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Camelid farming, production, reproduction, health, and welfare

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Camelid farming, production, reproduction, health, and welfare

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Editorial: Camelid farming, production, reproduction, health, and welfare

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Editorial on the Research Topic
Camelid farming, production, reproduction, health, and welfare

The Food and Agriculture Organization (FAO) year 2024 was dedicated to camelids (1), recognizing their growing role both as livestock and companion animals. To celebrate with FAO these species, this Research Topic entitled "*Camelid farming, production, reproduction, health, and welfare*" encompasses a broad spectrum of scientific disciplines, from milk production and reproduction to health and genetics. This multidisciplinary approach underscores the imperative of the "One Welfare" approach—recognizing the interconnected wellbeing of animals, humans, and the environment (2)—and highlights the necessity to include camels within this paradigm, a species that has been historically overlooked in scientific research. Once known as the "ships of the desert," camelids are now recognized as the "livestock of the future" due to their multipurpose role (3). This evolving livestock and cultural paradigm emerges amid a growing global camelid population, presenting both opportunities and challenges in breeding strategies, food security, and animal welfare.

It is therefore fitting that this Research Topic introduces the first comprehensive protocol for assessing camel welfare under nomadic pastoral conditions (Padalino and Menchetti), alongside presenting the results of its initial application in Pakistan (Padalino et al.). The welfare assessment protocol adopts the established framework used in the Welfare Quality® and European Animal Welfare Indicators (AWIN) projects for evaluating the welfare of other livestock species, and it builds upon the pioneering work developed for camels reared under intensive systems (4). The protocol incorporates the "Four Principles" of animal welfare (i.e., Good Feeding, Good Housing, Good Health, and Appropriate Behavior) along with multi-level assessment, a standardized scoring system, and farm classification. However, substantial adaptations to the conventional structure of welfare assessment protocols were required to account for the specific characteristics of camelids and their management systems. The traditional herd-level assessment was combined with a comprehensive interview with the caretaker, and additional management- and animal-based indicators were introduced to address context-specific challenges. Padalino et al. applied this protocol to 510 camels managed by nomadic pastoralists

in Pakistan, revealing that extensively managed animals achieved higher overall welfare scores than their intensively farmed counterparts, principally due to enhanced freedom of movement and richer social interactions (reflecting high scores in "Appropriate Behavior"). Nonetheless, critical welfare risks persisted even in pastoral systems, including seasonal variability in food availability, predators, and lack of shade and shelter. Moreover, access to adequate veterinary care was a significant concern, especially in remote regions. Regardless of the specific outcomes, the implementation of the protocol in Pakistan has demonstrated its value as an effective tool for identifying key animal welfare concerns. However, more data is needed to understand the welfare level of dromedaries kept for different purposes in other parts of the world.

Alongside the resilience of extensive farming systems, this Research Topic also highlights significant shifts in camel breeding practices, particularly with regard to milk production. In our Research Topic, the interest in camel milk emerges as multidimensional, with potential spanning its health benefits, the modernization of nutrition and milking practices, and advances in genetic selection. Its therapeutic potential is widely recognized by local communities, but scientific evidence is needed to substantiate these claims. In this Research Topic, Behrouz et al. examined the effects of camel milk on various indicators of inflammation and oxidative stress in a model of cigarette smoke -induced chronic obstructive pulmonary disease. Treatment with camel milk led to a reduction in total and differential white blood cell counts, serum levels of TNF-α, and malondialdehyde concentrations in both serum and various tissues of the animal models. At the same time, it increased the levels of antioxidant enzymes and thiol compounds. The evidence thus indicates that camel milk exerts anti-inflammatory effects, by modulating the immune response, and mitigates oxidative stress, by enhancing antioxidant defenses and protecting cells from free radical-induced damage. The same authors also propose avenues for refining both the experimental model and future lines of investigation. Nonetheless, their study reinforces the idea that scientific research can substantiate the reputation of camel milk as the "white desert gold."

With an emphasis on both nutritional interventions and milking technologies, the papers of Faraz et al. and Atigui et al. published under this Research Topic provide crucial insights into the optimization of camel milk output. Faraz et al. carried out a 45-day pilot study to examine the effects of a postbiotic derived from Saccharomyces yeast supplementation on milk yield and composition of camels raised in a semi-intensive manner. As compared to other groups, the group with higher supplementation had the highest milk yield and fat content, indicating a significant improvement in milk yield, especially in camels receiving greater dosages of postbiotics. Atigui et al. approach camel milk production from an alternative perspective, focusing on machine milking. While promising for improving farm profitability, the implementation of this technique requires specific adaptations to conventional technologies due to the camel's unique physiology and behavior. By figuring out the vacuum level required to open the teat sphincter (VLOTS) and recording the anatomical reactions of the teats during machine milking, Atigui et al. investigated the mechanical components of camel milking concurrently. Only 42% of teats opened below 70 kPa, indicating significant intra- and inter-animal heterogeneity in teat responsiveness. The publications collectively highlight the significance of combined approaches—nutritional improvement and customized mechanical procedures—to raise dairy camel milk yield and animal welfare.

As expected, several studies of this Research Topic focused on biotechnology to enhance camel reproduction, one of the fields most studied in camel science (5). In particular, El-Sokary et al. compared the efficiency of vitrification protocols for camel oviductal isthmus aggregates, focusing on the effects of aggregate size, cryoprotectants, cryodevices, post-thaw viability, and sperm-binding capacity. The research team completed five different experiments to achieve optimal preservation outcomes, and cryopreserving oviduct cell aggregated up to 150 μm in diameter using a 7 M cryoprotectant concentration, and 0.25 mL straw cryodevices were recommended. The second study of Mahdy and Nasr Eldeen presents for the first time a case of a completely divided female genital tract in a she-camel (i.e., uterus didelphys). This article presents a summary of the literature in other species, and a unique and rare pictures of uterous didelphys in a she-camels. It is worth noting that apparently the genital tract seems normal, until the persistence of the median walls of the Müllerian ducts along their entire length is detected. The wall results in two cervices and two separate uterine bodies. As this pathology is congenital, it may impair fertility. More studies on selective breeding should be performed, considering the advances made in the genetics of this species.

Three studies in this Research Topic explore innovative genetic strategies to improve camel breeding, with a focus on adaptation, lactation performance, and locomotion. A comprehensive genomic analysis of Awarik dromedaries from southwestern Saudi Arabia uncovered 66 and 53 candidate selection regions through iHS and nSL scans, encompassing a total of 308 genes (Almathen). Selection signals were particularly strong on chromosomes 15 and 16, with a dense cluster on chromosome 15 overlapping the TRNAI-AAU gene. Additional hotspots were identified on chromosomes 3, 2, 7, and 14, as well as large regions on chromosomes 11 (200 kb) and 9 (325 kb). Functional annotation of highlighted genes—such as BAG5, septin 7, SLC13A1, PCED1B, BMPR1B, ZAR1, JAKMIP2, and NOTCH2—points to diverse biological roles including olfaction, immune regulation, insulin secretion, reproduction, and cellular signaling. These findings not only deepen our understanding of adaptive mechanisms in desert environments, but also underscore the genetic value of locally adapted populations for resilient breeding and conservation under climate pressure. Complementing these genomic insights, a second study introduced the CamelBell No. 1 SNP array, the first functional 1K liquid SNP chip developed specifically for lactation performance in Bactrian camels (Guo et al.). Using RNA-seq data from 125 lactating females, the researchers selected 1,002 informative loci and validated the chip in 24 individuals, achieving >99% SNP call rates and 100% genotyping consistency. The chip was further tested on 398 camels from six breeding areas in Northwest China, confirming a shared genetic basis for milk-related traits. This

tool provides a robust technical foundation to accelerate marker-assisted selection and represents a scalable platform for modern dairy camel breeding. Addressing an uncharted domain, a third study (Pastrana et al.) applied curve-estimation regression and canonical discriminant analysis to link form and function in camel locomotion. The cubic model was identified as the most suitable mathematical function to represent camel locomotion. Angularity and mechanical forces at distal limbs, pelvis inclination, hump-to-body proportionalities, post-neutering effects, and the velocity–acceleration profiles of the scapula, shoulder, carpus, hip, and foot emerged as decisive predictors of gait proficiency. These traits govern weight absorption, elastic energy storage, and propulsion efficiency, while generic animal or environmental variables proved negligible—likely buffered by innate desert-adapted features such as pacing gait and broad foot pads. The resulting criteria inform selective breeding for leisure and racing performance and even open avenues for camel-assisted therapy. Taken together, these three studies underscore a growing paradigm shift: from descriptive to predictive camel breeding. Whether through genotyping tools or phenotype assessment, these methods offer pathways to improve camel productivity, functionality, and welfare while preserving adaptive genetic traits critical for sustainability in desert ecosystems.

At least but not last, the Research Topic includes novel articles addressing health issues. One notable contribution is the comprehensive investigation of subclinical mastitis in dairy camels by Jama et al. in Ethiopia. Mastitis is a well-known problem in dairy cattle, but its dynamics in camels have been less studied. In this survey of 244 lactating camels, the authors found a 10.6% prevalence of subclinical mastitis, detected by California Mastitis Test. This data suggests that about 1 in 10 she-camels in dairy herds may carry hidden udder infections. The dominant pathogen isolated was *Staphylococcus aureus*, followed by *Streptococcus agalactiae* and *S. dysgalactiae*. Worryingly, antibiotic sensitivity testing revealed that many isolates of these bacteria had limited susceptibility to common drugs—for instance, only 44.7% were susceptible to oxytetracycline and 36.7% to tetracycline. Such findings hint at emerging antimicrobial resistance in camel mastitis pathogens, likely exacerbated by imprudent antibiotic use. Indeed, interviews with herders revealed widespread use of traditional remedies or over-the-counter drugs without veterinary guidance. The study also pointed to basic hygiene issues in these semi-intensive farms: two-thirds of owners allowed calves and other livestock to commingle and did not practice proper udder hygiene during milking. The implication is clear: improving camel health requires not just treating infections, but raising awareness and management standards among camel owners. The authors conclude that combating camel mastitis will necessitate alternative therapies (given drug resistance), thorough herder training, and better farm hygiene practices. Parasitic diseases are another concern addressed in the Research Topic. An emerging parasitology study by Al-Shaebi et al. documented the presence of *Eimeria rajasthani*—a coccidian parasite—in dromedary camels in Saudi Arabia. Through morphological and molecular analysis, the researchers confirmed this specific *Eimeria* species, which historically was reported in Indian camels, now in Arabian herds. Coccidiosis in camels is often under-recognized, but as the authors note, it can cause severe diarrhea and weight loss, especially in young animals; adults tend to develop immunity from prior exposure. The identification of *E. rajasthani* in a new region underscores the need for vigilance in camel health management. It suggests that parasite ranges may be shifting or simply that diagnostic efforts are catching up. With improved diagnostic tools, veterinarians can better monitor and control such parasitic infections (e.g., via strategic deworming or coccidiostats in young camels), ultimately reducing morbidity in camel calves.

The papers included in this Research Topic highlight the growing multidisciplinary interest in the dromedary camel, pointing on the interconnection among camel productivity, health, and welfare, as has already happened in other livestock species. However, the unique physiological and ethological characteristics of camelids—along with their distinct production contexts—necessitate significant adaptations, both in scientific methodology and in practical applications. For this reason, our compilation should be seen not as a conclusion, but rather as a starting point for a line of research that holds considerable potential for future scientific and cultural advancement.

Author contributions

AF: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CI: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. BP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Determination of breeding criteria for gait proficiency in leisure riding and racing dromedary camels: a stepwise multivariate analysis of factors predicting overall biomechanical performance

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To date, the biomechanical dynamics in camelids have not been addressed, although it might be a factor that can affect selection and breeding in this species. Therefore, the aim of this article is to conduct curve fitting and discriminant canonical analysis to identify the mathematical function that best captures the dynamics of camel locomotion and to study the impact of kinematic, morphometric, physiological, and phaneroptic variables on gait performance in leisure riding and racing activities in dromedaries, respectively. The cubic function emerged as the most suitable mathematical model to represent the locomotive behavior of camels. Various factors were found to play a pivotal role in the athletic performance of leisure riding and racing dromedary camels. Concretely, angular measurements at the distal fore and rear extremity areas, pelvis inclination, relative volume of the hump, impact forces of the front limbs, post-neutering effects, and the kinematic behavior of the scapula, shoulder, carpus, hip, and foot are the factors that greatly impact gait performance in leisure riding and racing camels. The biomechanical performance at these specific body regions has a profound impact on weight absorption and minimization of mechanic impact during camel locomotion, static/dynamic balance, force distribution, energy of propulsion, movement direction and amplitude, and storage of elastic strain in leisure riding and racing dromedaries. In contrast, other animal- and environment-dependent factors do not exert significant influence on camel gait performance, which can be attributed to species-specific, inherited adaptations developed in response to desert conditions, including the pacing gait, broad foot pads, and energy-efficient movements. The outcomes of our functional data analysis can provide valuable insights for making informed breeding decisions aimed at enhancing animal functional performance in camel riding and racing activities. Furthermore, these findings can open avenues for exploring alternative applications, such as camel-assisted therapy.

KEYWORDS

gait performance, quantitative genetics, curve estimation regression models, discriminant analysis, breeding criteria, dromedary camel

1 Introduction

Although dromedary camels (*Camelus dromedarius*) have been present in the Iberian peninsula since the Roman period (1), the Canarian archipelago is the only Spanish national territory where history, culture, and ecology of these animals have been strongly rooted for more than 600 years (2). The oldest records of the presence of dromedaries in the archipelago coincide with the historical chronicles relating to the process of European colonization of the eastern islands (3). Due to its anatomical-physiological characteristics and its outstanding performance in fatigued draft works in the arid territories of the islands (4, 5), the camel has managed to perpetuate itself in them until the present.

For centuries, the possession of a camel among local farmers was considered a symbol of status and social prosperity as well as a reinforcement of the family livelihood. Unfortunately, the mechanization of agriculture from the last third of the 20th century led to the progressive substitution of camels in rural labor and the migration of entrepreneurs to the tourism sector. Thus, the initiation of the activity of the National Paradors in the late 1950s and the emergence of tourism as an economic activity in the archipelago contributed to the regression of the camel census in rural areas and their functional reorientation. That is, the main productive niche of the camel in the Canary Islands has since become tourist leisure, in which both animal behavior and physical conformation/performance have a notable impact (6).

Considering the census of active breeders, the risk status assigned to the breed was endangered, a condition still in force today (Order AAA/251/2012; Spanish Ministry of Agriculture, Food and Environment). The lack of knowledge about the productive potentials and the underutilization of local animal genetic resources at risk of extinction places them in a scenario of special vulnerability. Although the potential of this camel breed for milk production purposes is preliminary evaluated, camelback leisure riding continues to be the most profitable activity for this camel breed, which has also expanded its geographical distribution to other European countries during the last three decades in an attempt to mitigate the risk of extinction under the potential occurrence of local stochastic phenomena. In this context, the investigation and promotion of the current and future potential of these resources to ensure the sustenance of human beings and the balance of the local environment will help promote existing productive niches and define new ones.

The observable traits expressed by an organism are mainly governed by the inter-locus interaction (epistasis) of alleles for multiple single genes (7). In livestock scenarios, the magnitude and impact of such complex genetic interactions are driven to a great extent both by historical and current uses of animal species and the design and purposes of breeding programs. Indeed, epistatic genes are agreed to be a major feature in the genetic architecture of evolutionarily transcendent phenotypic variation (8). For the particular case of the relationships between physical attributes and gait performance in domestic animal species involved in moderately energy-demanding sports activities, scarce empirical information does exist. As such, selective breeding schemes may fail to reach genetic improvement and interfere with the adaptive natural history of the animals (9).

In the particular case of the Canarian camel, the present study pursues the biomechanical characterization of the breed by means of 2D video captures. Previous related research has performed basic analyses of the biomechanics of camel gaits (10–12) and the elastic extension of tendons (13), identified some environmental factors (e.g., sex and age) affecting the racing performance (14), and studied the morphology of some parts of the distal skeleton (10, 15–17) and the pedal anatomy (18). However, no reference literature reports the exercise- and animal morphometrics and phaneroptic-related factors that are potentially responsible for oscillations in the functional performance of camel locomotion to any degree of accuracy. Thus, the current research primarily aims to perform individual curve estimations through regression analysis to identify the model that best fits camel biomechanics when performing leisure riding or racing activities, for which camel pace is the most prevalent gait (19). Afterward, such curve fitting or functional data analysis approach will solve the need for stakeholders and breeders to identify a model to describe the dynamics of camel locomotion and detect the location and magnitude of differences in gait performance within the evaluated function (20). Following the theoretical body by Saastamoinen and Barrey (21) on the genetics of horse locomotion, this study also hypothesizes that the locomotor performance in dromedary camels is the overall result of the combination of conformational, physiological, and behavioral heritable traits. Indeed, the selection supported by these characters could overcome the generally low heritability of performance traits (22). Hence, after identifying the mathematical model that best describes camel locomotion, this study pursues the identification of the kinematics, animal morphometrics, physiological, and phaneroptic-related variables that significantly impact the gait proficiency of dromedaries in leisure riding and racing activities. The results obtained will aid in refining animal selection strategies for athletic performance in leisure riding and racing activities, as well as in the exploration of different alternatives in which to venture (i.e., assisted therapy) and with which to reinforce the tasks of functional valorization for its sustainable conservation. The methodology proposed is also expected to be translatable in a comparative manner to other camel populations involved in athletic and draft activities, for which these activities constitute the most profitable niches.

