle), with extensive exploitation, dual-purpose herds (meat and milk), Brahman breeds, manual milking, and little technology.

The bacteria were stored in Brain Heart Infusion broth (ICC, OXOID) with 10% glycerol at a temperature of –20°C. For their reactivation, 50 µL of each strain were inoculated in 1 mL of ICC, incubated at 37°C for 24 hours. Then they were inoculated on 5% Sheep Blood Agar (ASC, OXOID) and on MacConkey agar (MC, OXOID), incubating the agar plates at 37°C for 24 hours. Only 30 strains from 8 farms were successfully reactivated without contamination, while 16 of them were classified as gram-negative bacteria and 14 as gram-positive. The pure growth of the bacteria was verified, and their identification was carried out again using API 20E (Biomériux®, Marcy l'Etoile, France) for gram-negative bacteria, while for *Staphylococcus* gram staining was used, catalase, coagulase DNase, (OXOID).

Antimicrobial susceptibility phenotypic test

Resistance patterns were determined by the agar diffusion method, according to the protocol established by the Clinical Laboratory Standards Institute (CLSI), *(Uddin et al.*, 2018). In plates with Müller Hinton agar (OXOID), the bacterial inoculums from the previously isolated colonies were seeded on the surface. Bacterial suspensions were prepared with a turbidity of 0.5 Mc Farland, to obtain a concentration equivalent to 1.5 x 10^8 cfu/mL. They were inoculated with a swab soaked in the suspension, using a conventional striation. The plate with the already seeded agar was allowed to dry for 5 minutes, and later the antibiotic discs for gram-negative bacteria were placed. These were: amoxicillin plus clavulanic acid (AMC 30), ceftriaxone (CRO 30), gentamicin (120), cephalexin (CL 30) and trimethoprim sulfamethoxazole (SXT 25). For gram-positive bacteria (*Staphylococcus*), in addition to the above antibiotics, vancomycin (VA 30) and oxacillin (OX 1) were also added. The plates were incubated at 37°C for 24 h, the inhibition halos were measured, and the results were recorded as resistant (R), intermediate (I) and sensitive (S) referring to the parameters already established for each antibiotic.

Molecular detection of resistance genes

A colony of each previously purified strain was diluted in 200 µL of nuclease-free water in 1.5 mL vials, vortexed for 20 seconds. Then the protocol described by the manufacturer (QIAamp DNA Mini Kit QIAGEN, Germany) was used.

For the identification of ESBL genes from enterobacteria, the following primers were used: *blaSHV*: forward (TGGTTATGCGTTATATTCGCC) and reverse (GGTTAGCGTTGCCAGTGCT) with an amplicon size of 868 bp; *blaCTX:* forward (TCTTCCAGAATAAGGAATCCC) and reverse (CCGTTTCCGCTATTACAAAC) with an amplicon size of 909 bp; *blaTEM*: forward (TCCGCTCATGAGACAATAACC) and reverse (TTGGTCTGACAGTTACCAATGC) with an amplicon size of 931 bp (Asir et al., 2015). The reaction mixture for the detection of the corresponding genes was carried out in a final volume of 15 µL, with the following elements: 3 µL of genomic DNA; 7.5 µL of master mix; 1 µL of forward; 1 µL of reverse; 2.5 µL of nuclease-free water.

For the detection of the *mecA* gene in *Staphylococcus* spp. forward (TGGCTATACGTGTCACAATCG) and reverse (CTGGAACTTGTTGAGCAGAG) primers were used with an amplicon size of 310 bp (Vannuffel et al., 1998). The reaction mixture for the detection of the *mecA* gene was carried out in a final volume of 15 µL, with the following elements: 3 µL of genomic DNA, 7.5 µL of master mix; 1 µL of forward; 1 µL of reverse; 2.5 µL of nuclease-free water.

The amplifications were performed in the Applied Byosystem 2720 thermocycler following the program that consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 denaturation cycles at 95°C for 30 seconds, hybridization at 58°C for 30 seconds, and extension at 72°C for 1 minute. The final extension

was performed at 72°C for 7 minutes. PCR products were visualized by agarose gel electrophoresis (2% w/v) run using TBE buffer at constant power, 110 W, for 2 hours and stained with ethidium bromide.

