

BOOKLET





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Volume 16 (4); July 2026

Research Paper

Effect of bentonite-ginger combination on Ouled Djellal ewes' productivity in periparturient physiological stage

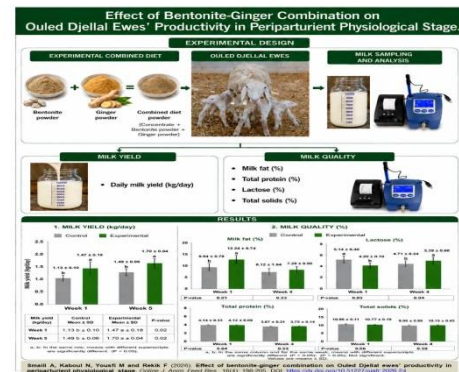
Smaili A, Kaboul N, Yousfi M and Rekik F.

Online J. Anim. Feed Res., 16(4): 198-205, 2026; pii: S222877012600024-16
DOI: <https://dx.doi.org/10.51227/ojafr.2026.24>

Abstract

This study evaluated the effects of dietary supplementation with a combination of 5% bentonite (clay) and 2% ginger on the productive performance of Ouled Djellal ewes during the periparturient period, from eight weeks before lambing to twelve weeks after lambing. Twelve clinically healthy Ouled Djellal ewes, with a mean age of 3.75 ± 1.22 years, an average body weight of 67.50 ± 8.63 kg, and a mean body conditions score (BCS) of 2.54 ± 0.54 were randomly allocated to two groups ($n = 6$ per group). Both groups received a basal diet consisting of alfalfa hay and wheat straw *ad libitum* supplemented with concentrate. The control group received the basal concentrate without additives, whereas the experimental group received concentrates supplemented with 5% bentonite and 2% ginger. The experiment was conducted at the demonstration station of the Technical Institute of Livestock Breeding, Ain M'lila, Algeria. The results showed that no statistical significant differences were observed in body condition score between the two groups throughout the experimental period. However, milk production was significantly higher in the supplemented group during the first and fifth weeks of lactation ($P = 0.02$), increasing by 30.10% and 14.10%, respectively, compared with the control group. This effect was no longer significant during the eighth week of lactation ($P > 0.05$). Regarding milk composition, total protein and total solids did not differ significantly between treatments. In contrast, milk fat content was significantly higher (+36.91%; $P = 0.01$) and lactose concentration was significantly lower (-22.38%; $P = 0.03$) in the supplemented group during the first week of lactation. During the fourth week, lactose was the only milk component that differed significantly between treatments, showing a 14.44% increase ($P = 0.04$). No significant differences in milk composition were observed during the second and twelfth weeks of lactation. Dietary supplementation with the bentonite-ginger combination had no significant effect on lamb birth weight or average daily gain throughout the suckling period. The absence of significant effects on lamb growth may be related to the inclusion levels of the additives used in the present study.

Keywords: Feed efficiency; Gestation; Lactation; Natural additives; Prebiotic; Sheep.



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Research Paper

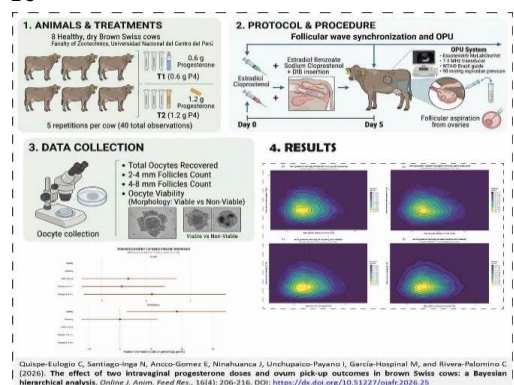
The effect of two intravaginal progesterone doses and ovum pick-up outcomes in brown Swiss cows: a Bayesian hierarchical analysis

Quispe-Eulogio C, Santiago-Inga N, Ancco-Gomez E, Ninahuanca J, Unchupaico-Payano I, García-Hospital M, and Rivera-Palomino C.

Online J. Anim. Feed Res., 16(4): 206-216, 2026; pii: S222877012600025-16
DOI: <https://dx.doi.org/10.51227/ojafr.2026.25>

Abstract

This study evaluated the effect of two intravaginal progesterone doses on follicular recruitment, oocyte recovery, and oocyte viability in Brown Swiss cows undergoing ovum pick-up. Eight dry cows were allocated to two treatments: T1 (0.6 g progesterone) and T2 (1.2 g progesterone), with four cows per treatment and five repeated sessions per animal. The responses analyzed were the numbers of 2–4 mm follicles, 4–8 mm follicles, total recovered oocytes, recovery probability, and oocyte viability. Count data were analyzed with negative binomial models, whereas recovery and viability were modeled as binomial responses, with cow included as a random effect. Overall, T1 showed higher adjusted posterior means for the number of 2–4 mm follicles per ovum pick-up (OPU) session (7.62 vs. 5.36 follicles), 4–8 mm follicles per OPU session (4.17 vs. 3.27 follicles), and total recovered oocytes per OPU session (4.75 vs. 3.56 oocytes) compared with T2. Oocyte



viability, expressed as an adjusted posterior probability, was also higher in T1 than in T2 (0.750 vs. 0.566, equivalent to 75.0% vs. 56.6%). In contrast, the adjusted posterior probability of oocyte recovery was nearly identical between treatments (0.403 vs. 0.402, equivalent to 40.3% vs. 40.2%). Among the evaluated outcomes, oocyte viability showed the clearest treatment difference, with an odds ratio of 2.50 and a posterior probability of superiority of 0.989 in favor of T1. Under the conditions of this study, the 0.6 g progesterone protocol was associated with a more favorable follicular response and better oocyte viability, while oocyte recovery efficiency remained essentially unchanged. Keywords: Cumulus-oocyte complexes; Follicular synchronization; Oocyte competence; Posterior inference; Reproductive hormones.

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Research Paper

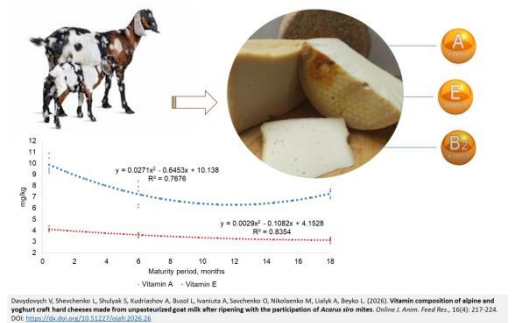
Vitamin composition of alpine and yoghurt craft hard cheeses made from unpasteurized goat milk after ripening with the participation of *Acarus siro* mites

Davydovych V, Shevchenko L, Shulyak S, Kudriashov A, Busol L, Ivaniuta A, Savchenko O, Nikolaenko M, Lialyk A, Beyko L.

Online J. Anim. Feed Res., 16(4): 217-224, 2026; pii: S222877012600026-16
DOI: <https://dx.doi.org/10.51227/ojaf.2026.26>

Abstract

Craft goat cheeses made from unpasteurized milk are considered elite food products with high nutritional and biological value for humans, which contributes to growing market availability and necessitates the assessment of their quality, authenticity and biological value. This study used hard craft cheeses, Alpine and Yoghurt, which ripened with the activity of *Acarus siro* mites and produced from unpasteurized goat milk on a local organic farm. The contents of retinol, tocopherol and riboflavin were determined in the cheeses after 7 days (young cheese), 6 months (mature cheese), and at the final stage of ripening (12 months for Alpine and 18 months for Yoghurt). In Alpine cheese during 12 months of ripening, the retinol content ranged from 5.04 to 5.30 mg/kg, the tocopherol content from 2.00 to 2.34 mg/kg and the riboflavin content from 2.19 to 2.48 mg/kg. The ripening period did not significantly affect the retinol and riboflavin content but increased the tocopherol content by 15.42 - 16.42%. In Yoghurt cheese, the retinol content ranged from 4.60 to 5.24 mg/kg, while the tocopherol content ranged from 2.18 to 2.33 mg/kg, and was not affected by the ripening period. The riboflavin content increased from 1.78 mg/kg to 2.58 mg/kg during ripening. The retinol content in the dry matter of both cheeses decreased with increasing ripening time, whereas the tocopherol content decreased only in Yoghurt cheese. To statistically assess the effect of ripening time on the vitamin content of the hard craft cheeses, one-way analysis of variance (ANOVA) was used. This analysis showed that the retinol concentration in the dry matter of both cheeses decreased with increasing ripening time, while the tocopherol concentration increased only in Yoghurt cheese. The results of the study indicate the high biological value of hard cheeses made from unpasteurized goat milk as sources of vitamins A, E, and B2. Moreover, the use of dispersion analysis in combination with microbiological and physicochemical characteristics allows evaluating and confirming their quality, authenticity, and biological value. Keywords: Alpine cheese, Maturation, Retinol, Riboflavin, Tocopherol, Yoghurt cheese.



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Short Communication

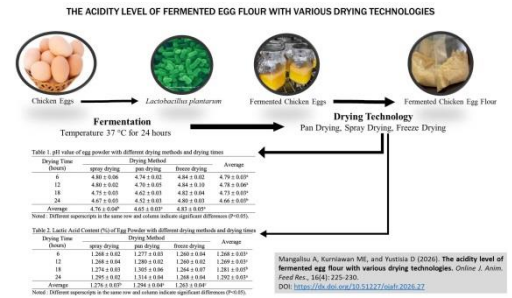
The acidity level of fermented egg flour with various drying technologies

Mangalisu A, Kurniawan ME, and Yustisia D.

Online J. Anim. Feed Res., 16(4): 225-230, 2026; pii: S222877012600027-16
DOI: <https://dx.doi.org/10.51227/ojaf.2026.27>

Abstract

Commonly, egg fermentation technology is carried out using *Lactobacillus plantarum* bacteria. Eggs that have undergone the fermentation process can be stored for a long time using the drying method. The results of fermentation in the drying process are expected to reduce the Maillard reaction. The purpose of this study was to identify the application of the right drying technology in fermented eggs on the stability of the acidity level of egg flour. This study was conducted experimentally using a Completely Randomized Design (CRD) factorial pattern of 3 × 4 treatments with 5 replications each. The treatment was carried out using the spray drying method, pan drying, freeze drying with a drying time of 6,12,18 and 24 hours. The acidity level can be measured by testing parameters for pH values and lactic acid levels using the titration method. The results showed that increasing drying time and different drying methods in fermented egg flour could maintain the pH value of eggs and the optimum at a drying time of 6 hours with the freeze drying method. Lactic acid levels tend to be stable because the fermentation product uses freeze drying which can



maintain the lactic acid levels of fermented eggs. The acidity level of fermented egg flour can be seen from the stability of the pH value and lactic acid levels from the drying process. The best pH value and lactic acid content were obtained using the freeze drying method with a drying time of 24 hours.

Keywords: Eggs, Fermentation, Drying, Flour, Lactic acid.

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Research Paper

In silico analysis of the promoter region of prolactin gene in cattle to understand its gene regulation

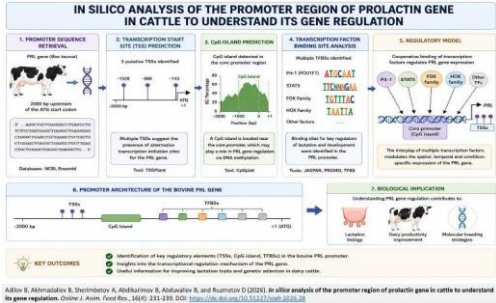
Adilov B, Akhmadaliev B, Sherimbetov A, Abdikarimov B, Abduvaliev B, and Ruzmetov D.

Online J. Anim. Feed Res., 16(4): 231-239, 2026; pii: S222877012600028-16
DOI: <https://dx.doi.org/10.51227/ojafr.2026.28>

Abstract

Prolactin (PRL) is a key endocrine regulator of lactation, mammary gland growth, and systemic metabolic homeostasis in cattle. The transcriptional architecture of bovine *PRL* regulation remains incompletely characterized, despite its physiological importance. In this study we used an in silico Transcription Start Site (TSS) prediction, CpG island, and TFBS profiling approach to analyze the ~5 kb upstream regulatory region of the *Bos taurus PRL* gene. By employing Neural Network Promoter Prediction under stringent criteria, we identified three highly reliable TSSs located at -4478, -2123, and -517 base pairs relative to the translational start codon, indicating a multi-TSS promoter complex that facilitates alternative transcript initiation. Canonical CpG islands were absent, consistent with tissue-specific, fine-tuned transcriptional control. TFBS analysis revealed a densely populated, hierarchically organized promoter enriched for pituitary-specific POU1F1 motifs, hormone-responsive ESRR and RARA elements, signal-dependent STAT5 and AP-1 sites, and developmental transcription factors such as NANOG, HOXD9, and FOXI3. High-affinity TFBS clusters, including cis-regulatory modules of activators and repressors (like PRDM1) suggest combinatorial control and dynamic transcriptional responsiveness. These findings give mechanistic insights into the transcriptional complexity of bovine *PRL* locus and built an essential resource base on functional genomics studies to enhance efficiency of lactation and endocrine resilience in cattle.

Keywords: *Bos taurus*, Lactation, Mammary gland growth, Prolactin gene, Promoter region, Transcription start sites.



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Research Paper

Development of whey protein and pectin-based film enriched with lemon essential oil for food packaging: effects on mechanical properties, antioxidant, and biodegradability

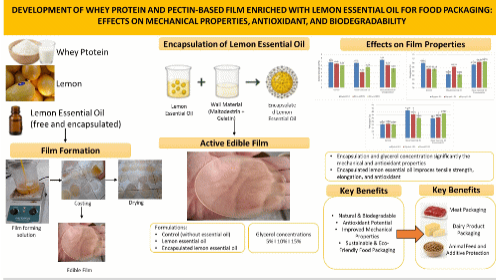
Fahrullah F, Kisworo D, Wulandani BRD, and Noersidiq A.

Online J. Anim. Feed Res., 16(4): 240-251, 2026; pii: S222877012600029-16
DOI: <https://dx.doi.org/10.51227/ojafr.2026.29>

Abstract

The employment of conventional plastics in food packaging has been demonstrated to exert a detrimental effect on the environment, owing to the fact that these plastics are non-biodegradable. Furthermore, there is a possibility that microplastics will accumulate within the food chain. The objective of this study is to develop an edible film that has been enriched with lemon essential oil and glycerol. The aim of the enrichment is to improve the film's mechanical properties, antioxidant content and biodegradability. The present study was conducted experimentally, employing a completely randomized factorial design with five replicates. Factor A consisted of control film; film with added lemon essential oil; and film with added encapsulated lemon essential oil. Factor B consisted of glycerol concentrations (5%; 10%; and 15%). The findings demonstrated that the incorporation of lemon essential oil and varying glycerol concentrations exerted a substantial impact ($P < 0.01$) on the tensile strength, elongation, and antioxidant content of whey-pectin-based edible films. The tensile strength values ranged from 4.23 to 7.54 MPa, the elongation ranged from 62.49 to 68.76%, and the antioxidant content ranged from 20.84 to 42.25%. The biodegradability of the film was demonstrated by the observation of a satisfactory degradation rate over a 30-day period following burial in soil. The edible film, composed of whey-pectin and fortified with encapsulated lemon essential oil and 10% glycerol, exhibited optimal tensile strength, elongation, antioxidant properties, and biodegradability. This renders it a sustainable and environmentally friendly solution for food packaging.

Keywords: Biodegradable, Essential Oil, Glycerol, Packaging, Whey.



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Assessment of nutritional potentials of palm kernel cake/rice husk-based diets supplemented with fibrolytic enzyme on performance, carcass characteristics and organ weight of broiler chickens

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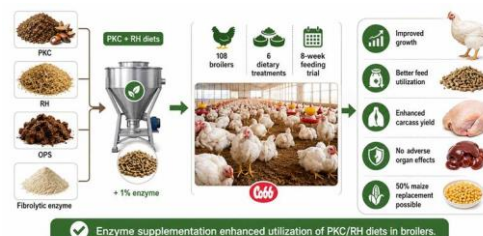
Online J. Anim. Feed Res., 16(4): 252-260, 2026; pii: S222877012600030-16

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Abstract

The prohibitive costs of conventional feedstuffs have necessitated the search for other alternatives. Palm kernel cake (PKC) and rice husk (RH) are potential feed resources. PKC contains about 20% crude protein and about 50% nitrogen free extractives (NFE), whilst RH contains about 38% NFE. The use of these ingredients is limited in monogastric diets due to their high content of crude fibre, particularly non starch polysaccharides. The main objective of this study was to evaluate the nutritional potential of PKC/ RH based diets, supplemented with exogenous fibrolytic enzymes on performance and carcass/organ weight characteristics of Cobb strain broiler chickens. Six experimental diets were formulated such that diets 1 and 2 contained 0% PKC/RH, and 0% oil palm slurry (OPS), whilst diets 3 and 4 contained 25% PKC, and diets 5 and 6 incorporated PKC at 50%, respectively. The OPS was also included in the diets 3 – 6 at 4% level to boost their primary energy levels, whilst the feed enzyme was added to the diets 2, 4, and 6 at 1% inclusion level. A total of 108, one-day-old chickens were randomly distributed to the six dietary groups, in a 3 × 2 factorial arrangement. At the end of trial, results showed that all performance parameters in enzyme-treated factorial sets were significantly ($P < 0.05$) better than those of zero enzyme-treated groups. Similarly, all carcass/organ weight parameters, except weights of thigh/drumstick were also significantly ($P < 0.05$) better in the enzyme treated groups. The results suggest that PKC/RH based diets supplemented with feed enzymes at 1% inclusion level, could replace 50% of maize in broiler chickens' diets without any deleterious effect on performance, carcass/organ weight characteristics.

Keywords: Broiler chicken, Feed enzyme, Nutritional potentials, Palm kernel cake, Rice husk.



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EFFECT OF BENTONITE-GINGER COMBINATION ON OULED DJELLAL EWES' PRODUCTIVITY IN PERIPARTURIENT PHYSIOLOGICAL STAGE

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✉ Supporting Information



ABSTRACT: This study evaluated the effects of dietary supplementation with a combination of 5% bentonite (clay) and 2% ginger on the productive performance of Ouled Djellal ewes during the periparturient period, from eight weeks before lambing to twelve weeks after lambing. Twelve clinically healthy Ouled Djellal ewes, with a mean age of 3.75 ± 1.22 years, an average body weight of 67.50 ± 8.63 kg, and a mean body conditions score (BCS) of 2.54 ± 0.54 were randomly allocated to two groups ($n = 6$ per group). Both groups received a basal diet consisting of alfalfa hay and wheat straw *ad libitum* supplemented with concentrate. The control group received the basal concentrate without additives, whereas the experimental group received concentrates supplemented with 5% bentonite and 2% ginger. The experiment was conducted at the demonstration station of the Technical Institute of Livestock Breeding, Ain M'lila, Algeria. The results showed that no statistical significant differences were observed in body condition score between the two groups throughout the experimental period. However, milk production was significantly higher in the supplemented group during the first and fifth weeks of lactation ($P = 0.02$), increasing by 30.10% and 14.10%, respectively, compared with the control group. This effect was no longer significant during the eighth week of lactation ($P > 0.05$). Regarding milk composition, total protein and total solids did not differ significantly between treatments. In contrast, milk fat content was significantly higher (+36.91%; $P = 0.01$) and lactose concentration was significantly lower (−22.38%; $P = 0.03$) in the supplemented group during the first week of lactation. During the fourth week, lactose was the only milk component that differed significantly between treatments, showing a 14.44% increase ($P = 0.04$). No significant differences in milk composition were observed during the second and twelfth weeks of lactation. Dietary supplementation with the bentonite–ginger combination had no significant effect on lamb birth weight or average daily gain throughout the suckling period. The absence of significant effects on lamb growth may be related to the inclusion levels of the additives used in the present study.

Keywords: Feed efficiency; Gestation; Lactation; Natural additives; Prebiotic; Sheep.

INTRODUCTION

In Algeria, meat production relies mainly on sheep farming, which represents 81% of the total livestock population (M.A.D.R, 2020), but this sheep population faces chronic feed shortages (Slimani et al., 2021). Natural rangelands have deteriorated due to recurrent droughts and now provide only limited forage resources for livestock, dominated by poorly digestible woody shrubs with low nutritional value. This problem has prompted many researchers (Ouachem and Soltane, 2009; Kaboul and Ouachem 2012) to seek solutions based on the incorporation of natural products to improve nutrient digestibility and utilization in lignified forages (Ouachem et al., 2008). Among natural additives, we can include the use of clay (Duval, 1993; Kahouli et al., 2020), as well as phytobiotics (Alloui, 2011; Kahouli et al., 2023) and, particularly, ginger as a medicinal plant (Gupta et al., 2025; Paudel et al., 2025).

In this context, the present study focuses on the use of clay and the medicinal plant ginger as phytobiotic included at dietary levels of 2% and 5%, respectively. The objective is to evaluate the impact of incorporating these two products on the performance of ewes at the end of gestation and during lactation, and on their offspring. It should be noted that to the best of our knowledge similar studies are not reported on combined supplementation with clay and ginger in ewes from late gestation to the end of lactation.

MATERIALS AND METHODS

Location, period of the experiment and ethical consideration

The experiment was conducted at the T.I. Bre Ain M'lila Demonstration Station in Oum El Bouaghi province, Algeria, located in the semi-arid region of eastern Algeria, over a period of six months from February to July 2019. All procedures involving the ewes, including diet modification with natural additives, housing, and handling, were performed in strict compliance with Algerian national regulations on animal health and welfare. The trial relied exclusively on non-invasive zootechnical measurements and was supervised by certified zootechnicians and veterinary staff.

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Animal material, feed and additives

Twelve clinically healthy Ouled Djellal ewes in late gestation were selected for the experiment. The average age of the ewes was 3.75 ± 1.22 years, with an average live weight of 67.50 ± 8.63 kg and a body condition score (BCS) of 2.54 ± 0.54 . The control ration (C) consisted mainly of roughage (alfalfa hay supplemented with wheat straw ad libitum) and 500 g/head/day of concentrate feed, composed of corn, bran, soybean meal, vitamins, minerals, phosphate and limestone. Water was provided ad libitum. Powdered ginger and bentonite (clay) were purchased commercially.

Experimental protocol

The 12 ewes were randomly allocated to two experimental groups (n = 6 per group):

- One control group (C) receiving the basal ration without additives.
- One experimental group (E) receiving the basal ration supplemented with 5% bentonite (clay) and 2% ginger.

Both groups underwent a 15-day adaptation period before the start of the experiment to adapt to their respective diets.

Feed analyses

Chemical analyses of the feed and supplemented diets focused on the determination of their chemical composition and nutritive value, in accordance with the guidelines of the French Standardization Association (AFNOR, 1982) and the Association of Official Analytical Chemists (AOAC, 1990). The analyses included determination of dry matter (DM), organic matter (OM), mineral matter (MM), total nitrogen (TN), crude fiber (CF), calcium (Ca) and phosphorus (P). The energy value of the diet, expressed in French INRA units as UFL (Milk Feed Unit) was calculated using the equations proposed by Soltner (1986). The protein value, expressed as Protéines Digestibles dans l'Intestin (PDI) or metabolizable protein (PDI), was calculated according to the equations proposed by INRA (2010).

Table 1 - Nutritional value of feed served to sheep.

Feed	DM (%FM)	OM (%DM)	F (%DM)	MM (%DM)	CP (%DM)	CF (%DM)	Ca (%DM)	P (%DM)
Alfalfa hay	89.48	89.87	0.45	10.13	20.85	23.91	0.16	0.31
Wheat straw	93.33	90.46	3.11	09.54	03.59	29.74	0.12	0.29
Concentrate	86.20	89.65	0.60	10.35	16.39	01.44	0.05	0.49

DM: dry matter; FM: fresh matter; OM: organic matter; F: fat; MM: mineral matter; CP: crude protein; CF: crude fiber; Ca: calcium; P: phosphorus.

Body condition score and body weight

The body condition score (BCS) and body weight of the ewes were recorded in the morning during the eighth and third weeks before lambing and during the second, fourth, eighth and twelfth weeks after lambing. BCS was assessed using the 0-5 scale developed by Russel et al. (1969), based on palpation of the lumbar region. Each ewe was weighed using appropriate livestock scale (Marechalle-pesage, PM 150, France) with a maximum capacity of 200 kg and an accuracy of ± 500 g. The lambs were also monitored for growth performance. They were weighed at birth, week during the first month of life, and subsequently at 8 and 12 weeks of age using a precision scale (Dahongying, China) with a maximum capacity of 30 kg and an accuracy of ± 5 g. Average daily gain (ADG) was calculated as follows:

$$\text{ADG (g)} = \frac{\text{weight (g) at (n) days} - \text{weight (g) at 0 days}}{(n) \text{ days}}$$

Milk yield

The milk yield of each ewe during the first, fifth and eighth weeks of lactation was estimated using the equations developed by Torres-Hernandez and Hohenboken (1980), which predict milk production based on the weight gain of suckling lambs and litter size.

Table 2 - Regression equations to predict milk production per day relative to ADG and correlation coefficient (r) according to Torres-Hernandez and Hohenboken (1980)

N° of week	Single lambing		Double lambing	
	Equation	r ²	Equation	r ²
1	MY= 0.17+5.06×ADG**	0.65	MY= 0.03+4.98×ADG**	0.69
5	MY= 0.92+3.03×ADG*	0.30	MY= 1.65+0.61×ADG	0.01
8	MY= 0.42+3.45×ADG*	0.33	MY= 0.63+1.89×ADG*	0.20

MY: Milk Yield; ADG: Average Daily Gain; (** P < 0.01); (* P < 0.05).

Milk analyses

Milk samples were collected by manual milking in the morning after temporary separation of the ewes from their lambs. Samples were collected during the first, second, fourth, and twelfth weeks of lactation into labelled glass tubes and transported to the laboratory in a cooled container to preserve milk quality and prevent acidification. Milk composition, including fat (FM%), total protein (TP%), lactose (L%) and total solids (TS%), was determined using an ultrasonic milk analyser (BQC®, Ultrasonic Milk Analyzer), which automatically aspirates the milk sample through a probe and displays the analytical results digitally.

Statistical analyses

Statistical analyses were performed using STATISTICA version 10 (StatSoft Inc., USA). Differences between the control and experimental groups were evaluated using Student's t-test. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

To ensure that the experiment was not influenced by pre-existing differences between the treatment groups, the body weight and body condition score the ewes were evaluated before the start of the experiment. As shown in Table 3, no significant differences were observed between the control and experimental groups in either ewe body weight (EW) or body condition score (BCS) before lambing.

Table 3 - Ewes Weight (EW) and Body Condition Score (BCS) of ewes before and after lambing.

Treatments		Control	Experimental	P-value
Before lambing	EW -8 week	65.83±9.60	60.67±10.67	0.44
	BCS -8 week	2.58±0.66	2.50±0.45	0.84
	EW -3 week	69.17±8.06	65.00±10.02	0.47
	BCS -3 week	2.17±0.60	2.25±0.61	0.84
After lambing	EW +2 week	58.00±8.69	56.42±9.30	0.78
	BCS +2 week	1.67±0.41	1.83±0.52	0.61
	EW +4 week	53.33±7.47	50.83±9.56	0.56
	BCS +4 week	1.67±0.26	1.50±0.45	0.47
	EW +8 week	52.42±6.57	52.08±7.30	0.93
	BCS +8 week	1.67±0.26	1.58±0.38	0.70

EW: Ewes Weight (Kg); BCS: Body Condition Score; $P > 0.05$: insignificant difference.

Although no statistically significant differences were detected throughout the experimental period, the BCS results revealed interesting trends. Eight weeks before lambing, the control group exhibited a 3.2% higher BCS than the Clay-Ginger group. However, by three weeks before lambing, the supplemented group had surpassed the control group by 3.7%, maintaining this advantage until the second week postpartum, when the difference reached 8.7%. During the fourth and eighth weeks of lactation, BCS values were again slightly higher in the control group by 11.3% and 5.7%, respectively.

Although supplementation with the Clay-Ginger combination did not significantly increase BCS, it appeared to reduce the mobilization of body fat reserves due to transition period. This effect may reflect an improved energy status during the physiologically demanding period extending from three weeks before lambing to two weeks after lambing. Similar observations were reported by [Kahouli et al. \(2020\)](#), who evaluated 5% clay supplementation under comparable experimental conditions using Ouled Djellal ewes from late gestation to weaning. Likewise, [Chemmam et al. \(2009\)](#) reported that nutritional supplementation during late gestation and early lactation significantly reduced body weight and BCS losses in Ouled Djellal ewes grazing natural pastures.