2 Materials and methods

2.1 Animal sample and data purge

A total of 130 Canarian dromedaries (72 male and 58 female camels; aged between 18 months and 35 years) were included in this study. These camels were located in three representative breeding locations within Spain: Huelva (Doñana National Park, coordinates

36.972330, −6.427498), Almería (coordinates 36.902180, −2.429520), and Fuerteventura (coordinates 28.186777, −14.158361). Gait evaluation was performed on all the animals, but data collection and extraction were only feasible in those animals that, first, were able to develop any of the three specific gaits performed by dromedaries as described in the literature, namely, walk, pace, and gallop; and second, from which two complete stride cycles could be collected and extracted. Animals that did not fulfill the aforementioned requirements were discarded from the database for further analysis. All animals were deemed healthy after clinical examination by a trained practitioner. No signs of pain or thoracolumbar vertebral alterations were observed.

As a result of this purging process, the number of animals producing data to be considered in statistical evaluations is as follows: 82 animals for walk, 97 for pace, and 10 for gallop. There were some animals ($n = 60$) that could be evaluated for more than one type of gait. The reduced number of animals that engaged in galloping patterns may be ascribed to the fact that, as described in the literature (18, 23, 24), these animals are inner energetic savers and hence rarely engage in faster patterns of movements except for very concrete situations in which their life may be compromised or threatened, or if trained to do it so as it happens in trained, racing dromedaries. In fact, according to Tharwat et al. (19), although at the beginning of the race most camels gallop, they frequently switch between pacing and galloping throughout the competition. It is intriguing to note that camels can maintain a pace that is nearly as swift as their gallop.

2.2 Biometric variables and force mathematical determination

The development of gaits has been reported to considerably depend not only on the biometry of dromedaries but also on the relative forces that the animals are able to develop. For these reasons, the following variables were considered in the present study.

First, live weight was calculated using the formula by Boujenane (25). Afterward, pull force (maximum and minimum), load force (maximum and minimum), and maximum and minimum power were calculated following the methods described by Delgado et al. (26).

Smoothened movement parameters are adjusted to trigonometric functions based on specific anatomical regions in both the forelimbs and hindlimbs. Given the implication of angles shared between joints, eight angles were considered: angle 1 = cranial angle of the scapula-midway between acromion and head of humerus; angle 2 = midway between acromion and head of humerus-olecranon; angle 3 = olecranon-carpus; angle 4 = carpus-fetlock (metacarpophalangeal joint) on the forelimb; angle 5 = iliac crest-greater trochanter of the femur; angle 6 = greater trochanter of the femur-stifle (knee) joint; angle 7 = knee joint-tarsus; and angle 8 = tarsus-fetlock (metatarsophalangeal joint) on the hindlimb.

The calculation of hump volume ($HV = 0.07\,L \times B \times H$), hump weight ($HW = 0.45\,H - 13.8$), the ratio HW/HV (fat density), the proportion HV/BW, and the proportion HW/BW was performed using the methods described by Bengoumi et al. (27). Additional indexes reported to condition the quality of movement and kinetics were also calculated, including chest index, cephalic index, pelvic index, dactilothoracic index, weight in cannon index, conformation index, chest height index, and compactness index, upon the methods described by Susana Lopes et al. (28).

The units of measurement for biokinematics variables were set using Dunbar et al. (29) as a reference.

2.3 Experimental setup and video recording

The image collection occurred on a level and solid open ground. Specific lighting conditions were selected to ensure that the animal was not situated in shadowed areas or under lighting that could distort the image capture. Additionally, the color of the animal was taken into account to prevent any potential distortion or misalignment caused by the background color.

To maintain consistency, the camera was positioned at a standardized height of 1 m on a camera stand. This setup was situated 4 m away from the center of balance of the camel. This configuration allowed for capturing the entire animal within the frame during the evaluation. To ensure proper alignment, we followed the guidelines presented by Iglesias et al. (30), which involved marking standard lines on the ground before taking photographs to confirm the animal's correct positioning.

The image capture process utilized a digital camera (Sony DSC-RX100 SENSOR CMOS Exmor 1.0, 20.1 MP, F1.8–4.9, Zoom 20–100, Optical Zoom 3.6×, 3″ LCD Screen with Image Stabilizer; Sony Electronics, San Diego, CA, United States) in its standard mode. Cameras are strategically positioned to capture the observations without interfering with the animal's movement along the evaluation track. Dromedaries were recorded from two different views (side view and front view). The images were saved using the Joint Photographic Experts Group (JPEG) compression format.

Before recording, body length and height were measured, and masking tape squares (5 cm × 5 cm) were affixed to the dromedaries' bodies to serve as joint tracer reference points for data analysis. These markers were placed on the following left-side anatomical landmarks of the dromedaries: cranial angle of the scapula, midway between acromion and head of the humerus (shoulder joint), olecranon (elbow joint), carpus and fetlock (metacarpophalangeal joint) on the forelimb, the iliac crest, greater trochanter of the femur (hip), stifle (knee) joint, point of the hock (tarsus), and fetlock (metatarsophalangeal joint) on the hindlimb, as shown in Figure 1. A trained operator visualized all video sequences and placed video tracers from video analysis software Kinovea software (Kinovea version 0.9.5; Kinovea Org., France). The same operator performed the potential replacement of joint tracers along the video sequences when a disagreement between the real course and the course of the point of the software occurred.

A wooden pole (100 cm) was positioned parallel to the gait plane to provide a horizontal reference and calibration. The camera, mounted on a tripod, was adjusted to minimize parallax and ensure level alignment. Video footage was collected while the dromedaries were engaged in their regular exercise routines, encompassing walking, pacing, and galloping.

2.4 Qualitative and quantitative stride-based gait analyses

The recording duration corresponds to the time taken by the animal to complete the circuit for each of the three gaits examined

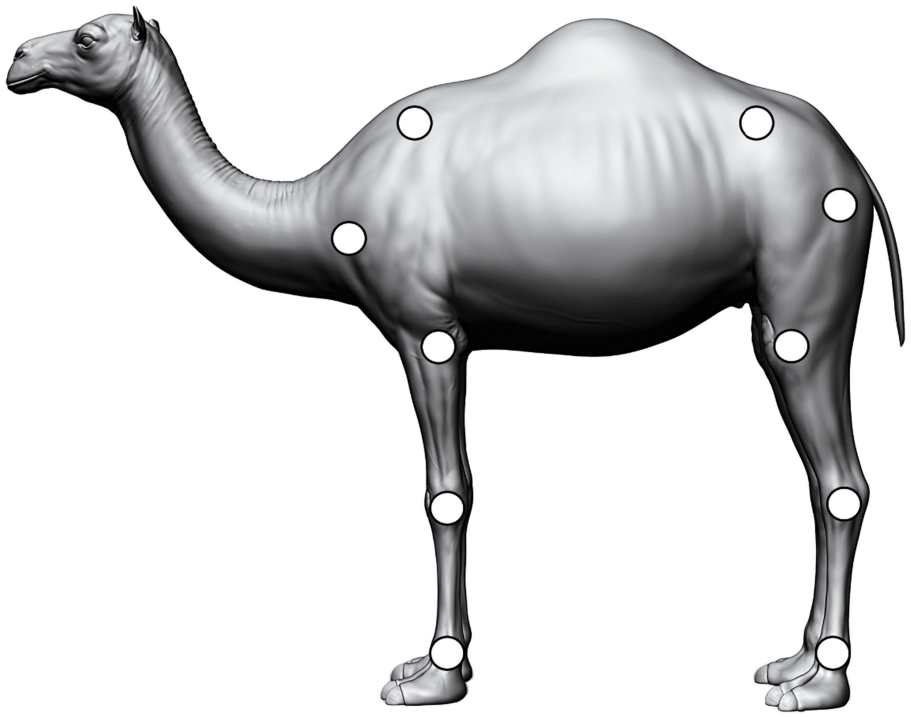

FIGURE 1
Graphical illustration of the anatomical disposition of masking tape squares (5 cm × 5 cm) at the dromedaries' bodies to serve as joint tracers reference points for gait analysis.

(walking, pacing, and galloping), as shown in Figure 2. Two complete stride cycles were considered for each animal. Stride-based gait analysis generally considers the contact of the trailing hindfoot as the starting point of the stride cycle (31). In the relative limb phase, the fraction of a stride in which the left forefoot, right hindfoot, and right forefoot touchdown was calculated following the left hindfoot touchdown. It was calculated by counting the frames of the filmed motion sequences based on the method by Hildebrand (32).

The individual locomotion process is assessed using a qualitative linear scale (1 to 5 points) described by Navas González et al. (33). Gaits characterized by a lack of uniformity, which may be indicative of an absence of balance, cadence, or harmony, and poor limb development were attributed a score of 1. In contrast, animals receiving a score of 5 were observed to exhibit a harmonious, rhythmic, and seamlessly coordinated gait, with their bodily movements demonstrating a high degree of synchronicity.

Kinovea software (Kinovea version 0.9.5; Kinovea Org., France) was used to analyze video sequences and kinematic data extraction. With Kinovea, images are calibrated using coordinate scales from the pictures, and the collected data undergo leveling through three smoothing methods. Eleven kinematic variables were considered to characterize each of the gaits performed by the camels as a default. Biomechanics parameters were as follows: acceleration, horizontal acceleration/position/velocity, total distance, total horizontal/vertical displacement, velocity, and vertical acceleration/position/velocity. An experienced researcher digitized anatomical landmarks to define body segments, including the lower lip, ear, withers (third thoracic vertebra), and tail base (dock). Segments' axes, such as the head, neck, and trunk, were established using these landmarks. Quantitative analysis was performed on trials where the head, neck, and trunk remained aligned in the sagittal plane. Various linear and angular variables related to the head movement, including displacement and velocity, were calculated using specialized software tools.

2.5 Definition of cubic regression equations and coefficients

Individual curve estimation regression analysis to identify the model that best describes camel biomechanics parameters (acceleration, horizontal acceleration/position/velocity, total distance, total horizontal/vertical displacement, velocity, and vertical acceleration/position/velocity) was performed using the *Curve Estimation* routine of the *Regression* package of SPSS Statistics for Windows, Version 25.0, IBM Corp. (2017). The *Curve Estimation* routine in SPSS software produces curve estimation regression statistics and related plots for 11 different curve estimation regression models, such as linear, quadratic, compound, growth, logarithmic, cubic, S, exponential, inverse, power, and logistic,. A generalized minimum adjusted R^2 value of over 0.7 was required for the model to be determined valid to capture data variability. The mathematical model that best fitted (comparatively superior average values of individual R^2; >0.7) the dynamics of locomotion of study animals was the cubic model. In these regards, parameters β0, β1, β2, and β3 of the curve for the variables of acceleration, horizontal acceleration/position/velocity, total distance, total horizontal/vertical displacement, velocity, and vertical acceleration/position/velocity, respectively, for each of the limbs were considered as independent variables in discriminant analysis.

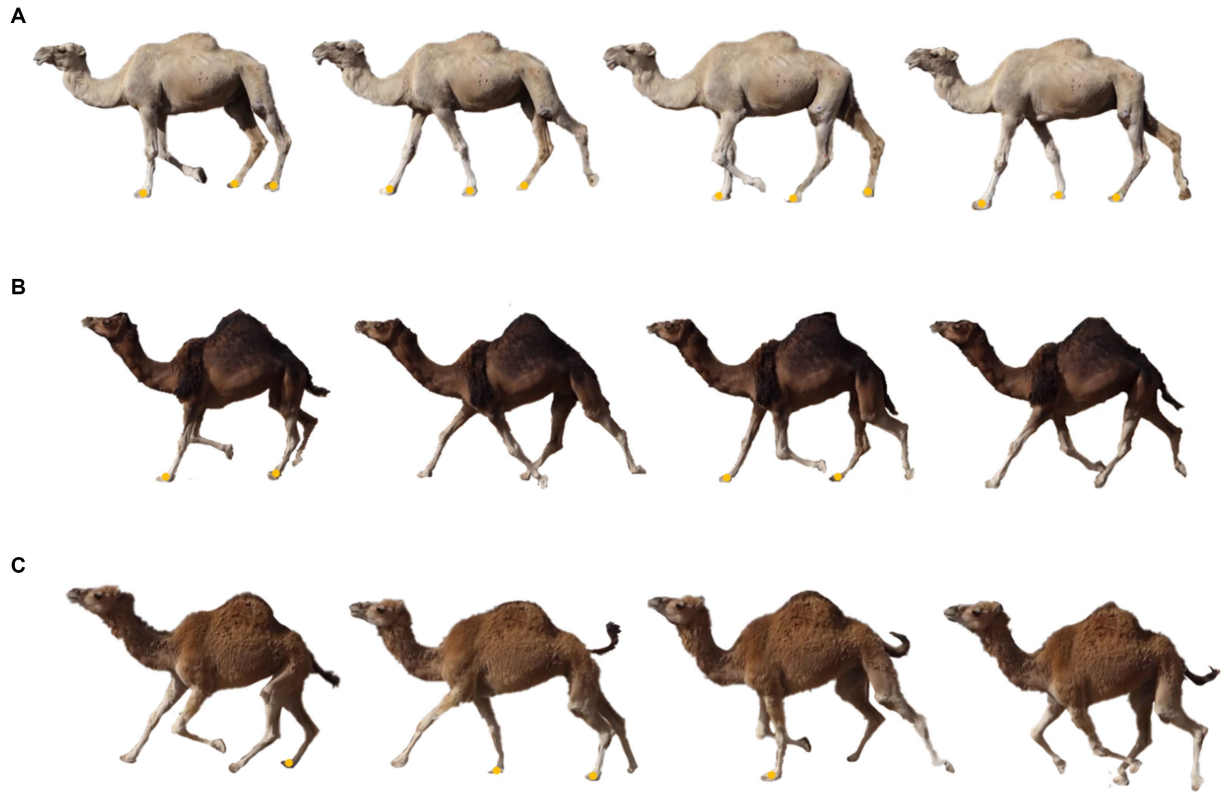

FIGURE 2
Graphical representation of the gait cycle during the walk **(A)**, pace **(B)**, and gallop **(C)** in dromedary camels. Yellow dots indicate which limbs are on the ground at each phase of the dromedary gait cycle.

In a cubic regression model, the equation takes the form:

$$y = \beta 0 + \beta 1x + \beta 2x^2 + \beta 3x^3 + \epsilon$$

Where:

$\beta 0$: This is the intercept term and represents the predicted value of y when x is 0. However, the interpretation of this term might not be as straightforward in some cases, depending on the range of your data and the context of your problem.

$\beta 1$: This coefficient represents the change in the predicted y for a one-unit change in the linear term x. It captures the linear relationship between x and y, just like in a simple linear regression model.

$\beta 2$: This coefficient represents the effect of the squared x on the predicted y. It indicates whether there is a curvature in the relationship between x and y. A positive $\beta 2$ suggests an upward curvature, while a negative $\beta 2$ suggests a downward curvature.

$\beta 3$: This coefficient represents the effect of the cubed x on the predicted y. It introduces another level of curvature to the relationship between x and y. A positive $\beta 3$ suggests that the curvature continues to be upward, while a negative $\beta 3$ suggests a downward curvature.

2.6 Statistical analysis

Following the methodology by González Ariza et al. (34), a discriminant canonical analysis was first used to develop a tool that enables the identification of the qualitative individual performance at locomotion (1 to 5 points; clustering criterion), determining whether linear combinations of biomechanics-related and biometrics variables, force and cubic regression coefficients, and categories for the variables "sex", "coat color", "coat particularities", "eye color", "neutering status", "owner", and "training regime" (actively or not actively involved in desensitization protocols) (explanatory variables) describe within- and between-population group clustering patterns. The discriminant canonical analysis is a statistical method that maximizes the discriminatory power of the variables, enhancing the accuracy of the results even with a relatively reduced number of observations (e.g., endangered livestock breeds). This technique has a notable ability to handle multicollinearity and identify the most influential variables that contribute to its accuracy, especially in situations where limited sample sizes may pose challenges for other statistical approaches.

The chi-squared automatic interaction detection (CHAID) decision tree method was used for classification, prediction, interpretation, and manipulation of the observation for the aforementioned independent or explanatory variables discretely categorized into the qualitative evaluation levels of gaits considered as the dependent variables of this analysis.

To assess the reliability of the CHAID decision tree, cross-validation is conducted to measure the predictive performance when applied to new data samples compared to the training sample. This assessment helps determine how well the model generalizes to unseen data. To validate the CHAID decision tree and evaluate whether the selected predictors effectively explain differences across the five qualitative evaluation levels of gaits, 10-fold cross-validation is utilized.

3 Results

3.1 Discriminant canonical analysis model reliability

After 325 rounds of multicollinearity analyses, the variables retained in the discriminant canonical analyses are those presented in Table 1.

As unbalanced samples and a considerable number of variables were used, the approximation of Rao for Wilks' Lambda test overcomes the robustness of other tests, such as Pillai's trace test statistic in the case of heterogeneous variance. Wilks' Lambda statistic was highly statistically significant when Gait qualitative scale levels (Wilks' Lambda statistic: 0.001; F (Observed value): 4.202; F (Critical value): 3.258; df1: 736; df2: 7; $p = 0.025$) were considered clustering criteria. Hence, the validity of the discriminant canonical analysis is ensured.

Five of the thirteen functions revealed after the discriminant analysis were reported to be significant for their discriminant ability (Table 2). The discriminatory power of the F1 function was the highest in comparison to that reported for the rest of the functions (eigenvalue of 6568.067; Figure 3), significantly explaining 89.72% of the variance. This framework makes F1 to F4 significantly explain 100% of the variability.

3.2 Canonical coefficients and loading interpretation

The different variables studied in this research were ranked according to their discriminating ability. A test of equality of group means of the independent variables involved in the present discriminant canonical analysis was used, as shown in Table 3. Standardized discriminant coefficients measure the relative weight of each independent variable across the significant discriminant functions (Supplementary Table S1).

A Press' Q value of 17.19 ($n = 189$; $n' = 15$; $K = 5$) was computed for qualitative gait evaluation levels. Thus, predictions can be considered to be better than chance at 95% (35).