Statistical analysis

The results were analyzed as relative frequencies with their respective 95% confidence intervals. The Fisher exact test was applied to determine the significant association between categorical variables.

Results

In the present study, gram-negative bacteria represented 16/30 (53.33%, 95% CI: 33.82–72.85) of the bacterial species analyzed, resulting in 6/16 belonging to the genus *Escherichia coli*, 6/16 corresponding to the genus *Enterobacte*r spp. and 4/30 for the genus *Serratia* spp. On the other hand, gram-positive bacteria represented 14/30 (46.66%, 95% CI: 27.15–66.18), resulting in the genus coagulase cegative *Staphylococcus* (SCN) with the highest frequency with 12/14, while *Staphylococcus aureus* strains were 2/14. In the antimicrobial resistance profile of gram-negative strains analyzed, it was possible to identify that the bacterial isolates presented greater resistance to amoxicillin/clavulanic acid with 16/16 strains analyzed, while resistance was also found for cephalexin with 6/16 (Figure 1). In addition, 6/16 strains with multiresistance (AMC and CL) were identified. The most effective antibiotics were gentamicin, ceftriaxone and trimethoprim sulfamethoxazole, to which no resistant gram-negative strain was found. In gram-negative bacteria, a significant difference was observed in the frequency of resistance between antibiotics ($P < 0.001$).

In the case of antibiotics for *Staphylococcus* spp., 10/14 strains resistant to oxacillin, 8/14 strains resistant to AMC and 2/14 to vancomycin were identified. The most effective antibiotics were gentamicin and trimethoprim sulfamethoxazole, to which no resistant gram-positive strain was found (Figure 2). In gram-positive bacteria, a no significant difference was observed in the frequency of resistance between antibiotics ($P = 0.981$).

Two antimicrobial resistance genes were detected in the gram-negative isolates related to ESBL. The *blaSHV* gene was found in 8/16 strains, of which, 4 were found in *Enterobacter* spp., 2 in *E. coli*, and 2 in *Serratia* spp. The *blaTEM* gene was detected in 4/16 strains, of which 2 were identified in *Enterobacter* spp. and 2 in *Serratia* spp. (Figure 3). The *blaCTX* gene was not detected in any of the 16 strains analyzed. Molecular analyses for the detection of the *mecA* gene in *Staphylococcus* spp. were negative in all the isolates (Table 1).

Discussion

Within the isolated and reactivated strains, the species of *Staphylococcus* spp. and *E. coli* were found, which most frequently cause mastitis in cattle, similar to a study conducted in 2012 in western Nicaragua found that the most frequent bacteria in bovine mastitis were CNS with 55% and *S aureus* with 25% (Rivera Varela & Tórrez Cálix, 2012). The high frequency of *Staphylococcus* spp. may be influenced by the fact that the samples were taken in the dry months (January-March) since, as described, cows are more prone to this bacterial genus in the dry season, as reflected in the study carried out in Mexico in which they found a higher frequency for *S. aureus* in the dry season with 23.6%, in relation to the rainy season with 3.2% (Adame-Gómez et al., 2021). Strains such as *E. coli* and gram-negative bacteria found in bovine mastitis may be associated with poor hygienic-