Although no significant differences were observed, [Abo Bakr \(2019\)](#) reported lower body weight loss during early lactation in ewes supplemented with different forms and doses of ginger (3 or 6 g/head/day of ginger powder or 2 ml/head/day of ginger oil). Similar findings were reported by [Shams Al-Dain and Jarjis \(2015\)](#) in dairy cows receiving 75 or 150 g/head/day of ginger powder.

According to available literature and the researchers knowledge no available studies prior to present study have evaluated the combined supplementation of clay and ginger in ewes from late gestation through the end of lactation. However, studies conducted in dairy cattle have demonstrated beneficial effects of lay supplementation. [Karatzia et al. \(2013\)](#) reported significantly higher BCS ($P < 0.05$) in dairy cows supplemented with 200 g/day of zeolite compared with controls. Likewise, [Garzón et al. \(2017\)](#) observed highly significant improvements ($P < 0.01$) in BCS in cows receiving 2% zeolite before and after calving.

Overall, clay supplementation may improve body condition indirectly by modifying ruminal fermentation patterns, altering volatile fatty acid production, and increasing starch flow to the intestine, thereby improving the animal's energy balance ([Katsoulos et al., 2006](#); [Karatzia et al., 2013](#)).

Milk production

The clay-ginger supplementation significantly increased milk yield during the first ($P = 0.02$) and fifth ($P = 0.02$) weeks of lactation by 30.1% and 14.1%, respectively, compared with the control group. During the eighth week, milk yield remained higher in the supplemented group, although the difference was not sufficiently significant ($P = 0.06$) (Table 4).

Table 4 - Estimated milk production for the 1st, 5th and 8th weeks, according to the Torres-Hernandez and Hohenboken (1980).

Milk Yield	Control	Experimental	P-value
1 st week (kg/d)	1.13 ^b ± 0.10	1.47 ^a ± 0.18	0.02
5 th week (kg/d)	1.49 ^b ± 0.06	1.70 ^a ± 0.04	0.02
8 th week (kg/d)	0.94 ± 0.05	1.08 ± 0.04	0.06

a, b: In the same row, means with different subscripts are significantly different ($P < 0.05$); ($P > 0.05$): insignificant difference.

These results are partially consistent with those reported by Abo Bakr (2019), who observed significant increases ($P < 0.05$) in milk yield at four weeks of lactation in ewes supplemented with different forms and doses of ginger. The greatest increase was obtained with 3 g/day of ginger powder, followed by 2 ml/day of ginger oil and 6 g/day of ginger powder. At eight weeks, however, the increase was no longer significant, whereas total milk production over the three-month lactation period remained significantly higher than that of the control group. Similarly, Al-Musodi and Jaafar (2019) reported that supplementation of Iraqi goats with 60 g of ginger powder per kilogram of concentrate significantly increased milk production during the fourth, fifth, and sixth weeks after kidding. Kholif et al. (2012) also demonstrated that supplementation with 2 ml/day of ginger oil significantly increased milk production ($P < 0.05$) throughout the first 90 days of lactation in Damascus goats, beginning on the seventh day after kidding, resulting in an 18.9% increase compared with the control group.

In contrast to the present findings, Kahouli et al. (2020) reported a significant increase in milk production during the eighth week of lactation (+ 29.40%; $P < 0.05$) in ewes receiving kaolinite, whereas significant effect was observed during the first week. Helal and Abdel-Rahman (2010) found that supplementation with 4% bentonite significantly increased milk yield by 7.7% and fat-corrected milk (4% fat) by 10.7%, possibly due to increased retention time of feed in the rumen. Similarly, Abdel Mawla et al. (1998) reported that sodium bentonite supplementation (6 or 12 g/head/day) had no significant effect during the second week of lactation but significantly increased milk production during the fourth ($P < 0.05$) and sixth ($P < 0.01$) weeks. Tayeb (2017) also reported significant improvements in daily milk yield following supplementation with 2% bentonite combined with either 2% or 2.5% urea. Likewise, Ammar and Al-Hafz (2019) observed significant increases ($P \leq 0.05$) in weekly and monthly milk production in Awassi ewes receiving 20 or 30 g/head/day of ginger. In dairy cows, Shams Al-Dain and Jarjis (2015) reported significant increases ($P \leq 0.05$) in milk yield of 30.9% and 58.2% following supplementation with 75 and 150 g/head/day of ginger powder, respectively. The increase in milk production reported in these studies has been attributed to several mechanisms:

1. Reduced methane production, decreased acetate concentration, increased propionate production, and higher total volatile fatty acid concentrations, resulting in improved energy efficiency (Patra and Saxena, 2010; Kholif et al., 2012).
2. Improved digestibility of fiber and dry matter (Nanon et al., 2014; Abo Bakr, 2019).
3. Enhanced nitrogen and energy utilization, leading to improved feed efficiency and animal health (Giannenas et al., 2013).
4. Stimulation of bile acid synthesis and pancreatic enzyme activity, thereby improving lipid digestion and nutrient absorption (Kumar et al., 2014).
5. Increased retention time of feed within the rumen (Helal and Abdel-Rahman, 2010).
6. The duration of dietary supplementation (Katsoulos et al., 2006).

Milk quality

The effects of the combined natural additives (Clay-Ginger) on milk composition are presented in Table 5.

During the first week of lactation, milk fat (MF) content was significantly higher in the experimental group than in the control group ($12.24 \pm 0.74\%$ vs. $8.94 \pm 0.78\%$; +36.91%; $P = 0.01$). In contrast, the control group exhibited a significantly higher lactose concentration than the supplemented group ($5.14 \pm 0.40\%$ vs. $4.20 \pm 0.10\%$; +22.38%; $P = 0.03$). No significant differences were observed between the groups in total protein or total solids. During the fourth week of lactation, lactose was the only milk component that differed significantly between treatments, showing a 14.44% increase compared with the control group ($P = 0.04$).

Overall, no significant differences in milk composition were observed between the two groups during the second and twelfth weeks of lactation (Table 5).

Table 5 - Milk quality (Milk Fat, Total Protein, Lactose and Total Solid) during the 1st, 2nd, 4th and 12th weeks.

Milk components		Milk Fat (%)	Total Protein (%)	Lactose (%)	Total Solid (%)
Treatments					
1 st week	Control	08.94 ^b ± 0.78	4.14 ± 0.13	5.14 ^a ± 0.40	10.96 ± 0.11
	Experimental	12.24 ^a ± 0.74	4.12 ± 0.09	4.20 ^b ± 0.10	10.77 ± 0.19
	P-value	0.01	0.64	0.03	0.06
2 nd week	Control	6.54 ± 2.30	3.77 ± 0.15	6.08 ± 1.25	10.21 ± 0.40
	Experimental	7.24 ± 2.12	3.91 ± 0.20	4.48 ± 0.42	10.56 ± 0.53
	P-value	0.64	0.40	0.10	0.41
4 th week	Control	6.12 ± 1.64	3.67 ± 0.31	4.71 ^b ± 0.24	09.96 ± 0.88
	Experimental	7.28 ± 0.90	3.75 ± 0.14	5.39 ^a ± 0.66	10.15 ± 0.43
	P-value	0.23	0.53	0.04	0.59
12 th week	Control	3.98 ± 2.16	3.91 ± 0.21	5.47 ± 0.35	10.63 ± 0.60
	Experimental	2.96 ± 0.65	3.90 ± 0.12	5.59 ± 0.51	10.64 ± 0.34
	P-value	0.33	0.98	0.35	0.97

a, b: In the same column, means with different subscripts are significantly different ($P < 0.05$); ($P > 0.05$): insignificant difference.

Unlike the present findings for the first week of lactation, [Al-Musodi and Jaafar \(2019\)](#) reported no significant effects ($P > 0.01$) of supplementing Iraqi dairy goats with ginger powder (60 g/kg concentrate) on milk fat, protein, or lactose during the same period. However, they observed highly significant increases ($P < 0.01$) in milk fat during the third and fourth weeks, milk protein during the fourth and fifth weeks, and lactose during the fourth, fifth, and sixth weeks, all in favour of the ginger-supplemented diet. Similarly, [Abo Bakr \(2019\)](#) reported that supplementation with different forms and doses of ginger (3 or 6 g/day of ginger powder and 2 ml/day of ginger oil) had no significant effect on the percentages of total protein, total solids, or lactose during the first four weeks of lactation. However, supplementation with 6 g/day of ginger powder significantly increased ($P < 0.05$) milk fat content, whereas 3 g/day of ginger powder and 2 ml/day of ginger oil had no positive effect compared with the control group. During the second month of lactation, no significant differences were observed. Nevertheless, supplementation with 2 ml/day of ginger oil resulted in significant decreases ($P < 0.05$) in milk fat and total solids during the final four weeks and over the entire 12-week lactation period. Likewise, [Kholif et al. \(2012\)](#) reported a significant reduction ($P < 0.05$) in milk fat content in goats receiving ginger oil supplementation compared with the control group, whereas milk protein concentration was significantly higher in the supplemented animals.

No significant differences were observed in lactose concentration. [Hendawy et al. \(2019\)](#) reported significant improvements ($P < 0.05$) in milk fat and total solids in ewes supplemented with ginger powder, whereas milk protein and lactose concentrations remained unaffected. Similarly, [Ammar and Al-Hafz \(2019\)](#) found no significant effects of ginger powder supplementation on milk fat, protein, or lactose content in ewe milk. The findings of [Kahouli et al. \(2020\)](#) who evaluated 5% kaolinite supplementation in Ouled Djellal ewes, differ from those of the present study. They reported no significant differences between the supplemented and control groups in milk fat, lactose, or protein concentrations during the first week of lactation. Likewise, [Ouachem et al. \(2012\)](#) reported that supplementation with 5% marl significantly increased ($P < 0.05$) milk protein concentration by 15% in Arbia goats but had no effect on milk fat or lactose.

[Salem and El-Shewy \(2001\)](#) reported that supplementing goat diets with 3% bentonite from parturition until the sixth week of lactation significantly increased ($P < 0.05$) milk fat and protein percentages. In sheep, however, [Tayeb \(2017\)](#) observed that supplementation with 2% bentonite significantly affected only milk fat content ($P < 0.05$). Other studies have reported highly significant effects ($P < 0.01$) of bentonite supplementation on milk fat and lactose concentrations in goats ([Abdel Mawla et al., 1998](#)) and on milk fat and total solids in ewes ([Helal and Abdel-Rahman, 2010](#)). Suggested that the improvement in milk fat content may result from increased acetate production associated with enhanced ruminal microbial activity following an improved immune response.

[Kholif et al. \(2012\)](#) also attributed changes in milk fat concentration to increased milk yield and to the effects of ginger essential oil supplementation, although these effects were not always statistically significant. Similar conclusions were reported by [Abo Bakr \(2019\)](#). [Helal and Abdel-Rahman \(2010\)](#) proposed that improvements in milk protein content result from enhanced microbial protein synthesis in the rumen. A similar explanation was provided by [Ouachem et al. \(2012\)](#), who attributed the increase in milk protein concentration to the stimulatory effect of marl on ruminal microbial activity. According to Ahmide and Abouzogaya (2009) cited by [Shams Al-dain and Jarjeis \(2015\)](#) the higher lactose concentration observed in cows supplemented with ginger root may be associated with reduced blood glucose concentrations.

Newborn weight and average daily gain

No significant effect ($P > 0.05$) of Clay–Ginger supplementation was observed on lamb birth weight compared with the control group. Similarly, no significant differences in average daily gain (ADG) was detected throughout the suckling period between lambs born to supplemented and control ewes. Despite the absence of statistically significant differences, lambs born to supplemented ewes exhibited a clear numerical improvement in growth during the first four weeks of life. Growth rates were approximately 18%, 22%, 29%, and 36% higher than those of the control group during the first, second, third, and fourth weeks, respectively. However, this trend was reversed during the later stages of the suckling period. At the eighth and twelfth weeks, growth performance of lambs from the supplemented group was approximately 27% and 19.5% lower, respectively, than that of the control group (Table 6).

CONCLUSION AND RECOMMENDATION

This study demonstrated the following fundamental findings:

- Although the combined supplementation with clay (5%) and ginger (2%) did not significantly affect the body condition score (BCS) of the ewes, it appeared to reduce the mobilization of body fat reserves during the last three weeks of gestation and the first two weeks of lactation.
- Combined supplementation significantly increased milk production during the first and fifth weeks of lactation, whereas no significant effect was observed during the eighth week.
- Milk fat content was significantly higher in the supplemented group during the first week of lactation; however, no significant differences were detected during the subsequent weeks.
- Total solids differed significantly between the groups during the first week of lactation, with higher values in the control group, whereas no significant differences were observed during the remaining weeks.
- Lactose concentration differed significantly during the first and fourth weeks of lactation, with higher values in the control group; no significant differences were detected at the other sampling times.
- No significant effects of Clay–Ginger supplementation were observed on lamb birth weight or average daily gain (ADG), although lambs from supplemented ewes exhibited higher numerical growth rates during the first four weeks of life.

The results suggest that dietary supplementation with a combination of clay and ginger may provide beneficial effects on the productive performance of ewes during the transition period, particularly with respect to milk production and the conservation of body reserves. However, further studies involving larger sample sizes and detailed investigations of nutrient digestibility, ruminal fermentation characteristics, and rumen microbial populations are required to clarify the mechanisms underlying these effects.

DECLARATIONS

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Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contribution

A. Smaili: Conceptualization, methodology, data collection, manuscript drafting, data analysis, interpretation. N. Kaboul: Conceptualization, methodology, literature review, data analysis. M. Yousfi: Methodology, Statistical analysis, supervision, manuscript drafting, interpretation. F. Rekik: Conceptualization, methodology, critical revisions. All authors read and approved the final manuscript.

Ethical consideration

All animal care and experimental procedures, including dietary modifications, housing, and handling, strictly complied with the Algerian national regulations on animal health and welfare (Law No. 88-08 of January 26, 1988, amended and supplemented by Law No. 19-03 of July 17, 2019). All experimental phases were conducted under the direct supervision of certified zootechnicians and local veterinary services.

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Competing Interests

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Abbreviations

M.A.D.R: Ministry of Agriculture and Rural Development; T.I.Bre: Technical Institute of Breedings; BCS: Body Condition Score; AFNOR: French standardisation association; AOAC: Association of Official Analytical Chemists; INRA: National Institute for Agricultural Research; UFL: Milk Forage Unit; PDI: Proteins that can be Digested in the Intestine; ADG: Average Daily Gain.

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THE EFFECT OF TWO INTRAVAGINAL PROGESTERONE DOSES AND OVUM PICK-UP OUTCOMES IN BROWN SWISS COWS: A BAYESIAN HIERARCHICAL ANALYSIS

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 Supporting Information



ABSTRACT: This study evaluated the effect of two intravaginal progesterone doses on follicular recruitment, oocyte recovery, and oocyte viability in Brown Swiss cows undergoing ovum pick-up. Eight dry cows were allocated to two treatments: T1 (0.6 g progesterone) and T2 (1.2 g progesterone), with four cows per treatment and five repeated sessions per animal. The responses analyzed were the numbers of 2–4 mm follicles, 4–8 mm follicles, total recovered oocytes, recovery probability, and oocyte viability. Count data were analyzed with negative binomial models, whereas recovery and viability were modeled as binomial responses, with cow included as a random effect. Overall, T1 showed higher adjusted posterior means for the number of 2–4 mm follicles per ovum pick-up (OPU) session (7.62 vs. 5.36 follicles), 4–8 mm follicles per OPU session (4.17 vs. 3.27 follicles), and total recovered oocytes per OPU session (4.75 vs. 3.56 oocytes) compared with T2. Oocyte viability, expressed as an adjusted posterior probability, was also higher in T1 than in T2 (0.750 vs. 0.566, equivalent to 75.0% vs. 56.6%). In contrast, the adjusted posterior probability of oocyte recovery was nearly identical between treatments (0.403 vs. 0.402, equivalent to 40.3% vs. 40.2%). Among the evaluated outcomes, oocyte viability showed the clearest treatment difference, with an odds ratio of 2.50 and a posterior probability of superiority of 0.989 in favor of T1. Under the conditions of this study, the 0.6 g progesterone protocol was associated with a more favorable follicular response and better oocyte viability, while oocyte recovery efficiency remained essentially unchanged.

Keywords: Cumulus-oocyte complexes; Follicular synchronization; Oocyte competence; Posterior inference; Reproductive hormones.

INTRODUCTION

In vitro embryo production is an important reproductive tool for accelerating genetic progress in cattle (Chinarov, 2024; Dogan and Yenilmez, 2024). Within this process, ultrasound-guided transvaginal follicular aspiration, or ovum pick-up (OPU), plays a central role because it determines the number of oocytes available for later stages such as maturation, fertilization, and embryo development (Sakaguchi, 2025; Unchupaico-Payano et al., 2025). The success of OPU depends not only on how many oocytes are recovered, but also on their biological competence, since both quantity and quality influence the final efficiency of in vitro embryo production (Jemal, 2022).

Follicular wave synchronization and pre-OPU hormonal treatments are commonly used to improve the ovarian response of donor cows by creating a more favorable endocrine environment (Hayden et al., 2025). Previous studies have shown that synchronization may influence not only the number of follicles available for aspiration, but also oocyte recovery and the developmental competence of cumulus-oocyte complexes, depending on the hormonal combination used, including progesterone, estradiol, gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), or equine chorionic gonadotropin (eCG) (Payano et al., 2024; Unchupaico-Payano et al., 2025). Current evidence also indicates that both the number and the quality of recovered oocytes remain central to the success of ovum pick-up and in vitro production (OPU-IVP) programs, which explains the continued interest in improving endocrine protocols before aspiration (Salek et al., 2025; Sakaguchi, 2025). Even so, an important question remains unresolved. Although progesterone-based protocols are widely applied in practice, the specific effect of changing the intravaginal progesterone dose on follicular recruitment, oocyte recovery, and oocyte viability is still not well defined. This distinction matters because a protocol may increase follicle numbers or oocyte yield without improving the biological quality of the recovered cumulus-oocyte complexes (COCs), whereas in other cases the main benefit may be reflected in oocyte competence (Lemseffer et al., 2022). For this reason, evaluating only total yield is not sufficient; it is also necessary to examine how treatment affects each biological stage that precedes embryo production.

An additional point concerns the statistical treatment of OPU data. These responses are not simple Gaussian outcomes (Perez et al., 2017), but rather counts and conditional variables measured repeatedly in the same cows (Motta

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et al., 2024). Despite this, studies in bovine reproduction have often relied on linear methods or conventional comparisons between treatments (Ciftci and Dinc, 2023). A hierarchical Bayesian framework is better suited to this type of data, since it allows each outcome to be analyzed according to its distributional structure, incorporates between-cow heterogeneity, and estimates treatment effects together with their posterior uncertainty (Feldes et al., 2024). For this reason, the present study examined the effect of two intravaginal progesterone doses on follicular recruitment, oocyte recovery, and oocyte viability in Brown Swiss cows undergoing OPU through a hierarchical Bayesian approach.

MATERIALS AND METHODS

Study area

The study was carried out at the cattle unit of the Faculty of Zootechnics, Universidad Nacional del Centro del Perú, located on the left bank of the Mantaro Valley, in the district of El Mantaro, Jauja province, Junín, Peru. The experimental herd consisted of Brown Swiss cows raised under highland conditions at approximately 3,320 m above sea level, about 36.5 km from the city of Huancayo (Senamhi, 2025). Throughout the experimental period, the animals were maintained under an exclusive alfalfa grazing system. Overall, this Andean production setting, together with an alfalfa-based feeding system, represents a suitable context for evaluating reproductive protocols in dairy cattle under the environmental and management conditions of the central Peruvian highlands.

Animals and distribution

The experiment included eight clinically healthy Brown Swiss cows in the dry period from the cattle unit of the Faculty of Zootechnics, Universidad Nacional del Centro del Perú. The animals were distributed into two treatments, with 4 cows per treatment. Treatment 1 corresponded to the bovine intravaginal device (DIB) with 0.6 g of progesterone, while treatment 2 corresponded to the DIB with 1.2 g of progesterone. Five repetitions were performed on each cow, obtaining a total of 20 repetitions per treatment and 40 experimental observations in total. For follicular wave synchronization, on day 0 the corresponding DIB was inserted, and 1.2 mg of estradiol benzoate and 0.524 mg of sodium cloprostenol were administered (Payano et al., 2024). Subsequently, on day 5, oocyte collection was performed via ovum pick-up (OPU) using an EsaoteVet® MyLabOneVet ultrasound scanner, equipped with a 7.5 MHz microconvex transducer, employing a WTA® Brazil follicular aspiration guide and a pressure of 90 mmHg. The evaluated variables were the number of 2 to 4 mm follicles, the number of 4 to 8 mm follicles, the total number of recovered oocytes, and oocyte viability (Unchupaico-Payano et al., 2025).

Data collection

The study variables were: number of 2 to 4 mm follicles, number of 4 to 8 mm follicles, total number of recovered oocytes, and oocyte viability, following the methodology used by Payano et al. (2024). Data collection was performed during each ovum pick-up (OPU) session. Prior to aspiration, through transvaginal ultrasound evaluation, the follicles present in both ovaries were identified and counted, classifying them according to their diameter into two categories: 2–4 mm follicles and 4–8 mm follicles. Subsequently, during follicular aspiration, cumulus-oocyte complexes (COCs) were recovered, which were quantified to obtain the total number of recovered oocytes per repetition. Oocyte viability was determined immediately after recovery, through observation under a stereoscope, considering the morphological characteristics of the cumulus and the cytoplasm. The COCs were classified into quality categories, and for the analysis, they were grouped into viable and non-viable oocytes. COCs with adequate morphology and structure compatible with biological competence were considered viable, while those with deficient morphology or signs of degeneration were classified as non-viable. All records were obtained individually per cow and per repetition, thus generating the database for statistical analysis.

Statistical analysis

The statistical analysis was performed under a Bayesian hierarchical approach, considering the repeated structure of the data, with 5 repetitions per cow and 4 cows per treatment, for a total of 40 experimental observations. The treatment was included as a fixed effect, with two levels: T1 (0.6 g of progesterone) and T2 (1.2 g of progesterone), while the cow was incorporated as a random intercept to capture the biological heterogeneity among animals.

The count variables, corresponding to the number of 2–4 mm follicles, number of 4–8 mm follicles, and total number of recovered oocytes, were modeled using negative binomial distributions due to the discrete nature of the data and the possible presence of overdispersion. For these responses, a logarithmic link function was used, so that, for each observation i in cow j , a model of the form was specified:

$$Y_{ij} \sim \text{NegBin}(\mu_{ij}, \phi), \log(\mu_{ij}) = \beta_0 + \beta_1 \text{Treatment}_{ij} + u_j$$

where β_0 represents the intercept, β_1 the treatment effect and u_j the random effect associated with cow j , with $u_j \sim N(0, \sigma_u^2)$.

The probability of oocyte recovery was analyzed as a binomial response conditional on the total number of observed follicles, whereas oocyte viability was modeled as a binomial response conditional on the total number of recovered oocytes. In both cases, a logit link function was used. Thus, the models were formulated as:

$$R_{ij} \sim \text{Binomial}(F_{ij}, p_{ij}), \text{logit}(p_{ij}) = \alpha_0 + \alpha_1 \text{Treatment}_{ij} + u_j$$

for recovery, where R_{ij} is the number of recovered oocytes and F_{ij} the total number of follicles; and:

$$V_{ij} \sim \text{Binomial}(O_{ij}, q_{ij}), \text{logit}(q_{ij}) = \gamma_0 + \gamma_1 \text{Treatment}_{ij} + u_j$$

for viability, where V_{ij} is the number of viable oocytes and O_{ij} the total number of recovered oocytes.

Inference was performed using Markov chain Monte Carlo (MCMC) sampling implemented in Stan through the brms package in R. Weakly informative prior distributions were used for the fixed coefficients and variance components in order to regularize estimation without imposing strong restrictions on the parameters. Model convergence was assessed through the number of divergent transitions, the $\hat{R}$ statistic, and the effective sample size for the bulk and tails of the posterior distribution. Additionally, trace plots of the chains and the marginal posterior distributions of the treatment effects were graphically inspected.

From the posterior distributions, posterior means, medians, 95% credible intervals, posterior probabilities of superiority $P(T1 > T2)$, rate ratios for the count variables, and odds ratios for the binomial variables were obtained. Likewise, population-level adjusted values for each treatment and posterior absolute contrasts between T1 and T2 were calculated in order to interpret the results both on the relative scale and on the original biological scale. R Studio version 4.5.2 was used.

RESULTS

Data structure

Table 1 summarizes the descriptive results of the variables evaluated in each treatment group. Both T1 and T2 included 20 observations, indicating a balance dataset. In T1 (0.6 g), the means and standard deviations were 4.75 ± 2.34 for recovered oocytes, 3.60 ± 2.30 for viable oocytes, 1.15 ± 0.88 for non-viable oocytes, 7.60 ± 2.76 for 2–4 mm follicles, 4.15 ± 2.08 for 4–8 mm follicles, and 11.75 ± 2.67 for total follicles. In T2 (1.2 g), the corresponding values were 3.55 ± 2.33 , 2.0 ± 1.65 , 1.55 ± 1.05 , 5.55 ± 3.39 , 3.30 ± 2.08 , and 8.85 ± 3.33 , respectively. For the variables derived, recovery was very similar in both treatments, reaching 0.410 in T1 and 0.417 in T2. Oocyte viability, however, was higher in T1 (0.72) than in T2 (0.48). These descriptive values offer a preliminary overview of the response pattern in each before the statistical analysis.

Table 1 - Structure of the analyzed dataset by treatment and cow

Variable	T1 (0.6 g)	T2 (1.2 g)	p-value
Number of observations	20	20	—
Recovered oocytes, n/session	4.75 ± 2.34	3.55 ± 2.33	0.112
Viable oocytes, n/session	3.60 ± 2.30^a	2.00 ± 1.65^b	0.016
Non-viable oocytes, n/session	1.15 ± 0.88	1.55 ± 1.05	0.200
2–4 mm follicles, n/session	7.60 ± 2.76^a	5.55 ± 3.39^b	0.043
4–8 mm follicles, n/session	4.15 ± 2.08	3.30 ± 2.08	0.204
Follicles count	11.75 ± 2.67^a	8.85 ± 3.33^b	0.004
Oocyte recovery, %	41.0	41.7	0.949
Oocyte viability, %	75.8 ^a	56.3 ^b	0.008

Values are presented as mean $\pm$ standard deviation for count variables and as percentages for oocyte recovery and viability. For count variables, p-values were calculated using Welch's two-sample t-test. For oocyte recovery and viability, p-values were calculated using a two-proportion z-test based on aggregated counts. Different superscript letters within the same row indicate statistically significant differences between treatments ($p < 0.05$). When treatments share the same letter, no statistically significant difference was detected. OPU: ovum pick-up.

Convergence diagnostics

Table 2 presents the convergence diagnostics for the hierarchical Bayesian models. No divergent transitions were detected in any of the fitted models, which suggests stable posterior sampling. In all cases, the maximum R value was 1.00, indicating good convergence and adequate mixing of the chains. The minimum effective sample sizes were also satisfactory, ranging from 2024 to 3648 for the bulk of posterior distribution and from 2711 to 3937 for the tails. Overall, these results support the computational stability of the models and the reliability of the posterior estimates for treatment effects. Posterior predictive checks further showed that the fitted models were able to reproduce the general shape of the observed distributions for the five responses analyzed (Figure 1).

Table 2 - Convergence diagnostics of the Bayesian hierarchical models fitted for each response variable.

Response variable	n_divergent	Max rhat	Min bulk ESS	Min tail ESS
2–4 mm follicles	0	1.00	2185	2711
4–8 mm follicles	0	1.00	3648	3937
Oocyte recovery	0	1.00	2024	2805
Oocyte viability	0	1.00	3418	3844
Ovocitos count	0	1.00	3514	3739

Divergent transitions indicate the number of problematic MCMC transitions. max Rhat represents the maximum potential scale reduction factor; values close to 1.00 indicate adequate convergence. min bulk ESS and min tail ESS indicate the minimum effective sample sizes for the central and tail regions of the posterior distribution, respectively.