Centroids from different levels for qualitative description of gait performance considered in this study are calculated. The relative position of each centroid is determined by substituting the mean value for the observations depicted in each of the five discriminant functions significantly detected (F1 to F5). The results for the functions at the centroids are reported in Table 4.

3.3 Data mining CHAID decision tree

The CHAID decision tree is presented in Supplementary Figure S1.

4 Discussion

Following the premises of Maloiy et al. (24), who stated that locomotory performance is essentially modulated by heritable characters, in lesser proportion by training, for both camels and equines, the present study has investigated which biomechanical and animal morphometric, physiology, and phaneroptic-related variables influence gait performance in dromedary camels leisure riding and racing activities. The mathematical function that had the best fit to a series of data points obtained from the analysis of different gaits (walking, pacing, and galloping) in the studied dromedary camels was identified as a preliminary requirement to effectively study these potential associations. The mathematical model that best fitted (comparatively superior average values of individual R^2; ≥ 0.7) the dynamics of camel locomotion was the cubic function, whose end behavior at the graphs is determined by the sign of their leading coefficients. These results are in accordance with previous research studies aimed at the mathematical and physical modeling of animal-like locomotion through the use of bioinspired robots (36–39), rigid body models (40), and video analysis (41).

The posterior examination of the statistical relationships between the coefficients of the individual curves of movement and other animal attributes and traits with gait performance made possible the identification of those variables that most largely influence this functional character. On the one hand, the kinematic parameters "vertical velocity", "total vertical displacement", "horizontal velocity", "vertical acceleration", "acceleration", "horizontal position", and "horizontal acceleration" appeared to exert a significant effect on the good performance of camel's gaits when performing leisure riding and racing activities. In addition to the benefits of good speed and acceleration for covering a specific distance in the shortest time possible (42, 43), the "vertical displacement" and the "horizontal position" of certain body regions during locomotion play a fundamental role in the physical performance of these animals. The vertical displacement determines both the shape and magnitude of the impulse created (vertical jump) and weightlifting performance (44, 45). In regards to the horizontal position, this variable could be an indirect reliable measure of joint rotation and skeletal impairments, thus an indicator of stabilization, alignment, and muscular force of the different body areas implicated in locomotion (46, 47). Therefore, overall athletic performance in leisure riding and racing dromedary camels may be the result of the sum of joint powers and other different sports performance characteristics such as velocity, acceleration, jumping, and change of direction.

Such performance characteristics are known to be intrinsically modulated by linear and angular morphometric measurements, as well as animal physiology, in several vertebrate species (48–57). Specifically, based on our results, superior performance in camel gait is likely to be affected by the angle between the carpus and the fetlock on the forelimb ("angle 4"), the angle between the iliac crest and the greater trochanter of the femur ("angle 5"), the angle between the stifle joint and the tarsus ("angle 7"), the proportion hump volume/body weight ("HV/BW"), the weight in cannon index, the chest height index, the body ratio, and the neutering status of the animals.

Similar to other quadrupedal animals that are bred for athletic activities, such as horses and dogs, the skeletal structure and deviations of the vertical line of carpus/tarsus joint, either medially or laterally, cranially or caudally, would significantly predispose the camel to carpus/tarsus lameness and splints, thus negatively affecting the gait score (58–60). Additionally, several authors confirmed the prominent role of the fore and hind fetlock joint angles, stifle and hock joint angle, and pelvis inclination on weight absorption, energy of propulsion, and storage of elastic strain in riding horses (61–64). Closely related to pelvis inclination, camels are known to be the only animal species that bend the thigh when moving (65). In addition,

TABLE 1 Summary of the value of tolerance and VIF after multicollinearity analysis of biomechanical performance traits in Canarian camel breed.

Statistic	Tolerance ($1-R^2$)	VIF (1/Tolerance)
Angle 4	0.607	1.646
Proportion HV/BW	0.538	1.859
B3-Scapula-Horizontal Acceleration	0.517	1.934
Angle 7	0.516	1.939
Angle 5	0.511	1.958
Chest height index	0.469	2.131
Weight in the cannon index	0.447	2.238
B1-Iliac crest-Horizontal Acceleration	0.443	2.255
B2-Fore fetlock-Vertical Acceleration	0.432	2.315
B2-Scapula-Horizontal velocity	0.425	2.353
B1-Hind fetlock-Vertical velocity	0.424	2.361
B2-Carpus-Total Vertical displacement	0.393	2.547
B1-Knee-Vertical acceleration	0.391	2.557
B3-Shoulder-Acceleration	0.367	2.727
B2-Hip-Horizontal Velocity	0.355	2.820
B2-Elbow-Acceleration	0.354	2.824
Neutered-No	0.344	2.908
B2-Shoulder-Horizontal position	0.342	2.927
B2-Scapula-Horizontal position	0.337	2.964
B2-Iliac crest-Horizontal velocity	0.321	3.117
B2-Scapula-Total vertical displacement	0.317	3.154
B1-Hip-Horizontal position	0.316	3.166
B2-Shoulder-Vertical velocity	0.315	3.177
B2-Iliac crest-Horizontal position	0.308	3.243
B1-Knee-Total vertical displacement	0.295	3.388
B2-Shoulder-Horizontal velocity	0.292	3.429
B2-Fore fetlock-Acceleration	0.291	3.442
B3-Hip-Vertical velocity	0.286	3.497
Body ratio	0.285	3.514
B2-Hip-Total vertical displacement	0.275	3.641
B1-Hip-Horizontal Acceleration	0.273	3.666
B1-Carpus-Vertical velocity	0.260	3.844
B2-Iliac crest-Total vertical displacement	0.257	3.897
B2-Hip-Vertical acceleration	0.255	3.925
B3-Knee-Vertical velocity	0.254	3.941
B2-Hind fetlock-Vertical acceleration	0.250	3.995
B1-Elbow-Vertical acceleration	0.249	4.021
B1-Elbow-Vertical velocity	0.247	4.050
B3-Iliac crest-Vertical velocity	0.240	4.166
B2-Elbow-Horizontal velocity	0.238	4.208
B2-Knee-Acceleration	0.234	4.276
B1-Scapula-Vertical acceleration	0.231	4.321
B1-Hind fetlock-Total vertical displacement	0.217	4.606
B2-Tarsus-Horizontal velocity	0.212	4.713

Interpretation thumb rule: VIF $\geq$ 5 (highly correlated); 1 < VIF < 5 (moderately correlated); VIF > 1 (not correlated).

TABLE 2 Canonical discriminant analysis efficiency parameters to determine the significance of each canonical discriminant function.

Test functions	Canonical correlations	Eigenvalue	Variance discrimination (%)	Bartlett's statistic	*p*-value
1 through 13	1.000	6568.067	89.720	2281.745	0.001
2 through 13	0.999	525.554	7.179	1459.868	0.001
3 through 13	0.997	153.996	2.104	873.964	0.001
4 through 13	0.993	72.981	0.997	402.406	0.001
5 through 13	0.000	0.000	0.000	0.000	0.001
6 through 13	0.000	0.000	0.000	0.000	1.000
7 through 13	0.000	0.000	0.000	0.000	1.000
8 through 13	0.000	0.000	0.000	0.000	1.000
9 through 13	0.000	0.000	0.000	0.000	1.000
10 through 13	0.000	0.000	0.000	0.000	1.000
11 through 13	0.000	0.000	0.000	0.000	1.000
12 through 13	0.000	0.000	0.000	0.000	1.000
13	0.000	0.000	0.000	0.000	1.000

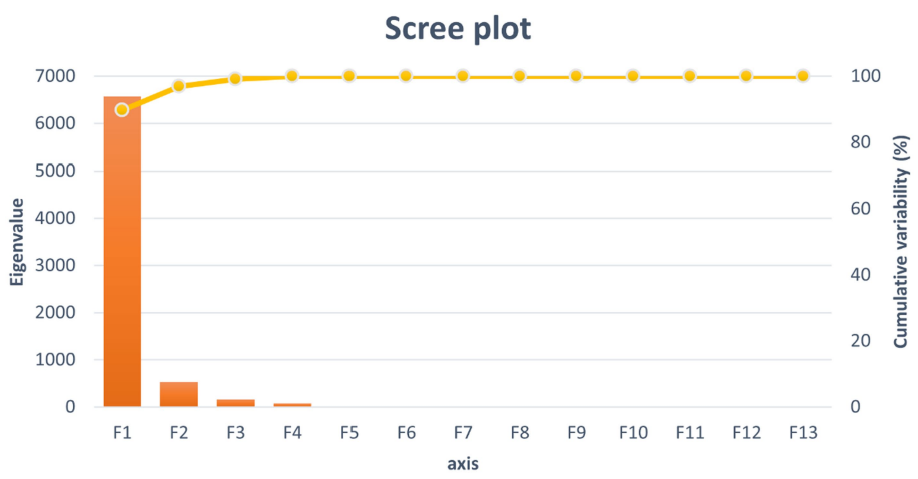

FIGURE 3
Canonical variable functions and their percentages of self-explained and cumulative variance.

chest development ("chest height index") and tallness ("body ratio") are reported to affect the static/dynamic balance and physical resistance in sport horses (66–69) and athletes (70).

Furthermore, if the hump of a camel is mechanically compared to the size and position of a rider on a horse, it could be inferred that the relative weight and dimensions of the hump (relation HV/BW) have a significant effect on camel locomotion given its influence on body balance, force distribution, and location of the center of gravity, analogous to the effects that the rider-horse-saddle aggregation has on equine gait (71). Moreover, the weight in the cannon index is expected to have a prominent effect on camel locomotion, as the study of the evolutionary history of these animal species has recently revealed. Through the analysis of the vertical ground reaction force with body mass in camelid species, Clemente et al. (72) found that camels have large foot contact areas due to broad fat pads. This condition would decrease the loading rate, thus leading to lower peak foot pressures and musculoskeletal stresses on the limbs. It also helps maintain proportional forces as animals grow in body mass or accelerate. These authors also encountered that dromedary camels load their forelimbs more than their hindlimbs, revealing the higher peak forces in forelimbs and, thus, their major functional relevance in this animal species' locomotion. Hence, this adaptive trait may be correlated with the afore-discussed prominent role of the alignment of the distal part of the forelimbs and the fore fetlock joint angle on the weight absorption and minimization of mechanic impact during camel locomotion.

In conclusion, the correlation between physiology and function was revealed by the significant impact of the neutering status of the camels on gait performance. In fact, different reports have discussed the advantages of castration in dromedary camels. Some authors highlight the larger effect of castration on the body development in this species if carried out after the animals have reached sexual maturity (73, 74). Concretely, gelded camels are larger, more robust, and more enduring (75, 76). In addition, Iglesias Pastrana et al. (77) remarked an increased effect of neutering on camel organic development and behavior if the animals are castrated not only when they have reached their sexual

TABLE 3 Results for the tests of equality of group means to test for difference in the means across sample groups after the removal of redundant variables.

Variable	Wilk's Lambda	*F*	*p*-value	Rank
B1-Elbow-Vertical velocity	0.769	13.822	<0.0001	1
B2-Iliac crest-Horizontal velocity	0.825	9.752	<0.0001	2
Neutered-No	0.845	8.411	<0.0001	3
B2-Shoulder-Horizontal velocity	0.864	7.244	<0.0001	4
Proportion HV/BW	0.867	7.080	<0.0001	5
B2-Hind fetlock-Vertical acceleration	0.873	6.672	<0.0001	6
Weight in cannon index	0.882	6.154	0.000	7
B2-Carpus-Total vertical displacement	0.891	5.635	0.000	8
B1-Knee-Total vertical displacement	0.903	4.954	0.001	9
B1-Carpus-Vertical velocity	0.910	4.566	0.002	10
B3-Shoulder-Acceleration	0.911	4.504	0.002	11
B1-Hind fetlock-Total vertical displacement	0.913	4.410	0.002	12
B1-Hip-Horizontal acceleration	0.917	4.172	0.003	13
B3-Knee-Vertical velocity	0.918	4.107	0.003	14
B2-Scapula-Total vertical displacement	0.923	3.855	0.005	15
B2-Elbow-Horizontal velocity	0.924	3.786	0.006	16
Body ratio	0.934	3.255	0.013	17
B2-Tarsus-Horizontal velocity	0.935	3.201	0.014	18
B3-Hip-Vertical velocity	0.937	3.083	0.017	19
Angle 5	0.943	2.771	0.029	20
B1-Iliac crest-Horizontal acceleration	0.943	2.756	0.029	21
Angle 7	0.945	2.692	0.033	22
B2-Scapula-Horizontal velocity	0.946	2.613	0.037	23
B2-Hip-Horizontal velocity	0.947	2.552	0.041	24
B2-Hip-Total vertical displacement	0.950	2.446	0.048	25
B3-Scapula-Horizontal acceleration	0.950	2.437	0.049	26
Angle 4	0.952	2.325	0.058	
B2-Knee-Acceleration	0.953	2.272	0.063	
B1-Hip-Horizontal position	0.954	2.235	0.067	
B2-Fore fetlock-Acceleration	0.954	2.211	0.069	
B2-Hip-Vertical acceleration	0.954	2.198	0.071	
B3-Iliac crest-Vertical velocity	0.957	2.082	0.085	
B2-Iliac crest-Horizontal position	0.958	2.037	0.091	
B1-Hind fetlock-Vertical velocity	0.965	1.683	0.156	
B2-Iliac crest-Total vertical displacement	0.965	1.662	0.161	
B2-Elbow-Acceleration	0.965	1.658	0.162	
B2-Shoulder-Horizontal position	0.970	1.437	0.223	
B1-Elbow-Vertical acceleration	0.970	1.400	0.236	
Chest height index	0.971	1.378	0.243	
B2-Shoulder-Vertical velocity	0.974	1.237	0.297	
B1-Knee-Vertical acceleration	0.981	0.904	0.463	
B2-Scapula-Horizontal position	0.981	0.874	0.480	
B1-Scapula-Vertical acceleration	0.983	0.781	0.539	
B2-Fore fetlock-Vertical acceleration	0.993	0.314	0.869	

Df1 = 4; Df2 = 184.

TABLE 4 Functions at the centroids for the 13 discriminant functions detected in this study.

Function/Gait qualitative evaluation level	1	2	3	4	5
F1	−476.738	−52.340	40.573	−54.118	134.521
F2	108.758	−29.110	4.553	−11.968	62.230
F3	22.288	10.686	6.627	−17.247	−25.252
F4	1.326	−16.244	3.854	3.913	−23.801
F5	0.000	0.000	0.000	0.000	0.000
F6	0.000	0.000	0.000	0.000	0.000
F7	0.000	0.000	0.000	0.000	0.000
F8	0.000	0.000	0.000	0.000	0.000
F9	0.000	0.000	0.000	0.000	0.000
F10	0.000	0.000	0.000	0.000	0.000
F11	0.000	0.000	0.000	0.000	0.000
F12	0.000	0.000	0.000	0.000	0.000
F13	0.000	0.000	0.000	0.000	0.000

maturity but have also been initiated in the training protocol for their functionality (e.g., leisure riding). Hence, apart from the benefit of body growth and endurance, castration also has effects on camel behavior, which ultimately constitutes a further criterion of notable interest for functional selection in working animals.

However, other morphometric, phaneroptic, and performance-type variables such as the angle between the cranial side of the scapula and the shoulder joint ("angle 1"), the angle between the shoulder joint and the olecranon ("angle 2"), the angle between the olecranon and the carpus ("angle 3"), the angle between the greater trochanter of the femur and the stifle joint ("angle 6"), the angle between the point of the hock (tarsus) and fetlock (metatarsophalangeal joint) on the hindlimb ("angle 8"), body weight, hump volume, hump weight, density of fat, proportion hump weight/body weight, chest index, cephalic index, pelvic index, dactilothoracic index, conformation index, compactness index, pull force, load force, power, sex, age, coat color and particularities, eye color, owner, training regime, and type of gait did not have a significant discriminatory effect on camel gait performance in leisure riding and racing activities.

First, the aforementioned singular function of fore fetlock joint angles, stifle and hock joint angle, and pelvis inclination on weight absorption, energy of propulsion, and storage of elastic strain in riding animals could be explaining the lack of large impact of the remaining angular measurements evaluated for its potential implication in camel locomotion in the present study. Concerning the effects of body weight, hump volume and weight, and the proportional relationship between different body regions in camel gait performance, the absence of significant affectation of this functional trait by the mentioned factors partially resembles the results of de Oliveira Bussiman et al. (78) for Brazilian horses. However, this contrasts with the results reported by Pratt-Phillips and Munjizun (79) and Gómez et al. (80). These authors support the effects of body mass index, body composition profile, and harmony of physical development on the exercise performance of sport horses, probably due to a combined effect between inflammation-type responses and the impacts of excessive weight carriage on limb health. Then, the contrasting results obtained for camels can be ascribed to different species-specific adaptive traits.

Fatty tissue in camels is mostly concentrated on the hump to improve heat removal through the skin, and the hump size is related to the general reserve status (body condition score) of the animals (81). For this reason, the hump volume in relation to the body weight, and neither the volume and weight of the hump nor the body weight considered separately, significantly influences the locomotion of these animals. Such a condition derives from the implication of the proportionality between the hump volume and the body weight for maintaining the static/dynamic balance of the body of the camel. In addition, in the case of hump weight, this variable greatly depends on the specific fatty acid composition (82), so a heavy hump does not necessarily mean a large volume hump. Apart from that, body balance is strongly controlled by the aforementioned functionality of the broad foot pads, which are responsible for solving the differential impact of the animal's weight and the speed of movement on the locomotor pressures that affect the musculoskeletal system forces. Finally, in the case of the variables that relate different measurements corresponding to the dimensions of the trunk and extremities, their non-significant influence could also be explained by purely evolutionary reasons. Camels are provided with relatively narrow bodies, long legs, no flank fold that makes the movement of hindlimbs difficult, a less rounded abdomen to allow the hindlimbs freer motion, and limbs more closely to the midline to minimize oscillation between strides, as a result of natural selection for energy efficiency of the movement (9, 83). In close relationship with this last statement, from a pure evolutionary physiology background, pull force, load force, and power may have had a non-significant effect on camel gait performance.