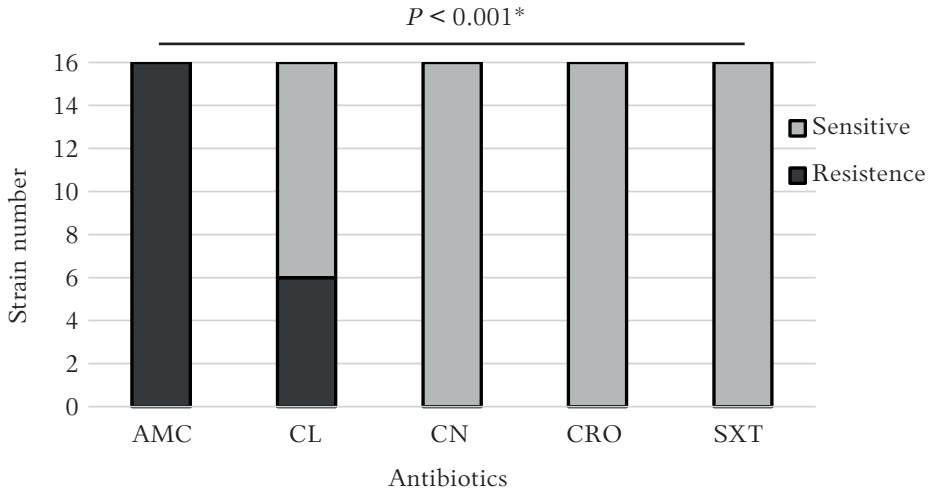

Fig. 1. Antimicrobial resistance profile of 16 gram-negative strains isolated from cows with mastitis in western Nicaragua

Amoxicillin/clavulanic acid (AMC), cephalexin (CON), gentamicin (CN), ceftriaxone (CRO), trimethoprim sulfamethoxazole (SXT).

* Significance according to the Fisher exact test

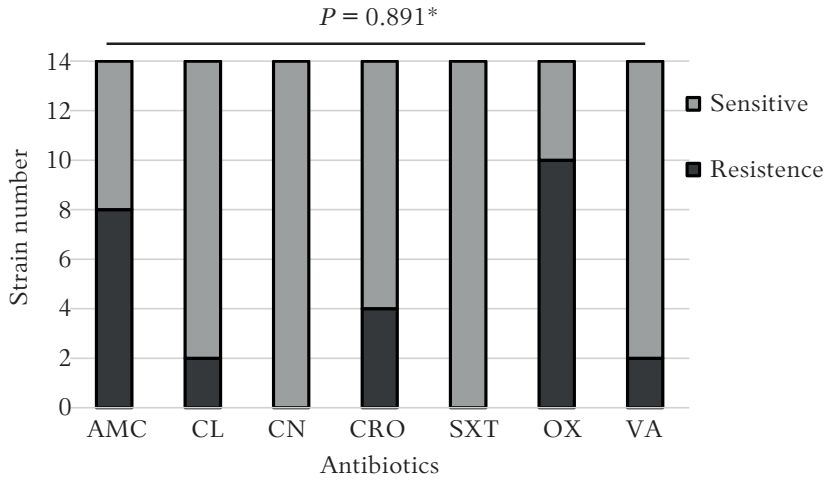

Fig. 2. Antimicrobial resistance profile of 14 *Staphylococcus* spp. strains isolated from cows with mastitis in western Nicaragua
Amoxicillin/clavulanic acid (AMC), cephalexin (CON), gentamicin (CN), ceftriaxone (CRO), trimethoprim sulfamethoxazole (SXT), oxaciline (OX), vancomicine (VA).
* Significance according to Fisher's exact test