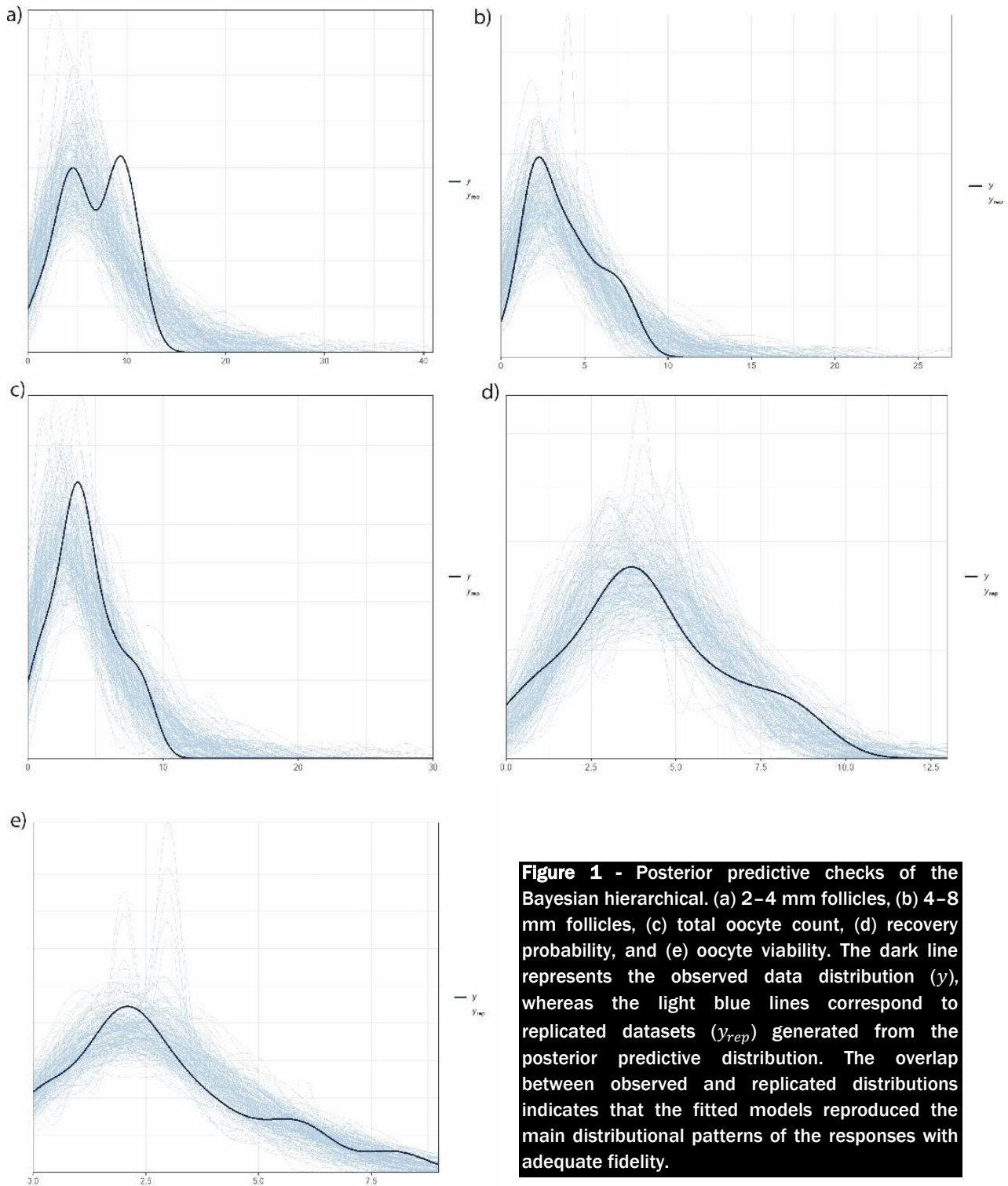


Figure 1 - Posterior predictive checks of the Bayesian hierarchical. (a) 2–4 mm follicles, (b) 4–8 mm follicles, (c) total oocyte count, (d) recovery probability, and (e) oocyte viability. The dark line represents the observed data distribution (y), whereas the light blue lines correspond to replicated datasets (y_{rep}) generated from the posterior predictive distribution. The overlap between observed and replicated distributions indicates that the fitted models reproduced the main distributional patterns of the responses with adequate fidelity.

Posterior treatment effects

Table 3 shows the posterior effect of T1 (0.6 g) relative to T2 (1.2 g) for each response variable. On the model's linear scale, the posterior mean was positive for 2-4 mm follicles (0.353), 4-8 mm follicles (0.244), oocyte viability (0.845), and total oocyte count (0.290), whereas the effect on recovery was essentially zero (0.000692). The posterior probability that the effect of T1 was greater than zero was 0.931 for 2-4 mm follicles, 0.842 for 4-8 mm follicles, 0.989 for oocyte viability, and 0.878 for total oocyte count.

By contrast, the corresponding value for recovery probability was 0.502, which suggests no clear directional difference between treatments. For ease of interpretation, count outcomes were expressed as rate ratios and binomial outcomes as odds ratios. For 2-4 mm follicles, the mean posterior rate ratio was 1.47, with a 95% credible interval between 0.874 and 2.36; for 4-8 mm follicles, the rate ratio was 1.32 (0.782-2.07); and for total oocytes, the rate ratio was 1.38 (0.797-2.21).

In the case of recovery, the mean posterior odds ratio was 1.03 (0.595-1.67), reflecting a response centered very close to the null value. In contrast, viability showed the most pronounced relative difference between treatments, with a mean posterior odds ratio of 2.50 and a 95% credible interval from 1.10 to 4.92, providing the clearest posterior evidence of a treatment effect among all evaluated responses.

Figure 2 presents the population-posterior adjusted means and 95 % credible intervals for each treatment on the original scale of the response variable. T1 (0.6 g) showed higher adjusted means than T2 (1.2 g) for 2-4 mm follicles, 4-8 mm follicles, total recovered oocytes, and oocyte viability. By contrast, recovery probability was virtually the same in both treatments. These results indicate that the main differences between protocols were concentrated in follicular response, total oocyte yield, and especially viability, while recovery remained stable.

Figure 3 presents the posterior absolute contrasts between treatments, expressed as T1 (0.6 g) minus T2 (1.2 g), on the original scale of each variable. Accordingly, the results can be interpreted not only by the direction of the difference, but also by its practical magnitude in counts or percentage points. In the count variables, the contrasts were located at positive values for 2-4 mm follicles, 4-8 mm follicles, and total oocytes, indicating that the adjusted posterior means of these responses tended to be higher in T1 than in T2.

Among them, the greatest absolute shift was observed in 2-4 mm follicles, followed by the total number of oocytes and, to a lesser extent, by 4-8 mm follicles. In the variables expressed as probability, the contrast for recovery remained very close to zero, reflecting an almost null absolute difference between treatments. In contrast, oocyte viability showed the greatest shift on the probability scale, with a clearly wider positive contrast than the rest of the variables, indicating a substantial difference in percentage points in favor of T1.

Joint posterior patterns among biological responses

Figure 4 summarizes the joint structure of the posterior distributions of the treatment effects on pairs of key biological responses, expressed on the transformed scale as rate ratios or odds ratios of T1 (0.6 g) relative to T2 (1.2 g). In all panels, the dotted lines at 1 delimit the null value, so that the regions of greatest density located to the right and/or above that point indicate posterior shifts compatible with a greater relative response under T1. This representation makes it possible to evaluate simultaneously not only the direction of the treatment effect on each variable, but also the way in which these responses tend to shift jointly within the posterior space.

In panel a), corresponding to the joint density between the rate ratio for 2-4 mm follicles and the rate ratio for total oocytes, the greatest posterior concentration is located in the upper-right quadrant, with both axes above the null value. This pattern suggests that the highest posterior density was concentrated in the region where T1 was associated with both a greater availability of small follicles and a higher total oocyte yield.

In panel (b), which compares the odds ratios for recovery and viability, a different pattern emerged: posterior density remained centered near the null value for recovery but was clearly shifted above 1 for viability. In biological terms, this reflects that the joint posterior signal is concentrated on a practically stable recovery between treatments, accompanied by relatively greater oocyte viability under T1.

Panel c), corresponding to the joint density between the rate ratio for 4-8 mm follicles and the rate ratio for total oocytes, also presents its main posterior core in the upper-right region, although with a somewhat wider dispersion than that observed for 2-4 mm follicles. This suggests that the joint shift between medium follicles and oocyte yield was positive, but with greater relative uncertainty than that observed for the early follicular response.

Finally, in panel d), which relates the rate ratio for 2-4 mm follicles to the odds ratio of viability, the posterior mass is again concentrated above the null value on both axes, showing that the shift toward a greater presence of small follicles tends to coexist with a favorable shift in the viability of the recovered oocytes. Taken together, the figure reinforces that the most consistent posterior treatment patterns were located in the combination of early follicular recruitment, oocyte production, and especially oocyte viability, whereas recovery remained comparatively more stable in the joint response space.

Table 3 - Posterior treatment effects of T1 (0.6 g progesterone) relative to T2 (1.2 g progesterone).

Response variable	Posterior effect, mean $\pm$ SD	95% CrI	Pr(T1 > T2)	Transformed effect	95% CrI	Interpretation
2–4 mm follicles	0.353 $\pm$ 0.247	-0.134 to 0.857	0.931	Rate ratio = 1.47	0.874 to 2.36	Tendency in favor of T1
4–8 mm follicles	0.244 $\pm$ 0.250	-0.245 to 0.726	0.842	Rate ratio = 1.32	0.782 to 2.07	Tendency in favor of T1
Oocyte recovery probability	0.00692 $\pm$ 0.258	-0.519 to 0.511	0.502	Odds ratio = 1.03	0.595 to 1.67	No relevant difference
Oocyte viability	0.845 $\pm$ 0.380	0.0985 to 1.59	0.989	Odds ratio = 2.50	1.10 to 4.92	Clear evidence in favor of T1
Total recovered oocytes	0.290 $\pm$ 0.259	-0.227 to 0.792	0.878	Rate ratio = 1.38	0.797 to 2.21	Tendency in favor of T1

SD: posterior standard deviation; CrI: credible interval; Pr(T1 > T2): posterior probability that the treatment effect favors T1 over T2. Rate ratios are reported for count outcomes, whereas odds ratios are reported for binomial outcomes. For transformed effects, the null value is 1.00. In this Bayesian framework, posterior probabilities and 95% credible intervals provide the inferential evidence for treatment comparison.

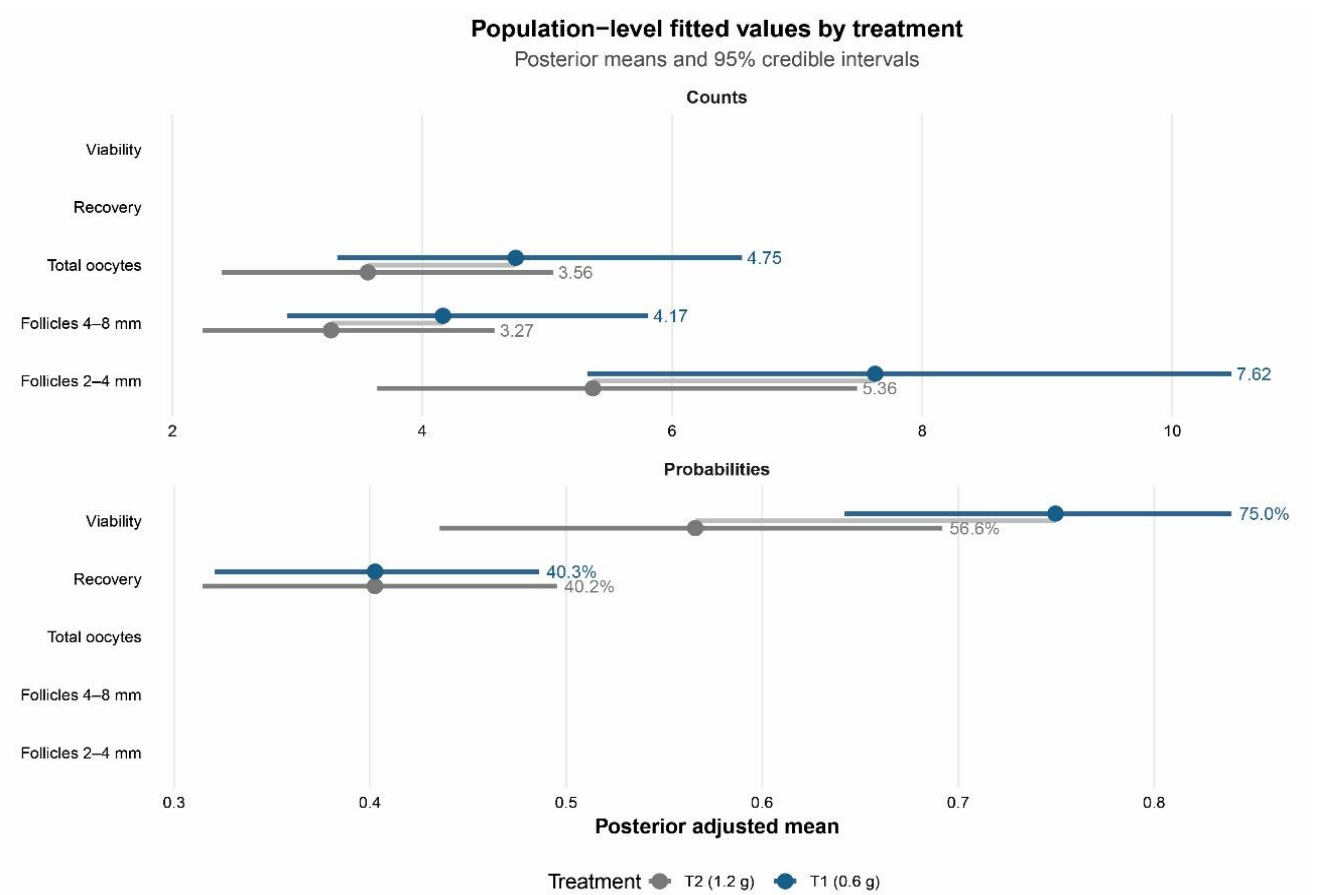


Figure 2 - Population-level fitted values by treatment.

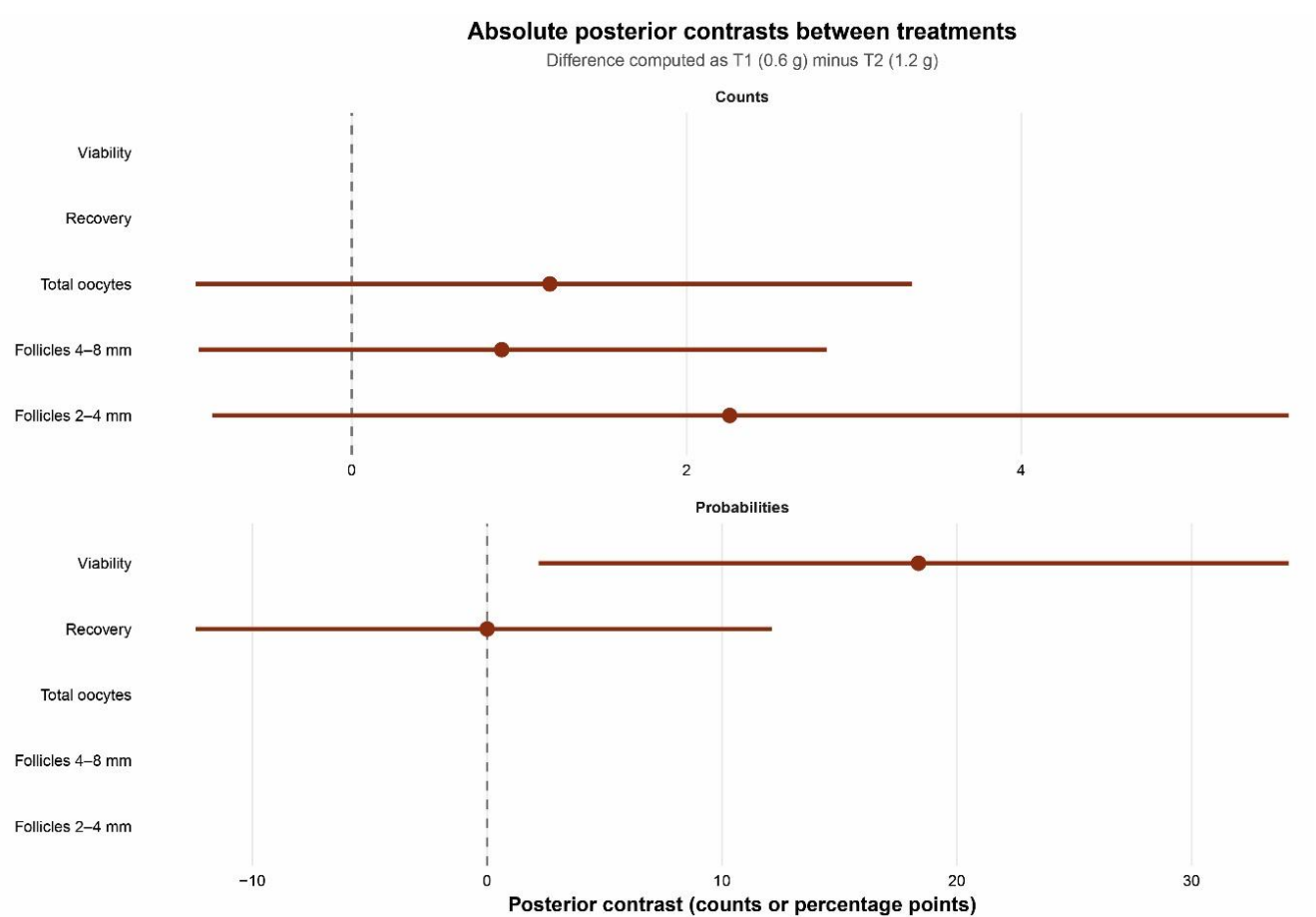


Figure 3 - Absolute posterior contrasts between treatments.

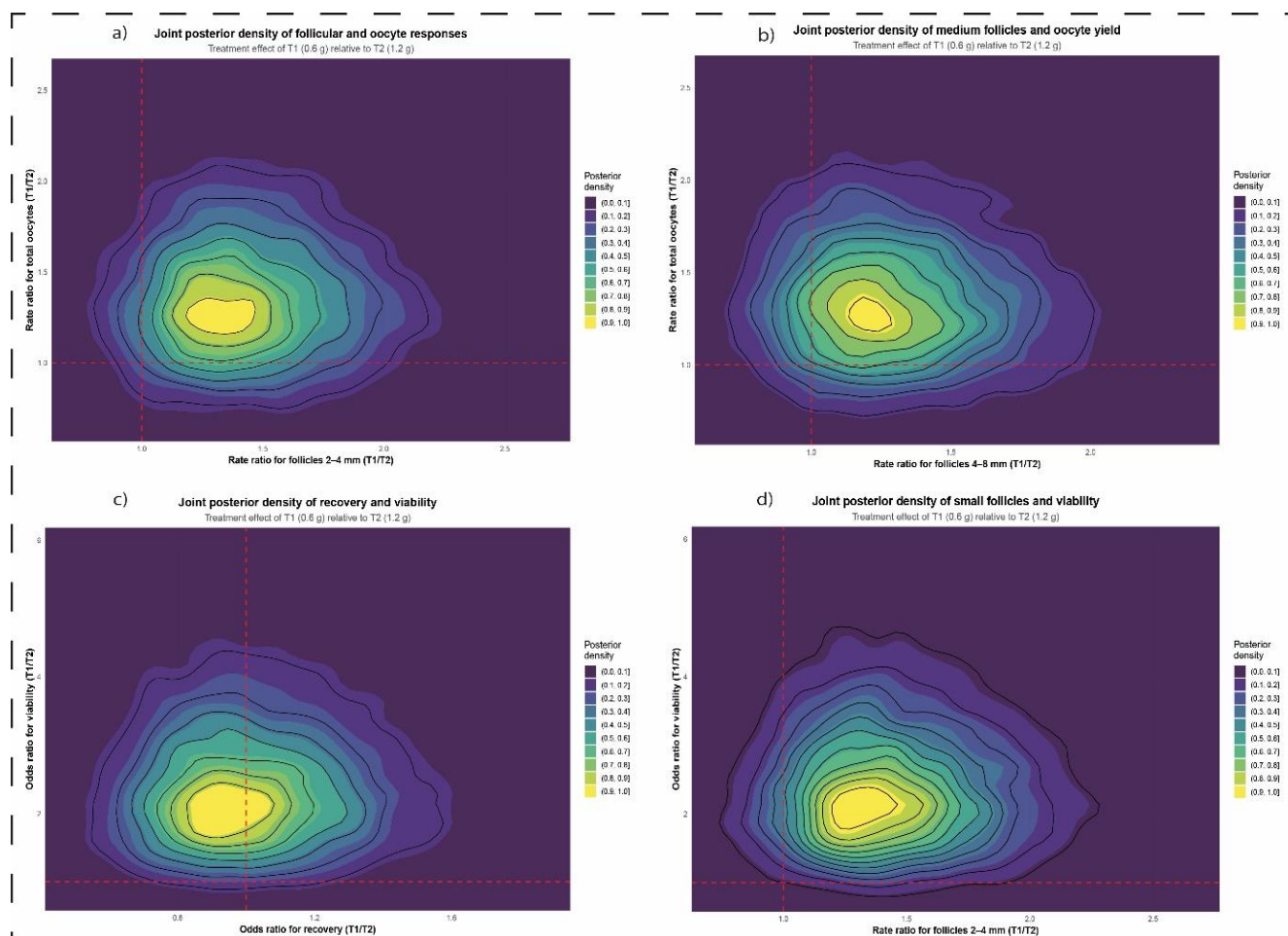


Figure 4 - Joint posterior patterns among follicular, oocyte, recovery, and viability responses under two intravaginal progesterone doses in Brown Swiss cows. (a) Joint posterior density between the rate ratio for 2–4 mm follicles and the rate ratio for total oocytes. (b) Joint posterior density between the rate ratio for 4–8 mm follicles and the rate ratio for total oocytes. (c) Joint posterior density between the odds ratio for recovery and the odds ratio for viability. (d) Joint posterior density between the rate ratio for 2–4 mm follicles and the odds ratio for viability.

DISCUSSION

The main finding of this study was that the protocol with 0.6 g of progesterone shifted the biological response toward higher adjusted values of 2–4 mm follicles, 4–8 mm follicles, and total oocytes, whereas the most marked signal was observed in oocyte viability; in contrast, the probability of recovery remained practically unchanged between treatments. This same structure was repeated in the absolute contrasts and in the joint posterior density maps, where the greatest separation between treatments was concentrated in the follicular response and, above all, in viability, rather than in recovery per follicle.

This pattern is biologically consistent with the literature, as pointed out by [Motta et al. \(2024\)](#), indicating that synchronization of the follicular wave before OPU modifies the distribution of follicular sizes and may improve oocyte competence, although the exact response depends on the protocol and the type of outcome evaluated. In Angus cows subjected to a protocol with an intravaginal progesterone device, estradiol benzoate, and cloprostenol, synchronization increased the number of medium follicles, but did not modify the total number of follicles or the number of recovered COCs, and even reduced the recovery rate ([Anchordoquy et al., 2024](#)). Complementarily, a recent study in Braford cows explicitly proposed that follicular synchronization prior to OPU improves oocyte competence and that the response of cows with a greater taurine component still requires further elucidation ([Unchupaico-Payano et al., 2025](#)). Taken as a whole, these studies suggest that endocrine treatments before OPU do not lead to a uniform improvement in every indicator; rather, their effect may be expressed differently across follicular size, recovery, and oocyte quality.

The results suggest that the lower progesterone dose may have favored an ovarian environment more supportive of early follicular recruitment, especially in the 2–4 mm category, and to a lesser extent, total oocyte yield. This interpretation is consistent with the known role of progesterone in regulating LH pulse Frequency and, together with estradiol, modulating gonadotropin secretion and follicular wave dynamics ([e Silva et al., 2025](#); [Garcia et al., 2025](#)). Previous evidence indicates that lower progesterone concentrations during follicular growth may be associated with increased LH

pulsatility and higher estradiol concentrations, while the combined action of estradiol and progesterone suppresses FSH and LH, induces atresia of the dominant follicle, and synchronizes the emergence of a new wave (Gomez-León et al., 2020). In this sense, a lower progesterone load may have reduced gonadotropic suppression while still maintaining protocol control, which could explain the greater availability of small aspiratable follicles (Hugues and Durnerin, 1998). Still, because progesterone, LH, and estradiol were not directly measured, this mechanism should be viewed as a biologically plausible interpretation rather than a demonstrated effect.

The clearest finding of this study was the difference observed in oocyte viability. Compared with the other responses, viability showed the strongest separation between treatments on both the absolute and relative scales, and this pattern was also evident in its joint posterior distribution with follicular response. This suggests that the main advantage of the 0.6 g protocol was not a higher mechanical efficiency of aspiration, but an improvement in the biological quality of the recovered oocytes. This interpretation is in line with previous evidence showing that synchronization before OPU may enhance oocyte competence (Garcia et al., 2025), and with studies reporting that exogenous progesterone can improve oocyte quality even when later in vitro outcomes do not change substantially (Payano et al., 2024). In Sahiwal cows, for instance, progesterone treatment increased follicular population, recovery, and the proportion of better-quality oocytes, although it had no effect on nuclear maturation, cleavage, or blastocyst formation (Nawaz et al., 2022). In the same direction, recent work has increasingly viewed synchronization as a strategy to improve competence, not only quantity (Lopes et al., 2019). In contrast, recovery probability remained largely unchanged between treatments. This finding is relevant because it suggests that progesterone dose did not materially affect the proportion of oocytes recovered per aspirated follicle, even though differences were observed in other responses. From both a biological and an operational perspective, this pattern is not unexpected, since oocyte recovery depends not only on the endocrine environment but also on technical and physical factors such as follicle accessibility, aspiration pressure, flow rate, operator handling, and, in some cases, follicular size (Rose, 2014; Anchordoquy et al., 2024). In fact, the literature shows inconsistent findings regarding oocyte recovery when protocols preceding OPU are modified: some studies have reported that synchronization reduced the recovery rate, whereas others have found that recovery may vary more with follicular size or aspiration technique than with the hormonal protocol itself (Seneda et al., 2001; Márquez-Moya et al., 2025). Consequently, the present pattern, stability in recovery together with improvement in viability, reinforces the idea that the main effect of the treatment was more related to oocyte competence than to extraction efficiency.

Within the conditions evaluated in this study, reducing the progesterone load in the intravaginal device from 1.2 g to 0.6 g did not reduce oocyte recovery efficiency. Instead, the lower-dose protocol was associated with a favorable posterior shift toward a greater number of aspiratable follicles, higher total oocyte yield, and improved oocyte viability. From an applied perspective, these findings suggest that the optimization of pre-OPU protocols should not be based only on the total number of recovered structures, but also on how the treatment influences follicular recruitment, oocyte yield, and biological competence (Sarwar et al., 2020). Therefore, the main contribution of the present study is not merely the comparison of two progesterone doses, but the identification of a potential biological advantage of the lower-dose protocol, mainly reflected in the functional quality of the recovered oocytes.

CONCLUSION

Treatment T1 (0.6 g of progesterone) showed a more favorable response than T2 (1.2 g) in follicular recruitment, total number of oocytes, and especially in oocyte viability, the latter being the variable in which the most consistent posterior evidence of biological superiority was observed. No relevant differences were observed between treatments in the probability of oocyte recovery, indicating that both protocols showed similar behavior in the efficiency of obtaining oocytes per aspirated follicle. The hierarchical Bayesian approach made it possible to adequately model the repeated structure of the data, the heterogeneity among cows, and the specific nature of each response, providing more robust and biologically interpretable inference on the effects of the two progesterone doses in Brown Swiss cows subjected to ovum pick-up (OPU).

DECLARATIONS

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

Carlos Quispe-Eulogio contributed to the study methodology. Neale Santiago-Inga was responsible for data collection. Edith Ancco-Gomez performed the laboratory procedures. Jordan Ninahuanca contributed to manuscript writing and statistical analysis. Ide Unchupaico-Payano and Milagros García-Hospinal contributed to conceptualization and methodological development. Cecil Rivera-Palomino was responsible for the critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript.

Consent to publish

The publication of this manuscript has been approved by all authors.

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Competing Interests

There is no competing interest among the authors regarding the publication of this article.

Ethical approval

The experimental procedures involving animals were conducted in accordance with animal welfare and ethical handling standards applicable to bovine reproductive research. All procedures related to animal handling, hormonal treatment, and ovum pick-up were conducted under veterinary supervision, with special attention to minimizing stress and ensuring proper care of the donor cows throughout the study. The protocol was reviewed and approved by the competent institutional authority under letter N. 006-GRJ-DRAA-AAC-PERU-2025.