Within the same phylogenetic background, sex, age, coat color and particularities, eye color, owner, training regime, and type of gait did not influence in a significant manner the physical performance of camels. When walking, camels move their ipsilateral limbs in unison ("pacing gait", as referred to in the literature). Tracing back camel evolutive history, pacing gait is agreed to have been developed about 20 million years ago (84). Apart from the fact that the legs of camels cannot collide at this pacing gait, the stride length, speed, and kinematics efficiency are higher when compared to other gaits, such as regular trots. However, pacing gait made the animal more prone to lateral instability. This way, camels have developed a broad foot pad and a digitigrade stance to abate

such relative lack of instability (18, 65). Contrary to horses, camels are known to be the only ungulate species that always use this pattern of leg movements independent of speed (18). Moreover, this is a highly conserved ancestral trait (85). Hence, the underlying genetic basis of camel gaits, which is elementally implicated in the sort of neural impulses from the spine to the extremities (86) and presumably free of epistatic interactions from genes that control coat and eye color associated traits, may be extrapolated to the whole range of physical activities that are feasible for camels to perform. This last hypothesis could be confirmed in future approaches aimed at studying which genomic variants are statistically associated with an enhanced or decreased physical performance.

Then, these statements are contrary to the results presented by Al-Shorepy (14) for racing camels, Entin (87) for racehorses and Greyhound dogs, Vicente et al. (88) for classical riding horses, and Navas González et al. (33) for assisted-therapy donkeys, who reported a significant effect of sex and age on animal functional performance but agreed with Senefeld et al. (89), who support the idea that large differences in athletic performance do not exist between sexes in animals. Regarding the association between coat and eye color with different psychological constructs that are transcendental for animal trainability and safe interaction, the molecular basis of these desirable traits has been identified mainly for dogs and horses (90–93), and some inferences are discussed by Iglesias Pastrana et al. (77) for dromedary camels. Furthermore, a non-negligible impact of rider fitness and interaction with the animal (94), training regime (95, 96), and type of gait (97) on exercise performance are also recognized in the literature on sport horses and dogs. Such controversy could definitively be ascribed to methodological issues (e.g., type and number of potential influencing factors considered, and statistical treatment of data), as well as to animal species and breed-mediated effects.

In summary, rather than the morphology of a specific region of the body, which is correlated with the weight of the animal (98), or factors such as sex, age, and phaneroptic-related variables, known to affect athletic performance in some animal species, a reduced number of angular morphometric measurements, definite body proportions that can affect the general balance of the body, the weight supported by structures with notable involvement in the damping of the mechanical forces produced during locomotion, post-neutering effects, and the particular mobility of specific joints are the factors that should be considered when selecting leisure riding and racing dromedary camels for gait proficiency. More specifically, based on the data mining decision tree, the variables that serve to discriminate with greater accuracy between leisure riding and racing camels for gait performance are the neutering status, with the advantage for neutering, and the kinematic behavior of the scapula, shoulder, carpus, hip, and foot, anatomical structures of singular importance for propulsion, movement direction, and limb support in quadrupeds, as cited above.

With special emphasis on kinematics, the horizontal position of the scapula, the total vertical displacement of the carpus, the horizontal velocity of the shoulder, and the horizontal acceleration of the hip in neutered animals, the horizontal and vertical acceleration of the scapula, and the total vertical displacement of foot in non-neutered animals are specific discriminating parameters among good and poor performance camels. It can also be observed that coefficient 2 is more relevant in castrated animals, while coefficients 1 and 3 are more discriminative among non-castrated animals. Based on the graphic behavior of the cubic function, coefficients 1 and 3 for curves of movement could be related to the direction and force of the movement, while coefficient 2 would be with the amplitude of the movement, suspension duration, and speed (99). From the viewpoint of athletic performance, these results may be indicative of better dressage/beauty and endurance riding performance for intact and castrated animals, respectively. In any case, the selection of the animals should be complemented by the use of behavior evaluation tools. Indeed, the common practice of castration in the camel breed studied in the present research consists of the neutering of the animals once they are initiated in the domestication protocol for leisure riding activities and are sexually mature. It is pursued that animals have reached proper general organic maturity by the mediation of plasma androgens (mainly within brain structures) while also avoiding harmful levels of these hormones from an ethological perspective (77, 100).

With regard to the quantitative evaluation of kinematic parameters, better gait performance in leisure riding and racing camels is achieved when the horizontal position of the scapula is greater than 0, the total vertical displacement of the carpus is greater than −3.78, the horizontal velocity of the shoulder is less than or equal to 2.25, and the horizontal acceleration of the hip is greater than 5.74, for neutered animals. For non-neutered animals, optimal gait performance is observed when the acceleration of the scapula is less than or equal to 0 as well as the total vertical displacement of the foot greater than −2.24. Elementally, the total vertical displacement of the carpus, as an anatomical structure significantly involved in forelimb mechanical support and cushioning, would act as a corrector for the horizontal position of the scapula as well as the horizontal velocity of the shoulder, thus achieving greater general stability, harmony, and energy efficiency of the movement (101). In the case of the horizontal acceleration of the hip, the higher its value, the greater the propulsion force and amplitude of the movement. Contrastingly, for intact camels, a lower acceleration of the scapula and a greater vertical displacement of the distal region of the hindlimbs determine a more harmonious and efficient movement. These values, being interpreted on account of the relative significance of coefficients 1 and 3 for this animal subgroup, reflect the relevance of the rear quarters in maintaining the dynamic balance of the body while limiting as far as possible a wide acceleration at the anterior third, in animals presumably more apt for riding/dressage activities. These functionalities make rear quarters broad, deep, and heavy to support load carriage while moving in an efficient way (102).

Concerning the exploration of new potential functional niches, the results of the present study can assist the tasks of camel valorization and selection for their active participation in animal-assisted therapy since those biomechanical and physiological traits of the animals that most influence locomotor performance, regardless of the type of gait, are identified. On the contrary, for equines, since the cadence of movements can vary between gaits, it may be necessary to look at different and varied selection criteria depending on the type of gait involved in each of the assisted activities in which these animals would be involved. Furthermore, camels are less flighty than horses (103), and the pelvic movement of a person when riding a camel is reported to be very similar to natural human pelvic movements during walking (104); thus, therapeutic applications of camelback riding apart from mere leisure are clearly plausible.

Ultimately, some limitations can be identified in this study. Specifically, future applied studies are encouraged to correlate biomechanical traits with physiological indicators, such as activity-dependent hematological and biochemical parameters, to enhance dromedary camel breeding programs for physically demanding activities not only from a genetic improvement perspective but also to ensure the maintenance of optimal welfare status of the animals.

5 Conclusion

Individual curve estimation regression analysis plays a pivotal role in synthesizing complex datasets by constructing validated mathematical descriptions of locomotive behavior in camels. The posterior study of the relationships among morphology, physiology, phaneroptic, and gait performance-related traits made possible the identification of those factors that largely influence gait proficiency in dromedary camels; thus, these criteria should assist breeding decisions for camels dedicated to different athletic activities. Angularity and mechanical forces at distal fore and rear extremity areas, inclination of the pelvis, specific proportionalities affecting the general balance of the body, neutering-mediated effects, and the velocity, acceleration, position, and displacement of particular body regions significantly affect the overall athletic performance in dromedary camels. More specifically, the kinematic behavior of the scapula, shoulder, carpus, hip, and foot, as these anatomical structures primarily control the take-off of the rear limbs and the impact forces of the front limbs, has a prominent impact on the locomotor performance in dromedary camels. Other animal and environment-dependent variables do not influence camel gait performance, probably due to the mixed effect of different species-specific adaptive traits inherited in response to desert environment demands, such as the pacing gait, broad foot pads, and energy efficiency of the movement.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

All farms included in the study followed specific codes of good practices and therefore, the animals received humane care in compliance with the national guide for the care and use of laboratory and farm animals in research. Written consent from the owners was obtained for their participation. The study was conducted in accordance with the Declaration of Helsinki. The Spanish Ministry of Economy and Competitivity through the Royal Decree Law 53/2013 and its credited entity, the Ethics Committee of Animal Experimentation from the University of Córdoba, permitted the application of the protocols present in this study as cited in the 5th section of its 2nd article, as the animals assessed were used for credited zootechnical use. This national Decree follows the European Union Directive 2010/63/UE, from the 22 September of 2010.

Author contributions

CI: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. FN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EC: Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. CM: Data curation, Formal analysis, Funding acquisition, Resources, Software, Validation, Visualization, Writing – review & editing. JD: Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1297430/full#supplementary-material

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Determination of prevalence of subclinical mastitis, characterization of intra-mammary infection-causing bacteria, and antibiotic susceptibility in dairy camels in Jigjiga City, Somali region, Ethiopia

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Background: Subclinical mastitis in camels, an inflammation of the udder without visible signs, can reduce milk quality and raise bacteria levels. Regular monitoring of camel milk is crucial for consumer safety.

Methods: A cross sectional study was conducted in Jigjiga city, Ethiopia to investigate the prevalence and characteristics of subclinical mastitis in she-camels. The study included 244 lactating she-camels from three privately-owned camel dairy farms, and a questionnaire survey was conducted with 60 camel owners.

Results: The overall prevalence of subclinical mastitis in she-camels was 10.6% (26/244), with no significant difference among the studied dairy farms. Risk factors that influenced the result of California Mastitis Test (CMT) included age and udder and leg hygiene. The study revealed that *S. aureus* was the most prevalent bacterium among the isolated bacteria, with a prevalence rate of 34.5%. This was followed by *S. agalactiae*, *S. dysgalactiae*, and *Pasteurella multocida*, with prevalence rates of 29.8, 19.4, and 16.2%, respectively. Among the isolated bacteria, 84.5% were sensitive to Erythromycin, 60% to Streptomycin, 44.7% to Oxytetracycline, and 36.7% to Tetracycline. Interviews with camel owners revealed that 66.7% used mixed herd grazing methods and reported feed shortage. Treatment practices for sick camels included modern veterinary drugs, traditional medicines, or a combination of both. The owners of camel dairy farms did not maintain proper hygiene practices during milking, such as not using soap when washing hands.

Conclusion: Addressing camel mastitis necessitates access to alternative drugs, comprehensive herder training, and enhanced management practices.

KEYWORDS

antibiotic sensitivity, antimicrobial resistance, camel, California mastitis test, *Staphylococcus aureus*, udder health, Jigjiga, Ethiopia

1 Introduction

The one-humped camels (*Camelus dromedarius*) demonstrate remarkable adaptation to arid and semi-arid environments, enabling them to not only survive but also thrive. They possess the ability to produce milk even in the midst of severe droughts, a crucial advantage over cattle, sheep, and goats, which often suffer high mortalities under such conditions. Consequently, dromedaries play a vital role in sustaining the livelihoods of pastoralists, particularly in the pastoral areas of Africa (1). Milk is a very nutritional food that is rich in carbohydrate, proteins, fats, vitamins, and minerals. However, milk and milk products of dairy cows and camel can harbor a variety of microorganisms and can be important sources of food borne pathogens to humans (2). Mastitis, a prevalent disease among camels, poses a significant threat to both the health of these animals and the economic well-being of the pastoralists. As it can result in substantial financial losses, combatting mastitis becomes paramount in safeguarding the livelihoods of pastoralists (3).

Mastitis can occur in two main forms: clinical and subclinical. Clinical mastitis shows visible signs and abnormalities in the animal's udder and milk, while subclinical mastitis leads to reduced milk production without obvious symptoms (4). Diagnosing subclinical mastitis can be particularly challenging because it often lacks visible signs, making it easy for the condition to go unnoticed. This can lead to a prolonged presence of the disease within the herd, causing higher economic losses compared to clinical mastitis. Farmers may not be aware of the issue until it has already had a significant impact on milk quality and production. Therefore, early and targeted testing of the milk is essential to detect and address subclinical mastitis promptly, ultimately minimizing economic losses and ensuring the overall health and productivity of the herd (5).

In Ethiopia, the dairy industry faces significant obstacles due to various diseases associated with livestock farming practices, with mastitis being the primary concern. The effects of mastitis on this sector are extensive and encompass several areas. These include the temporary or permanent impairment of milk production capacity, a decline in milk quality, milk wastage resulting from antibiotic drug residues, increased expenses related to veterinary care and labor, a reduced productive lifespan of the animals, diminished value of meat after slaughter, and losses arising from reduced overall dairy product output (6).

Reports of sub-clinical mastitis in camels have been documented in various regions of Africa, including Egypt (7), Somalia (8), and Kenya (9). Very recent studies conducted in two pastoral districts in southern Ethiopia, reported a prevalence of sub-clinical mastitis in dairy cows, camels, and goats as 33.3, 26.3, and 25.0%, respectively; and quarter-level prevalence of sub-clinical mastitis in cows, camels and goats as 17.6, 14.5, and 20%, respectively (10). Sub-clinical mastitis in Ethiopia has been over-looked, with limited research and information available on its prevalence, geographic distribution, impact on milk quality, and related consumer risks. There is a need to study and understand the risk factors and negative effects of mastitis on milk and milk products to improve prevention and control measures (11). Particularly, there is very limited knowledge about sub-clinical mastitis in camels, including its causes and occurrence. However, cases of mastitis in camel have recently been reported in Ethiopia (12).

Limited information exists on the antimicrobial resistance (AMR) of pathogens in camel milk, but a study conducted in southern Ethiopia found that *S. aureus* isolates in camel milk were resistant to multiple drugs. The use of antimicrobials in dairy farms, particularly in food animal production, has been connected to an increased resistance to tetracycline in *S. aureus* and *E. coli* strains causing mastitis. This practice is widely acknowledged as a contributing factor to the development of antimicrobial resistance (10). The objective of this study was to determine the prevalence of camel sub-clinical mastitis and identify the associated risk factors. With great emphasis on isolation, characterization, and determination of antibiotic sensitivity of the bacteria causing sub-clinical mastitis in and around Jigjiga City, Fafan Zone, Somali Region, Ethiopia.

2 Materials and methods

2.1 Description of the study area

The study was conducted in and around Jigjiga city, Fafan zone, Somali regional state. Fafan administrative zone, is located in the northern part of the Ethiopian Somali Region, at 9°20′ North latitude; 45°56′° East longitude, about 630 km East of Addis Ababa. It covers a total land area of 40.86 km^2 with altitude ranging from 500 to 1,650 m above sea level (masl). The area receives a precipitation ranging from 300 to 600 mm *per annum* and an average daily temperature of 16–20°C. Agro-pastoralist is a dominant production system in Fafan zone. The estimated livestock population of the zone is 248,435 cattle, 666,130 sheep, 503,881 goats 72,390 camels, and 10,548 poultry (13) (Figure 1).

2.2 Study design

A cross-sectional study was conducted from October 2021 to June 2022, on lactating female camels of local breed (*Camelus dromedarius*) reared either under intensive or extensive systems.

2.3 Study population

The study population consisted of lactating female camels from Jigjiga's commercial dairy farms. The camels included in the study were all indigenous breeds of one humped camel (*Camelus dromedarius*) selected from three privately owned camel dairy farms (Dhaygel: $n = 81$, Barkomal: $n = 131$, Suleka: $n = 32$) that followed a

Abbreviations: AMR, Antimicrobial resistance; CLSI, Clinical and Laboratory Standards Institute; CMT, California mastitis test; MASL, Meters above sea level; SORPARI, Somali Region Pastoral Agro-pastoral Research Institute.

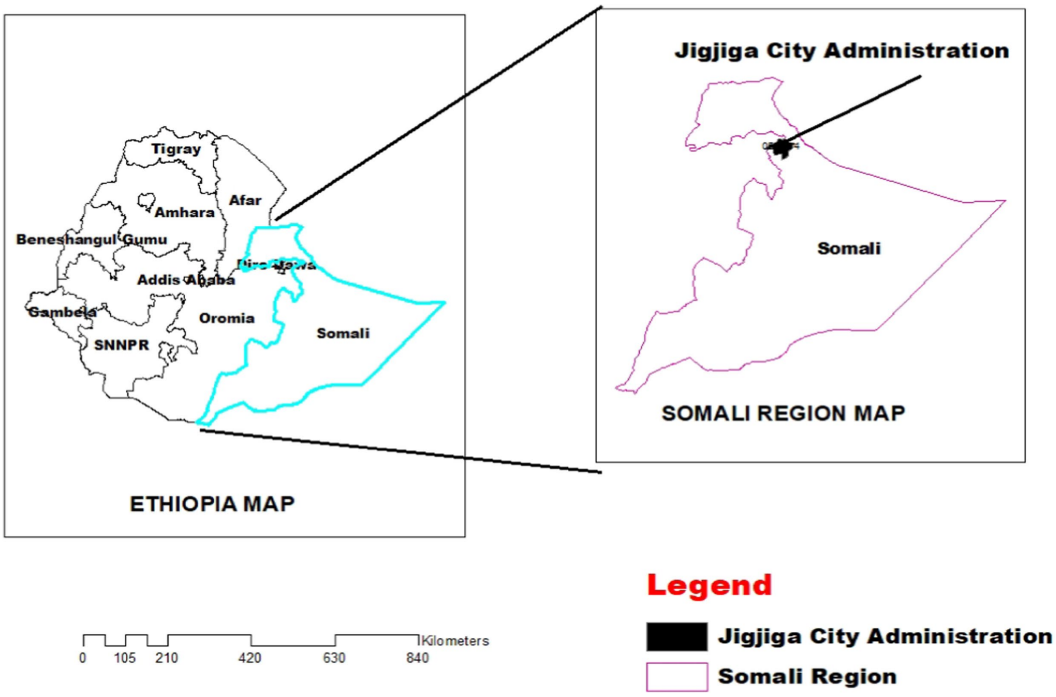

FIGURE 1
Map of the study area.

semi-intensive farming system. Semi-intensive camel dairy farming in the Somali region combines aspects of intensive and extensive farming practices to optimize milk production from camels. This system includes regular milking, supplementary feeding, proper housing and management, breeding management, healthcare and disease management, as well as water and pasture management to ensure high-quality milk production while maintaining the health and wellbeing of the camels. The age of the animal was determined by examining dentation and owners records. In addition, A group of 60 specifically chosen camel owners, along with three owners/managers/attendants of camel dairy farms, were interviewed for the study.