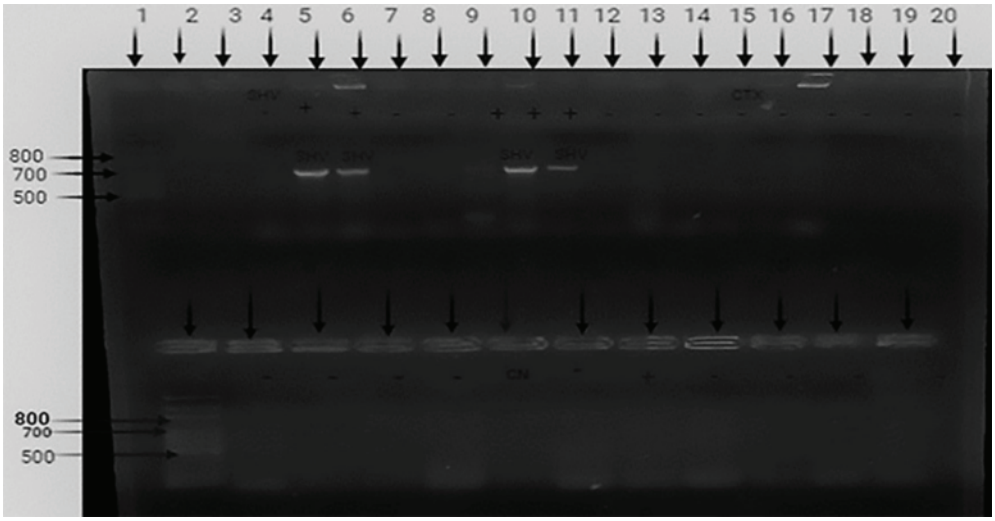

Fig. 3. Electrophoresis and identification of *blaSHV* genes in bacteria isolated from bovine mastitis in Nicaragua
Molecular weight marker (1), *Serratia* spp. positive to SHV (5), *Enterobacter* spp. positive to SHV (6), *Enterobacter* spp. positive to SHV (10). SHV-positive *Escherichia coli* (11).

sanitary practices at the time of handling the animals and their work environment. Being environmental pathogens, they have a greater possibility of causing clinical mastitis (Das et al., 2017).

In the analysis of the antimicrobial resistance profile, it was shown that AMC was the least effective (24/30). Furthermore, gram-negative bacteria showed a greater resistance to AMC with 16/16, while the gram-positive resistance frequency was lower with 8/14. This agrees with what was reported in 2012 in *La Paz Centro* in which sensitivity to AMC was only observed in 35.7% of the strains isolated from the milk of cows with mastitis (Chavarría Narváez and Meléndez Martínez, 2012). This can be attributed to the fact that this antibiotic is sold freely and is the most used in the country to empirically treat infections in humans and animals.

In this study, the bacterial strains did not show resistance to gentamicin (CN), similar to what was reported in a study carried out in the municipality of *La Paz Centro* in 2012, in which it was reported that the antibiotic gentamicin was the most effective against bacteria isolated from milk of cows with subclinical mastitis (Chavarría Narváez and Meléndez Martínez, 2012). This is because aminoglycosides are widely used in Nicaragua for the treatment of mastitis and there are no restrictions regarding their veterinary use.

The gram-positive bacteria were *S. aureus* (2/14) and SCN (12/14). In these bacteria, a high resistance to the antibiotic oxacillin was observed (10/14), which is a high result compared to a study carried out in the municipality of León in 2016, in which 18% of oxacillin-resistant *S. aureus* isolated from the

Table 1. Detection of genes for resistance to extended spectrum betalactamases (ESBL) and methacillin (MRSA) in bacteria isolated from cows with mastitis in Western Nicaragua

Strains	Bacterias	Resistance genes			
		blaSHV	*blaCTX*	*blaTEM*	*mecA*
35	*E.coli*	Negative	Negative	Negative	x
26	*E.coli*	Negative	Negative	Negative	x
38	*E.coli*	Negative	Negative	Negative	x
28	*E.coli*	Negative	Negative	Negative	x
34	*E.coli*	Positive	Negative	Negative	x
25	*E.coli*	Positive	Negative	Negative	x
31	*Enterobacter* spp.	Negative	Negative	Negative	x
22	*Enterobacter* spp.	Negative	Negative	Negative	x
11Mc	*Enterobacter* spp.	Positive	Negative	Negative	x
18Mc	*Enterobacter* spp.	Positive	Negative	Negative	x
3223	*Enterobacter* spp.	Positive	Negative	Positive	x
23	*Enterobacter* spp.	Positive	Negative	Positive	x
9	*Serratia* spp.	Negative	Negative	Negativo	x
4	*Serratia* spp.	Negative	Negative	Negativo	x
13	*Serratia* spp.	Positive	Negative	Positive	x
14	*Serratia* spp.	Positive	Negative	Positive	x
65	*Staphylococcus aureus*	x	x	x	Negative
68	*Staphylococcus aureus*	x	x	x	Negative
12Mb	Coagulase negative *Staphylococcus*	x	x	x	Negative
14Mb	Coagulase negative *Staphylococcus*	x	x	x	Negative
71	Coagulase negative *Staphylococcus*	x	x	x	Negative
79	Coagulase negative *Staphylococcus*	x	x	x	Negative
2	Coagulase negative *Staphylococcus*	x	x	x	Negative
17	Coagulase negative *Staphylococcus*	x	x	x	Negative
4mb	Coagulase negative *Staphylococcus*	x	x	x	Negative
6mb	Coagulase negative *Staphylococcus*	x	x	x	Negative
81	Coagulase negative *Staphylococcus*	x	x	x	Negative
84	Coagulase negative *Staphylococcus*	x	x	x	Negative
83	Coagulase negative *Staphylococcus*	x	x	x	Negative
86	Coagulase negative *Staphylococcus*	x	x	x	Negative