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VITAMIN COMPOSITION OF ALPINE AND YOGHURT CRAFT HARD CHEESES MADE FROM UNPASTEURIZED GOAT MILK AFTER RIPENING WITH THE PARTICIPATION OF *Acarus siro* MITES

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Supporting Information



ABSTRACT: Craft goat cheeses made from unpasteurized milk are considered elite food products with high nutritional and biological value for humans, which contributes to growing market availability and necessitates the assessment of their quality, authenticity and biological value. This study used hard craft cheeses, Alpine and Yoghurt, which ripened with the activity of *Acarus siro* mites and produced from unpasteurized goat milk on a local organic farm. The contents of retinol, tocopherol and riboflavin were determined in the cheeses after 7 days (young cheese), 6 months (mature cheese), and at the final stage of ripening (12 months for Alpine and 18 months for Yoghurt). In Alpine cheese during 12 months of ripening, the retinol content ranged from 5.04 to 5.30 mg/kg, the tocopherol content from 2.00 to 2.34 mg/kg and the riboflavin content from 2.19 to 2.48 mg/kg. The ripening period did not significantly affect the retinol and riboflavin content but increased the tocopherol content by 15.42 - 16.42%. In Yoghurt cheese, the retinol content ranged from 4.60 to 5.24 mg/kg, while the tocopherol content ranged from 2.18 to 2.33 mg/kg, and was not affected by the ripening period. The riboflavin content increased from 1.78 mg/kg to 2.58 mg/kg during ripening. The retinol content in the dry matter of both cheeses decreased with increasing ripening time, whereas the tocopherol content decreased only in Yoghurt cheese. To statistically assess the effect of ripening time on the vitamin content of the hard craft cheeses, one-way analysis of variance (ANOVA) was used. This analysis showed that the retinol concentration in the dry matter of both cheeses decreased with increasing ripening time, while the tocopherol concentration increased only in Yoghurt cheese. The results of the study indicate the high biological value of hard cheeses made from unpasteurized goat milk as sources of vitamins A, E, and B₂. Moreover, the use of dispersion analysis in combination with microbiological and physicochemical characteristics allows evaluating and confirming their quality, authenticity, and biological value.

Keywords: Alpine cheese, Maturation, Retinol, Riboflavin, Tocopherol, Yoghurt cheese.

INTRODUCTION

Cheese is a food product that simultaneously provides healthy microflora, nutrients (protein, fat, amino acids, fatty acids, mineral components), and a number of biologically active substances, including fat- and water-soluble vitamins (Iakubchak et al., 2024; Kukhtyn et al., 2024; Savchenko et al., 2024). Hard cheeses made from unpasteurized goat milk are of particular value, as they are characterized not only by valuable nutritional components but also by distinctive flavour characteristics, for which they are highly valued by consumers (Awad and Abdou, 2024).

Craft hard cheeses are becoming increasingly popular among goat cheeses. They are made using proprietary technologies and have a unique taste and aroma. Such properties are conferred by various biologically active compounds, in particular volatile fatty acids, organic acids, peptides, amino acids and vitamins. A significant amount of these compounds enters the cheese with the milk, but during ripening, they are also formed through lipolysis, proteolysis and glycolysis. It is known that cheese proteins can be hydrolyzed to form peptides and amino acids, and cheese lipids can serve as precursors of conjugated linoleic acid as well as certain fat-soluble vitamins (Vázquez-García and Martín del Campo, 2023; Sadvari et al., 2025).

It is believed that cheeses are characterized by an optional balance of vitamins for the human body. Lactic acid bacteria participate in vitamin synthesis. For example, *Limosilactobacillus reuteri* can synthesize riboflavin (vitamin B₂) in

an amount of 18.36 µg/ml when cultured in the presence of the fermentative bacterium *Streptococcus thermophilus*, which is used in the production of many cheeses (Spacova et al., 2022) and other food products (Rogoskii et al., 2020; Bal-Prylypko et al., 2024). A similar property has also been found in certain strains of *Lactococcus cremoris*, *Lactococcus lactis*, and *Limosilactobacillus fermentum* (Langa et al., 2024). The vitamins found in cheese include vitamins A, E, D, K, B₂, B₆, and B₁₂ (Zhou et al., 2022).

The presence of such a complex composition of biologically active compounds in cheese contributes to its probiotic properties (Jena and Choudhury, 2023; Bustos et al., 2025), and its anti-carcinogenic, antioxidant and anti-inflammatory effects (Yerlikaya, 2023; Zhang et al., 2023). Thus, cheese consumption reduces the risk of cardiovascular, digestive and nervous system diseases and improves human quality of life (Saleem et al., 2024).

A limited number of studies have investigated the influence of the ripening process and period on the vitamin content of cheeses, and these have mainly focused on cheeses produced using industrial technologies, whereas craft cheeses have been scarcely studied in this respect. There is almost no information on the influence of ripening period on the vitamin content of hard cheeses made from unpasteurized goat milk and ripened with the participation of acarid mites. Therefore, the aim of this study was to determine the contents of retinol, tocopherol and riboflavin in hard craft cheeses made from unpasteurized goat milk during the ripening process with the participation of acarid mites.

MATERIALS AND METHODS

Animals

Unpasteurized goat's milk was used to produce hard cheeses. Milk was sourced from Anglo-Nubian goats at Eco-Farm Zhuravka in the Kyiv region.

Experimental Design

Research on the vitamin composition of hard craft cheeses made from unpasteurized goat milk and ripened with the participation of *Acarus siro* mites was conducted from May 2023 to January 2025. Alpine and Yoghurt cheeses were made from the milk of Anglo-Nubian goats at Eco-Farm "Zhuravka" in the Kyiv region, Ukraine. The technology for cheese production is described in our previous work (Davydovych et al., 2025a).

Sampling

To study the vitamin content, average samples of both types of cheese weighing 200 g were selected from 5 batches of each cheese type at different stages of ripening: 7 days (young cheese), 6 months (mature cheese), 12 months (old-mature cheese) for Alpine cheese, and 18 months (old-mature cheese) for Yoghurt cheese.

Sample analysis

Determination of vitamin A (retinol) content in cheeses was performed after saponification with an alcoholic KOH solution in the presence of the antioxidant pyrogallol. Following saponification, vitamin A was extracted from the cheese samples with diethyl ether, which was separated from the aqueous phase. The extract was then purified by passing it through anhydrous sodium sulfate to remove residual water. The next step involved concentration by evaporating the ether on a rotary evaporator, after which the residue was dissolved in chloroform. The resulting solution was used for the quantitative determination of vitamin A. Measurements were carried out using a KFK-3-01 photoelectric photometer at a wavelength of 620 nm against chloroform. A standard retinol solution (Vitamin A) 95% (Thermo Fisher Scientific, 1 g, USA) was used to construct the calibration curve in accordance with DSTU 4687:2006.

Determination of vitamin B₂ (riboflavin) in cheeses was based on the ability of riboflavin solutions to produce a yellow coloration and exhibit yellow-green fluorescence under ultraviolet light, the intensity of which depends on the concentration of riboflavin in the solution. For this purpose, a weighed sample was boiled in a hydrochloric acid solution, and the pH was adjusted to 6.0–6.5 using a saturated sodium carbonate solution. The filtered solution was transferred into the cuvette of a Fluorat-02-2M liquid analyzer, and fluorescence intensity was measured. In parallel, the fluorescence intensity of a standard working solution was measured, and sodium hydroxide solution was added to quench riboflavin fluorescence in accordance with DSTU 4687:2006.

Determination of vitamin E (tocopherol) content in cheeses was carried out through the oxidation of tocopherols with ferric chloride and subsequent determination of the formed ferrous iron as a pink-red complex with 2,2'-dipyridyl. For the analysis, saponification was performed using an alcoholic KOH solution in the presence of pyrogallol. After saponification and extraction, the dry residue was dissolved in benzene and treated with the iron–dipyridyl reagent. Colorimetric measurements were performed using a KFK-3-01 photoelectric photometer at a wavelength of 520 nm against a reagent blank, in accordance with DSTU 4687:2006.

Statistical analysis

The results of the study were statistically processed using one-way analysis of variance, information technologies for data analysis, and modelling of ripening processes. Digital methods for collecting, processing and visualizing data were

applied, which allowed for high accuracy and reproducibility of research. Correlation and regression analyses of Alpine and Yoghurt cheeses were also performed. A comparison of the indicators was carried out within each cheese type over the ripening period. The analyses were performed using Microsoft Excel 2016 and XLSTAT (Addinsoft, Paris - 2017). The data in the tables are presented as $x \pm SD$ (mean $\pm$ standard deviation). Differences between the indicators were considered significant at $P < 0.05$ using the Tukey's test with Bonferroni correction.

RESULTS AND DISCUSSION

The ripening period of hard Alpine cheese did not affect the content of retinol or riboflavin, whereas the content of tocopherol increased with age, reaching 2.32 mg/kg (by 15.42%) after 6 months and 2.34 mg/kg (by 16.42%) after 12 months compared with young cheese aged 7 days (Table 1). Analysis of the dry matter of Alpine cheese showed that the contents of tocopherol and riboflavin did not depend on the ripening period, whereas the retinol content decreased, in particular, to 7.29 mg/kg (by 22.03%) after 6 months, and to 7.46 mg/kg (by 20.21%) after 12 months compared with cheese aged 7 days (Table 2).

Correlation analysis showed a strong inverse relationship between the retinol content in the dry matter of Alpine cheese and the ripening period ($r = -0.775 \pm 0.119$, $P < 0.01$). The regression line confirmed this relationship and was best described by a second-degree polynomial (Figure 1).

During the ripening of hard Yoghurt cheese, no changes in the concentration of retinol or tocopherol were observed. However, the riboflavin concentration after 18 months increased to 2.58 mg/kg, which exceeded the values in cheese aged 7 days by 44.94% and those in cheese aged 6 months by 35.08% (Table 3).

In the dry matter of Yoghurt cheese, specific patterns of vitamin accumulation were observed. In particular, the retinol content decreased to 7.24 mg/kg (by 26.72%) after 6 months of ripening and to 7.31 mg/kg (by 26.01%) after 18 months compared with young cheese aged 7 days (Table 4).

A similar pattern was observed for tocopherol, and its content in the dry matter of Yoghurt cheese decreased to 3.67 mg/kg (by 10.71%) after 6 months of ripening and to 3.14 mg/kg (by 23.60%) after 18 months compared with the 7-day-old cheese. In contrast, the riboflavin concentration in the dry matter of this cheese did not depend on the ripening period. To confirm the statistical significance of the detected changes in vitamin content during ripening, a one-way analysis of variance (ANOVA) was used, which allowed the establishment of a significant effect of ripening duration on tocopherol content ($P < 0.05$), whereas no significant effect was observed for riboflavin. Thus, a moderate inverse relationship was found between the retinol content in the dry matter and the ripening period of Yoghurt cheese ($r = -0.640 \pm 0.145$, $P < 0.01$), as well as a strong inverse relationship between the tocopherol content in the dry matter and the ripening period of Yoghurt cheese ($r = -0.890 \pm 0.086$, $P < 0.001$). In both cases, the regression line was best described by a second-degree polynomial (Figure 2).

Table 1 - Vitamin content in Alpine cheese during maturation, $x \pm SD$, mg/kg.

Vitamin name	Cheese maturation period		
	7 days	6 months	12 months
Retinol	5.30 ± 0.22	5.04 ± 0.26	5.29 ± 0.08
Tocopherol	2.01 ± 0.16^b	2.32 ± 0.07^a	2.34 ± 0.11^a
Riboflavin	2.23 ± 0.25	2.19 ± 0.23	2.48 ± 0.61

Note: different superscript letters indicate a significant difference between values in a row of the table ($P < 0.05$) using the Tukey test with Bonferroni correction

Table 2 - Vitamin content in dry matter of Alpine cheese during maturation, $x \pm SD$, mg/kg

Vitamin name	Cheese maturation period		
	7 days	6 months	12 months
Retinol	9.35 ± 0.48^a	7.29 ± 0.35^b	7.46 ± 0.09^b
Tocopherol	3.53 ± 0.23	3.35 ± 0.10	3.31 ± 0.14
Riboflavin	3.93 ± 0.47	3.17 ± 0.36	3.52 ± 0.87

Note: different superscript letters indicate a significant difference between values in a row of the table ($P < 0.05$) using the Tukey test with Bonferroni correction

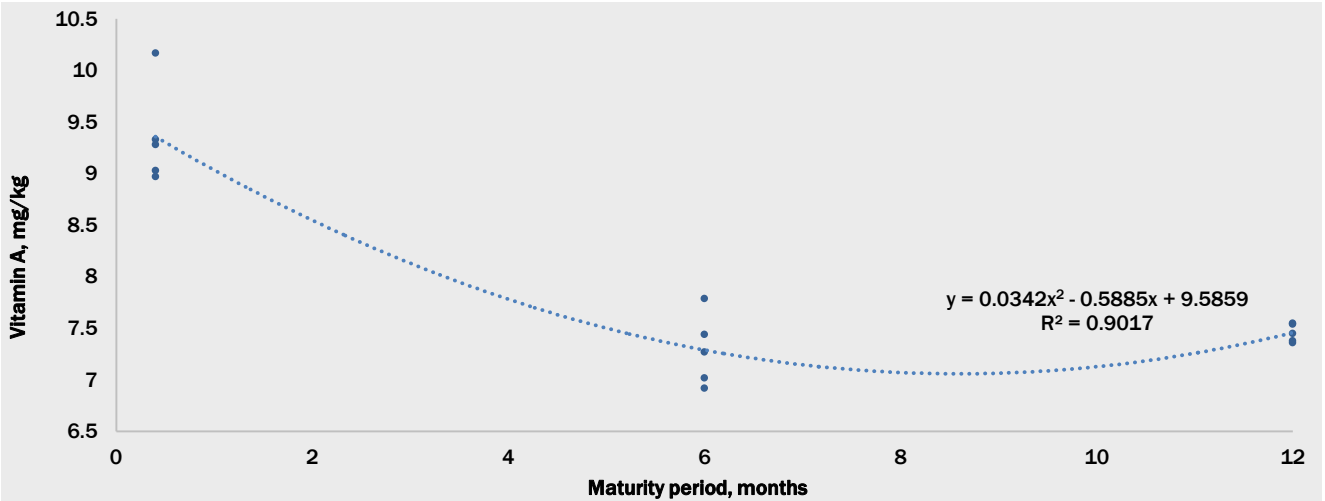


Figure 1 - Dependence of retinol content in dry matter of Alpine cheese on maturation period, n =15

Table 3 - Vitamin content in Yoghurt cheese during maturation, x ± SD, mg/kg				
Cheese maturation period		7 days	6 months	12 months
Vitamin name				
Retinol		5.24 ± 0.26	4.62 ± 0.68	5.11 ± 0.23
Tocopherol		2.18 ± 0.15	2.33 ± 0.09	2.21 ± 0.13
Riboflavin		1.78 ± 0.08 ^b	1.91 ± 0.36 ^b	2.58 ± 0.19 ^a
Note: different superscript letters indicate a significant difference between values in a row of the table (P < 0.05) using the Tukey test with Bonferroni correction (Bonferroni CE (1936))				

Table 4 - Vitamin content in dry matter of Yoghurt cheese during maturation, x ± SD, mg/kg				
Cheese maturation period		7 days	6 months	12 months
Vitamin name				
Retinol		9.88 ± 0. 75 ^a	7.24 ± 1.02 ^b	7.31 ± 0.36 ^b
Tocopherol		4.11 ± 0.19 ^a	3.67 ± 0.14 ^b	3.14 ± 0.20 ^c
Riboflavin		3.35 ± 0.17	3.01 ± 0.85	3.68 ± 0.32
Note: different superscript letters indicate a significant difference between values in a row of the table (P < 0.05) using the Tukey test with Bonferroni correction (Bonferroni CE (1936)).				

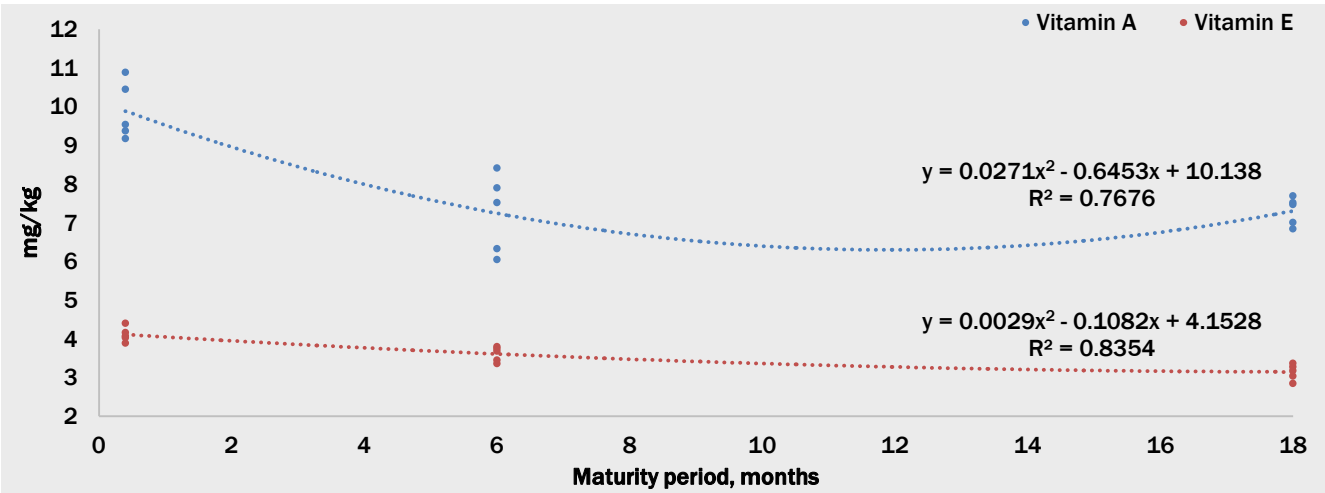


Figure 2 - Dependence of the content of retinol and tocopherol in the dry matter of Yoghurt cheese on the maturation period, n=15.

During the ripening of hard craft cheeses, their chemical components (Jakabová et al., 2023; Čapla et al., 2024), particularly protein and fat, play an important role, affecting cheese yield and its organoleptic characteristics (Davydovych

et al., 2025b). In addition, all fat-soluble biologically active compounds, such as vitamins, are concentrated in fat. The content of vitamin A in the dry matter of Alpine and Yoghurt hard goat cheeses made from unpasteurized milk decreased during ripening, which may be due to the influence of physicochemical factors that contributed to oxidation processes during prolonged ripening.

One of the important factors that may have influenced the loss of vitamin A in the cheeses is the activity of *A. siro* mites. Thus, by feeding evenly on the rind of Alpine and Yoghurt hard cheeses, acarid mites could have increased the aeration not only of the surface but also of the deeper parts of the cheese core thereby increasing moisture loss and, consequently, affecting the solubility and distribution of both water-soluble and fat-soluble components with antioxidant properties (Davydovych et al., 2025a). The increased oxygen supply to the surface and deeper layers of the cheese matrix could, in turn, have intensified retinol oxidation. However, this assumption requires further verification.

It is practically impossible to compare the dynamics of retinol content in goat cheeses produced by other manufacturers because they are made using different technologies, including milk obtained from goats of different breeds and raised under different feeding systems. It is believed that the vitamin A content of goat milk cheeses is influenced by salting. According to the study by do Oriente et al. (2020), the vitamin A content was 3.87 ng/ml in salted cheeses compared with 1.27 ng/ml in cheeses produced without salt. However, these data were obtained from soft cheeses that were not subjected to a long ripening period, which does not allow a direct comparison with our results. It is more appropriate to compare our findings with published data on Italian hard and semi-hard cheeses. The retinol content was reported to be 2.5–2.6 mg/kg in *Caciocavallo Palermitano* hard cheese made from raw cow's milk after 60 days of ripening, 2.5–2.7 mg/kg in *Pecorino siciliano* hard cheese made from raw sheep's milk after 210 days, 2.1–2.9 mg/kg in *Casizolu del Montiferru* hard cheese made from raw cow's milk after 180 days, and 2.9–4.1 mg/kg in *Caprino Nicastrese* semi-hard cheese made from raw goat milk after 20 days (Di Trana et al., 2022). In contrast, the retinol content in Alpine and Yoghurt hard cheeses made from unpasteurized goat milk exceeded these values throughout the ripening period, which may be attributed to regional and seasonal production conditions, as well as differences in breed, animal feeding regimen, and the botanical composition of pasture vegetation.

For the quantitative analysis of changes in retinol content during the ripening period, univariate regression analysis was used. This analysis identified a statistically significant trend toward a decrease in retinol concentration in the dry matter of the cheese over time. The regression model was best described by a second-degree polynomial with significant coefficients ($P < 0.05$), indicating the nonlinear nature of these changes and allowing prediction of this parameter at later stages of ripening.

In addition to vitamin A, vitamin E is one of the most important fat-soluble vitamins in cheeses and also has antioxidant properties. The source of vitamin E in cheeses is animal milk, in which α -tocopherol is the predominant form. The tocopherol content of cheeses reflects their quality and biological value for humans. As an antioxidant, vitamin E plays an important role during cheese ripening by preventing the oxidation of other biologically active substances. A study of authentic Njeguški cheese showed that the content of α -tocopherol in cheese made from goat milk exceeded the corresponding values in the same cheese made from cow's and sheep's milk. At the end of the ripening period, the tocopherol content was 15.73 mg/kg in goat cheese, 10.39 mg/kg in cow cheese, and 11.52 mg/kg in sheep cheese (Bittante et al., 2022). Analysis of the tocopherol content indicates significant differences among hard and semi-hard Italian cheeses made from cow's, sheep's and goat's milk. It was found that hard *Caciocavallo Palermitano* hard cheese made from raw cow's milk contained 6.0–7.2 mg/kg of tocopherol after 60 days of ripening, *Pecorino Siciliano* hard cheese made from raw sheep's milk contained 3.6–5.8 mg/kg after 210 days of ripening, *Casizolu del Montiferru* hard cheese made from raw cow's milk contained 7.0–11.8 mg/kg after 180 days of ripening, and *Caprino Nicastrese* semi-hard cheese made from raw goat milk contained 7.2–15.0 mg/kg after 20 days of ripening (Di Trana et al., 2022). In another study, α -tocopherol levels in *Pecorino* and *Casu Axedu* cheeses were 2.0 and 3.0 mg/kg, respectively, on a natural moisture basis. At the same time, the researchers reported that sheep's milk contained more tocopherol than cow's or goat's milk. In our study, significantly lower tocopherol contents were obtained in Alpine and Yoghurt cheeses than in Njeguški cheese and the Italian cheeses described above. However, most of our results were similar to those reported for *Pecorino* and *Casu Axedu* cheeses. The decrease in tocopherol concentration in the dry matter of Yoghurt cheese was probably caused by oxidation, whereas no such trend was observed in Alpine cheese.

No decrease in tocopherol content was found in soft goat cheeses made from unpasteurized milk and ripened with the participation of noble white mold for 40 days, or in brined Feta cheese ripened for 30 months (Da-vydovych et al., 2026). Therefore, it can be assumed that the moisture content of cheese plays an important role in vitamin preservation. The rate of tocopherol oxidation in hard goat cheeses ripened with the participation of acarid mites may, to some extent, depend on the intensity of rind damage and the duration of ripening, as in the case of vitamin A.

A similar pattern was confirmed in a study of *Pasta-filata* cheese ripened for 30 or 60 days at a temperature of 14–16 °C, in which a decrease in the levels of γ -tocopherol and β -carotene was observed. However, the concentration of α -tocopherol did not change (Marino and Schadt, 2016). This may indicate a significant influence of the cheese microbiome on vitamin E levels (Davydovych et al., 2025a). The results of another study indicated that the tocopherol content of

ruminant milk used for cheese production depends on the feeding regimen, the presence or absence of grazing, the breed, and the season of production, thereby supporting our assumption (Martini et al., 2021).

Taking into account the content of B vitamins in cheeses is an important criterion not only for their biological value but also for their authenticity. Since B vitamins are water-soluble, a significant proportion is lost during whey separation from the curd in cheese production, although this process depends on the salt content of the cheese mass (Ferroukhi et al., 2022). It is believed that lactic acid bacteria of the genera *Lactococcus* and *Streptococcus* not only influence the chemical composition and ripening process of cheeses (Sadvari et al., 2024a, 2024b) but also synthesize vitamins B₂ and B₆ during ripening, which is consistent with previous findings (Ferroukhi et al., 2022). The recommended daily intake of vitamin B₂ for adults is 1.1–1.3 mg. Thus, hard cheeses can provide approximately 10% of the recommended daily intake of vitamin B₂, as well as 20% of vitamin B₆ and 40% of vitamin B₁₂ (Rashidinejad et al., 2017; Sohail et al., 2024). The results of the riboflavin analysis in Alpine and Yoghurt cheeses are consistent with these findings. Unlike fat-soluble vitamins, B vitamins can be synthesized by microorganisms and their concentration therefore increases during the ripening of soft goat cheeses (Davydovych et al., 2026). However, in hard cheeses, the decrease in moisture content during ripening limits microbial biosynthesis. At the same time, riboflavin is probably less susceptible to degradation than the fat-soluble vitamins A and E, and its concentration in the dry matter of hard cheeses does not undergo significant fluctuations.

CONCLUSION

The ripening period significantly influenced the content of vitamins A, E and B₂ in Alpine and Yoghurt hard craft cheeses made from unpasteurized goat milk and ripened with the participation of mites. The tocopherol content was directly dependent on the age of Alpine cheese: its level increased to 2.32 mg/kg (by 15.42%) after 6 months and to 2.34 mg/kg (by 16.42%) after 12 months of ripening, whereas the levels of retinol and riboflavin remained stable. In the dry matter of Alpine cheese, the contents of tocopherol and riboflavin did not change, whereas the retinol content decreased to 7.29 mg/kg (by 22.03%) after 6 months and to 7.46 mg/kg (by 20.21%) after 12 months of ripening. During the ripening period of Yoghurt cheese, the riboflavin content increased by 35.08–44.94%, reaching a maximum of 2.58 mg/kg after 18 months, whereas the retinol and tocopherol contents remained unchanged. During the ripening of Yoghurt cheese, a decrease in the dry matter content of fat-soluble vitamins was observed. In particular, the retinol content decreased to 7.24 mg/kg (by 26.72%) after 6 months and to 7.31 mg/kg (by 26.01%) after 18 months. Likewise, the tocopherol content decreased to 3.67 mg/kg after 6 months and to 3.14 mg/kg after 18 months. Alpine and Yoghurt cheeses contain sufficient amounts of retinol, tocopherol, and riboflavin, which, together with microbiological and other physicochemical characteristics, indicate that they are biologically valuable products. The obtained results may serve as a basis for assessing the quality, nutritional value, and authenticity of craft hard goat cheeses made from unpasteurized milk. The research results were statistically analysed using one-way analysis of variance (ANOVA), implemented using information technology, to assess the significance of the effect of the ripening period on the contents of retinol, tocopherol, and riboflavin in the cheeses. This approach made it possible to identify statistically significant differences in vitamin composition according to the stage of cheese ripening and to confirm the biological dynamics of the formation or preservation of individual compounds.

DECLARATIONS

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Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contribution

Viktor Davydovych, Larysa Shevchenko: contribute to the research, data analysis, and manuscript writing.
Svitlana Shulyak: conceptualization, Investigation. Lesia Busol: data curation, writing – review & editing.
Mykola Nikolaenko: formal analysis, visualization. Anastasiia Lialyk: funding acquisition.
Andrii Kudriashov: methodology, software. Anastasiia Ivaniuta: project administration, supervision.
Lyudmila Beyko: writing – review & editing, resources.
Oleksandr Savchenko: validation, writing – original draft.

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Ethical consideration

No experimental procedures or manipulations involving animals were performed in this study. Therefore, ethical approval for animal experimentation was not required.

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Competing interests

The authors have not declared any competing interests.

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THE ACIDITY LEVEL OF FERMENTED EGG FLOUR WITH VARIOUS DRYING TECHNOLOGIES

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➤ Supporting Information



ABSTRACT: Commonly, egg fermentation technology is carried out using *Lactobacillus plantarum* bacteria. Eggs that have undergone the fermentation process can be stored for a long time using the drying method. The results of fermentation in the drying process are expected to reduce the Maillard reaction. The purpose of this study was to identify the application of the right drying technology in fermented eggs on the stability of the acidity level of egg flour. This study was conducted experimentally using a Completely Randomized Design (CRD) factorial pattern of 3 × 4 treatments with 5 replications each. The treatment was carried out using the spray drying method, pan drying, freeze drying with a drying time of 6, 12, 18 and 24 hours. The acidity level can be measured by testing parameters for pH values and lactic acid levels using the titration method. The results showed that increasing drying time and different drying methods in fermented egg flour could maintain the pH value of eggs and the optimum at a drying time of 6 hours with the freeze drying method. Lactic acid levels tend to be stable because the fermentation product uses freeze drying which can maintain the lactic acid levels of fermented eggs. The acidity level of fermented egg flour can be seen from the stability of the pH value and lactic acid levels from the drying process. The best pH value and lactic acid content were obtained using the freeze drying method with a drying time of 24 hours.