2.4 Questionnaire survey

During the farm visits, data were collected using a pretested semi-structured questionnaire administered through personal interviews at each time. The information gathered included farm biodata and herd management practices of camel owners, such as the production system (pastoral or agro-pastoral), the purpose of keeping livestock (milk, meat, income, or social prestige), camel herd grazing methods (mixed with other species or grazing separately), cleaning the house (yes or no), milking practices (with or without calf suckling), washing hands before milking (yes or no), and cleaning milking equipment (yes or no).

2.5 Sample size determination

The sample size was determined using the formula described by Thrusfield (14), considering an expected prevalence of 18.1%, an absolute precision of 5%, and a 95% confidence interval. However, to increase precision, the sample size was increased to 244 lactating camels that were sampled in this study.

$$n = \frac{(1.96)^2 P(1-P)}{d^2} \; n = \frac{(1.96)^2 0.181(1-0.181)}{0.05^2} = 228$$

Where: P = expected prevalence
n = required sample size
d = desired absolute precision

2.6 Sampling technique

First, the udder was cleaned to remove any dirt or debris. Following this, each teat was wiped with a clean cloth and disinfected using 70% alcohol. The initial streams of milk were discarded, and approximately 10–20 mL of milk was collected from each quarter (5–10 mL in each teat) in sterile universal bottles with unique labels. Risk factors, such as age, lactation stage, parity, production system, source of water, and udder and leg hygiene, were all documented.

2.7 California mastitis testing

To assess the clinical form of mastitis, milk from each quarter was examined using a strip cup, and any observable changes in color, odor, and consistency were recorded. Additionally, the presence of subclinical mastitis was determined using the California mastitis test (CMT) following the prescribed procedures outlined by Ferronatto et al. (15). The CMT results were interpreted subjectively according to the categories of negative, trace, 1+, 2+, or 3+, as outlined by Adkins

and Middleton (16). Using the CMT, camels were classified as positive for SCM if they had readings of (1+, 2+, or 3+), while negative and trace readings were considered as negative.

2.8 Bacteriological laboratory examination and antimicrobial susceptibility testing

Bacteriological examination and AMR testing were conducted following the methods described by Quinn et al. (17) and the Clinical and Laboratory Standards Institute (CLSI) guidelines (18). For the analysis, a loopful of milk sample was streaked onto tryptose blood agar base supplemented with 5% sheep blood agar (Oxoid, United Kingdom) and MacConkey Agar using the quadrant streaking technique for each quarter. After incubation, the cultural growth macro-and micro-scale characteristics were evaluated, examining distinctions of each colony including colony morphology, hemolysis, and pigment production. Gram staining was performed on pure culture colonies to analyze their staining reaction and cellular morphology under a light microscope at 100× magnification. To avoid confusion in the Gram stain reaction, a potassium hydroxide (KOH) test was also conducted. For further examination, mixed colonies and Gram-negative bacteria were sub-cultured on sheep blood and MacConkey (Oxoid, Hampshire, United Kingdom) agar plates. Pure cultures of single colony types from both blood and MacConkey agar were transferred onto nutrient agar-medium for a series of primary tests including Catalase, Oxidase, Motility, and Fermentative-Oxidative tests, as well as secondary tests like triple sugar iron agar, citrate utilization test, methyl red test, and indole test, following standard procedures (19, 20).

Isolated bacterial colonies was transferred to a tube with sterile normal saline, creating a homogenous suspension adjusted to a turbidity equivalent to a 0.5 McFarland standard. The bacterial suspension were then inoculated onto Muller-Hinton agar using a sterile swab to cover the entire surface, with plates left to dry at room temperature. Prepared plates was checked for sterility and incubated overnight at 37°C before antimicrobial disks were placed on the media surface. A variety of commonly used antibiotics were selected for susceptibility testing in accordance with CLSI criteria. Standard antimicrobial impregnated disks (HiMedia Mumbai, India) were used, including Erythromycin 15 µg, Streptomycin 10 µg, Oxytetracycline 30 µg, and Tetracycline 30 µg. The zone of inhibition diameters around the disks were measured with rulers, and isolates were classified as susceptible, intermediate, or resistant following CLSI standards. Isolates resistant to three or more antimicrobial subclasses were considered multidrug resistant (21).

2.9 Data analysis

The data obtained from laboratory investigations and questionnaire survey were organized in a Microsoft Excel spreadsheet and analyzed using STATA (version 16; Stata Corp LP, College Station, TX, United States). Descriptive statistics were used to determine the prevalence of subclinical mastitis. The associations between subclinical mastitis and various factors (such as location/farm, age, lactation stage, parity, and production system, sources of water, and udder and leg hygiene) were evaluated using the chi-square test (χ^2). The odds ratio (OR) was utilized to indicate the level of association between risk factors and SCM occurrence, with 95% confidence intervals provided. Variables with a p value <0.25 in univariate logistic regression analysis were included in a multivariate logistic regression analysis to control for potential confounding variables, and adjusted odds ratios were calculated. The model's goodness-of-fit was assessed through backward elimination, where variables were sequentially removed starting from the least influential until the removal of a variable had a significant impact on the dependent variable. Collinearity between variables was checked using standard error, and model fitness was evaluated through the Hosmer and Lemeshow test and Omnibus test. A 95% confidence level was maintained throughout the data presentation, and a p value less than 0.05 (i.e., $p < 0.05$) was considered statistically significant.

3 Results

3.1 House hold questionnaires and dairy camel farm owners interview survey

In the study area, the main feed sources for dairy camels were natural pasture and crop residue, as stated by 58.3% of respondents. Feed shortage problems were reported by 98.3% of dairy camel owners. Out of 60 respondents, 85% were aware of clinical mastitis in lactating she camels, but none were aware of subclinical mastitis in apparently health she camels. When treating mastitis, 65% used modern veterinary drugs and 25% relied on traditional medicines, particularly experienced camel owners. Most respondents (93.3%) had access to veterinary services. The summarized survey results are presented in Tables 1, 2, with a bar column chart (Figure 2).

3.2 Prevalence of subclinical mastitis

In the study area, the overall prevalence of subclinical mastitis in she-camels was 10.6% (95% CI: 0.067–0.146) with 26 out of 244 individuals affected. Among the three camel dairy farms examined, Suleka had the highest prevalence at 12.5% (95% CI: 0.003–0.246), followed by Dhaygel and Barkomal with prevalence of 11.1% (95% CI: 0.041–0.181) and 9.9% (95% CI: 0.047–0.151), respectively. However, there were no significant differences in the prevalence of subclinical mastitis among the different dairy farms ($p > 0.05$) (Table 3).

She camels in the 5–7 years age category were found to be at a greater risk compared to those aged 2–4 and over 7 years. Poor hygiene of the udder and legs were identified as significant risk factors for subclinical mastitis in she-camels, with poor udder and leg hygiene being the most affected factors ($p < 0.05$). However, factors such as location/farm, lactation stage, parity production system, and sources of water for the farms did not display a statistically significant association with the occurrence of subclinical mastitis in the study areas ($p > 0.05$) (Table 4).

3.3 Bacterial species isolation analysis

In the 26 cultured samples, a total of 510 bacterial colonies were found. The most common species was *Staphylococcus aureus*, comprising 34.5% of the isolated bacteria. Other bacteria like *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Pasteurella multocida* were also present, with proportions of 29.8, 19.4, and 16.2%, respectively (Table 5).

TABLE 1 Dairy camel owners' socio-economic profile and herd management.

Categories	Proportions (%)
Educational level	
Illiterate	45.0
Primary school	21.8
Religious knowledge	28.3
Secondary school	5.0
Livestock production system	
Pastoral	15.0
Agro—pastoral	85.0
Purpose of keeping camels	
Milk	60.0
Social and cultural values	10.0
Income generation	18.3
All the above purposes	11.6
Stage of lactation	
Early (First 3 months)	30.0
Mid (4–6 months)	45.0
Late (7 months onward)	25.0
Camel herd grazing methods	
Mixed with other animals	66.7
Grazing camels alone	33.3
Major feed sources for dairy camels	
Natural pasture	41.7
Natural pasture and crop residue	58.3
Feed shortage problems	
Yes	98.3
No	1.7
Source of water for camels	
Well	21.6
Dam/pond	51.6
River	18.3
Spring	8.3
Distance to watering point	
At home	5.0
Less than 1 km	13.3
More than 1 km	81.6
Frequency of watering	
Freely available	11.6
1 day interval	3.3
2 days interval	33.3
3 days interval	38.3
Once in a week	13.3
Housing system	
Together with other spps	66.7
Separately alone	33.3
Do you clean camel house	
Yes	81.7
No	18.3

(Continued)

TABLE 2 Major constraints of camel milk production and handling practices in three dairy farm camels in Somali region, Ethiopia.

Categories	Proportion (%)
Major constraints to she camel milk production	
Lack of Vet. Services	13.33
Fodder and Vet. Service	18.33
Shortage rangeland and vet. Services	65
Marketing, shortage of rangeland and vet. Service	3.34
Type of milking practices	
Few suck before milking	81.7
Milk without suck	18.3
Where do you milk the camel	
Outside the barn	78.3
Inside the barn	21.7
Washing hands before and after milking	
Yes	75
No	25
Washing with Soap	
Yes	16
No	84
Types of materials used for milking	
Wood container	68.3
Plastic container	15
Both	16.7
Purpose of camel milk production	
Selling	41.7
Home consumptions	3.3
Both of the above	55
Do you clean the milking equipment regularly	
Yes	96.7
No	3.3
How to consume camel milk	
Raw	96.7
Fermented state	3.3

3.4 Antibiotic sensitivity test (Kirby-Bauer disk diffusion method)

The study found that *Staphylococcus*, *Streptococcus*, and *Pasteurella* species isolates had higher susceptibility to erythromycin compared

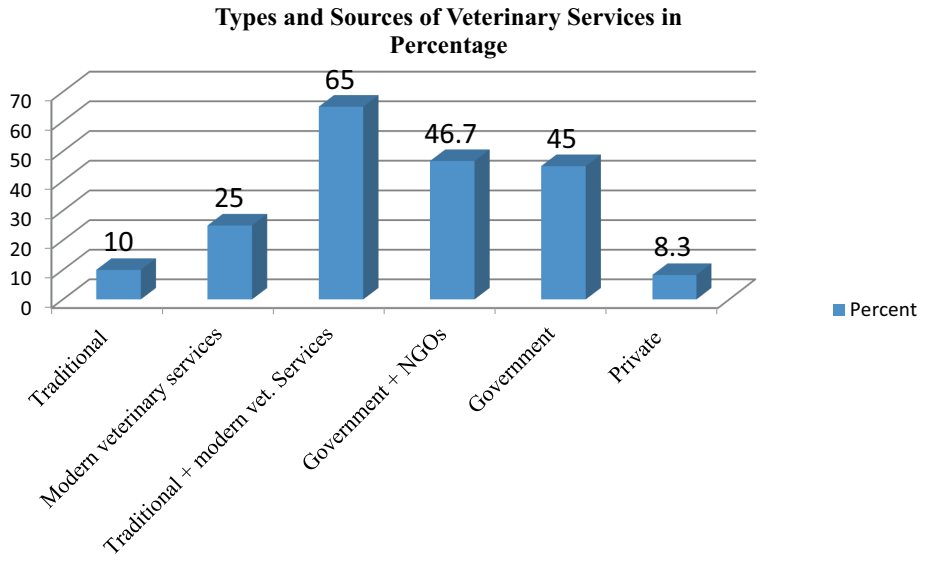

FIGURE 2
Category and sources of animal health service delivery system in the study area.

TABLE 3 Prevalence of sub-clinical mastitis in the she-camels from the three dairy farm camels in Somali region, Ethiopia.

Camel dairy farms	Number of examined	Number of positives	Prevalence (%)	χ^2	*p*-value
Dhaygeel	81	9	11.1		
Barkomal	131	13	9.9		
Suleka	32	4	12.5	0.20	0.90
Total	244	26	10.6		

to other antibiotics tested. Erythromycin was the most effective antibiotic, with high susceptibility among the identified bacterial species. Streptomycin was the second most effective antibiotic. In contrast, oxytetracycline and tetracycline antibiotics showed low sensitivity among the identified bacterial species. The resistance profile against tetracycline was relatively high among the bacteria tested (Table 6).

4 Discussion

The prevalence of subclinical mastitis in she camels in this study (10.6%) aligns with previous studies by Mohamud et al. (8) who reported 9.8% in Somalia, and Juboori et al. (22) who reported 11.67% in the UAE. Similarly, the findings are comparable to Balemi et al. (10) who reported 14.5% in Ethiopia. However, Geresu et al. (12) found a slightly higher prevalence of 18.1% in Southern Ethiopia. On the other hand, the camel mastitis prevalence found in the present study is relatively lower than the reported 59.8% in the Afar Region of Ethiopia (23), 76% in pastoral areas of eastern Ethiopia (24), 18.5% in Abu Dhabi, United Arab Emirates (25) and 34.7% in Borena zone of Oromia Regional State (26). Contrastingly, Almaw and Molla (27) reported a lower incidence of 2.1% for subclinical mastitis in lactating she camels in northeastern Ethiopia, which is below the prevalence indicated in the current study. The higher prevalence of those research studies may be attributed to differences in the management systems of camels.

The 5–7 years age group showed a significant difference in subclinical mastitis compared to other age groups, aligning with previous studies on the correlation between age of she camels and sub-clinical mastitis prevalence by Geresu et al. (12), which found a significant difference in sub-clinical mastitis among different age groups ($p < 0.05$). Additionally, our study found that udder and leg hygiene measures significantly influenced the prevalence of camel subclinical mastitis. Nevertheless, lactation stage is not significantly associated with the occurrence of camel mastitis in the study areas, with ($p > 0.05$) consistent with the findings of Mahboob et al. (28).

In addition, Alebie et al. (29) found that neither parity nor lactation stages were statistically significant in the occurrence of camel subclinical mastitis in lactating she camels from Dubti district, Afar Regional State, Northeastern Ethiopia, which is consistent with our current results. However, Mogeh et al. (30) reported a significant difference in camel subclinical mastitis across lactation stage and parity ($p < 0.05$) among lactating dromedary camels in and around Hargeisa, Somaliland. Variation in research methodologies and types of bacteria examined across previous studies may contribute to the differences in findings and discrepancies in reported rates of subclinical mastitis.

The findings of the study regarding the respondents' knowledge and practices related to mastitis in she camels are worth discussing. It is noteworthy that a significant majority of respondents (85%) were aware of clinical mastitis in lactating she camels, indicating a basic understanding of this condition. However, the complete lack of familiarity

TABLE 4 Multivariable logistic regression analysis of factors associated with subclinical mastitis in lactating camels from three dairy farms in the Somali region of Ethiopia.

Risk factors	No of examined	No of positive, *n* (%)	OR [95%CI]	χ^2	*p*-value
Location/Farm					
Barkomal	131	13 (9.9)			
Dhaygel	81	9 (11.1)	1.1		
Suleka	32	4 (12.5)	1.3	0.2	0.904
Age					
2–4 years	132	7 (5.3)			
5–7 years	36	13 (36.1)	10.1		
>7 years	76	6 (7.8)	1.5	21.8	0.0000***
Lactation stage					
Early	144	17 (11.8)			
Late	100	9 (9)	0.73	0.5	0.48
Parity					
Few (1–3)	162	14 (8.6)			
Many (≥4)	82	12 (14.6)	1.8	1.97	0.16
Production system					
Extensive	113	13 (11.5)			
Semi-intensive	131	13 (9.9)	0.84	0.16	0.69
Source of water					
Tap water	32	4 (12.5)			
Well	212	22 (10.4)	0.81	0.13	0.722
Udder and leg hygiene					
Good	170	1 (0.5)			
Medium	51	7 (13.7)	26.8	88.41	
Poor	23	18 (78.2)	608.4		0.0000***

TABLE 5 Distribution of isolates and individual prevalence of bacterial species isolated from she camels in three dairy farm camels in Somali region, Ethiopia.

Name of the farm	*Staphylococcus aureus, n (%)*	*Streptococcus agalactiae, n (%)*	*Streptococcus dysgalactiae, n (%)*	*Pasteurella spp., n (%)*
Barkomal	31 (6.1)	46 (9.0)	27 (5.2)	0 (0)
Dhaygel	80 (15.6)	51 (10.0)	29 (5.6)	40 (7.8)
Suleka	65 (12.7)	55 (10.7)	43 (8.4)	43 (8.4)
Total	176 (34.5)	152 (29.8)	99 (19.4)	83 (16.2)

TABLE 6 *In vitro* susceptibility test of bacterial profiles from camel milk samples with subclinical mastitis in somali region dairy farms.