X: No test performed.

milk of cows with subclinical mastitis were reported (Thompson Bello and Ingram Oporta, 2017). This represents an increase in resistance; therefore, vigilance must be maintained in the use of veterinary antibiotics on livestock farms in the area.

Studies of resistance genes in bacteria in Nicaragua have been carried out on strains isolated from humans related to the hospital environment (Dretler et al., 2020; Sandoval-Rojas et al., 2022); there is no precedent for the detection of resistance genes in bacteria isolated from bovine mastitis.

In the analysis for the identification of genes associated with ESBL in gram-negative bacteria, it was shown that the *blaSHV* gene was the most frequent and the *blaTEM* gene was less frequent. The presence of ESBL in enterobacteria coincides with a study carried out by Timofte et al., where they show that *E. coli* obtained from milk samples from cows with mastitis could harbor *SHV-12* β-lactamases (Timofte et al., 2014). In another study, six ESBL-producing *E. coli* strains were also identified as carriers of the *blaTEM-1* gene; 3 also carried *blaCTX-M* genes and 3 carried *blaSHV* genes (Filioussis *et al.,* 2020). The finding of the study *blaSHV* gene was frequent in *E. coli*, *Enterobacter* and *Serratia* spp. different from what was described by Bradford, where

he points out that *SHV* β-lactamase is found more frequently in *K. pneumoniae*, because this bacterium has a greater ability to survive in the environment (Bradford, 2001). The ESBLs are enzymes that are phenotypically characterized by conferring resistance to penicillins and cephalosporins, including third and fourth generation ones. The frequency of these genes produced by enterobacteria is due to the disproportionate use of these families of antibiotics throughout the field of veterinary medicine, mainly in cattle (Álvarez Almanza, 2010).

Regarding the methacillin genotypic analysis, the *mecA* gene was not identified in any of the *Staphylococcus* spp. isolates. This coincides with the low methacillin frequency found by Monistero et al., who were able to detect the *mecA* gene in only 1.6% (2/120) *Staphylococcus aureus* isolates from different countries (Monistero et al., 2018). The low frequency of *Staphylococcus* spp. with *mecA* gene found in this study differs from those found by Velásquez et al. (2020), who detected the *mecA* gene in 26.7% of *S. aureus* strains, which in turn found resistant strains to cefoxitin that did not carry the *mecA* gene. Similarly, *mecA*-positive strains were sensitive to the same B-lactam antibiotic (Jiménez Velásquez et al., 2020).

In this study, high phenotypic resistance to OX was found in SCN and *S. aureus*; however, the *mecA* gene was not found in any of these strains, which has also been reported in SCN isolated from mastitis, in which 26.67% of the *mecA* negative isolates were found ti be resistant to oxacillin (Nayel et al., 2020). The discrepancy between phenotypic and genotypic tests could be linked to the presence of some other marker associated with methicillin resistance, such as the *mecC* homologous gene, which is located between the *mecA* gene and the AME genes, which could explain why many MRSA strains can be resistant to different antimicrobials (Aqib et al., 2018). It is *mecA* that causes resistance to methicillin in *S. aureus* isolates; however, it has been shown that some methicillin-resistant (MRSA) strains of *S. aureus* do not carry this gene but rather the new resistance gene called *mecC*, which is a homologue of *mecA* (Cikman et al., 2019).

Conclusion

Gram-negative bacteria showed greater resistance to amoxicillin, identifying the antimicrobial resistance genes encoding ESBL (*blaSHV* gene and *blaTEM* gene), with the exception of the *blaCTX* gene, while gram-positive bacteria showed significant resistance to oxacillin. However, the *mecA* gene encoding MRS was not identified.

Conflicts of interest

The authors declare they have no conflicts of interest.

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