Keywords: Eggs, Fermentation, Drying, Flour, Lactic acid.

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INTRODUCTION

One of poultry product that is full of easily digestible nutrients is eggs. Although eggs provide necessary lipids, balanced amino acids, and a number of minerals and vitamins, they are a food item that is susceptible to contamination by bacteria and breakage (Vitkova et al., 2024). Drying eggs into flour offers many advantages, including ease of storage and distribution, protection against microbial growth (low water activity), reduced weight per volume of whole eggs, and a longer shelf life. Using egg flour as an additive to other food products is easier than using fresh eggs (Abreha et al., 2021).

One of the most popular techniques for preserving food is drying, which lowers the water content to decrease damage from reactions and microorganisms (Casella et al., 2020) which is documented in egg drying (Alp and Bulantekin, 2021; Ekpo et al., 2022). There are multiple methods for drying different egg products, such as spray drying, pan drying, and freeze drying. Amelia et al. (2013) found that for drying small particles, the freeze-drying method works better than spray drying and is advised for both particle integrity and the amount of solids produced.

The primary issue with producing egg flour, particularly when using the drying method, is the product's brownish color shift, which is known as the Maillard Reaction (Martins et al., 2000). This issue affects how egg flour goods look in terms of color and odor as well as the deterioration of their functional qualities. The reaction between the aldehyde group of carbohydrates and the amino group of the protein that makes up the egg is what gives it its brown hue (Baynes et al., 2007). Products made using fermentation technology can lessen the Maillard process.

Food safety studies must be conducted on fermented chicken eggs before they are used as health food products. Identifying food items that are safe to eat, particularly fermented eggs, and recognizing antioxidants as a feature of food quality (Mangalisu et al., 2022). Given the numerous advantages of antioxidant content, it is intriguing to investigate the presence of anthocyanin molecules as a natural antioxidant source in fermented egg flour. The advantages of using egg flour in industry include a longer shelf life, ease of storage and transportation due to its floured form, and hygiene and safety. The use of egg flour offers significant flexibility and economic advantages on an industrial scale, especially for food manufacturers who prioritize efficiency without sacrificing product quality (Uhlmann and Hertel, 2016).

Assessing lactic acid content and viable lactic acid bacteria counts to ensure microbiological safety and functional quality of dried egg products. Determining lactic acid and total lactic acid bacteria levels is important for evaluating the microbiological stability and safety of fermented food products, including those in dry or floured form. Food products with low water content still allow certain microorganisms to survive during storage, making microbiological analysis and total titratable acidity important parameters in food quality assurance (Odu et al., 2017; Såde et al., 2016).

Measurements of the egg flour's total bacteria, acidity, functional properties, and antioxidant quality are made while it is being stored at room temperature. A study on the identification of fermented egg flour antioxidants using different drying procedures was carried out in light of the aforementioned studies.

MATERIALS AND METHODS

Materials

The equipment used in this study were sample tubes, test tubes, petri dishes, erlenmeyers, micropipettes, tips, syringes, analytical scales, beakers, incubators, biuret, spatulas, autoclaves, colony counters, vortexes, pH meters, magnetic stirrers, PCR Hoods, tube racks, tube shakers, vortex mixers, measuring flasks, water bath shakers, spray dryers, ovens, and freeze dryers. The materials used were fresh chicken eggs, MRS (Man Rogosa Sharpe) agar, aluminum foil, tomato juice, distilled water, alcohol, 0.89% NaCl, 0.1 N NaOH, PP indicator (phenolphthalein), pH 4 and pH 7 buffer solutions.

Experimental design

This research was conducted experimentally using a Completely Randomized Design (CRD) with a factorial of 3 x 4 with 5 replications each. This research uses *Lactobacillus plantarum* in the fermentation process and then carries out drying treatment. The treatments consisted of Factor A is the drying method treatment A1: spray drying, A2: pan drying, A3: freeze drying. Factor B is the drying time treatment B1: 6 hours, B2: 12 hours, B3: 18 hours, B4: 24 hours.

Culture propagation

Lactobacillus plantarum FNCC 0027 was stored on De Man Ragosa Sharpe (MRS) agar. Culture propagation was performed by creating subcultures. Subcultures were prepared by transferring the culture stock to MRS broth (OXOID CM0359) with 20% tomato extract added and incubated for 24 hours (Pramono et al., 2003). The culture stored in MRS broth was inoculated at 10% concentration into egg white containing 20% tomato extract to produce a working culture (Legowo et al., 2013). Gram staining was performed on the starter culture to determine the morphology of *Lactobacillus plantarum*.

Making fermented chicken eggs

Egg samples were cleaned using clean water. The eggs were then fumigated using Calcium Permanganate (KP) flour and formalin in a closed room for 5 minutes and successively cleaned using a wet cloth, chlorine solution and wiped with alcohol using cotton. The eggs were wrapped in aluminum foil and pasteurized at 60 °C for 3.5 minutes (Froning et al., 2002) then separated from the shell and placed into sample bottles. The sample bottles were first cleaned using warm water and sterilized. A 200 ml sample was homogenized and then sterilized using ultraviolet by placing it in a PCR Hood for 15 minutes. The sterilized sample was added with 10% working culture *Lactobacillus plantarum* and then homogenized with a tube shaker, the sample was then fermented at 37 °C for 24 hours (Nahariah et al., 2015).

Making fermented egg flour

Fermented chicken eggs were dried using three drying methods. Fermented eggs were dried using a co-current spray dryer (A1). Pan drying was performed at 45 °C (A2). Freeze drying was performed by drying to a thickness of 3.2 mm at -40 °C, with the pressure on the freeze dryer set at 0.036 psi or 0.0025 bar (A3). The dried eggs were then ground. Grinding was carried out using a blender for 2 minutes. The resulting egg flour was then sieved using an 80-mesh sieve. The purpose of this sieving was to produce a uniform egg flour (Koswara, 2009). The flour samples were then tested for acidity level (as measured by a pH meter) and lactic acid level (as determined by the titration method) are the parameters that are tested.

Statistical analysis

Using the SPSS 23 data processing program, the acquired data were examined using Analysis of Variance based on Completely Randomized Design (CRD) (3 × 4) with a factorial design. Additionally, proceed with the Least Significant Difference (LSD) test if the treatment exhibits a significant effect.

RESULTS AND DISCUSSION

Tables 1 (pH value) and 2 (lactic acid content) show the degree of acidity of fermented chicken egg flour with various drying techniques and drying durations. Different drying times and techniques have a substantial ($P < 0.05$) impact on the pH value of fermented egg flour, and there is an interaction between the two, according to analysis of variance. The pH values in each treatment of the freeze drying method and various drying durations at 24 hours were substantially different

($P < 0.05$), according to the results of the Least Significance Different (LSD) additional test. Because the fermentation product involves freeze drying, which may keep the pH value of fermented eggs, the pH value tends to be stable.

Table 1 demonstrated how varying drying times and techniques on fermented egg flour can preserve the pH level of the eggs, with the freeze drying method working best at a drying duration of six hours. After drying, the pH level of fermented egg flour is comparatively stable because spray drying inhibits microbial and enzyme activity. The pH value is nearly identical to what it was before to drying. The pH stays constant between 6 and 12 hours of drying since there is little humidity and little microbial activity. Because fermented eggs are exposed to moisture or oxidation, there are significant pH changes that take place between 18 and 24 hours of drying. This situation is thought to be caused by a decrease in the pH value of the product with citric acid before the fermentation stage and, in addition, an increase in the amount of acid in the product during the fermentation stage. It has also been reported that *Lactobacillus* converts glucose into organic acids (mainly lactic acid) and lowers the pH during fermentation (Doblado et al., 2003).

Table 1 - pH value of egg flour with different drying methods and drying times

Drying time (hours)	Drying method	Spray drying	Pan drying	Freeze drying
6		4.80 ± 0.06 ^a	4.74 ± 0.02 ^b	4.84 ± 0.02 ^a
12		4.80 ± 0.02 ^a	4.70 ± 0.05 ^b	4.84 ± 0.10 ^a
18		4.75 ± 0.03 ^b	4.62 ± 0.03 ^c	4.82 ± 0.04 ^a
24		4.67 ± 0.03 ^b	4.52 ± 0.03 ^c	4.80 ± 0.03 ^a

Noted: Different superscripts in the same row indicate significant differences ($P < 0.05$).

Table 2 - Lactic acid content (%) of egg flour with different drying methods and drying times

Drying time (hours)	Drying method	Spray drying	Pan drying	Freeze drying
6		1.268 ± 0.02 ^b	1.277 ± 0.03 ^a	1.260 ± 0.04 ^b
12		1.268 ± 0.04 ^b	1.280 ± 0.02 ^a	1.260 ± 0.02 ^b
18		1.274 ± 0.03 ^b	1.305 ± 0.06 ^a	1.264 ± 0.07 ^c
24		1.295 ± 0.02 ^b	1.314 ± 0.04 ^a	1.268 ± 0.04 ^c

Noted: Different superscripts in the same row indicate significant differences ($P < 0.05$).

Pan drying is the process of applying low to medium temperatures to a pan or flat surface. Compared to spray drying, this procedure takes longer. The slower procedure and lower drying temperature result in partial deactivation of certain bacteria and enzymes. After drying, this results in pH shifts. During drying, pH variations are more likely to happen, particularly if drying is not flawless. Because of moisture or slower chemical activity, the pH may alter somewhat over the course of 18 to 24 hours.

Freeze-drying is the technique of freezing fermented eggs and then using a sublimation process (a straight transition from solid to gas without going through the liquid phase) to extract the water. Because the drying process occurs at a low temperature and no major chemical changes occur, freeze drying preserves the pH value of the fermented eggs quite well. Due to the extremely low water content that is produced by freeze drying, the pH is extremely stable for 6 to 24 hours. Fermentation causes the pH to drop. This happens as the pH value drops due to an increase in the number and metabolism of lactic acid-producing bacteria brought on by longer incubation times. The pH of the fermentation medium may drop as a result of the buildup of lactic acid created by lactic acid bacteria. The concentration of H⁺ emitted throughout the fermentation process is the computed pH value (Legowo et al., 2013).

Because of the metabolic process that is aided by the availability of nutrients, the growth rate of bacteria is correlated with temperature. The supply of nutrients declines at a particular temperature due to the fast growth rate. Bacterial growth rate is directly influenced by temperature because temperature determines enzymatic activity and cellular metabolism. At optimum temperatures, metabolic activity increases, resulting in faster nutrient consumption, which can ultimately lead to substrate limitations at certain growth stages (Zwietering et al., 1991; Nedwell, 1999). Increasing the temperature to the optimum point increases the rate of bacterial growth and metabolic activity, so that nutrient consumption occurs more quickly and can cause substrate limitations at certain growth phases (Membré et al., 2005).

Different drying times and techniques had a substantial ($P < 0.05$) impact on the amount of lactic acid in fermented egg flour, and there was an interaction between the two, according to analysis of variance. Each treatment of the freeze drying method and different drying times at 24 hours had substantially different amounts of lactic acid ($P < 0.05$), according to the results of the Least Significance Different (LSD) further test. Because the fermentation product involves freeze drying, which can preserve the lactic acid levels of fermented eggs, the lactic acid levels are generally constant.

Spray drying is a widely used drying method for fermented products because it can rapidly convert liquid ingredients into flour through contact with hot air. In this process, fermented egg flour containing lactic acid is atomized into small droplets and dried in a very short time. Although the inlet air temperature is relatively high, the short thermal contact time minimizes the degradation of organic compounds, including lactic acid. Several studies have reported that fermented organic acids are relatively stable during the spray drying process due to the short residence time of the particles in the drying chamber. Consequently, the lactic acid content in the final product does not change significantly compared to before drying (Gharsallaoui et al., 2007).

Furthermore, spray-dried products have low moisture content and water activity, which suppresses microbial activity and chemical reactions during storage. These conditions contribute to the stability of lactic acid and other fermentation metabolites in fermented egg flour. Changes in lactic acid levels after drying are generally more influenced by the initial fermentation conditions than the drying process itself. Therefore, the stability of lactic acid in fermented egg flour tends to be maintained during storage, although slow chemical degradation can still occur over a longer period of time due to oxidative reactions and interactions between food components (Barbosa-Cánovas et al., 2007; Mujumdar, 2014).

Drying using a pan (pandrying) or drying with a tray (tray drying) is a conventional drying method that takes place on a flat surface at low to moderate temperatures for a relatively long time. Compared to spray drying, this method has a slower rate of heat and mass movement so that the material is exposed to heat for a longer time. These conditions can increase the opportunity for physical and chemical changes to occur, such as protein denaturation, lipid oxidation, and changes in fermentation components. In addition, the stability of fermented compounds is influenced by temperature and heating time; the longer the exposure to heat, the greater the possibility of chemical changes and loss of heat-sensitive components (Santivarangkna et al., 2008; Mujumdar, 2014).

In addition to temperature, drying time and humidity conditions also affect lactic acid stability. In conventional drying, lactic acid retention tends to be lower than in spray drying because longer exposure to heat allows oxidation and other chemical changes to occur. Once the product's moisture content decreases, microbial activity is inhibited, thereby increasing lactic acid stability. However, in high humidity conditions or poor packaging systems, lactic acid degradation can still occur during storage due to residual chemical reactions and absorption of water vapor from the environment (Barbosa-Cánovas et al., 2007).

Freeze-drying (or lyophilization) is a drying method that involves first freezing the material, then removing the water through sublimation, a process where ice changes phase to water vapor without passing through the liquid phase. This process occurs at low temperatures and vacuum pressure, minimizing thermal damage to chemical and biological components (Rey, 2010). Compared to thermal drying methods, freeze-drying is more effective in maintaining the stability of bioactive compounds, including lactic acid and fermentation metabolites, because it does not involve intense heat exposure that can trigger chemical degradation (Ratti, 2001).

The stability of lactic acid in freeze-dried products generally remains high due to low water activity and minimal oxidative reactions during the drying process. Dry, low-temperature environmental conditions inhibit microbial activity, enzymes, and other chemical reactions, so that lactic acid content remains relatively unchanged after the drying process (Barbosa-Cánovas et al., 2007). Generally, the method of drying, as well as the method of pretreatment, has a strong effect on the final product. However, thermal processing before drying differs the samples in comparison to non-thermal processing. Furthermore, even the sonication and pulsed electric field cause a similar effect as cell-wall rupturing, and structure changes, the mechanism is different, and this resulted in different properties for the samples (Raybak et al., 2022).

CONCLUSIONS

Drying technologies have different effects on the acidity level of fermented flour, particularly regarding lactic acid stability and total titratable acid values. Freeze-drying methods demonstrate the best ability to maintain product moisture because the process takes place at low temperatures and vacuum pressure, thus minimizing thermal degradation of lactic acid. Consequently, the characteristics of the lactic acid content and acidity of the product remain relatively stable after drying. Future studies should investigate the combined effects of fermentation conditions and drying technologies on the physicochemical, microbiological, and functional properties of fermented egg flour. Particular attention should be given to optimizing fermentation parameters, including fermentation duration, and drying conditions to maximize lactic acid retention while preserving protein functionality. Research should assess the shelf life during storage under different packaging conditions. Finally, pilot-scale production and techno-economic analyses should be conducted to support the commercial development of fermented egg flour as a functional food ingredient.

DECLARATIONS

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Data availability

The data used and/or analyzed in this study are available from the corresponding author on reasonable request through the research results.

Author contributions

All authors contributed substantially to this research. Contributions included study design, experimental execution, data analysis, interpretation of results, drafting the manuscript, and final revision of the manuscript. All authors have read and approved the final version of the manuscript for publication.

Consent for publication

All authors have approved the final version of the manuscript and agreed to the publication of this article.

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Ethical considerations

This research did not involve human subjects or laboratory animals directly, requiring specific ethical approval. All research procedures were conducted in accordance with applicable laboratory standards and research guidelines.

Competing interests

The authors declare that they have no competing interests in the research or publication of this article.

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IN SILICO ANALYSIS OF THE PROMOTER REGION OF PROLACTIN GENE IN CATTLE TO UNDERSTAND ITS GENE REGULATION

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Supporting Information



ABSTRACT: Prolactin (PRL) is a key endocrine regulator of lactation, mammary gland growth, and systemic metabolic homeostasis in cattle. The transcriptional architecture of bovine PRL regulation remains incompletely characterized, despite its physiological importance. In this study we used an in silico TSS, CpG island, and TFBS profiling approach to analyze the ~5 kb upstream regulatory region of the *Bos taurus* PRL gene. By employing Neural Network Promoter Prediction under stringent criteria, we identified three highly reliable TSSs located at -4478, -2123, and -517 base pairs relative to the translational start codon, indicating a multi-TSS promoter complex that facilitates alternative transcript initiation. Canonical CpG islands were absent, consistent with tissue-specific, fine-tuned transcriptional control. TFBS analysis revealed a densely populated, hierarchically organized promoter enriched for pituitary-specific POU1F1 motifs, hormone-responsive ESRRA and RARA elements, signal-dependent STAT5 and AP-1 sites, and developmental transcription factors such as NANOG, HOXD9, and FOXI3. High-affinity TFBS clusters, including cis-regulatory modules of activators and repressors (like PRDM1) suggest combinatorial control and dynamic transcriptional responsiveness. These findings give mechanistic insights into the transcriptional complexity of bovine PRL locus and built an essential resource base on functional genomics studies to enhance efficiency of lactation and endocrine resilience in cattle.

Keywords: *Bos taurus*, Lactation, Mammary gland growth, Prolactin gene, Promoter region, Transcription start sites.

INTRODUCTION

In cattle, the genetic and regulatory basis of economically important traits such as milk production has attracted considerable attention. The bovine prolactin (PRL) gene has been extensively investigated because of its associations with milk yield, fertility, and lactation performance. Several studies have reported polymorphisms within the PRL gene and their associations with milk production traits (Motmain et al., 2022; Ilie et al., 2023). In addition, previous studies have characterized specific regulatory variants and promoter polymorphisms of the bovine PRL gene. However, most of these studies have focused primarily on genetic associations and sequence polymorphisms rather than on the comprehensive regulatory architecture of the PRL promoter (Brym et al., 2007).

To date, information regarding putative transcription start sites, the distribution of transcription factor binding motifs, and the presence of CpG islands within the bovine PRL promoter remains limited. Therefore, a comprehensive in silico characterization of the bovine PRL promoter is still lacking. Recent advances in genomics and bioinformatics have made it possible to predict promoter regions, transcription factor binding sites (TFBS), CpG islands, and conserved regulatory motifs using computational approaches without requiring immediate experimental validation (Matys et al., 2006; Fornes et al., 2020).

Such analyses are useful for generating hypotheses regarding regulatory mechanisms prior to experimental validation. Therefore, the present study aimed to perform a comprehensive in silico characterization of the bovine PRL promoter region and to identify putative regulatory elements that warrant further experimental validation and functional investigation.

MATERIALS AND METHODS

Identification of transcription start sites

The genomic sequence of the bovine PRL gene was retrieved from the NCBI Gene database (Gene ID: 280901; RefSeq transcript accession: NM_173953.2) based on the *Bos taurus* reference genome assembly ARS-UCD1.2. According to the NCBI reference genome, the PRL gene is located on chromosome 23 (NC_037350.1:35332693–

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35341308) on the forward (+) strand. A 5-kb genomic region upstream of the translation start codon (ATG) of the reference transcript was extracted (NC_037350.1:35327693–35332692) and used for promoter analysis. The 5-kb upstream region was selected to include both proximal and potential distal regulatory elements that may contribute to transcriptional regulation. Putative transcription start sites (TSSs) were predicted using the Neural Network Promoter Prediction (NNPP) server (https://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001). NNPP employs a time-delay artificial neural network trained on experimentally validated eukaryotic promoter sequences to identify potential promoter regions and putative TSSs based on sequence characteristics and promoter-associated motifs. Predictions were performed using the default parameters with a minimum promoter prediction score threshold of 0.80.

Transcription factor binding site prediction

The transcription factor binding site (TFBS) prediction was done from the AnimalTFDB v4.0 platform (https://guolab.wchscu.cn/AnimalTFDB4/#/TFBS_Predict), a broad animal transcription factors and regulatory annotations repository (Shen et al., 2023). AnimalTFDB integrates genome-wide TF repertoires from Ensembl (release 105) and provides a TFBS prediction module based on curated motif libraries across multiple species. The TFBS identification was based on a position weight matrix (PWM)-based scanning implemented in AnimalTFDB. This prediction module includes quality and computationally inferred TF binding motifs from several public databases like HOCOMOCO, TRANSFAC, JASPAR, CIS-BP, and hTFtarget (Kulakovskiy et al., 2018; Fornes et al., 2020). These motifs provide high-confidence transcription factor binding profiles. The input nucleotide sequence is scanned against the integrated motif datasets using PWM-based scoring algorithms which assess the potential of TF–DNA interactions through sequence similarity to established binding motifs (Stormo, 2013). AnimalTFDB provides a motif score and p-value for each predicted binding site. The score reflects the log-likelihood ratio of the observed sequence against a background model, and the p-value describes the probability of obtaining a match with the same or better strength by chance. For TFBS prediction, motifs with p-values $\leq 1 \times 10^{-4}$ and motif match scores exceeding the cut-off values recommended by AnimalTFDB were retained for analysis, whereas motifs with $P \leq 1 \times 10^{-5}$ were considered high-confidence predictions. These criteria are commonly used in genome-wide motif scanning to reduce false-positive predictions (Fornes et al., 2020). Unless otherwise stated, the default background nucleotide frequencies were used for TFBS prediction by AnimalTFDB. The predicted TFBSs were annotated with the names of transcription factors, genomic coordinates, orientation of strands, and metrics of statistical significance.

Identification of CpG Islands in promoter regions

CpG islands were determined using a strict computational scheme within the promoter regions. The criteria included a CpG island with a GC content of at least 50%, an observed to expected CpG ratio of ≥ 0.60 , and an island minimum length of 200bp (Takai and Jones, 2002; Long et al., 2013). CpG islands were identified using CpG Island Searcher (CpGi130) program at http://www.bioinformatics.org/sms/cpg_island.html (Takai and Jones, 2002).

RESULTS

Identification of TSSs

Neural Network Promoter Prediction (NNPP) analysis predicted three putative transcription start sites (TSSs) within the *Bos taurus* PRL promoter region (Table 1). These putative TSSs received NNPP scores of 0.98, 0.94, and 0.84, suggesting the presence of promoter-like sequence features. Positional mapping relative to the translational start codon (ATG) indicated that the predicted putative TSSs were located approximately at –4478 bp, –2123 bp, and –517 bp upstream. However, these predictions should be considered putative and require experimental validation or support from public transcriptomic datasets. Multiple TSSs may be alternative transcription starts, with a possible implication for complex regulation of PRL expression in cattle. The proximal TSS with –517 bp and a higher prediction score is presumably representing the core promoter region while the more distal sites may function as auxiliary or tissue-specific regulatory elements. These findings, therefore, implied a multiple-TSS architecture of the PRL promoter in cattle, which could integrate differential transcriptional control mechanisms with potential functional and physiological implications in lactation and endocrine regulations in cattle.

Table 1. Number and predictive score value for cattle PRL gene TSSs.

Promoter region name	No. of TSS Identified	Predictive score at cutoff value of 0.8	Distance from start codon (ATG)
Bos_taurus_pro-PRL	3	0.98, 0.94, 0.84	–4478, –2123, –517

Identification of CpG Islands

Analysis of the PRL promoter revealed no detectable CpG islands. Despite comprehensive screening using established prediction algorithms and criteria for CpG island identification (length ≥ 200 bp, GC content $\geq 50\%$, observed-to-expected CpG ratio ≥ 0.6), this region did not exhibit characteristics consistent with canonical CpG islands.

Common motifs and transcription factors

Transcription Factor Binding Site Landscape of the *Bos taurus* Prolactin 5 kb Promoter. The promoter region of the *Bos taurus* PRL gene (5kb) contains a high density of transcription factor binding sites (TFBS) that are functionally diverse and hierarchically organized based on an in silico analysis. Motif prediction was conducted via several highly confident databases (TRANSFAC, JASPAR, hTFtarget, CIS-BP, HOCOMOCO), and all predicted TFBS were mapped with respect to the start codon (ATG) to allow for spatial characterisation of regulatory elements. Overall, an estimated 76 high-confidence TFBS were identified in the promoter region (Table 2), and the average density of TFBS (~16 sites per kb) indicates that the PRL promoter is highly compact and rich in regulatory elements. Additionally, the TFBS distribution in the PRL promoter is not evenly distributed; two regions of enrichment were identified: a distal regulatory region (–5000 to –3000 bp) and a mid-promoter region (–3000 to –1000 bp), both of which contain most of the predicted binding sites. In contrast, the proximal region of the promoter (–1000 to –1 bp) has a lower density of TFBS but does contain high concentrations of core promoter elements. This non-random pattern of organization indicates that the PRL promoter has a modular regulatory architecture, with the distal and mid-promoter regions acting as enhancer-like domains with multiple TFBS clustered together, whereas the proximal region is involved in supporting the initiation of transcription. Distribution of transcription factor binding sites (TFBS) by functional category revealed a non-uniform pattern. Hormone receptor-associated factors, including ESRRA, RARA, and NR4A1, constituted the largest proportion (31.6%). This was followed by POU1F1 (17.1%) and the STAT/JUN group (11.8%). SP1 accounted for 6.6%, and developmental transcription factors comprised 10.5% of the predicted TFBS. The remaining 22.4% of predicted TFBS were classified as “Other TFs”, representing transcription factors with diverse or unclassified biological functions (Figure 1).

Table 2 - Identified Transcription Factor Binding Sites (TFBs) within the PRL promoter region.