	Staphylococcus aureus		*Streptococcus agalactiae*		*Streptococcus dysgalactiae*		*Pasteurella multocida*		Total	
Antibiotics	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Erythromycin	176/176	100	136/152	89.4	61/99	61.6	58/83	69.8	431/510	84.5
Streptomycin	93/176	52.8	110/152	72.3	54/9	54.5	49/83	59	306/510	60
Oxytetracycline	65/176	36.9	76/152	50	45/99	45.5	42/83	50.6	228/510	44.7
Tetracycline	54/176	30.6	66/152	43.4	34/99	34.3	33/83	39.8	187/510	36.7

N, Number of sensitive bacteria/evaluated number of bacteria.

with subclinical mastitis in seemingly healthy she camels among the respondents highlights a gap in knowledge that could potentially impact the health management of the camel herd. The treatment practices reported by the respondents also provide insights into the current approaches taken in managing mastitis. The fact that 65% of respondents turned to modern veterinary drugs for treating mastitis suggests a reliance on evidence-based interventions. On the other hand, the 25% of respondents who preferred traditional medicines and sought advice from experienced camel owners indicate a potential reliance on indigenous knowledge and practices. This brings to light the blending of modern and traditional approaches in managing mastitis in she camels within the community. Furthermore, the high percentage of respondents (93.3%) having access to veterinary services is an encouraging finding as it indicates the potential for professional guidance and support in managing mastitis cases. This accessibility to veterinary services can contribute to improved diagnostic and treatment outcomes for mastitis in she camels. These findings are consistent with the report by Seligsohn et al. (9).

The study findings indicate that a majority of farmers (75%) claim to wash their hands before milking. However, an even larger proportion (96.7%) regularly clean the milking equipment. It is worth noting that a significant number of farmers (84%) do not use soap during handwashing. This highlights a potential gap in hygiene practices that may require further attention and education among farmers. The absence of proper hygiene standards during milking processes could be a contributing factor to the spread of subclinical mastitis in camel herds Wang et al. (31).

The prevalence of *Staphylococcus aureus* and *Streptococcus agalactiae* in the current study, at 34.5 and 29.8% respectively, aligns with the results of Wubishet et al. (26), who reported 38.0 and 27.5% prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* isolates in mastitis-positive lactating she camels from the Borena Zone, Oromia Regional State, Ethiopia. Nonetheless, Compared to the 34.5% found in the current study, Eyassu and Bekele (32) reported a significantly lower percentage of *Staphylococcus aureus* (4.2%).

The present study has shown a high level of multi-drug resistance against commonly used drugs such as Tetracycline and Oxytetracycline. However, Erythromycin was found to be the most effective antibiotic, with high susceptibility among the isolated bacterial species, this is in line (33). Based on the results of the antibiotics sensitivity test in the current study, it can be concluded that the preferred antimicrobial drugs for treating mastitis in dairy camels should be Erythromycin first, followed by Streptomycin and Oxytetracycline in descending order.

5 Conclusion

The present study revealed a high prevalence of sub-clinical mastitis in dairy camels, indicating it as a significant health problem. Among the risk factors considered, age and hygienic measures such as udder and leg hygiene showed a significant association with the prevalence of sub-clinical mastitis in lactating she camels. The major bacterial species identified as the cause of mastitis in the study area were *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Pasteurella multocida*. The antibiotic sensitivity test indicated that Erythromycin was the preferred drug for treatment, while Oxytetracycline and Tetracycline showed the least efficacy in the study area. Based on the findings, it is recommended to focus on various aspects to address camel mastitis. These include conducting further research, utilizing traditional knowledge, increasing the availability of alternative drugs, providing comprehensive training, and implementing improved management practices. These measures aim to reduce the prevalence and transmission of the disease.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were approved by ethics committee of Somali region pastoral and agro-pastoral research institute (SORPARI). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MJ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. HH: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. ZD: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. AA: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The first protocol for assessing the welfare of dromedary camels (*Camelus dromedarius*) kept under nomadic pastoralism

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There is no protocol to measure the welfare level of dromedary camels (*Camelus dromedarious*) kept under pastoralism—the predominant husbandry system of this species. This study therefore aimed to develop and describe a protocol for measuring welfare levels in dromedary camels kept under nomadic pastoralist conditions—. The indicators for each welfare principle (i.e., Good Feeding, Good Housing, Good Health, and Appropriate Behavior) were tailored to the specific conditions of camel pastoralism, drawing from the currently available protocol for assessing welfare in dromedary camels kept in intensive and semi-intensive systems. This adaptation was achieved using a structured literature search and Expert Knowledge Elicitation (EKE). The developed protocol, covering animal-, resource-, and management-based indicators, comprises two assessment levels: 'Caretaker-Herd level' and 'Animal level'. The Caretaker-Herd level is a face-to-face interview of about 10 min including 16 questions, spit into the four welfare principles, and a visual observation of applied animal handling practices. The 'Animal level' encompasses a behavioral observation and a visual clinical inspection of randomly selected individual dromedary camels, about 5 min/camel. The 'Animal level' includes 27 welfare indicators displayed for each welfare principle. The present study also includes the score for each indicator, the model for aggregating indicators' scores into compound indices for each welfare principle (PAI), and how to classify the herds based on the PAIs or to produce an overall welfare index for each herd. Even if the proposed protocol needs to be applied, refined, and validated, it is a first step toward a standardized method to collect data related to dromedary camel welfare kept under pastoralism. This framework may ultimately guide herd managers, animal health practitioners, experienced advisers, and lawmakers in fostering optimal conditions and proposing welfare standards for dromedary camels in pastoralist settings.

KEYWORDS

animal welfare, feeding, housing, health, behavior, camels, compound welfare indices

1 Introduction

Large camelids or Old World camels (*Camelus dromedarius*), dromedary or one-humped camel, and Bactrian or two-humped camel) are known for their resilience in arid and semi-arid landscapes. The assumed camel's adaptive resilience in hostile settings may keep the notion of 'animal well-being' disregarded and considered independent of human influence. In recent decades, however, the global population of large camelids has surged due to factors like climate change-induced desertification and the demand for sustainable meat and milk production (1). This shift has led to significant changes in camel breeding practices (1, 2). Concretely, growing social and economic interests in dromedary camel husbandry at intensive and semi-intensive production systems have significantly appeared on the scene during the past three decades (3). This situation has parallelly promoted an increase in the scientific actions that are implemented, and which deal with almost any discipline applied to this animal species (1). Nonetheless, applied scientific studies on the effects of different housing systems and handling practices, on camel behavior, health, and welfare are scarce (4–7). Hence, both the traditional and currently changing dynamics of camel breeding necessitate an objective assessment of their impact on the camel's well-being for their long-term sustainability.

A pioneer protocol for the assessment of welfare in dromedary camels has been recently set up by Padalino and Menchetti (8). It applies to dromedary camels kept at intensive and semi-intensive housing systems and develops a model considering overall welfare indices (9). This protocol involves a combination of indicators, evaluated at three levels, namely Caretaker-level, Herd-level, and Animal-level. These indicators align with animal welfare principles ('Good Feeding,' 'Good Housing,' 'Good Health,' and 'Appropriate Behavior') based on the Welfare Quality® and European Animal Welfare Indicators (AWIN) projects (10, 11). In addition, to include indicators of positive welfare, in the protocol also the Five Domains model was considered (12). However, this protocol cannot be applied to dromedary camels kept under pastoralism, as in these pastoralist nomadic environments, animal husbandry methods still notably diverge from those employed in modern semi-intensive and intensive camel farming systems.

The majority of dromedary camels are raised under nomadic pastoralist conditions in the arid and semi-arid ecosystems of Africa and Asia (13, 14). Pastoralism involves the practice of raising livestock for subsistence, with practices varying from agropastoralism (a blend of plant cultivation and herding) to predominantly herding animals (15). Concretely, dromedary camel pastoralism holds profound social and economic significance for local human livelihoods. Socially, it forms the backbone of many communities, shaping cultural practices and traditions. The communal nature of camel herding often strengthens social bonds, as communities collaborate in managing herds and sharing resources. Economically, camel pastoralism provides a sustainable source of income through the sale of camel milk, meat, hides, and wool. Camels' ability to thrive in harsh environments makes them invaluable also for transportation and agricultural activities, enhancing productivity and enabling communities to access remote markets (16, 17). Dromedary camels raised in pastoralist conditions, as more aligned with their natural behaviors, have not raised, up to date, many welfare concerns from the community and the policy-makers (7). However, like all the animals kept in extensive systems, they can face other several challenges that can influence their homeostasis and thus impact both production and welfare (18). Dromedary camels kept in these semi-arid regions deal with unpredictable rainfall cycles, alternating between dry and rainy seasons. Forage availability is inconsistent, leading to periods of hunger during the dry season and potential starvation in droughts. Limited access to drinking water in arid areas also forces dromedaries to endure thirst, especially during the dry season when watering points are distant. Night enclosures, if present, are basic, exposing the animals to thermal and predator stresses (7). Furthermore, remote nomadic pastoral areas often lack veterinary services and essential drugs, resulting in regular occurrences of diseases, parasites, and associated pain and distress (19). However, neither empirical data nor a tool to objectively assess and score the welfare status of dromedaries reared in pastoralist nomadic environments exist up to the present.

To fill this gap of knowledge, this study aimed to develop and describe a novel protocol for assessing welfare in adult dromedary camels reared in pastoralist nomadic environments. Welfare principles and indicators were adapted from the existing protocol for assessing welfare in dromedary camels raised in intensive and semi-intensive systems (8) to suit the unique conditions of camel pastoralism.

2 Methods

2.1 Selection of welfare indicators

The currently available protocol for assessing the welfare of dromedary camels kept at intensive and semi-intensive systems (8) was used as the starting point. However, the nomadic nature of dromedary camel pastoralism poses a challenge. These animals typically do not inhabit a stationary pen but roam across varied landscapes even within the same day in response to the caretaker's strategic decisions mostly regarding foraging locations and water sources. Hence, to effectuate this adaptation, a comprehensive literature search and a series of meetings, during which several unstructured Expert Knowledge Elicitations (EKEs) (20) were conducted.

In particular, a scientific literature search was conducted using the following keywords: 'camel welfare', 'camel feeding, 'camel behavior', 'camel housing', 'camel health', 'camel management', 'camel reproduction', and 'camel pastoralism'. ScienceDirect, PubMed, and Scopus were the research databases used for tracking recent academic publications. It is noteworthy that the literature review was confined to English-language publications and the last 20 years. Briefly, 7,589 peer-reviewed papers (i.e., 7,456 research studies, 116 reviews, and 17 book chapters) were retrieved and the authors screened them, excluding those that were not related to animal welfare indicators applied under an extensive husbandry system. Given the scarcity of species-specific literature, the researchers also took into account peer-reviewed papers on other species, with particular emphasis on those aimed to develop and apply welfare protocols in horses and ruminants that are kept in groups and in extensive husbandry systems (21, 22).

During the meetings, a small group of experts ($n = 10$), selected from the authors' network (see acknowledges for details on them)

based on their experience in camel behavior and animal welfare, discussed and developed the recording sheets using EKEs. Starting from the recording sheets published and applied previously for the assessment of the welfare of dromedary camels kept under intensive and semi-intensive systems (8, 9), different animal-, resource-, and management-based indicators were kept or adapted in accordance with prevalent practices in the literature (23–25). While the previous protocol for dromedary camels kept in intensive systems (8) has three levels of investigation, only two levels of investigations, namely 'Caretaker-Herd level' and 'Animal level', were agreed upon for the current protocol. For each level of investigation, the experts agreed on a variety of management- resource- and animal-based welfare indicators for each welfare principle ('Good Feeding', 'Good Housing', 'Good Health, and 'Appropriate Behavior') according to the previous protocol (8) and the Welfare Quality® and AWIN methods (10, 11). However, as in the previous protocol (8), indicators more related to a positive welfare state were also selected and included. The discerning selection of welfare indicators adhered to the tenets of reliability (capability to yield consistent outcomes across various time points or when conducted by different assessors) and feasibility (time and cost efficiency), as expounded in the scientific literature for diverse species (26, 27). For instance, indicators necessitating extensive laboratory analyses, such as metabolic profiling, were not included to ensure feasibility. Additionally, considerations of animal welfare and operator safety led to the exclusion of invasive indicators or those requiring physical contact, acknowledging the potential stress these procedures could induce in untamed camels. Animal-based indicators are predominant at the 'Animal level', whereas the 'Caretaker-Herd level' primarily encompasses resource- and management-based indicators. The developed protocol, including the Caretaker-Herd level and Animal level recording sheets, was then piloted on a small number of herds ($n = 5$) on a total of 60 animals in Egypt to refine the questions and to test the reliability and feasibility of the chosen indicators.

2.2 Scoring of indicators, model for aggregation of indicators' measures, and system for classification of herds

Starting from the scoring systems proposed by Menchetti et al. (9) a 3-step process was retained useful for this protocol to aggregate the scores of each welfare indicator in each principle and assessment level into compound indices. The two systems for herd categorization proposed by the previous paper (9) (i.e., one based on the score profiles of the partial indices of the welfare principles and one on the categorization into tertiles of the total score; see paragraph 3.4) were also discussed and agreed upon using EKEs.

3 Results

The logical sequence for on-field data collection is detailed in Figure 1. Prior to conducting assessment activities, assessors are required to undergo standardized training that comprehensively covers the entire protocol. This training should include the description and application of each indicator, the order for data collection, possible constraints in protocol application, and adherence to sanitary rules.

The assessment must be scheduled to align it with pastoralists' routines. If the assessor cannot communicate in the same language, a native speaker must contact the herd manager/camel herd owner and plan a meeting to conduct the welfare assessment. During this meeting, the welfare protocol's objectives and methods must be elucidated, and permission to implement the assessment protocol must be obtained. It is crucial to clarify that the welfare assessment poses no risk to the involved dromedaries and caretakers, and all procedures are non-invasive. To minimize those risks, the assessment of dromedary camels should occur from a distance to ensure the animals are unaware of or undisturbed by the assessor's presence, the dromedary camels should only be approached gently, and the assessment must be stopped if a camel displays behavior that poses a danger to people or the animal itself. Assessors should keep this conversation concise to prevent subjective influence on results (28).

To ascertain the number of dromedary camels for assessment, it is imperative to first identify and discard those animals that are suffering from severe or acute pathological processes and are under treatment. After that, the minimum number of animals to be randomly selected for assessment at each herd can be calculated, taking into account the number of adult animals (i.e., > 3 years old) that are present within the herd and adhering to the AWIN's guidelines for the selection of small ruminants (28), and as already reported in the other protocol (8) (Table 1).

3.1 Dromedary camel welfare assessment at the caretaker and herd levels

Table 2 shows the recording sheet at the Caretaker-Herd level. The assessment at the Caretaker-Herd level includes a face-to-face interview with the herd manager/owner (i.e., the person in charge of the management of the herd under pastoralism conditions). The face-to-face interview is specifically composed of 16 close-ended queries that explore various aspects, including feeding, watering and health practices, housing conditions, and the human-animal relationship. As a part of the assessment at this level, the assessors must also observe from a distance when caretakers (s; i.e., the person involved mainly in the handling, feeding, and watering practices of the animals) are handling the camels. The assessors should focus on the attitudes and manners of the caretakers, and whether some equipment (e.g., stick) is used and the manner it is used, aligning with the principles outlined in the OIE Terrestrial Animal Health Code (42). Assessment at the 'Caretaker-Herd level' should take approximately 10 min.

The recording sheet (Table 2) shows also the scoring for each possible answer, in line with the literature (9). Briefly, a 0–2 scale was used, namely 0 = good welfare, 1 = compromised welfare, and 2 = low welfare. In the case of welfare indicators represented as a binary response (e.g., presence/absence), only scores of 0 (good welfare) and 2 (low welfare) are considered, to keep the same weight for each indicator and in agreement with the previous scoring systems (9, 10).

As in the previous protocol (8), before starting the interview (Table 2), the assessor should record the environmental parameters (at least air temperature and relative humidity) using a weather station, and the lux with a lux meter. On the recording sheet, the assessor should also note down the date, time of the day, season, and location. In regards to the location, it is important to understand whether the animals have been there for some days or are just in transit.

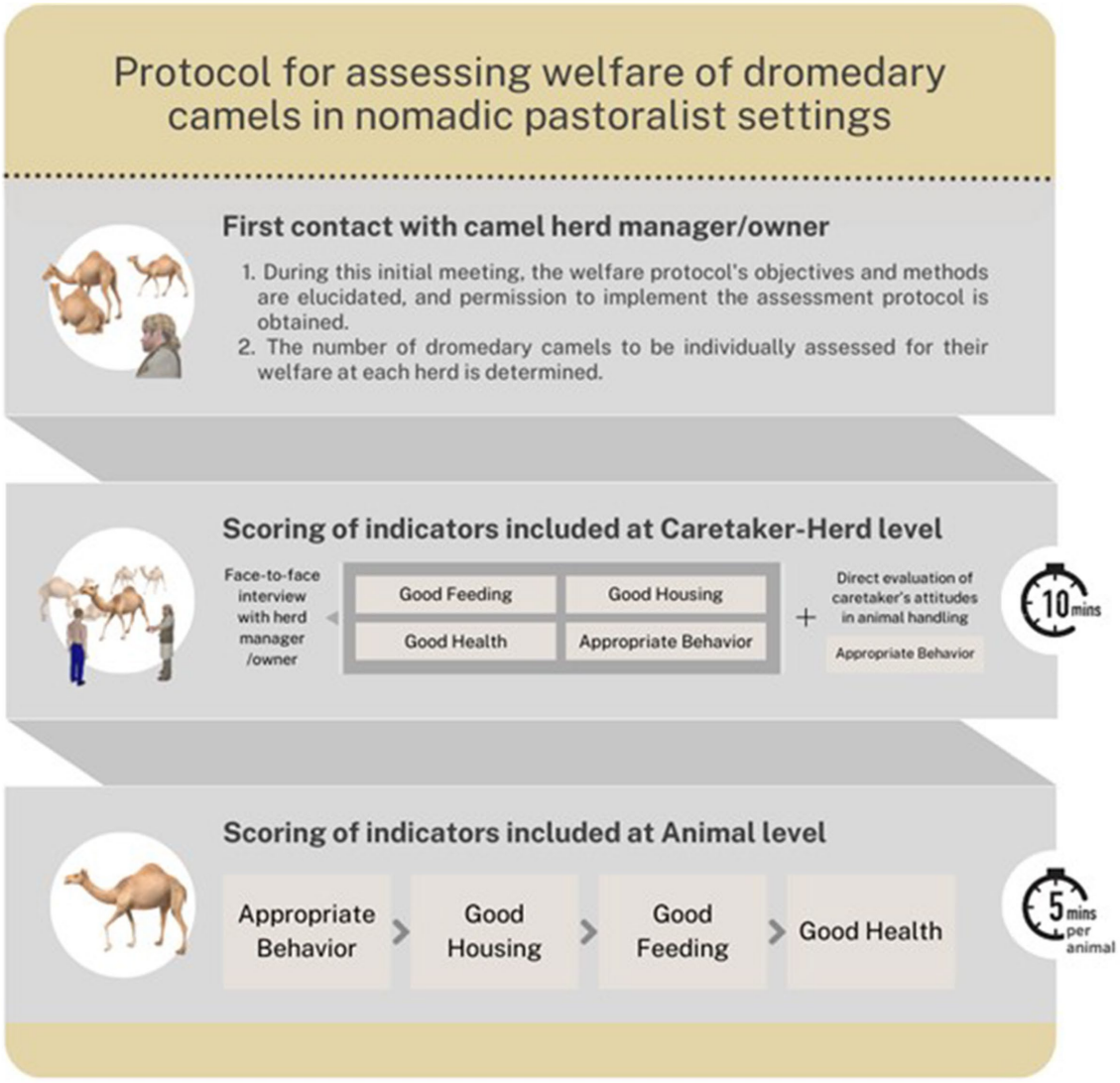

FIGURE 1
Graphical representation of the sequential data collection and processing during the on-field application of the protocol to assess the welfare of dromedary camels in nomadic pastoralist settings.

3.2 Dromedary camel welfare assessment at the animal level

Table 3 shows the recording sheet for the assessment at the Animal level. The assessment of each individual dromedary camel should take approximately 5 min per each dromedary.

Concerning the flow of steps at this assessment level, after having randomly chosen the animal to assess, a 3-min behavioral observation must be conducted without disturbing the camel. During this observation, the assessors must note down the indicators included in the Good Housing (i.e., access to shaded areas, risk injury and foreign bodies, and voluntary resting behavior), Good Feeding (i.e., food and water availability), and Appropriate Behavior (i.e., positive and aggressive camel-camel interactions, stereotypy, feeding and rumination). After the behavioral observation, the assessor must conduct the approaching test as in the other protocol (8). Briefly, the assessor approaches the camel gradually from the side, taking one step at a time to minimize stress and extending his/her arm and hand in a non-threatening manner. The test stops if the camel exhibits avoidance or aggressive behavior or when the tester successfully approaches and places a hand close to the camel's shoulder. Three different behavioral responses can be observed. Negative responses encompass defensive, anxious, avoidant, or aggressive behavior. A neutral response is characterized by the camel remaining calm and relaxed, paying no further attention to the test person. On the other hand, a positive response involves the camel approaching the test person with a positive interest, engaging in sniffing, and allowing touch or petting by the test person.

After this behavioral test, a thorough visual clinical inspection of the camel is carried out to determine its Body Condition Score (BCS) and the rest of the indicators of Good Housing (presence of ectoparasites, cleanliness, and physical restrain), as well as to check for clinical signs, presence of pain-induced practices, and injuries listed in the Good Health principle (Table 3). Body condition must

TABLE 1 Rule of thumb to determine the minimum number of dromedary camels to be individually assessed for their welfare at each herd.

Number of adult dromedary camels in the herd	Minimum number of dromedary camels to be individually assessed for their welfare
<15	All animals
15–19	13
20–24	16
25–29	19
30–34	21
35–39	24
40–44	26
45–49	28
50–59	29
60–69	32
70–79	35
80–89	37
90–99	39
100–124	41
125–149	44

be assessed using the 0–5 validate scale based on visual examination of the camel's ribs, ischial and coxal tuberosities, the hollow of the flank, and the recto-genital zone (43), and then scored on a three-point scale, considering both cachexia and obesity as a welfare concern (8). Concerning the presence of bleeding and open wounds (both shallow and deep wounds), it was agreed after the piloting that bleeding refers to the visible flow of blood from an injury or wound, whereas open wounds refer to injuries where the skin is compromised, exposing underlying tissues and not necessarily resulting in bleeding, and old scars, where the skin was not compromised anymore, must not be considered as wounds. In addition, to properly score the presence or absence of lameness, if the dromedary camel has remained in a resting position during the application of the protocol, it has to be asked, in a gentle manner, to stand up and walk for a few steps at the end of the assessment. This way, the camel's gait can be evaluated to determine if the animal can bear weight wholly or evenly, and if the course of movement is disturbed or not. By contrast, if the animal can only stand up with help or not at all and cannot bear weight on one leg or shows a relieving posture, assessing the camel in motion will not be necessary to confirm the presence of lameness.

At the end of the examination, the assessor should give his/her impression of whether the camel is in pain or not ('Evident pain'; Table 3). Currently, there are no sensitive scales for recognizing and scoring pain through physiological and behavioral responses in dromedary camels, but a composite pain scale based on the literature available on other mammal species has been recently proposed (44) and could be used as a possible reference.

Before moving to the assessment of a second animal, it is also suggested to mark the assessed one, to avoid reassessing it, as being free to move, it would be hard to track and recognize the assessed animals. It may be useful to have one of the caretakers available in case of the need to calm down a camel or for a more specific veterinary inspection in case of the identification of a possible disease that needs a more invasive diagnostic.

The recording sheet at the Animal level (Table 3) shows also the scoring for each possible answer; the scale used to score the indicators gathered at this assessment level is identical to the scale previously defined at the Caretaker-Herd level.

3.3 Model for aggregation of measures from welfare indicators

Figure 2 shows the 3-step process of aggregation of measures from welfare indicators applied to the current assessment protocol in line with the literature (9).

In the first step, scores are converted into partial indices (PIs). A total of 8 PIs per herd are calculated, namely: Good Feeding at the Caretaker-Herd level, Good Housing at the Caretaker-Herd level, Good Health at the Caretaker-Herd level, Appropriate Behavior at the Caretaker-Herd level, Good Feeding at the Animal level, Good Housing at the Animal level, Good Health at the Animal level, and Appropriate Behavior at the Animal level. In the calculation of PIs, the original 0–2 scale is transformed into a 0–100 scale, where 0 represents the lowest (i.e., unacceptable welfare) value and 100 the highest (i.e., optimal welfare). PIs are computed for each assessment level (i) and each principle (j) using the following formula:

$$\mathrm{PI}_{i,j} = 100 - \left(\frac{\sum_{m=1}^{n_{i,j}} (\text{Score of welfare indicator})_m \times 100}{k_{i,j}} \right)$$

where i is the assessment level i, j corresponds to the principle level j, n refers to the number of welfare indicators included in the j principle of the i level, and k is the highest possible total score of each principle j within each assessment level i.

The second step involves combining PIs into weighted sums, resulting in indices aggregated at each welfare principle (Principle Aggregate Indices or PAIs). Four different aggregate indices are obtained per herd: Good Feeding Index, Good Housing Index, Good Health Index, and Appropriate Behavior Index. These PAIs range from 0 (worst condition/unacceptable welfare) to 100 (best condition/optimal welfare) and offer an overall assessment for each welfare principle by herd including the scores obtained at the two levels of investigation. The relative weight of each level of assessment within the calculation of the PAIs was determined regarding the quality of the information provided by each of them. Specifically, a lower weight (20%) was attributable to the PIs of 'Caretaker-Herd level' given the fact that the recording sheet designed for this level of assessment primarily scores resource- and management-based indicators based on the responses provided by the caretaker, hence subjected to potential 'questionnaire bias' (45). The PAI for each principle j is calculated as follows:

$$\mathrm{PAI}_j = \left(\mathrm{PI}_{Caretaker-Herd,j} \times 0.20\right) + \left(\mathrm{PI}_{Animal,j} \times 0.80\right)$$

where j corresponds to the principle level j.

TABLE 2 Recording sheet to use during the dromedary camel welfare assessment at the Caretaker-Herd level.

Day:___, Time:____	Location	Season	Temperature:Humidity	Lux
Principle	Question/welfare indicator	Answer/observation	Scoring scale	Notes
Good feeding	How often do you feed the camels?	Grazing for around 10–12 h per day + supplementation	0	
		Only grazing for 10–12 h per day	1	
		Only grazing for less than 6–8 h per day	2	
	How often do you water the camels?	Always available	0	
		Available more than once daily	1	
		Available less than once daily	2	
	Total Observed Score for Good Feeding at Caretaker-Herd level			
Good housing (Environment)	Do camels have a resting place overnight?	Yes	0	
		No	2	
	How many adult animals do you have in your herd?[1,2]	<30 camels (*Small size*)	0	
		>30 camels (*Large size*)	2	
	Do the camels have access to shaded areas?	Free access during the whole day	0	
		For a short period of time per day	1	
		Never	2	
	Do you practice any type of predator control?[3]	Yes	0	
		No	2	
	Total Observed Score for Good Housing at Caretaker-Herd level			
Good health	Who routinely assesses the camel's health?	A veterinarian	0	
		A non-veterinarian	1	
		Not conducted	2	
	Who treats the camels when they are sick?	A veterinarian	0	
		A non-veterinarian	1	
		Not conducted	2	
	Are vaccinations routinely conducted?	Yes	0	
		No	2	
	Is deworming routinely conducted?	Yes	0	
		No	2	
		A non-veterinarian	1	
		Not conducted	2	
	What is the 1-year-old calf mortality rate?[4,5,6,7,8,10,11]	Below 10%	0	
		Over 10%	1	
		Records not available[9]	2	
	Do you identify your animals?	Yes, using non-invasive methods	0	
		Yes, using pain-induced practices	1	
		No[9]	2	
	Do your animals have the possibility to contact with other livestock herds (commingling)?	No	0	
		Ratherly	1	
		Yes	2	
	Total Observed Score for Good Health at Caretaker-Herd level			
Appropriate behavior	Do you have any aggressive/dangerous animals in your herd?	No	0	
		Yes, but only during the breeding season	1	
		Yes	2	

(Continued)

TABLE 2 (Continued)

Day:___, Time:____	Location	Season	Temperature:Humidity	Lux
	How many years of experience in handling camels do you have?	More than 10	0	
		Between 5 and 10	1	
		< 5 years	2	
	What is the ratio between number of caretakers and number of animals kept at the herd?	Ratio ≥ 0.05	0	
		Ratio < 0.05	2	
	Caretaker attitudes in animal handling[12,13]	Speaks, touch and/or whistles softly/quietly	0	
		Speaks, touch and/or whistles harshly/loudly	1	
		Speaking/shouting impatiently, forceful use of stick/hand	2	
	Total Observed Score for Appropriate Behavior at Caretaker-Herd level			

Questions are split accordingly with each welfare principle and possible answers are scored on a three-point scale where 0 is the best welfare condition. [1]Benaissa, Mimoune (29); [2]Schulte and Klingel (30); [3]Farah, Nyariki (31); [4]Kaufmann (32); [5]Nagy, Reiczigel (33); [6]Megersa, Regassa (34); [7]Wernery (35); [8]Jaji, Elelu (36); [9]Greene (37); [10]Nagy, Skidmore and Juhasz (38); [11]Ihuthia (39); [12]Waiblinger, Menke and Coleman (40); [13]Hultgren, Wiberg (41).

During the third and last step, PAIs are further combined to derive the Total Welfare Index (TWI) for each herd. The TWI, representing the overall assessment regardless of assessment level and welfare principle, ranges from 0 (poor welfare) to 100 (optimal welfare). All PAIs are combined with equal weights (25%) to calculate the TWI as follows:

$$\begin{aligned} \text{TWI} = &\left(\text{Good Feeding Index x } 0.25\right) + \\ &\left(\text{Good Housing Index x } 0.25\right) + \\ &\left(\text{Good Health Index x } 0.25\right) + \\ &\left(\text{Appropriate Behavior Index x } 0.25\right) \end{aligned}$$

While interpreting both PAIs and TWI, the assessors must take into account the location, the season and the environmental conditions (i.e., T and H), and whether reschedule other assessments during other climatic conditions and/or in other locations.

3.4 Criteria and welfare classes for the classification of the herds

Different welfare classes were delineated to classify the herds based on the scores of the four Principle Aggregate Indices (PAIs) as suggested by the Welfare Quality® Network (46, 47) and already applied in the protocol to assess the welfare of dromedary camels kept in intensive systems (9) (Table 4).

An example of possible graphic results of the classification of the herds based on the PAIs is reported in Figure 3. This classification is useful as it is easy to identify the welfare principles where the herds have some issues, so recommendations to enhance them can be suggested considering the welfare indicators that were inappropriate in that particular welfare principle.

Another classification of the herds can be performed using the TWI and statistical binning. Specifically, three classes can be established based on TWI tertiles, and they are labeled using a "traffic light" system: "green light" if the pen's TWI falls within the third tertile, "orange light" if it is in the second tertile, and "red light" if it is in the first tertile (9). However, to apply the binning method, the protocol needs to be applied to a specific population to accurately calculate the tertiles and compare the herds of the assessed population among them.

4 Discussion

This study developed and presented an original protocol to assess the welfare of adult dromedary camels, in any physiological states and during any seasons, kept under nomadic pastoralist conditions. This protocol was developed adapting the currently accessible protocol for assessing the welfare of dromedaries reared in intensive and semi-intensive systems (8, 9), using the literature and structured EKE. The main adaptation from the previous protocol was to eliminate a level of investigation, namely, the Herd level, where the majority of the parameters were on the pen where the animals were kept, which clearly is lacking under pastoralism. The recording sheet at the Caretaker and Herd levels were therefore joined. This integration captures the direct interactions between the caretaker(s) and the camels (i.e., animal handling and care practices) and the impact of the environmental factors that inherently shape the caretaker's decisions in regard to herds' transhumance, thus the overall well-being of the herd (48). The Caretaker-Herd level of assessment has been adapted from previously developed surveys focused on how camel caretakers manage their herds (49) and their proficiency in collecting long-term data of sufficient quality (32). Additionally, information regarding the effect of group size (29, 30), control of potential predators (31), the absence of an animal identification/traceability program (37), stock person actions, and caretaker/camel ratio (40, 41, 50) and calf mortality rate (33, 39) was retrieved and adapted from the specific literature. This conglomerate of information serves the purpose of investigating aspects of the management that are hard to capture with the instantaneous evaluation at the Animal level, considering that these animals keep moving based on the caretaker's decisions. The caretaker information is therefore crucial to evaluate the level of the welfare of the animals kept under that particular management. Moreover, information on the general management of the animals also helps to define longer-term challenges and opportunities for animals, in agreement with the most modern concepts of welfare (such as those

TABLE 3 Recording sheet to use during the dromedary camel welfare assessment at the Animal-level.

Principle	Welfare indicator	Observation	Scoring scale	Note
Good feeding	Food availability	Yes, and of good quality	0	
		Yes, but of low quality	1	
		No	2	
	Water availability	Yes, fresh and clean water is available	0	
		Yes, but of low quality (e.g., dirty, warm)	1	
		No	2	
	Body Condition Score (BCS)	BCS = 3 (good body condition)	0	
		BCS = 2 or BCS = 4 (Moderate body condition)	1	
		BCS = 0–1 or BCS = 5 (cachexia or obesity)	2	
	Total Observed Score for Good Feeding at Animal level			
Good housing (Environment)	Currently available shade	Yes	0	
		No	2	
	Risk of injury/foreign body (e.g., presence of rubbish and other foreign objects which could be eaten or could injury the camel)	No	0	
		Yes	2	
	Presence of ectoparasites	No	0	
		Yes	2	
	Camel coat cleanliness	Clean	0	
		Partially clean	1	
		Dirty	2	
	Tethered	No	0	
		Yes	2	
	Restrained into two/three legs	No	0	
		Yes	2	
	Hobbled	No	0	
		Yes	2	
	Voluntary resting behavior	Yes	0	
		No	2	
	Total Observed Score for Good Housing at Animal level			
Good health	Presence of bleeding	No	0	
		Yes	2	
	Presence of injury (open wounds)	No	0	
		Yes	2	
	Presence of swollen joints	No	0	
		Yes	2	
	Presence of lameness	No	0	
		Yes	2	
	Presence of skin disorders	No	0	
		Yes	2	
	Presence of discharge (nose, eye, vulva)	No	0	
		Yes	2	
	Presence of diarrhea	No	0	
		Yes	2	
	Presence of respiratory disorders	No	0	
		Yes	2	

(Continued)

TABLE 3 (Continued)

Principle	Welfare indicator	Observation	Scoring scale	Note
	Presence of other health disorders*	No	0	
		Yes	2	
	Presence of pain-induced management practices (cauterization, branding, nose pag, mutilation)	No	0	
		Yes	2	
	Evident pain	No	0	
		Yes	2	
	Total Observed Score for Good Health at Animal level			
Appropriate behavior	Positive social camel-camel interactions (cow-calf contact, allogrooming, sniffing)	Yes	0	
		No	2	
	Aggressive camel-camel interactions	No	0	
		Yes	2	
	Stereotypies	No	0	
		Yes	2	
	Feeding or rumination	Yes	0	
		No	2	
	Approaching test	Positive	0	
		Neutral	1	
		Negative	2	
	Total Observed Score for Appropriate Behavior at Animal level			

Indicators are split accordingly with each welfare principle and possible answers are scored on a three-point scale where 0 is the best welfare condition. *Please write down the specific health disorders observed.

of balance between positive and poor welfare) which emphasize the need to take into consideration the cumulative experiences over time (51). However, the data collected as a questionnaire are clearly reported information, so subject to bias and, although they assume a limited weight in the calculation of total welfare (i.e., 20%), must be reported as a possible limitation of the current protocol (9). Overall, the proposed protocol is a first step in the evaluation of welfare in dromedary camels under pastoralism, and it needs to be applied and refined. If it was used in multiple regions and countries, it would give data useful for the development of welfare standards.