TF	Source	location in the promoter	Strand	Score	P value	Mached sequence
POU1F1*	hTFtarget	-107	-	16.2022	1.47E-07	TTCATGAATATAT
POU1F1	CISBP	-2521	+	9.77692	4.91E-06	CTAATTAGG
POU1F1	hTFtarget	-393	-	13.427	8.96E-06	TTCTTGAATATAG
POU1F1	hTFtarget	-1160	-	13.2809	1.06E-05	GTAATGAATATAA
POU1F1	CISBP	-208	-	8.61062	1.58E-05	CTTATTCTATA
POU1F1	hTFtarget	-1866	+	12.7245	1.87E-05	TTGCATTATTAG
POU1F1	CISBP	-2754	+	8.48673	3.03E-05	ATAATTCTATT
POU1F1	hTFtarget	-1739	+	11.618	5.07E-05	CCATTGAATAAAC
POU1F1	hTFtarget	-4042	-	7.29592	5.89E-05	TTATGTACATTAAC
POU1F1	hTFtarget	-2832	-	7.0102	6.37E-05	ATATAGTAATTAGA
POU1F1	CISBP	-265	-	8.82308	6.64E-05	TTAATTAGA
POU1F1	hTFtarget	-2615	+	10.6458	7.73E-05	TTTCTGCATCATTAAAGG
POU1F1	hTFtarget	-4225	-	11.1011	7.74E-05	GAAATGAAATTTT
NR4A1	hTFtarget	-1225	+	15.0	2.21E-06	TAAAGGTCAC
NR4A1	hTFtarget	-736	-	12.7	2.13E-05	TTAAATGTCAAG
NR4A1	hTFtarget	-2165	-	12.4364	2.56E-05	TAAAATGTCATA
NR4A1	HOCOMOCO	-2339	+	10.824	3.21E-05	AAAATGGCA
NR4A1	hTFtarget	-365	+	10.9817	4.86E-05	AGGCAAATGTTA
NR4A1	HOCOMOCO	-1991	+	10.488	5.84E-05	GAAATGGCA
NR4A1	hTFtarget	-1572	+	10.6301	7.44E-05	AAATGGCAC
NR4A1	HOCOMOCO	-1182	+	10.232	8.96E-05	AGAAAGTCA
ESRRA	hTFtarget	-1226	+	15.5	3.45E-06	CTAAAGGTCACAGGC
ESRRA	hTFtarget	-926	-	14.6	6.86E-06	TTCAAGGGCACACAC
ESRRA	hTFtarget	-1512	-	13.3939	1.17E-05	GGGTCAAAATGCCAT
ESRRA	hTFtarget	-1081	-	12.187	2.42E-05	GGCCAAGGTGACA
ESRRA	hTFtarget	-1917	+	7.87755	3.57E-05	GGGTTCGCCAGAGTCA
ESRRA	hTFtarget	-2979	+	10.2653	6.82E-05	TCAGGTGAAGGGGACCTGG
ESRRA	hTFtarget	-843	+	5.06122	9.76E-05	GAGGGCAACGTATGTTG
RARA	hTFtarget	-2415	-	13.3776	1.94E-06	AGGTCAAATAACGTTCA
RARA	TRANSFAC	-1228	+	15.6053	2.95E-06	AACTAAAGGTCACAGG
RARA	hTFtarget	-1066	+	13.5606	1.03E-05	AGAGTGGCTTGAGGTCAGAG
RARA	hTFtarget	-2068	+	13.1712	1.30E-05	CAGTCCATGGGGTCAC
RARA	CISBP	-3159	-	5.82653	1.57E-05	AATTTCAAAGATCA

RARA	hTFtarget	-1511	-	6.44898	2.71E-05	TGGGTCAAAATGCCA
RARA	hTFtarget	-1918	+	5.52041	2.73E-05	TGGGGTCGCCCAGAGTCA
RARA	hTFtarget	-737	-	11.8333	3.14E-05	GGGGAGTTTAAATGTCAAGG
RARA	hTFtarget	-241	+	5.47959	6.56E-05	GATGTTCAATTCTGGTCA
Sp1	hTFtarget	-720	+	11.6966	4.29E-05	CCCATCCCAC
Sp1	TRANSFAC	-134	+	11.0843	5.46E-05	GCAAAAGGGAAGGG
Sp1	TRANSFAC	-814	-	10.6386	7.28E-05	TGAAGAGGGCTGGA
Sp1	hTFtarget	-1609	-	10.6923	7.47E-05	TCCCCTCCTTT
Sp1	hTFtarget	-3334	-	9.94231	9.95E-05	CTCCCACCCCTT
STAT5A	hTFtarget	-3290	+	14.4242	1.15E-05	TTCTAGGAAC
STAT5A	HOCOMOCO	-503	-	9.84848	9.69E-05	TACTAAGAAA
STAT6	HOCOMOCO	-790	-	13.973	7.86E-06	CTTCAAGAGAAATC
JUN	hTFtarget	-4794	-	11.4343	4.87E-05	CATTCCATTGTGGTGAGAGA
JUND	hTFtarget	-524	+	11.1327	5.25E-05	CTTACTCATCCTT
JUND	hTFtarget	-3485	+	11.0	5.73E-05	GCTGATTCATG
JUNB	hTFtarget	-420	-	10.4184	7.69E-05	GGTGTCTCATG
JUNB	hTFtarget	-1818	-	10.6364	7.74E-05	GCTGATTCACA
JUND	hTFtarget	-2063	+	7.93878	9.48E-05	CATGGGGTCACTAG
Blimp-1	TRANSFAC	-2026	-	19.303	9.36E-08	AAAAGGGAAAGTGA
Irx6	TRANSFAC	-677	+	15.8204	8.12E-07	TACATACATGTTAAAT
Hoxd9	TRANSFAC	-1301	+	13.4306	1.64E-06	GCAATAAAAA
Zfp445	TRANSFAC	-550	+	10.2787	2.87E-06	GAGTATAACAGGAAGTGA
Hic1	TRANSFAC	-3233	-	12.3103	3.00E-06	TGCCCCACCAATGCCATG
Blimp-1	TRANSFAC	-1548	-	15.3333	3.52E-06	GACAGTGAAAGTCT
Foxj3	TRANSFAC	-4009	-	14.8621	3.70E-06	ATAAACAAAAGCA
Lhx4	TRANSFAC	-2525	+	14.1503	3.73E-06	ATTTCTAATTAGGGATG
Evx2	TRANSFAC	-1304	-	14.597	3.89E-06	TTTTATTGCATT
Irx2	TRANSFAC	-677	+	14.3133	4.38E-06	TACATACATGTTAAAT
Zfp90	TRANSFAC	-891	-	15.4754	4.96E-06	AGGGAGTTCATA
Foxj3	TRANSFAC	-3056	+	14.0862	5.44E-06	CAAAACAAAACA
Sall3	TRANSFAC	-1024	+	14.0161	5.98E-06	TAACAGTTT
Nanog	TRANSFAC	-3915	+	12.8358	1.47E-05	TATCAAAAAACAATACAGGCA
Nanog	TRANSFAC	-3060	+	13.6866	5.99E-06	ATAGCAAAACAAAACAAAT
BACH2	hTFtarget	-1817	-	14.3818	6.61E-06	TGCTGATTCAC
Blimp-1	TRANSFAC	-3004	-	14.0788	7.34E-06	CACTTTCCC
Blimp-1	TRANSFAC	-2025	+	14.0788	7.34E-06	CACTTTCCC
Alx1	TRANSFAC	-2522	-	12.3152	7.98E-06	CCTAATTAGA
Nanog	TRANSFAC	-2565	+	13.3881	8.31E-06	ATAGATGAACAAAAGAAAAT
Zfp37	TRANSFAC	-1676	-	12.7869	8.35E-06	ATTTAAAGGTGC
Arid5a	TRANSFAC	-4207	-	13.1786	9.34E-06	GCAATATTGGAAGC
Blimp-1	TRANSFAC	-909	-	13.0642	1.11E-05	GGGGAAGTGACAGTGG
Foxj3	TRANSFAC	-3732	+	12.5862	1.15E-05	ACAAACAAAAGCA
Blimp-1	TRANSFAC	-3870	+	12.8624	1.20E-05	CTAAATGGGAAAGGAA

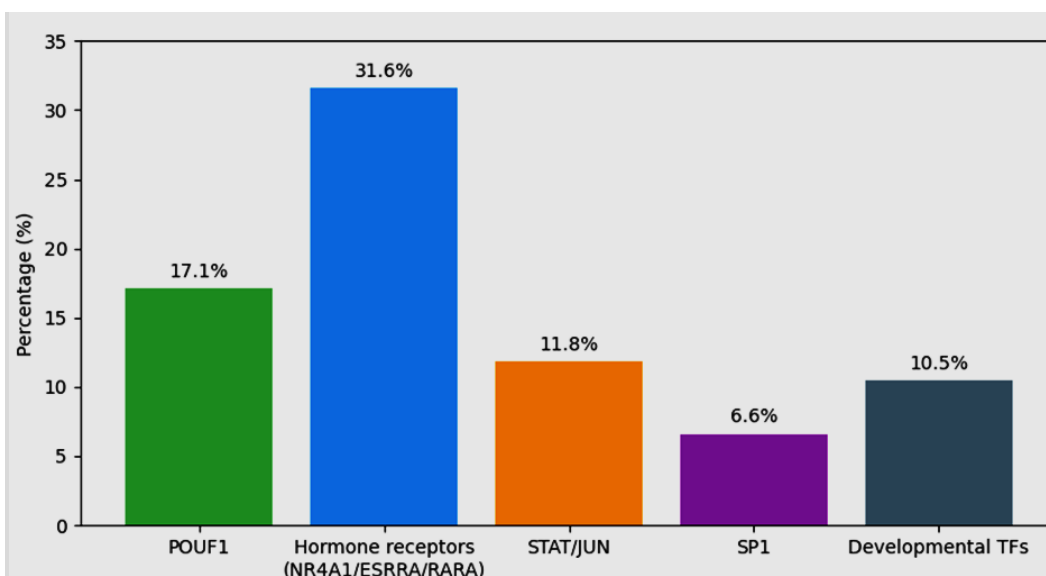


Figure 1 - Distribution of transcription factor binding sites (TFBS) by functional category. Bar chart showing the percentage of TFBS associated with different transcription factor families. Values indicate the relative contribution of each functional category to the total TFBS.

Hormone-responsive regulatory elements

One of the notable features of the PRL promoter was the presence of numerous predicted hormone-responsive elements, particularly those associated with nuclear receptors. Multiple putative binding sites for estrogen-related receptor alpha (ESRRA) were identified across a substantial portion of the PRL promoter (~-3084 to -841 bp). These predicted motifs exhibited relatively high scores (up to 15.5) and low P-values (as low as 3.45×10^{-6}), suggesting sequence similarity to known ESRRA binding motifs. The occurrence of predicted ESRRA motifs in the mid and proximal promoter regions may indicate potential regulatory regions; however, their functional significance and direct involvement in PRL transcription require experimental validation. In addition, several putative binding sites for retinoic acid receptor alpha (RARA) were identified throughout the promoter (~-2586 to -841 bp). A cluster of predicted RARA motifs was observed near -2586 bp (score = 13.38; $P = 1.94 \times 10^{-6}$). The co-occurrence of predicted ESRRA and RARA motifs may suggest potential interactions between regulatory pathways, although no functional relationship can be inferred from computational predictions alone.

Pituitary-specific and prolactin-regulatory factors

Multiple predicted POU1F1 (Pit-1) binding motifs were identified at several positions throughout the PRL promoter, spanning approximately -4894 to -776 bp. These putative motifs represent the largest group of predicted TFBS identified in the promoter region. The distribution of predicted POU1F1 motifs was not uniform, with apparent clusters observed in the distal (-4800 to -3800 bp) and mid-promoter (-2400 to -2200 bp) regions. The occurrence of these motif clusters may suggest potential cis-regulatory regions; however, their functional significance cannot be inferred from sequence analysis alone and requires experimental validation. In addition, several predicted NR4A1 binding motifs were identified within the -4600 to -2600 bp region, some of which were located near predicted POU1F1 motifs. The spatial proximity of these predicted motifs may indicate potential regulatory interactions; however, no functional relationship or coordinated regulatory network can be concluded based solely on computational predictions.

General transcription factors and signal-responsive elements

Numerous general transcription factor binding sites have been found in the PRL promoter, including SP1, STAT5A, STAT5B and JUN. Many of the SP1 binding sites occur predominately within a proximal promoter (approximately ~-720 to -130 bp) and correspond with the canonical function of SP1 in regulating regions of core promoters to promote basal levels of transcription. The presence of multiple SP1 motifs in close proximity suggests a strong basal transcriptional framework necessary for PRL expression. The presence of STAT5A and STAT5B binding sites at both distal (~-3290 bp) and proximal (~-503 bp) positions strongly supports the established role of STAT5 in prolactin signaling and cytokine-induced responses. Therefore, these binding sites play a role in the feedback mechanism regulating transcriptional activity and signal-dependent transcriptional activation. STAT5 binding sites are located throughout the promoter, indicating that STAT5 may regulate transcription through both direct binding as well as through long-distance interactions. JUN binding sites, which are part of the AP-1 complex, were identified in both distal (~-4794 bp) and proximal (~-524 bp) areas. The pattern of binding sites therefore indicates that PRL transcriptional activity may be regulated by external signals, including stress and inflammation and growth factors, and demonstrates that this gene is involved in broader physiological processes.

Developmental and pluripotency-associated factors

Various developmental and lineage-associated transcription factors (e.g., NANOG, FOXI3, IRX2, HOXD9) could putatively bind to the distal promoter region (~-4300 to -1700 bp), in addition to the classical prolactin regulators. Though these transcription factors are not traditionally linked to prolactin gene regulation, they provide evidence that the PRL promoter may contain evolutionarily-conserved regulatory elements that may be functional during certain developmental or physiological stages. Additionally, these motifs may also reflect latent regulatory potential that can be activated in non-canonical frameworks such as during early embryonic development or cellular reprogramming.

High-affinity and clustered binding regions

One of the main features of the PRL promoter was the presence of TFBS clusters located in two major regions (-4500 to -3000 bp and -2500 to -700 bp). Several predicted motifs for POU1F1, ESRRA, RARA, SP1, and NR4A1 were found in close proximity within these regions, suggesting potential cis-regulatory modules. The highest-scoring predicted PRDM1 (Blimp-1) binding motif was identified at approximately -2026 bp (score = 19.303, $P = 9.36 \times 10^{-8}$). This high-scoring motif may represent a potential regulatory element; however, its functional significance remains to be experimentally validated. The proximity of predicted activator (e.g., POU1F1) and repressor (e.g., PRDM1) motifs may indicate potential regulatory complexity, although no conclusions regarding promoter dynamics or regulatory interactions can be drawn from computational predictions alone.

DISCUSSION

Promoter analysis of the *Bos taurus* PRL gene predicted the presence of three putative transcription start sites (TSSs). The occurrence of multiple putative TSSs is consistent with the multi-TSS promoter architecture reported for many tissue-specific and developmentally regulated genes; however, the predicted TSSs identified in the present study require experimental validation or support from public transcriptomic datasets before being considered active transcription initiation sites. The proximal putative TSS located at -517 bp received the highest prediction score and may correspond to a potential core-promoter region responsible for basal transcriptional activity. In contrast, the more distal putative TSSs may represent alternative regulatory regions that could contribute to context-dependent regulation of PRL expression. The multi-TSS organization has been associated with dynamic transcriptional regulation and alternative promoter usage in other endocrine genes (Carninci et al., 2006; Haberle and Stark, 2018).

Similarly, the presence of multiple putative TSSs in the bovine PRL promoter may suggest the possibility of alternative 5' UTR isoforms; however, this hypothesis remains to be experimentally verified. Collectively, these observations suggest that the bovine PRL promoter may possess a multi-layered regulatory architecture involving multiple putative transcription initiation regions and diverse cis-regulatory elements. Using the classical criteria of CpG island length, GC content, and observed/expected CpG ratio (Takai and Jones, 2002; Long et al., 2013), this study did not identify a canonical CpG island within the analyzed PRL promoter sequence. Previous studies have shown that canonical CpG islands are commonly associated with housekeeping genes and permissive chromatin environments (Deaton and Bird, 2011), whereas tissue-specific promoters frequently lack classical CpG island features (Saxonov et al., 2006). Therefore, the absence of a canonical CpG island in the bovine PRL promoter may suggest that epigenetic regulation of this gene could involve mechanisms other than broad CpG island-associated methylation. However, no direct evidence regarding DNA methylation or chromatin organization was generated in the present study, and the potential contribution of local CpG methylation and chromatin modifications to PRL regulation remains speculative and requires further investigation. In silico analysis of the ~5 kb *Bos taurus* PRL promoter suggested a potentially complex regulatory architecture containing numerous predicted transcription factor binding motifs. Prolactin is a key regulator of mammary gland development, metabolism, immune function, and reproduction in cattle (Bole-Feysot et al., 1998; Zhou et al., 2025). The high density of predicted transcription factor binding sites (TFBS) observed in the present study may indicate the presence of multiple regulatory regions capable of integrating diverse physiological signals. However, the functional significance of these predicted motifs cannot be inferred from sequence analysis alone and requires experimental validation. Previous experimental studies have demonstrated that POU1F1 (Pit-1) plays an essential role in lactotroph differentiation and prolactin production (Li et al., 1990; Sönmez and Ünal, 2023).

In the present study, multiple predicted POU1F1 binding motifs were identified throughout the bovine PRL promoter, both proximally and distally. The apparent clustering of these predicted motifs may suggest potential cis-regulatory regions; however, their occupancy and regulatory function *in vivo* remain unknown. Similarly, although cooperative interactions among adjacent POU1F1 binding sites have been reported in other experimental systems (Dasen and Rosenfeld, 2001; Kawauchi et al., 2025), the present study did not evaluate transcription factor binding or promoter activity. Therefore, any relationship between the predicted POU1F1 motifs and PRL transcription should be regarded as a hypothesis requiring future experimental validation. Single nucleotide polymorphisms (SNPs) within regulatory regions have been reported to alter the binding affinity of transcription factors, including POU1F1, and may influence gene expression in certain biological contexts (Lü et al., 2010). Because prolactin plays an important role in mammary gland development and lactation, variation within predicted POU1F1 binding motifs could potentially affect PRL regulation. However, the present study did not evaluate genetic polymorphisms, gene expression, or production traits, and therefore no conclusions can be drawn regarding the functional effects of these predicted motifs on milk production or lactational performance. Future studies integrating polymorphism analyses, gene expression data, and phenotypic measurements will be necessary to determine the biological significance of these predicted regulatory elements. The presence of multiple predicted POU1F1 motifs in the PRL promoter may indicate potential regulatory regions; however, their functional roles remain to be experimentally validated.

The presence of multiple motifs known to have high-affinity for estrogen receptor α (ESR1 and ESRRA), as well as retinoic acid receptor- α (RARA), in the PRL promoter underscores the fundamental importance of hormone action in the expression of prolactin gene. Prolactin is known to be synthesized in response to the action of estrogen, especially during pregnancy and early lactation, when increasing levels of estradiol sensitize both the lactotrophs and the mammary epithelium to become maximally prepared for their functions (Freeman et al., 2000). There is evidence that, at the regulatory regions of the PRL gene, ESR1-binding is synergistic with Pit-1, thereby increasing the activity of the PRL promoter in the presence of estrogen. Furthermore, there is considerable evidence to support the conclusion that retinoic acid signaling via the activation of RAR/RXR heterodimers plays an important role in cell fate decision making for mammary epithelial cells and their differentiation into functional lactocytes (Hannan et al., 2023). The position of the putative binding sites for ESRRA and RARA in relation to the POU1F1 motifs suggests that hormones may also alter the basal transcriptional program mediated by pituitary-specific factors. In addition to being hormone responsive, the PRL

promoter contains putative binding sites for the transcription factors STAT5A/B and AP-1 (JUN/FOS), which may increase the responsiveness of the PRL promoter to extra-cellular signals such as cytokines, growth factors, and metabolic stress.

STAT5 is activated by the signal from the prolactin receptor and is an established downstream effector of this signaling pathway in the mammary gland, resulting in the activation of milk protein genes and enhancing the lactogenic pathways via a positive feedback mechanism (Liu et al., 1997; Rädler et al., 2017; Zhou et al., 2025). The presence of motifs that can bind to STAT5 at the PRL locus indicates that autoregulatory loops may be present at this locus and that prolactin itself could modulate its own expression in an indirect manner using receptor-mediated signaling pathways. In addition, stress signals, the availability of nutrients and growth factor signaling pathways can all modulate the activity of the AP-1 complex (also known as JUN, FOS, and all related family members) indicating that transcriptional activity of prolactin can potentially be affected by metabolic and environmental stressors (Shaulian and Karin, 2002).

Several predicted motifs corresponding to developmental transcription factors, including NANOG, IRX2, FOXI3, and HOXD9, were identified in the distal region of the bovine PRL promoter. However, these transcription factors are not currently known to participate in PRL regulation, and their predicted occurrence may reflect the limitations of motif-scanning approaches and the potential for false-positive predictions. Although members of the HOX and FOX transcription factor families have been implicated in developmental and tissue-specific regulatory processes in mammals (Golson and Kaestner, 2016; Hubert and Wellik 2023), there is currently no direct evidence supporting a role for NANOG, IRX2, FOXI3, or HOXD9 in the regulation of bovine PRL expression. Therefore, the biological significance of these predicted motifs remains uncertain and requires further investigation through analyses of sequence conservation, tissue-specific expression, and experimental validation in bovine pituitary and mammary tissues.

The occurrence of transcription factor-binding sites (TFBS) near each other, especially those that contain POU1F1, ESRRA, RARA, SP1 and STAT motifs, provides evidence for the presence of cis-regulatory modules (CRM) that serve as integrative hubs for grouping together different transcription factors (TFs). CRMs are being increasingly recognized as playing an essential role for context-dependent gene regulation, providing a means for multiple TFs to combine their efforts to influence levels of gene transcription (Spitz and Furlong, 2012). In the bovine PRL promoter, these CRMs may enable dynamic switching between activation and repression states in response to developmental stage, endocrine milieu, or physiological stress. Additionally, the finding of a high affinity PRDM1 (also known as Blimp-1) binding site suggests that there are repressive mechanisms in place in addition to activating platforms that provide another layer of regulation to prevent excessive PRL production (Minnich et al., 2016). These types of repressive modules are important to maintaining normal cellular homeostasis and preventing abnormal hormone production. Collectively, the architecture of the PRL promoter provides evidence that there is a multi-layered regulatory system that enables prolactin expression to be dynamically adjusted throughout pregnancy, parturition, and lactation. This ensures that mammary gland development, the production of milk and maintenance of overall metabolic homeostasis of the whole organism are coordinated in response to both intrinsic cues and external environmental conditions (Bauman and Currie, 1980). The distal clusters and CRMs that were found in this *in-silico* study may be good candidates to target for genetic selection or epigenetic modulation to enhance the efficiency of lactation and the resilience of dairy cattle. Functional validation of these elements through reporter assays, CRISPR gene editing, and chromatin accessibility profiling will be essential for elucidating their roles *in vivo*.

CONCLUSION

The present study provides an *in silico* characterization of the bovine PRL promoter and identifies several putative regulatory elements, including predicted transcription start sites and transcription factor binding motifs. These findings should be regarded as hypotheses for future research and require experimental validation to determine their functional significance in bovine PRL gene regulation.

DECLARATIONS

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Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contribution

Author's contribution should be explained in the title page as in the example. Initials of the authors must be in compliance with the following templates:

Conceptualization, study design, supervision, and critical revision of the manuscript: Bakhtiyor ADILOV;

Draft manuscript preparation, integration of all sections, and manuscript formatting: Boburbek AKHMADALIEV;
Data curation, genomic sequence retrieval from genomic databases, and transcription start site (TSS) analysis: Anvar SHERIMBETOV;
Transcription factor binding site (TFBS) prediction, bioinformatics analysis, and data visualization: Bobur ABDIKARIMOV;
CpG island analysis, statistical evaluation, and interpretation of epigenetic features: Bobir ABDUVALIEV;
Results interpretation, literature review, and drafting of the Discussion section: Dilshodbek RUZMETOV.

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Ethical consideration

This study was conducted using publicly available nucleotide sequence data and bioinformatic analyses. Since no live animals, human participants, or biological samples were involved, ethical approval and informed consent were not required.

Competing Interests

The authors have not declared any competing interests.

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DEVELOPMENT OF WHEY PROTEIN AND PECTIN-BASED FILM ENRICHED WITH LEMON ESSENTIAL OIL FOR FOOD PACKAGING: EFFECTS ON MECHANICAL PROPERTIES, ANTIOXIDANT, AND BIODEGRADABILITY

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ABSTRACT: The employment of conventional plastics in food packaging has been demonstrated to exert a detrimental effect on the environment, owing to the fact that these plastics are non-biodegradable. Furthermore, there is a possibility that microplastics will accumulate within the food chain. The objective of this study is to develop an edible film that has been enriched with lemon essential oil and glycerol. The aim of the enrichment is to improve the film's mechanical properties, antioxidant content and biodegradability. The present study was conducted experimentally, employing a completely randomized factorial design with five replicates. Factor A consisted of control film; film with added lemon essential oil; and film with added encapsulated lemon essential oil. Factor B consisted of glycerol concentrations (5%; 10%; and 15%). The findings demonstrated that the incorporation of lemon essential oil and varying glycerol concentrations exerted a substantial impact ($P < 0.01$) on the tensile strength, elongation, and antioxidant content of whey-pectin-based edible films. The tensile strength values ranged from 4.23 to 7.54 MPa, the elongation ranged from 62.49 to 68.76%, and the antioxidant content ranged from 20.84 to 42.25%. The biodegradability of the film was demonstrated by the observation of a satisfactory degradation rate over a 30-day period following burial in soil. The edible film, composed of whey-pectin and fortified with encapsulated lemon essential oil and 10% glycerol, exhibited optimal tensile strength, elongation, antioxidant properties, and biodegradability. This renders it a sustainable and environmentally friendly solution for food packaging.

Keywords: Biodegradable, Essential Oil, Glycerol, Packaging, Whey.

INTRODUCTION

The primary issue associated with the utilization of conventional plastic for food packaging pertains to its deleterious impact on the environment, a consequence of its non-biodegradable nature. Furthermore, it has been demonstrated that plastic contains reactive and toxic chemicals that have the potential to accumulate in the food chain in the form of microplastics (Butler et al., 2023). A solution that has been extensively developed is the creation of eco-friendly packaging made from natural materials (Fahrullah et al., 2022, 2024). A widely considered approach is the use of edible films based on natural biopolymers, such as proteins, polysaccharides, and lipids (Motelica et al., 2020; Reis & Gomes, 2023). Edible films have been shown to function not only as protective coatings for food products but also to carry active compounds that act as antioxidants and antimicrobials, thereby extending the shelf life of food items (Barbosa et al., 2021). This approach offers the ability to preserve food products by preventing microbial contamination and oxidative damage (Qian et al., 2021).

The combination of whey protein and pectin is considered to be a promising biopolymer matrix system, exhibiting the capacity to yield films that demonstrate favorable mechanical characteristics and intercomponent compatibility. A substantial body of research has been published on the use of whey protein as a film, and the results are very promising in terms of its potential as a barrier against moisture, aroma, lipids, and oxygen (Kumar et al., 2022). Lemon by-products have been identified as a potential alternative source of natural components for the manufacture of active packaging. This phenomenon can be attributed to the high concentration of functional compounds present in lemons, including vitamin C, fibre, carotenoids, essential oils, phenolic compounds, and pectin. The pectin content of lemons is a particularly attractive option in packaging formulations due to its superior physical and functional properties. Pectin's capacity to form gels, in conjunction with its biocompatibility and biodegradability, renders it a highly suitable biomaterial for a range of applications. Moreover, pectin is an excellent polymer matrix for film production because it is easily biodegradable (Karim et al., 2022; Aleksanyan, 2023; Djerri et al., 2025). Pectin also has water solubility, good flexibility, and effective barrier properties, which play an important role in inhibiting the oxidation process in food products during storage (Dobrucka et al., 2024).

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Essential oils are natural compounds derived from aromatic plants and have been used in food packaging (Anuradha and Bharadvaja, 2023). These compounds have been shown to enhance resistance to oxidation and mitigate the occurrence of mechanical degradation in biopolymer-based materials. However, not all types of essential oils are suitable for food packaging applications due to migration issues, as well as undesirable tastes and odours (Rehman et al., 2020; Suri et al., 2021). A potential solution to this issue could be the research and development of innovations such as encapsulation techniques. Encapsulation has been demonstrated to play a pivotal role in enhancing the stability of bioactive compounds (Kadmiri et al., 2021; Bae et al., 2022). This process effectively protects the compounds from external factors that can cause degradation, thereby maintaining their functionality. The incorporation of bioactive compounds into a protective matrix has been demonstrated to enhance stability, regulate release, control solubility, and augment bioavailability (Riseh et al., 2021; Zabot et al., 2022).

Beyond mechanical and functional aspects, the biodegradability of an edible film is a crucial parameter in assessing its environmental sustainability. Films derived from renewable biopolymers such as starch, protein, or chitosan have been shown to undergo microbial degradation and reduce plastic waste accumulation (Molavi et al., 2015; Gupta et al., 2022). Recent reviews also indicate that the sustainability of food films is not only related to materials and barrier properties, but also to their compostability and biological recyclability potential (Oliveira et al., 2025). In light of the mounting cognizance of the significance of environmental sustainability, the evolution of environmentally friendly and biodegradable edible films emerges as a pivotal stratagem for curtailing plastic waste within the food industry. The present study focuses on the development of natural-based edible films, with a particular emphasis on biodegradability. The objective is to enhance comprehension and to explore potential applications in a food packaging industry that is more environmentally friendly.

Whey, as a major by-product of the dairy industry, represents a significant component of livestock-based agro-industrial systems. The valorization of whey into biodegradable films not only addresses environmental concerns but also contributes to sustainable livestock production by converting dairy waste into value-added biomaterials. In livestock and animal feed industries, biodegradable and edible films have potential applications in the packaging of animal-derived products (Kandasamy et al., 2021), as well as in the protection of functional feed additives such as probiotics, enzymes, organic acids, and phytochemical compounds. The development of active films enriched with essential oils may also support hygienic handling, extended shelf life, and reduced contamination risk in feed and feed supplement distribution systems. Therefore, this technology is relevant not only to food packaging but also to sustainable livestock and feed-related industries (Chaudary et al., 2025). The present study aims to evaluate the effect of the incorporation of lemon essential oil, in both free and encapsulated forms, and varying glycerol concentrations, on the physical properties, antioxidant activity, and biodegradability of whey-pectin-based edible films.

MATERIALS AND METHODS

Materials

This study employed a range of materials for analysis, encompassing whey derived from cow's milk (Sigma-Aldrich, Germany), lemon, glycerol (Merck, Germany), gelatin (Greenara, Indonesia), silica gel, maltodextrin (Lihua Starch, China), distilled water, Tween 80, NaOH, ethanol, sodium tripolyphosphate, HCl, and CH₃COOH.

Sample preparation

The preparation of the whey-pectin-based film was undertaken through the utilization of a casting method. A total of 15 grams of whey and 1 gram of pectin were dissolved in 100 ml of distilled water. The solution is then subjected to continuous stirring at a temperature of 60 °C for a duration of 30 minutes, employing a hot plate stirrer. Subsequently, lemon essential oil and lemon essential oil encapsulation are introduced, with each constituting 5% of the total solution. In order to enhance the mechanical properties of the film, solutions of glycerol in the proportions of 5, 10, and 15% have also been incorporated. The resulting mixture solution was then transferred to a Petri dish and levelled. The film was then subjected to a drying process at a temperature of 50 °C for a duration of 24 hours, with the objective of achieving a hardened state. Subsequent to this, the film was stored within a desiccator at ambient temperature for a period of 24 hours prior to testing. Modification (Fahrullah et al., 2020).

Extraction of lemon essential oil

The extraction of lemon essential oil was accomplished through the utilization of a Heidolph Rotary Evaporator Hei-VAP Value Digital G3 (Heidolph Instruments GmbH & Co. KG, Germany). The grated lemon was subsequently placed in a rotating vessel containing ethanol. Subsequently, the vessel was attached to the evaporator unit, with the temperature maintained between 40-60 °C using a water bath. The utilization of a vacuum pump serves to reduce the pressure, thereby accelerating the evaporation process and facilitating more efficient separation of the essential oil. It is during this process that the solvent undergoes evaporation, with the resultant essential oil being collected in a suitable container. The essential oil obtained is then stored in dark glass bottles in a cool place to maintain its quality.

Extraction of pectin

Pectin was extracted from the waste residue produced during the lemon essential oil extraction process using an acidification method with an acid solution. The extraction process was conducted within a controlled environment, utilizing a hot hydrochloric acid solution at a temperature of 45°C and a pH of 1.25, with the objective of achieving the desired hydrolysis and dissolution of the pectin. Following the conclusion of the extraction process, an ethanol solution is introduced with the objective of reducing the solubility of pectin in the acid solution. This results in the pectin precipitating and separating from the liquid phase. The precipitated pectin is then collected through filtration, washed with ethanol to remove impurities, and then dried.

Lemon essential oil encapsulation

The encapsulation process, as outlined in the extant literature (Bezerra et al., 2016; Manaf et al., 2018), involves a two-stage procedure. The initial stage of the process is the preparation of an essential oil emulsion with a gelatin and maltodextrin solution. A total of 100 ml of gelatin solution was mixed with 8 ml of a lemon essential oil mixture and 6 ml of Tween 80, then homogenized at room temperature at a speed of 700 revolutions per minute for 30 minutes. Subsequently, 100 ml of maltodextrin solution was added and the homogenization process was repeated for a duration of 30 minutes. The second stage of this process was complex coacervation, which commenced with the addition of 2 ml of a 10% CH₃COOH solution until the pH had decreased to 4. Thereafter, the homogenization process was continued for a period of 60 minutes. The emulsion was left at a temperature of ±10°C for 30 minutes. Subsequently, 20 ml of sodium tripolyphosphate solution is introduced as a cross-linking agent, and the mixture is subjected to homogenization for a duration of 60 minutes. Subsequently, the pH is adjusted by the addition of a 20% NaOH solution until the pH reaches 9. Following this adjustment, the homogenization process is carried out for a period of 2 hours. The emulsion is then left at a temperature of ±10°C for 16 hours prior to being dried using a freeze dryer.

Measurement of tensile strength and elongation film

The tensile strength testing method, utilizing a Universal Testing Machine (UTM) (ASTM, 2018).

Antioxidant

The antioxidant content was analyzed using UV-Vis spectrophotometry and the DPPH method. A total of 1 g of sample was added to 9 ml of methanol, then vortexed. The sample was left for 24 hours to ensure optimal extraction. Subsequently, the sample underwent filtration using Whatman filter paper to facilitate the separation of the residue. Thereafter, 1 ml of the filtrate was collected and added to 2 ml of a 0.1 mM DPPH solution. Subsequently, the mixture was subjected to agitation until the desired homogeneity was achieved. Subsequently, the mixture was covered with aluminium foil and placed in an incubator in a dark environment for a period of 30 minutes. The rationale behind this procedure was to mitigate the potential for light interference, which could compromise the precision of the results. Subsequent to the incubation period, the absorbances were measured at a wavelength of 517 nm, employing a UV-Vis spectrophotometer. The results of the measurement of the degree of light absorption by the sample were recorded and utilized to calculate the antioxidant content in the sample using the following formula:

$$\text{Antioxidant (\%)} = ((A \text{ blank} - A \text{ sample}) / (A \text{ blank})) \times 100\% \quad (1)$$

Biodegradability testing

The biodegradability of the film was assessed by evaluating the physical changes that occurred in the composting environment. The samples were buried at a depth of 12–15 cm in the soil for a period of 30 days. Following a 30-day period, more in-depth observations were made to evaluate the level of film degradation. The samples were meticulously extracted from the soil to circumvent any potential disruption to the ongoing degradation process. A visual inspection of the films was conducted to ascertain any substantial alterations in their physical integrity, including damage, decomposition, or dissolution.

Statistical analysis

The present study employed a completely randomized design with a factorial pattern and five repetitions. The treatments comprised Factor A (control film; film with added lemon essential oil; film with added encapsulated lemon essential oil) and Factor B (5% glycerol; 10% glycerol; 15% glycerol). The data obtained were analyzed using Analysis of Variance (ANOVA), and if there were differences, they were followed up with Duncan's Multiple Range Test using SPSS 25 software.

RESULTS AND DISCUSSION

Tensile Strength

Tensile strength is a pivotal mechanical property in the assessment of material resistance to applied loads, particularly in the context of film and plastic materials. This property is defined as the capacity of a material to resist

tensile forces prior to the occurrence of damage or rupture. The tensile strength values of films with the addition of lemon essential oil and different glycerol concentrations are presented in Figure 1. The mean tensile strength values obtained ranged from 5.61 to 7.03 MPa. Glycerol, a hydrophilic plasticizing agent utilized in the production of this film, interacts with whey and pectin polymer chains through the formation of hydrogen bonds. It has been demonstrated that the presence of glycerol results in an increased mobility of the polymer chains, consequently leading to the formation of a more flexible film. However, this process concomitantly reduces the tensile strength, thereby rendering the material softer. Furthermore, the presence of a polar group in glycerol contributes to the stabilization of the dispersion of lemon essential oil within the water polymer matrix. In addition, lemon essential oil has been observed to aggregate (clump) into larger droplets. These substantial aggregates function as weak points within the film structure, thereby substantially diminishing the tensile strength of the material.

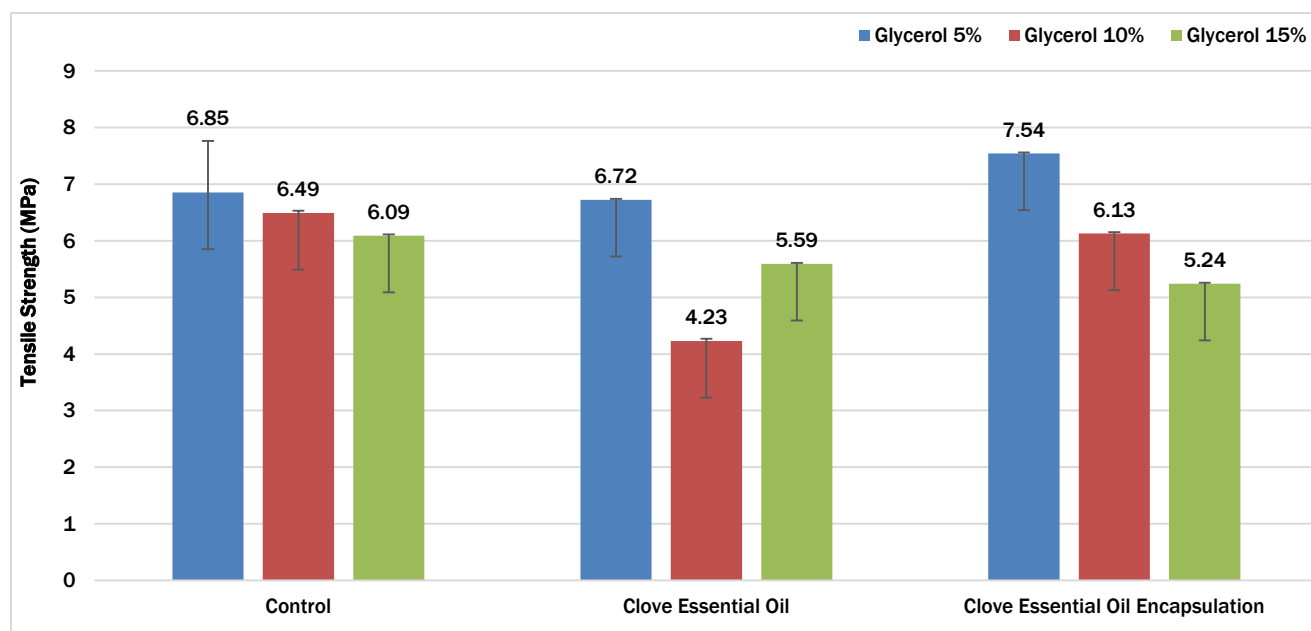


Figure 1 - Tensile strength (Mpa) of whey-pectin film with the addition of lemon essential oil and different glycerol concentrations

Whey-pectin films have been shown to possess the highest tensile strength in comparison to other treatments, a property attributable to the formation of a cohesive and dense polymer matrix through strong intermolecular interactions. It has been established that whey protein and pectin form a stable network through hydrogen bonds, electrostatic interactions and disulphide bonds (in whey protein). This results in the creation of a film that is resistant to external tensile forces (Pires et al., 2024). The addition of lemon essential oil resulted in a decline in tensile strength, which can be ascribed to the essential oil's role as a hydrophobic dispersed phase within a hydrophilic matrix (Sharma et al., 2021; Fahrullah et al., 2024, 2025). These oil droplets have the capacity to disrupt the continuity of the polymer network, and the oil also has a tendency to coat the polymer chains, thereby reducing the number of hydrogen bonds. Consequently, the internal cohesion of the film is reduced, rendering it more vulnerable to fracture under pressure, which in turn decreases its tensile strength (Atarés and Chiralt, 2016; Šuput et al., 2016). It has been demonstrated that the process of essential oil encapsulation leads to an enhancement in tensile strength, with a recorded maximum of 6.30 MPa being attained. It has been demonstrated that, by means of this technique, the primary whey-pectin matrix is capable of reconfiguring itself into a robust polymer-polymer interaction network (Akachat et al., 2025 a). The process of physical encapsulation involves the isolation of the hydrophobic oil core from the whey-pectin polymer matrix. Furthermore, the presence of these fillers within the film matrix has been shown to result in a more even distribution and improved integration into the overall polymer network (Reis and Gomes, 2023). This reduction in tensile strength is indicative of a decrease in the film's ability to withstand pressure when the glycerol content is increased. As demonstrated in Figure 2, elevated glycerol concentrations have been shown to diminish the intermolecular forces that facilitate mobility between molecular chains, consequently resulting in augmented elongation values (Akachat et al., 2025 b). It has been demonstrated that high tensile strength is obtained from lower glycerol concentrations, possibly due to the dominance of strong hydrogen bonds formed through polymer interactions that are greater than the tensile forces between polymers and glycerol (Geleta et al., 2020). This decline in tensile strength can be attributed to the weakening of intermolecular bonds and the reduction in internal cohesion. This results in an increase in the stretchability and flexibility of the film, accompanied by a decrease in its tensile strength (Butt et al., 2023).

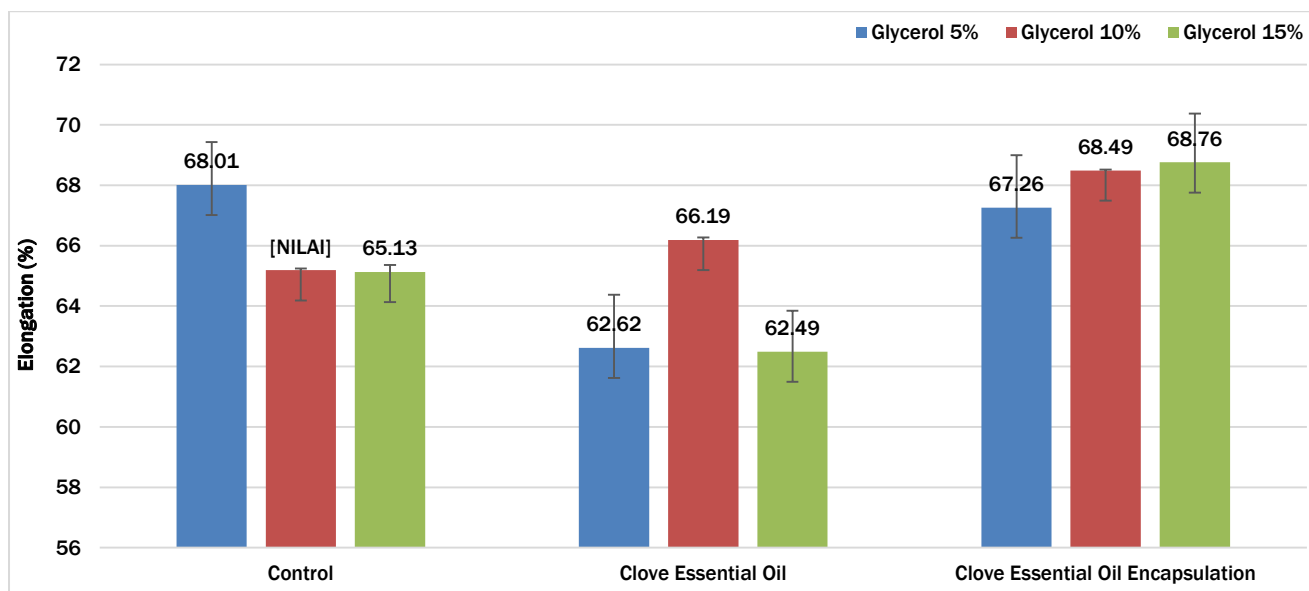


Figure 2 - Elongation (%) of whey-pectin film with the addition of lemon essential oil and different glycerol concentrations

Elongation

Film elongation is defined as the ability of a material to stretch or lengthen when subjected to tensile stress before breaking or rupturing. This property is significant in the assessment of materials' flexibility and resistance to mechanical deformation. The film elongation values resulting from the incorporation of lemon essential oil and varying glycerol concentrations are illustrated in Figure 2. The mean elongation value produced ranged from 63.76 to 68.17%. The interaction between glycerol and polymers can be influenced by essential oils, which have been shown to reduce the amount available for hydrogen bonding between glycerol and polymers, thereby facilitating more even dispersion of glycerol across multiple domains. The range of elongation values produced is indicative of a balance between the plasticizing effect of glycerol and the structural modification caused by the dispersion of lemon essential oil in the whey-pectin matrix. It has been demonstrated that specific interactions at certain concentrations optimize polymer chain mobility, resulting in films with significant stretchability (Lina et al., 2023). Lemon essential oil is hydrophobic and not fully soluble in the hydrophilic whey-pectin matrix; the oil tends to form large droplets that are not evenly dispersed in the polymer matrix. These oil droplets have the capacity to interfere with the bonds between polymer chains and to reduce the density of cross-links. This, in turn, can inhibit the uniform transfer of tension throughout the film when it is pulled. This phenomenon has been shown to result in the film's increased susceptibility to breakage, thereby reducing the overall elongation when compared to whey-pectin film (Ghadetaj et al., 2018). The utilization of encapsulated lemon essential oil has been demonstrated to result in an augmentation of the elongation value. This phenomenon can be attributed to the encapsulation process, which generates minute essential oil droplets that are uniformly dispersed throughout the polymer matrix (Mehta et al., 2022). This uniform dispersion enhances the compatibility between the hydrophobic phase (oil) and the hydrophilic phase (whey-pectin), thereby enabling the film matrix to undergo greater elastic stretching and to absorb pressure more effectively prior to rupture. This, in turn, leads to the attainment of the highest elongation value (Amjadi et al., 2021).

Glycerol, a hydrophilic polyol, functions as a plasticizer in the film matrix by reducing interactions between polymers. Glycerol has been shown to interact with whey and pectin through hydrogen bonds, thereby replacing the strong hydrogen bonds between polymer chains and increasing the distance between chains and the free volume in the matrix (Akachat et al., 2025 b). This increase in flexibility is accompanied by a corresponding decrease in rigidity, resulting in an enhancement of the elongation value, defined as the film's capacity to stretch prior to breaking (Hachity-Ortega et al., 2024). The dense whey-pectin film matrix is characterized by the presence of numerous hydrogen bonds and robust van der Waals forces between polymer chains. The presence of these bonds contributes to the film's rigidity and fragility. The incorporation of glycerol has been demonstrated to occur between the polymer chains, thereby increasing the distance between them. This has been shown to result in an increase in the free volume present within the film matrix. The polymer chains become freer to move (increased mobility) when the film is subjected to pressure (Asfaw et al., 2023; Agustín et al., 2024), due to the extra space and weaker interactions.

Antioxidant

The results of the study demonstrate that the antioxidant content of the film with the addition of lemon essential oil and different glycerol concentrations can be observed in Figure 3. This finding suggests that lemon essential oil is a

significant source of bioactive compounds, and that glycerol functions as a plasticizer capable of regulating the antioxidant activity it produces. Lemon essential oil, which contains main components such as limonene and geraniol, provides natural antioxidants (Bof et al., 2016; Song et al., 2018). In addition, glycerol affects the physical properties of the film, such as homogeneity, flexibility (Lau and Sarbon, 2022), and the distribution of active compounds in the whey-pectin polymer matrix. The control film (whey-pectin) exhibited an antioxidant content of 21.12%, while the addition of lemon essential oil to the film matrix resulted in a significant increase to 36.28%, thereby underscoring the role of bioactive compounds (limonene and geraniol) in neutralizing free radicals (Budiarto et al., 2024). Furthermore, formulations incorporating encapsulated lemon essential oil exhibited a 32.62% increase in antioxidant content. This discrepancy is presumably attributable to the controlled release mechanism of lemon essential oil in encapsulated form, which results in the active compounds being less available in the initial stages but more stable over the storage period (Himed et al., 2019). Research related to the effectiveness of using encapsulated lemon essential oil in films as a sustainable controlled release antioxidant strategy (Akachat et al., 2025 b) demonstrates that pectin-based films with encapsulated lemon essential oil not only increase antioxidant activity but can also improve the film's structural resistance to biological and water degradation. The encapsulation of essential oils represents a technological advancement that has the potential to address issues of volatility and enhance the release efficiency of antioxidant compounds (Khan et al., 2023).

As demonstrated in Figure 1, the incorporation of elevated levels of glycerol into whey-pectin films has been observed to result in a concomitant reduction in the antioxidant content. This phenomenon can be attributed, at least in part, to the indirect impact of glycerol on the rate of diffusion and release of active compounds, thereby influencing the overall antioxidant content of the films. It has been established that elevated glycerol concentrations have the capacity to diminish tensile strength and modulus. Conversely, they have been observed to augment elongation and water vapor permeability. This condition has been observed to accelerate the release of antioxidants, but it has also been demonstrated that excess glycerol can cause a rapid decline in their stability (Hachity-Ortega et al., 2024; Lau and Sarbon, 2022; Tafa et al., 2023).

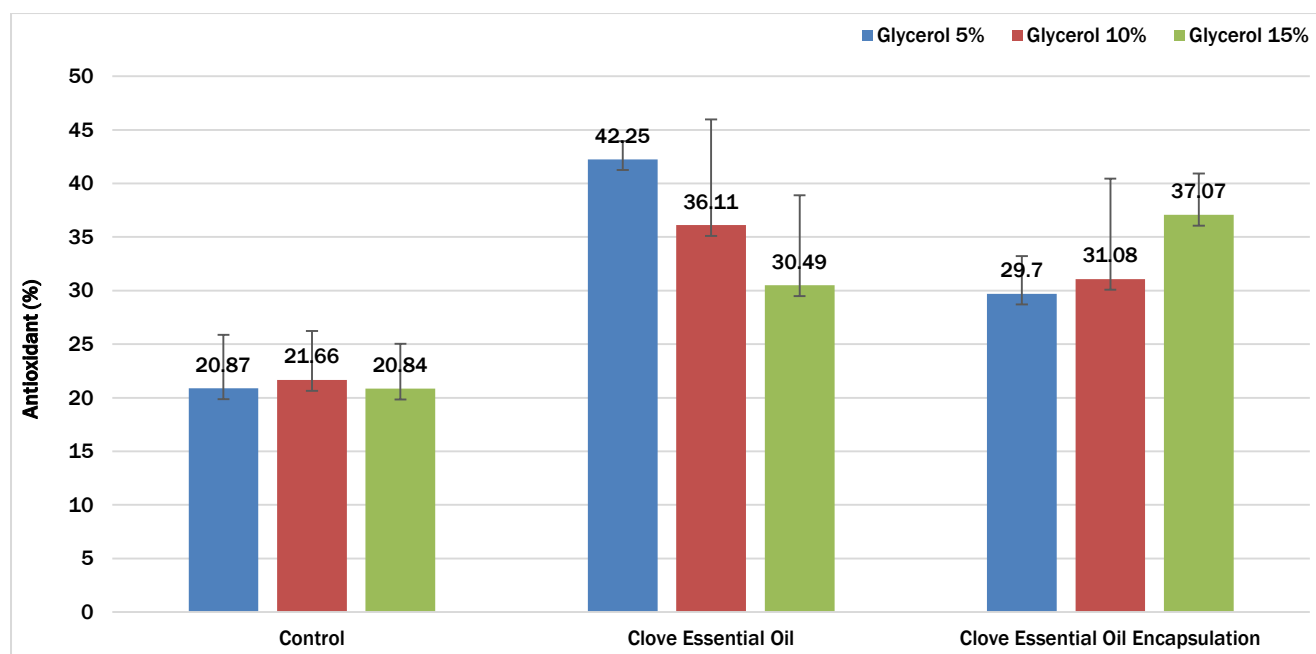


Figure 3 - Antioxidant (%) of whey-pectin film with the addition of lemon essential oil and different glycerol concentrations

Film biodegradability

The biodegradability of whey-pectin-based edible films was investigated to ascertain their capacity to undergo natural degradation through microbial activity in the environment. The findings of this investigation offer a comprehensive overview of the environmental sustainability of the film produced and its degradation rate when compared to conventional materials. The findings of the biodegradability test demonstrate that whey-pectin-based edible films exhibit favorable degradability within natural environments. The degradation process was observed through physical appearance, with the samples being exposed to weathering conditions for a period of 30 days in the soil. The biodegradability of whey-pectin edible film is demonstrated in Figure 4,







Figure 4 – (a) whey-pectin film with 5% glycerol; (b) whey-pectin film with 10% glycerol; (c) whey-pectin film with 15% glycerol; (d) whey pectin film with lemon essential oil and 5% glycerol; (e) whey pectin film with lemon essential oil and 10% glycerol; (f) whey pectin film with lemon essential oil and 15% glycerol; (g) whey pectin film with encapsulated lemon essential oil and 5% glycerol; (h) whey pectin film with encapsulated lemon essential oil and 10% glycerol; (i) whey pectin film with encapsulated lemon essential oil and 15% glycerol

The utilization of glycerol as a plasticizer in biopolymer films, including those derived from whey protein and pectin, has been demonstrated to enhance the flexibility (i.e. the elongation at break) of the resultant films. Concurrently, this increase in flexibility is accompanied by a reduction in film stiffness (Young's modulus), a phenomenon that can be attributed to the capacity of glycerol to disrupt the intermolecular bonds between polymer chains. This disruption leads to an augmentation in the openness and accessibility of the film structure to water and microorganisms. For instance, research on whey-based films indicates that the incorporation of glycerol leads to a reduction in inter-protein chain forces and an enhancement in film flexibility (Fahrullah et al., 2020; Pires et al., 2024). In a similar manner, pectin films fortified with glycerol have demonstrated that the glycerol concentration exerts an influence on the mechanical properties and permeability of the film (Akachat et al., 2025 b). It can be deduced that the increased flexibility and susceptibility to water and microorganism penetration of films with elevated glycerol levels (15%) results in a faster rate of biodegradation in comparison to films with lower glycerol concentrations (5%).

Furthermore, the incorporation of essential oils into the biopolymer film matrix exerts a dual effect: firstly, EOs can enhance the antimicrobial and antioxidant activity of the film, thereby reducing microbial contamination; however, secondly, from a structural standpoint, the addition of EOs frequently compromises the integrity of the film matrix due to the formation of droplets or dispersions within the oil phase, resulting in the creation of pores or channels. In films containing lemon essential oil, the rate of biodegradation tends to be slower in the early stages, as soil microbes require a longer time to attack the polymer structure of the film. However, over time and with increasing degradation of the encapsulation matrix, the essential oil begins to be released and its antimicrobial properties gradually decrease, allowing the biodegradation process to continue until the film is almost completely decomposed.

The process of essential oil encapsulation introduces an additional factor: namely, that the encapsulated essential oil is released in a controlled or gradual manner. This can increase the interaction of the film with environmental conditions (humidity, microbes) over a longer period of time, while also changing the structure of the film. Encapsulation can form a more heterogeneous microstructure and increase the interface area between the film, oil and environment, causing the film to degrade more quickly due to the many 'entry points' for degradation. The analysis of biopolymer films incorporating essential oils and plasticizers has revealed a correlation between the degree of porosity and the rate of degradation. It has been demonstrated that a more porous or disturbed film structure is associated with an accelerated rate of degradation. As demonstrated by Anis et al. (2021), the utilization of biopolymer-based films, incorporating a combination of plasticizers and essential oils, results in a reduction of film compactness and an escalation in the rate of mass loss (weight loss) during biodegradation tests.

From an environmental and biodegradation perspective, protein- and polysaccharide-based films offer inherent advantages due to the natural composition of their polymer chains, which are susceptible to biodegradation through microbial enzymes or through processes such as oxidation and hydration (Nikbakht et al., 2025). The combination of these polymers produces a more flexible, porous film that is open to the environment, facilitating the interaction of degradation microorganisms with the film. Consequently, visual changes show a reduced, damaged, partially lost film structure after 30 days, in line with active biodegradation expectations.

CONCLUSION

This study demonstrates that whey-pectin-based edible films incorporated with lemon essential oil and glycerol possess the potential to serve as an eco-friendly packaging solution. The incorporation of lemon essential oil has been demonstrated to enhance the antioxidant content and barrier effectiveness of the film, thereby mitigating its susceptibility to oxidation and microbial contamination. The utilization of glycerol as a plasticizer has been demonstrated to enhance the flexibility of the resultant film. The optimal treatment is a film comprising encapsulated lemon essential oil and 10% glycerol, which offers an optimal balance between tensile strength, elongation, antioxidant activity, and biodegradability. This film also exhibits good biodegradability, is more environmentally friendly than conventional plastics, and can be used for more sustainable food packaging. Future studies are recommended to evaluate the performance of this edible film under real food storage conditions using different food matrices, investigate its long-term storage stability and controlled release behavior of the encapsulated essential oil, and assess its scalability and economic feasibility for commercial food packaging applications.

DECLARATIONS

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Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contribution

FF, DK, BRDW: designed the research; FF, DK, BRDW, AN: investigation the research; FF, AN: conducted the research and analyzed data; All authors wrote the manuscript, had primary responsibility for final content and approved the final manuscript.

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Consent to Publish

The publication of this manuscript has been approved by all authors.

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Competing Interest

The authors have not declared any competing of interests.

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ASSESSMENT OF NUTRITIONAL POTENTIALS OF PALM KERNEL CAKE/RICE HUSK-BASED DIETS SUPPLEMENTED WITH FIBROLYTIC ENZYME ON PERFORMANCE, CARCASS CHARACTERISTICS AND ORGAN WEIGHT OF BROILER CHICKENS

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Supporting Information



ABSTRACT: The prohibitive costs of conventional feedstuffs have necessitated the search for other alternatives. Palm kernel cake (PKC) and rice husk (RH) are potential feed resources. PKC contains about 20% crude protein and about 50% nitrogen free extractives (NFE), whilst RH contains about 38% NFE. The use of these ingredients is limited in monogastric diets due to their high content of crude fibre, particularly non starch polysaccharides. The main objective of this study was to evaluate the nutritional potential of PKC/ RH based diets, supplemented with exogenous fibrolytic enzymes on performance and carcass/organ weight characteristics of Cobb strain broiler chickens. Six experimental diets were formulated such that diets 1 and 2 contained 0% PKC/RH, and 0% oil palm slurry (OPS), whilst diets 3 and 4 contained 25% PKC/RH, and diets 5 and 6 incorporated PKC/RH at 50%, respectively. The OPS was also included in the diets 3 – 6 at 4% level to boost their primary energy levels, whilst the feed enzyme was added to the diets 2, 4, and 6 at 1% inclusion level. A total of 108, one-day-old chickens were randomly distributed to the six dietary groups, in a 3 × 2 factorial arrangement. At the end of trial, results showed that all performance parameters in enzyme-treated factorial sets were significantly ($P < 0.05$) better than those of zero enzyme-treated groups. Similarly, all carcass/organ weight parameters, except weights of thigh/drumstick were also significantly ($P < 0.05$) better in the enzyme treated groups. The results suggest that PKC/RH based diets supplemented with feed enzymes at 1% inclusion level, could replace 50% of maize in broiler chickens' diets without any deleterious effect on performance, carcass/organ weight characteristics.

Keywords: Broiler chicken, Feed enzyme, Nutritional potentials, Palm kernel cake, Rice husk.

INTRODUCTION

Intensive broiler chicken production has the potential of mitigating the short supply of animal protein in Nigeria. This is because of the birds' fast growth, livability, and excellent ability to convert feed to meat. With good holistic management, a day-old broiler of about 40g can reach a live weight of 1.8kg to 2.5kg within 7 – 8 weeks.

Apart from beef, chicken is the most widely consumed meat product in the country. Its extensive acceptability is due largely to the complete nonexistence of traditional or religious restrictions regarding its marketing and consumption (Bashar et al., 2010). The fundamental constraint to intensive poultry farming in Nigeria is feeding cost, which constitutes over 70% of production cost (Ukorebi, 2022). Poultry feed manufacture is dependent on availability of feed ingredients, the major proportion of which are energy and protein sources. For broilers to convert feed into profitable products there must be adequate calorie intake, sufficient supply of amino acids in suitable proportion, and the provision of necessary vitamins and minerals (Ogunnusi et al., 2023; Alabi and Adedokun, 2025). In recent times, the major energy and protein sources for poultry diets formulation such as corn, soybean and groundnut are in competitive demand for human consumption and industrial uses (Erdaw et al., 2016; Hijati, 2010). Consequently, the cost of finished feed and the poultry products they support have become prohibitive. This underscores the need for animal- nutritionists to search for alternative non-conventional feed resources, which are cheap, and of little or zero demand for direct human consumption (Onyimanyi and Onukwafor, 2003).

A readily available potential feedstuff for compounding broiler chickens' diets is palm kernel cake (PKC), a by-product of palm kernel oil extraction. Research report by Ramin et al. (2010) showed that CP content of PKC varies between 10 – 19%. Onuh et al. (2012) observed that the proximate content of PKC varies considerably, which variability is traceable to: its source, method of oil removal, the proportion of endocarp remaining, and efficiency of oil extraction from the kernel (Adesehinwa, 2007). Broilers can tolerate up to 20% PKC in their diets without affecting their growth performance and feed efficiency (Anaeto et al., 2009).

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The digestion of non-starch polysaccharides (NSPs) of the cell wall of PKC in poultry is variable due to the low digestive enzymes activity and their tendency to create a viscous environment in the intestinal lumen (Yuan et al., 2017; Nguyen et al., 2021). However, it can be broken down with the help of enzymes produced by the caecal microflora or by supplementation of poultry diets with specific enzymes (Jozefiak et al., 2004; Yamsakul et al., 2025). There is a wide variation in optimum inclusion level of PKC in poultry diets. More research is needed to standardize this.

Another potential feed resource for broiler chickens production is rice husk (RH). Rice husk is a by-product of rice milling. Bassey et al. (2022) indicated that during milling of paddy, about 78% of weight is received as rice, broken rice, and bran, the rest (22% of paddy) is received as husk. The researchers pointed out that Nigeria produces around 4.3 million tons of paddy per year, giving rise to around 1.1 million tons of RH per year.

The proximate composition of RH can vary based on variety, soil conditions, fertilizer use, and processing methods. However, general ranges for key components have been established. Uzoamaka et al. (2023) reported the following percentage levels: Ash, 23.39 ± 0.19 ; Carbohydrate, 37.04 ± 0.82 ; Crude fibre, 25.74 ± 0.42 ; Crude lipid, 3.76 ± 0.42 ; Moisture, 7.93 ± 0.02 ; Crude protein, 1.85 ± 0.10 ; Energy (Kcal/100g), 189.40. There is paucity of data on RH research.

Most alternative feed ingredients proposed for compounding poultry diets contain Non-Starch Polysaccharides, NSPs which cannot be hydrolyzed by endogenous digestive enzymes of poultry. This is a major limiting factor in the utilization of NSPs in broiler chickens feed manufacture, as birds require quite a moderate amount of fibre in their diets for proper functioning of their gastro-intestinal tract. However, utilization by monogastric livestock has recently gained interest for various reasons, primarily to promote constant passage of materials through the gut, and to stimulate growth (Grieshop et al., 2001).

Addition of exogenous enzymes in poultry diets has become widely acceptable strategy to address the fibre component of diets particularly NSP viscosity, improve gut health, feed efficiency, and faecal quality, this makes it easier and a lot more beneficial to use non-conventional feedstuffs in the formulation of poultry diets (Bedford et al., 2024; Bedford and Apajalahti, 2024). Faniyi (2006) reported that enzyme supplementation of poultry diets resulted in hydrolysis of NSP into its component monosaccharides and disaccharides, which can be digested, absorbed, metabolized and utilized.

Exogenous fibrolytic enzyme (Nutrizyme) is an enzyme designed to improve the digestibility of pigs and poultry diets which are rich in fibre. It is a multi-enzyme complex which contains hemicellulase, phytase, arabinose, pectinase, amylase, β -glucanases, cellobiase, xylanase, pentosanase, and lipase. Information on enzyme treatment of chicken broiler diets PKC/RH to facilitate the liberation of their nutrients in the diets such that they will be efficiently utilized to enhance performance of birds is unavailable. This research was therefore designed to evaluate the effects of enzyme supplementation of PKC/RH based diets on performance of broiler chickens, and to provide reference data on the same.

MATERIALS AND METHODS

This study was carried out in the Animal Science unit of the Teaching and Research Farm of the Faculty of Agriculture and Forestry, Cross River University of Technology, Obubra Campus. The location lies along longitude $8^\circ - 9^\circ\text{E}$, and latitude $6^\circ - 7^\circ\text{N}$ of the Equator, with a warm weather and ambient temperature of about $21 - 30^\circ\text{C}$, and has an annual rainfall of 500 – 1070 mm (Mfam, 2002).

Animal care

This study was duly reviewed and approved by the Institutional Animal Care and use Committee of the University of Cross River State (UNICROSS8289). The animal experiment was performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory.

Sources of feed ingredients

Rice husk (RH) was collected from a rice milling plant, whilst oil palm slurry (OPS) was sourced from an oil palm mill in Ofodua, Obubra Local Government Area, all of Cross River State, Nigeria. Palm kernel cake and the commercial feed enzyme (Nutrizyme®) were purchased from a certified feed merchant in Calabar, Cross River State, and Uyo, Akwa Ibom State, respectively. All other feed ingredients were procured from Calabar.

Processing of some feed ingredients/ration formulation

PKC and RH were mixed in the ratio of 1:1, homogenized, and sun-dried for 3 – 4 days, thereafter, the percentage requirement of PKC/RH combo for each treatment diet was measured out and mixed with the quantity of OPS required for each diet. This strategy was adopted in order to use the PKC/RH as absorbent for OPS. The combo was dried again for another 3 – 5 days before other ingredients were subsequently incorporated according to the respective inclusion levels required in the diets (broiler starter and finisher diets). Apart from conditioning the feedstuffs to a safe moisture level for the final compound diets, the second sun-drying process also benefited from the sterilization properties of the ultra-violet radiation of the sun to destroy pathogenic organisms associated with oil palm slurry.

Six experimental diets were formulated such that diets 1 and 2 contained zero percent PKC/RH and OPS, while diets 3 and 4; 5 and 6 incorporated PKC/RH at 25% and 50%, respectively. OPS was also included in the test diets (diets 3 - 6) at 4% level to boost their energy levels, while the feed enzyme was added to diets 2, 4, and 6 at 1% inclusion level. A similar feed formula was used in compounding both starter and finisher diets. However, the finisher contained percentages of key ingredients as required in chicken finisher broilers diets (Tables 1 and 2).

Table 1 - Gross composition of experimental broiler starter diets (g/100g)

Treatment/Enzyme level		T ₁ ,0.00E	T ₁ ,1.00E	T ₂ ,0.00E	T ₂ ,1.00E	T ₃ ,0.00E	T ₃ ,1.00E
Ingredients							
Maize		50.00	50.00	37.50	37.50	25.00	25.00
PKC/RH		-	-	12.50	12.50	25.00	25.00
Soybean meal		36.03	36.65	37.12	37.76	36.29	36.91
Wheat offal		7.57	5.95	2.48	0.84	3.31	1.69
Fish meal		2.00	2.00	2.00	2.00	2.00	2.00
Bone meal		3.00	3.00	3.00	3.00	3.00	3.00
NaCl		0.30	0.30	0.30	0.30	0.30	0.30
L- Lysine		0.30	0.30	0.30	0.30	0.30	0.30
DL- Methionine		0.30	0.30	0.30	0.30	0.30	0.30
Vit/Tm premix*		0.50	0.50	0.50	0.50	0.50	0.50
Oil palm slurry		-	-	4.00	4.00	4.00	4.00
Nutrizyme®		-	1.00	-	1.00	-	1.00
Calculated nutrient composition							
Crude protein (%)		23.00	23.00	23.00	23.00	23.00	23.00
Crude fibre (%)		4.35	4.35	6.37	6.29	8.70	8.60
ME (kcal/kg Diet)		2888.59	2888.59	2758.77	2678.34	2479.11	2464.67

*To provide the following per kg of diet: Vit A, 10,000iu; Vit D₃, 2000iu; Vit E, 5iu; Vit K, 2mg; Riboflavin, 4.20mg; Folic acid, 0.5mg; Choline, 3mg; Mg, 56mg; Fe, 20mg; Cu, 10mg; Zn, 50mg; Co, 125mg.

Table 2 - Gross composition of experimental broiler finisher diets (g/100g)

Treatment/Enzyme level		T ₁ ,0.00E	T ₁ ,1.00E	T ₂ ,0.00E	T ₂ ,1.00E	T ₃ ,0.00E	T ₃ ,1.00E
Ingredients							
Maize		56.00	56.00	42.00	42.00	28.00	28.00
PKC/RH (1:1)		-	-	14.00	14.00	28.00	28.00
Full-fat soybean meal		31.28	32.03	32.45	32.04	31.34	32.08
Wheat offal		6.31	4.57	1.15	0.56	2.26	0.52
Fish meal		2.00	2.00	2.00	2.00	2.00	2.00
Bone meal		3.00	3.00	3.00	3.00	3.00	3.00
NaCl		0.30	0.30	0.30	0.30	0.30	0.30
L- Lysine		0.30	0.30	0.30	0.30	0.30	0.30
DL- Methionine		0.30	0.30	0.30	0.30	0.30	0.30
Vit/Tm premix*		0.50	0.50	0.50	0.50	0.50	0.50
Oil palm slurry		-	-	4.00	4.00	4.00	4.00
Nutrizyme®		-	1.00	1.00	1.00	1.00	1.00
Calculated nutrient composition							
Crude protein (%)		20.00	20.00	20.00	20.00	20.00	20.00
Crude fibre (%)		4.00	4.00	6.98	6.81	8.94	8.24
ME (kcal/kg Diet)		3130.48	3130.48	2917.89	2892.32	2670.50	2662.38

*To provide the following per kg of diet: Vit A, 10,000iu; Vit D₃, 2,000iu; Vit E, 5iu; Vit K, 2mg; Riboflavin, 4.20mg; Folic acid, 0.5mg; Choline, 3mg; Mg, 56mg; Fe, 20mg; Cu, 10mg; Zn, 50mg; Co, 125mg.

Experimental birds and design

A total of one hundred and eight (108) one day old broiler chicks of Cobb strain used for the trial were purchased from a reputable distributor in Calabar. The birds were randomly distributed into six groups of eighteen (18) birds each. In the starter phase of the experiment, the groups were randomly assigned to six dietary energy and single dietary protein rations in a 3x2 factorial arrangement involving 0, 25 and 50% inclusion of PKC/RH combo and two enzyme levels (0% and 1%). Each treatment was replicated three times with six (6) birds per replicate. Each replicate was housed in a separate pen in a Deep litter system. A similar arrangement was used in the finisher phase of the trial.

Management of experimental Birds

Brooding of birds was carried out until they were three weeks old, after which period, the brooding equipment were withdrawn feed and clean drinking water were provided ad libitum for all treatments throughout the experimental term of eight weeks. In addition, adequate prophylactic medications and vaccinations were carried out during the experiment.

Data collection

Performance data

All birds were weighed at the beginning of the trial, and subsequently on weekly bases. Daily feed intake was calculated as the difference between weight of feed offered from the beginning of a day, and the left over at the beginning of a new day. In all, data collected included average initial live weights, weekly average live weight and live weight gain, average daily feed intake, total average live weight and live weight gain, feed-to-gain ratio and mortality (Table 3).

Carcass/organ weight evaluation data

At the end of the trial, six birds per treatment were picked for carcass/organ weight analyses. These included two birds per replicate (one with the highest live weight, and another with the lowest). They were starved for twelve hours, of feed only, whilst being allowed access to clean drinking water free choice, this was to decongest their alimentary system. Thereafter, the birds were weighed and slaughtered by cervical bone dislocation, after which their jugular veins were severed, and the carcasses thoroughly bled. Before subsequent defeathering, the carcasses were scaled in hot water of about 80°C for about one minute. Defeathering of the carcasses was followed by their evisceration by cutting through the vents, and the viscera removed. The dressed carcasses were thereafter weighed. Major parts of the dressed carcasses including the breasts, thighs/drumsticks were sectioned out and accurately weighed using a sensitive electronic scale, and the weights expressed as percentages of respective live weights of birds used. Also, weights of internal organs including crop, proventriculus/gizzard, liver and pancreas were taken. These and the dressed carcass weights were similarly expressed as percentages of their respective live weights (Table 4).

Statistical analyses

All experimental data were subjected to a two-way analysis of variance as described by [Snedecor and Cochran \(1978\)](#). Where F-tests indicated significant treatment mean effects, the means were separated using the new Duncan's multiple range test as outlined [Steel and Torrie \(1990\)](#).

RESULTS

Performance

Performance characteristics of broiler chickens fed PKC/RH diets supplemented with exogenous enzyme (Nutrizyme) is shown on Table 3. Average body weight gain obtained for T₁, T₂, and T₃ factorial sets not supplemented with Nutrizyme® ranged from 1801.89g to 1941.11g whilst the treatment sets with treatment diets supplemented with 1% exogenous enzyme recorded 1955.55g to 2031.22g. There were significant ($P < 0.05$) treatment effects. Average daily weight gain data for the factorial set with zero enzyme supplementation ranged from 32.18g to 34.66g, whereas the set treated with Nutrizyme ranged from 34.92g to 36.27g. The results were significantly ($P < 0.05$) different among the treatment means. Average daily feed intake for zero enzyme factorial set were 82.18g (T₁), 93.81g (T₂), and 104.01g (T₃), and those of the enzyme treated counterpart were 71.89g, 76.37g, and 79.63g, respectively for T₁, T₂, and T₃; the data showed significant ($P < 0.05$) treatment effects. Feed-to-gain ratios recorded for zero enzyme factorial set ranged from 2.37 to 3.32 whilst the set supplemented with the feed enzyme ranged from 1.98 to 2.28; there were significant ($P < 0.05$) treatment effects.

Carcass and organ weights analysis

These are summarized in Table 4. Apart from weight of thigh/drumstick, all other carcass and organ weight parameters including live weights, dressed weights, dressing percentages, weights of breasts, crop, proventriculus/gizzard, weights liver, and pancreas showed significant ($P < 0.05$) differences among treatment means.

Table 3 - Effects of dietary levels of PKC/RH supplemented with feed enzyme on broiler chickens performance

Parameters	Treatment/Enzyme level						SEM	P. Value
	T ₁ , 0.00E	T ₁ ,1.00E	T ₂ , 0.00E	T ₂ 1.00E	T ₃ , 0.00E	T ₃ , 1.00		
Average initial weight (g)	38.89	38.89	38.89	38.89	38.89	38.89	0.00	P > 0.05
Average final weight (g)	1980.00 ^b	2070.11 ^a	1962.78 ^c	2065.83 ^a	1840.78 ^d	1994.44 ^b	61.59	P < 0.05
Average weight gain (g)	1941.11 ^b	2031.22 ^a	1923.89 ^{bc}	2026.94 ^a	1801.89 ^d	1955.55 ^b	61.59	P < 0.05
Average daily wt. gain (g)	34.66 ^b	36.27 ^a	34.36 ^{bc}	36.20 ^a	32.18 ^c	34.92 ^b	1.10	P < 0.05
Average feed intake (g)	4602.05 ^c	4025.59 ^f	5253.43 ^b	4276.47 ^e	5824.76 ^a	4459.49 ^d	197.91	P < 0.05
Average daily feed intake (g)	82.18 ^c	71.89 ^{cd}	93.81 ^b	76.37 ^c	104.01 ^a	79.63 ^c	3.36	P < 0.05
Feed conversion ratio	2.37 ^b	1.98 ^c	2.73 ^b	2.11 ^c	3.23 ^a	2.28 ^b	0.11	P < 0.05

^{a b c...} Means on the same row, with different superscripts are significantly different (P < 0.05).

Table 4 - Effects of dietary levels of PKC/RH supplemented with feed enzyme on carcass and organ weight characteristics of broiler chickens

Parameters	Treatment/Enzyme level						SEM	P. Value
	T ₁ , 0.00E	T ₁ ,1.00E	T ₂ , 0.00E	T ₂ 1.00E	T ₃ , 0.00E	T ₃ , 1.00		
Live weight (g)	2100.00 ^c	2466.67 ^a	2066.67 ^d	2266.67 ^b	2050.00 ^d	2166.67 ^c	25.78	P < 0.05
Dressed weight (g)	1450.00 ^c	1750.00 ^a	1283.33 ^d	1683.33 ^b	1150.00 ^e	1500.00 ^c	18.23	P < 0.05
Dressing %	69.05 ^{bc}	70.95 ^b	62.10 ^c	74.26 ^a	56.10 ^d	69.23 ^b	1.87	P < 0.05
Weight of thigh/drumstick (% Of live weight)	24.01	24.41	23.40	24.72	23.28	24.10	0.89	P > 0.05
Weight of breast (% of live weight)	28.62 ^b	30.82 ^a	26.10 ^c	28.95 ^b	26.01 ^c	28.21 ^b	0.95	P < 0.05
Weight of crop (% of live weight)	0.59 ^b	0.44 ^c	0.70 ^a	0.46 ^c	0.74 ^a	0.48 ^c	0.07	P < 0.05
Weight of proventriculus/gizzard (% of live weight)	2.79 ^b	2.78 ^b	4.30 ^a	2.79 ^b	4.33 ^a	2.81 ^b	0.09	P < 0.05
Weight of pancreas (% of live weight)	0.26 ^b	0.15 ^c	0.33 ^a	0.25 ^b	0.34 ^a	0.26 ^b	0.01	P < 0.05
Weight of liver (% of live weight)	1.84 ^b	1.74 ^c	2.94 ^a	1.76 ^c	2.97 ^a	1.78 ^c	0.02	P < 0.05

^{a b c...} Means on the same row, with different superscripts are significantly different (P < 0.05).

DISCUSSION

Performance characteristics

There were no significant differences ($P > 0.05$) across the treatment groups for average initial body weights. Experimental birds were homogenous in age, and average body weights, a fundamental requirement for the application of the Completely Randomized Design (CRD) in Animal experimentation

Birds in treatment groups whose diets were supplemented with Nutrizyme recorded the highest final body weights, whilst the least body weight gain was in a treatment group without enzyme supplementation (T_3 , 0%E). It was observed that average body weight gain, and average daily body weight gain followed a similar trend as observed in average final body weight gain. In general, PKC/RH treatment groups supplemented with Nutrizyme performed better with respect to the performance parameters so far discussed. It is suggested that this trend is traceable to the complementary role played by the exogenous enzyme in facilitating digestion, absorption and metabolism of NSPs in the test diets. This observation is corroborated by research reports (Douglas et al; 2000; Enberg et al; 2004), which stated that dietary inclusion of exogenous enzymes could compensate for the deficiencies of endogenous enzymes to speed up their catalytic and hydrolytic activities for increasing digestion of feed.

Sogbesan et al. (2006) posited that ability of an organism to utilize nutrients will positively influence its growth performance; also, the work of Ukorebi (2022) has shown that supplementation of feeds reduce digesta viscosity, enhance digestion of and absorption of nutrients especially fats and protein, improve metabolizable energy value of the diet, as well as alter microbial population in the tract, all of which are believed to enhance weight gain. This is in agreement with the reports of Alabi et al (2014): the researchers pointed out that addition of enzymes to address NSP viscosity can improve gut health, feed efficiency, improve fecal quality, and facilitate the use of low cost ingredients; they maintained that the benefits of using livestock feed enzymes is to increase availability of starches and proteins which are enclosed within the fibre-rich cell wall and to increase availability of phosphorous from phytate.

Average daily feed intake was significantly ($P < 0.05$) higher in the factorial set with zero enzyme dietary supplementation (T_1 0% E, T_2 0% E, and T_3 0% E). This is attributable to the lower metabolizable contents of diets of these treatments, as birds would normally continue in feed intake until their energy requirements are met.

Feed-to gain ratio was significantly ($P < 0.05$) low in T_1 , 1%E (1.98), followed by T_2 , 1%E (2.11), whilst in contrast, it was significantly ($P < 0.05$) higher in T_3 , 0%E (3.23). This trend further indicates better performance of birds placed on enzyme supplemented diets. Feed conversion ratio (FCR) of 2.20 is considered good in chicken broiler production; feed conversion ratios greater than 2.50 would suggest either feed wastage, low dietary energy, or poor-quality feed, among others.

In general, the relatively lower performance characteristics of birds in factorial sets without feed enzyme supplementation suggests that endogenous enzymes of the birds were incapable of hydrolyzing the dietary NSPs to liberate the nutrients they contain for the birds. Research reports (Kanengoni et al., 2002; Partenen et al., 2007) have shown that as the level of fibre in monogastric diets increase, digestibility of energy and Nitrogen decrease. In broilers, endogenous enzymes cannot adequately digest NSPs, therefore ingestion of high levels of soluble leads to digesta viscosity, low nutrient digestibility and absorption (Hijati, 2010; El-Wahab et al., 2024). Agreeably however, one may not also rule out the potential of certain anti-nutritional factors in NSPs to negatively impact performance of bird receiving diets containing them. Atasie and Akinhanmi (2009) reported that Palm kernel Meal (PKM) contain anti-nutritional properties such as β -mannan, galactomannan, Xylan, and arabino-Xylan which limit its inclusion in poultry diets. The aforementioned inhibitors can be dismantled by Nutrizyme the multi-enzyme complex of which contains hemicellulase, phytase, arabinases, pectinases, amylases, β -glucanases, cellobiase, Xylanase, pentosanase, lipase, among others.

Carcass and Organ weight characteristics

All carcass and organ weight characteristics excepting weights of thigh/drumstick were significantly ($P < 0.05$) affected by treatments. Live weights were highest in enzyme supplemented treatments (T_1 , 1%E; T_2 , 1%E; T_3 , 1%E), followed by T_1 , 0%E (conventional diet with zero enzyme inclusion). The dressed weights, dressing percentage and breast weights followed a trend similar to live weights. From the results obtained, it would seem that enzyme supplementation facilitated the breakdown of NSPs and anti-nutritional factors contained in them, thus making the feed safe and richer in nutrients than the factorial set without enzyme supplementation, thus making way for the desirable carcass/organ weight characteristics observed.

The weights of proventriculus and pancreas were highest in factorial sets with zero percent enzyme supplementation and lower in treatment groups where diets were supplemented with exogenous enzyme. This might have been consequent on the challenge on these organs to increase their activities in order to acclimate themselves to the bulky feeds they received. It is also suggested that: the crop increased in weight as a result of the need to increase its storage capacity cope with increased storage of the feed; increase in the weight of the proventriculus could be traceable to its increased activity to produce more enzymes to deal with the bulky feed; similarly, the increased grinding activity of the gizzard needed to attack the bulky feed led to increased muscular development of the organ. The challenge posed by intact PKC/RH based diets could negatively impact the pancreas due to its dual role as an exocrine gland and an endocrine gland. As an exocrine gland, it produces pancreatic juices containing amylase, lipase and protease to hydrolyze

carbohydrates, lipids and proteins; as an endocrine gland, the pancreas secretes hormones like insulin and glucagon that function in the regulation of glucose. With enzyme supplementation of PKC/RH based diets, the requirements for the digestion of NSPs are supplied thus reducing the workload on the pancreas. On the other hand, the structural increase in the size of the pancreas can be due to the 'struggle' to meet the enzyme requirement for dismantling the NSPs.

The significantly ($P < 0.05$) increased weights of livers in test diets with zero enzyme supplementation can also be associated with increased demand on it for its total "performance". The liver performs numerous functions, some of which include producing bile for digestion of lipids, metabolism of nutrients, filtering toxins, making essential proteins like clotting factors, and albumen, storing glucose and glycogen, fighting infections, and removing bacteria, detoxifying harmful and safely removing them, regulating blood chemistry, among others. To perform all its statutory functions coupled with antinutritional inadvertently present in dietary NSPs, as inhibitors in the diets, the liver has to increase its activities to match the demands on it, hence the increase in its size. The detailed interactions between the inclusion levels of PKC/RH and enzyme supplementation are shown in Tables 3 and 4.

CONCLUSION

The experiment showed that PKC/RH diets supplemented with exogenous fibrolytic enzyme (Nutrizyme) can replace up to 50% of maize in the diets of broiler chickens, both at starter and finisher phases. The diets were found to sufficiently support production without any deleterious effect on performance and carcass characteristics. The test diets not supplemented with the feed enzyme adversely affected weight gain, feed conversion ratio, and internal organs (crop, proventriculus/gizzard, pancreas and liver) of the birds. This has demonstrated that the use of commercial feed enzymes is beneficial in the utilization of high NSPs in chicken diets. In practical diets of broiler chickens therefore, Farmers could replace 50% of maize with PKC/RH (1:1 ratio), when the feed is supplemented with 1% Nutrizyme supplement (a kind of fibrolytic enzyme).

DECLARATIONS

Corresponding author

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Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethical approval

This study was duly reviewed and approved by the Institutional Animal Care and use Committee of the University of Cross River State (UNICROSS8289). The animal experiment was performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals; and the ARRIVE (Anima Research: Report of *In vivo* Experiments) guidelines.

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The author declares that no funds/grants were received during the research and for the preparation and publication of this manuscript.

Consent to publish

The author gives full consent for the publication of this manuscript by Online Journal of Animal and feed research.

Competing Interests

Author declares that there are no competing interests.

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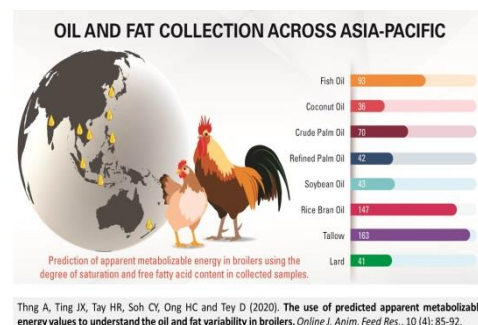
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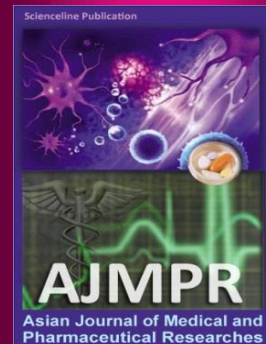
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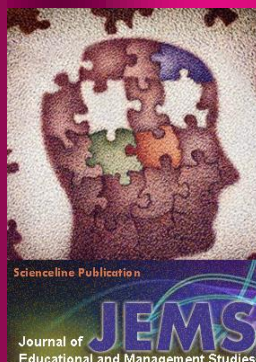
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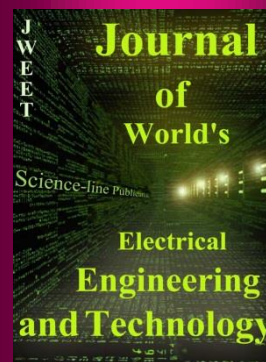
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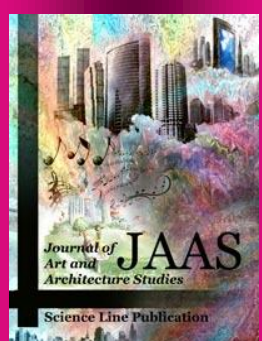
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