The principle of 'Good Feeding' takes into account criteria concerning the prevention of prolonged hunger and thirst, ensuring immediate access to a suitable diet and fresh water to uphold overall health and vitality. Prolonged hunger and thirst can arise when feed and water are not readily available, inaccessible, or fail to meet not only the nutritional needs but also the behavioral needs of grazing and browsing (27). The present protocol thus included indicators of effective availability of feed and water (Animal level) and feeding strategies such as the provision of dromedary camels with the possibility of grazing (Caretaker-Herd level) and to cover their needs using supplementation in case of drought and limited pasture quality. Under natural conditions, camels predominantly engage in grazing and rumination, selecting plants rich in water and minerals (52–54). Mineral salts are crucial for thermoregulation, metabolic health, and water retention in camels (55, 56). Despite gauging feed and water quantity and potential variety, these indicators may overlook nutritional content variations and water quality, both critical factors in preventing health issues (57). Seasonal fluctuations and unpredictable environmental conditions affecting forage and water accessibility and quality may not be fully captured, and individual dietary variations based on age, sex, physiological status, and functionality may not be addressed. Hence, the indicator 'Body Condition Score' (BCS), including at the 'Animal level', is vital for assessing the long-term welfare conditions of camels. BCS is a robust animal-based indicator for evaluating medium to long-term good feeding practices in livestock species, including camels (43, 58, 59). Because of the subjective nature of body condition scoring, standardized training for scorers is highly recommended to ensure consistency (60). Moreover, it is always to take into account that a low BCS could also be associated with health issues (9), and further research inquiries are encouraged to discern the welfare implications associated with each body condition scoring category in dromedary camels, considering factors such as age, physiological state, and rearing purpose. The bucket test, included in the previous available protocol for camel welfare assessment at intensive and semi-intensive systems (8), was considered to be not feasible in the present protocol due to practical constraints (i.e., lack of materials for the implementation of the test in pastoralist settings). Similarly, other reliable ABM, as the capillary refilling time was considered not feasible as many of the camels under pastoralism are unhandled and to evaluate the CRT the animals would have needed a containment. Another indicator, namely the sunken eyes proposed by Abdalla et al. (61) was excluded as it was considered not specific, as this is often present in dehydration status caused by health problems in dromedary camels (44). However, it would be good to find a better indicator of prolonged thirst/dehydration using non-invasive smart technologies.

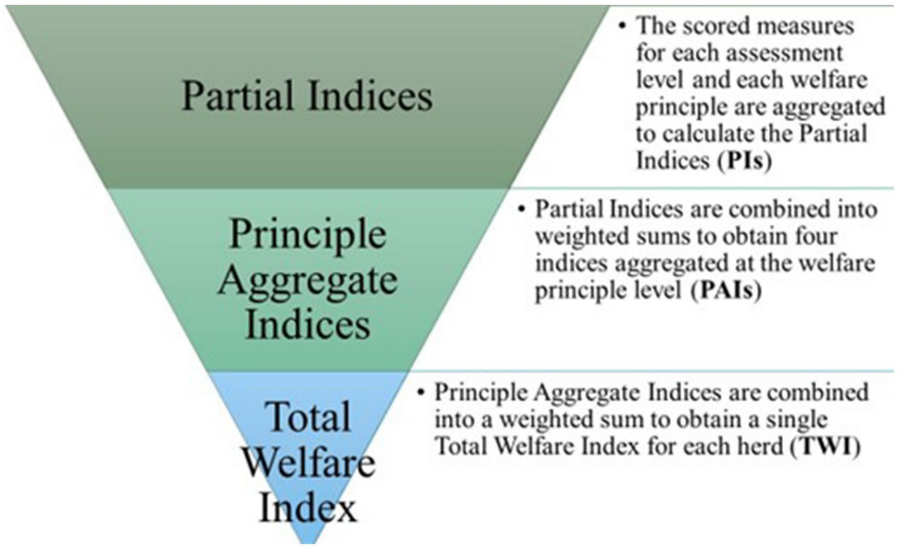

FIGURE 2
The three-step process of aggregation of measures from indicators collected to assess the welfare of dromedary camels kept under nomadic pastoralism conditions.

TABLE 4 Quantitative criteria and respective welfare categories for the classification of camel herds based on Principle Aggregate Indices (PAIs).

Parameter	Criteria	Welfare category
Principle Aggregate Indices (PAIs)	>60 for each PAI and > 80 for at least two PAIs	Excellent
	>30 for each PAI and > 60 for at least three PAIs	Satisfactory
	>20 for each PAI and > 30 for at least three PAIs	Unsatisfactory
	Failure to meet the abovementioned criteria	Unacceptable

The principle of 'Good Housing' encompasses criteria associated with herd structure and the environment where the animals are experiencing, namely comfort around resting, thermal comfort, and ease of movement. In extensive contexts, where camels exhibit a strong attachment to specific sleeping sites (30), access to suitable resting places overnight significantly contributes to their overall welfare. This provision helps reduce stress and ensure thermal comfort when sheltered areas do exist in the resting environment. Beyond these physical benefits, a resting area facilitates behavioral observations, enabling caretakers to monitor behavior and identify potential signs of distress, pain, or disease. Fenced resting areas also protect against predators, although pastoralists mostly engage in strategic grazing activities and constant surveillance to mitigate potential losses from predators (31, 62). Shaded areas accessible to camels during the day are further beneficial for thermal comfort in high-temperature environments, preventing heat stress (63, 64). Despite camels' adaptations to extreme temperatures, prolonged heat stress can result in decreased appetite, reluctance to rise, lethargy, and even death. Predisposing factors, including parasitism, lameness, weaning, inadequate nutrition, or obesity, have been listed as risk factors for heat stress (64). Providing the camels with a sheltered and fenced place with access to fresh water and food during the night, would not only enhance camel welfare but also help in the milking practices and the commercialization of the milk. Group size was also inserted as an indicator of good housing, as it significantly influences the general comfort of animals in terms of housing conditions. Where overnight resting areas or daytime shaded areas are small in relative proportion to the group size, not all animals will have free and adequate access to them. Indeed, limited space allowance can lead to a reduction of lying time in animals (65). In addition, overcrowding due to large group size can impair access to resting areas and feed resources, potentially resulting in aggressive interactions and increased risk of injury and distress (66). A group size larger than 30 animals, which doubles the average group size in natural populations (8–15 camels) (30), is also associated with a significantly increased risk of health issues (29). The latter is the reason for this threshold within the present protocol. However, as group size is largely dependent on managerial decisions, and it is the same for all the assessed herds, this specific indicator was included as a Good housing indicator at the Caretaker-Herd level.

Good Housing at the Animal level encompassed several indicators. To evaluate the impact of inadequate environment on resting and walking spaces in dromedary camel welfare (67), three specific indicators were included, namely risk of injury, presence of ectoparasites, and camel coat cleanliness. Risk of injury is a critical indicator in identifying potential hazards in the environment (e.g., the presence of sharp and protruding elements, damaged fencing areas, or elements used for animal handling/restraining, but also rubbish), then in preventing injuries and the ingestion of foreign bodies and promoting a safe living space for dromedaries. On the other hand, the presence of ectoparasites (e.g., ticks) and the cleanliness of camel coats are indicators that address the specific impact of the hygiene conditions of the living areas on the overall health and well-being of dromedary camels (63). Moreover, as good housing also responds to the guarantee of freedom of movement, some indicators on the restrain methods (e.g., presence of hobbles, restrained in three legs) were included. Movement control in camels is reported to influence

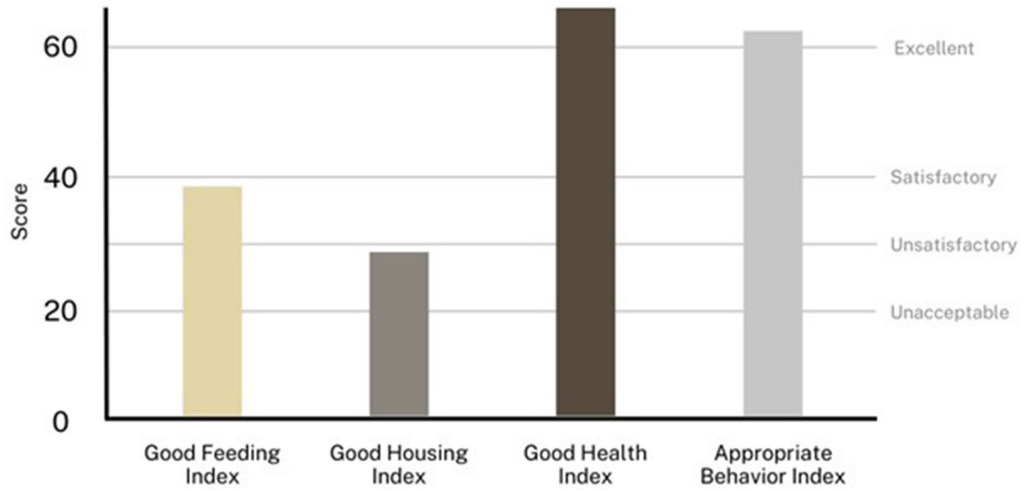

FIGURE 3
Example of classification of a herd having a score of 38 for the Good Feeding Index, 28 for the Good Housing Index, 65 for the Good Health Index, and 62 for the Appropriate Behavior Index. This herd is classified as Unsatisfactory, as all PAIs are above 20 but only three PAIs are above 30.

metabolism, benefiting feed digestion and nutrient absorption, thus the overall welfare and performance of camels (68). In pastoralist herds, the majority of camels can move freely for many hours a day. However, a small percentage may experience restricted movement on account of various factors based on individual health considerations and reproductive status (13, 69). For instance, some animals may need partial restraining due to illness, injury, or the necessity for specialized care. Additionally, limiting the movement of selected animals can aid in the strategic management of breeding programs, allowing for controlled interactions and monitoring. Although restraining can be beneficial in some instances, excessive restrictions lead to stress and discomfort due to the inhibition of movement and expression of natural behavior. Therefore, evaluating the ease of movement at the 'Animal level' allows for a focused examination of the welfare of the animals with restricted mobility within a herd.

Concerning the welfare principle of 'Good Health', the indicators included at the Caretaker-Herd level aimed to address the presence of injury, disease, pain, and pain induced by management procedures within nomadic pastoralist contexts. The inefficacy of traditional treatments, the absence of professional surgical interventions, and improper use of veterinary drugs have been listed as causes of mortality and calf mortality has been identified as one of the major welfare concerns under this type of management (13, 70). In fact, traditional husbandry practices and calf mortality rates continue to be among the major constraints affecting camel overall production in extensive pastoral settings (71). The present protocol therefore introduced indicators to scrutinize camel health care and management, particularly examining the regular monitoring of herd health (preventive and curative healthcare measures) and the expertise dedicated to camel well-being (i.e., routine involvement of veterinarians) and 1-year-old calf mortality. Concerning calf mortality in dromedary camels, these indices are reported to be quite variable (5.1–50%) due to the complex interplay of biological, environmental, and human-related factors that vary across different locations and contexts (32–36, 38, 39). Following the recommendations of Nagy et al. (38) and in virtue of the potential application of a Food Safety Management System (FSMS) to extensive livestock farming, we suggested a threshold of 10% for calf mortality rates to accurately assess the welfare of dromedary camel pastoralist herds. However, in comparison with other species, the rate is high and it could be used as an iceberg indicator (25). In addition, the absence of formal recording is considered the worst condition in terms of animal welfare within this protocol, as it may make it difficult for the proper implementation of corrective measures. Further indicators addressing animal identification and commingling (mixing camels from different herds or with other livestock species) provide a broader perspective on herd health dynamics. While an identification and traceability program is crucial for individual monitoring and minimizing the risks to public health and welfare (i.e., eradication programs) (37), commingling may expose animals to increased epidemiological risks (72). Besides, the procedures used for animal identification can induce pain; this condition is proposed to be addressed in the current protocol.

Additional indicators to be assessed at the Animal level for the principle of 'Good Health' were introduced. They are mainly clinical indicators that can signal the presence of a disease or anatomical irregularity. They are the same as the previous protocol (8) as they are easy to assess also under pastoralist conditions. While comprehensive, this indicator demands a holistic and expert understanding of camel health and the ability to differentiate between normal variations and abnormal conditions. Lastly, the presence of pain-induced management practices (i.e., cauterization, branding, nose peg, and mutilation) (73–75) and evident pain highlight the importance of assessing not only physical health but also the impact of human interventions on the psychological health of camels. Although a composite pain scale has been recently conceived based on the literature available on other mammal species (44), this needs application and validation. This validation would lead to the refinement of the specific animal-based indicator of 'Evident pain' included in the present protocol.

The principle of 'Appropriate Behavior' encompasses the evaluation of the domain of behavior, considering the possible effects that environment, con-specific, and humans may have on animal behavior

(76). Given that camels are herd animals, the assessment of social behavior constitutes a valuable measure of the general welfare of the herd. Positive social camel-camel interactions (i.e., cow-calf contact, allogrooming, and sniffing among others) provide a window into the animals' social cohesion, indicating positive affiliations that contribute to a harmonious herd structure. Conversely, aggressive camel-camel interactions highlight potential sources of stress or conflict within the herd. Identifying and understanding these aggressive behaviors is crucial for managing social dynamics and preventing injuries. Additionally, as reported by Padalino and collaborators (77), the manifestation of locomotor and oral stereotypies in camels is heightened by factors like inadequate living conditions. In extensive systems, moreover, the adequacy of time spent grazing and rumination might suggest a favorable welfare state, considering that in natural ecosystems these animals spend the majority of the time grazing and ruminating (78). In fact, feeding and rumination behavior might be related to the size of the group and the feeding/housing areas. Specifically, larger group sizes in relatively reduced areas could be expected to increase alert behavior and individual vigilance (i.e., aggressive behavior is more prevalent due to increased social interactions in reduced areas), and decrease eating time (65, 66, 79). The human-camel relationship can be evaluated using the approaching test previously applied in camels kept in intensive and semi-intensive conditions (8). The presented list of indicators could be further implemented in the near future including other behavioral traits, such as the Qualitative Behavioral Assessment (QBA), as in other species, after some specific validation studies on the term to use to evaluate camel affective states, both positive (e.g., calm, content) and negative (e.g., agitated, frustrated) emotional states (80) will be conducted.

Four indicators under the 'Appropriate Behavior' principle at the 'Caretaker-Herd level' were also included. One of the indicators focused on the presence of aggressive or dangerous animals, as aggressivity can be abnormal behavior led by inappropriate management. Moreover, it is well known that the presence of aggressive animals within a herd may impair the welfare of the other herd members, but it may be triggered by stressful situations and therefore not always observable in a short time window (81). As dromedary males can exhibit aggression during the breeding season (68), an intermediate score for this situation was created. The second indicator regarded the years of experience that caretakers have in handling camels, as lack of experience is a well-known welfare hazard (25). The third indicator focused on the ratio between the number of caretakers and the number of camels kept in the herd. The caretaker-to-animal ratio emerges as a critical factor influencing animal welfare, particularly linked to animals' responses to humans or the quality of human-animal relationships. As a reference, Des Roches (50) found that the proportion of cows that accepted being touched increased with the worker/cow ratio on the farm and the caretaker's years of experience. This phenomenon may be attributed to cows becoming more familiar with interacting with individuals exhibiting diverse appearances and gestures through the process of stimulus generalization. The fourth indicator focuses on caretaker attitudes during animal handling (40, 41). The OIE Terrestrial Animal Health Code (42) emphasizes the caretaker's responsibility for humane animal handling and care, necessitating adequate skills and knowledge to ensure adherence to animal welfare principles. Within the framework of human-animal interactions, the caretaker's attitudes depict the frequency and nature of engagements between the herd manager and the animals (82). Therefore, on-field, objective evaluation of caretaker's attitudes in camel handling, along with caretaker-to-animal ratio and behavioral tests proposed at the 'Animal level' (i.e., approaching test), is crucial for a comprehensive understanding of the human-animal relationship.

Finally, concerning the aggregation of measures from indicators to construct compound welfare indices, a model was adapted to be applied through the present protocol, following the methodology and conclusions by Menchetti et al. (9). However, based on the results of the applicability of the whole scoring and aggregation system, PAIs (indices that provide scores for each welfare principle) are concluded to be more useful and effective than LAIs (indices that provide scores for each evaluation level, i.e., Caretaker-Herd and Animal). At a practical level, PAIs could be used to identify the major issues constraining camel welfare, thus suggesting preventive, mitigating, and corrective actions to the animal caretakers (9). Therefore, LAIs were not included in the current protocol. The classifications of the herds based on the PAIs and the traffic light systems are in line with the literature, and data collection is required to see if the proposed thresholds can be applicable under pastoralism conditions. As reported in the literature (8–11), the findings of a welfare assessment should be interpreted as a snapshot influenced by the particular season and climatic conditions, and useful to suggest best practices to apply in a particular principle/domain and decide when to re-assess the herd to see if the welfare of the animals have improved.

This is a theoretical protocol and it has all the limitations already listed for similar papers (51, 76). In particular, the limited literature related to dromedary camel welfare forced the authors to look into the welfare assessment of other species kept under extensive husbandry systems, and this may require a refinement of the protocol after it is applied more times. Moreover, for the moment this protocol has been developed for adult dromedary camels, in any physiological state (pregnant and non-pregnant, lactating and non-lactating), a specific protocol for calves and pre-puberty animals should be developed, applied, and validated as it was done in other species (10). In the literature, welfare assessment protocols are tailored based on the age (young vs. adult animals) and the husbandry systems (intensive vs. extensive husbandry systems), more specific protocols based on seasons or physiological states could be more accurate but would require further studies. Data collections are also needed to refine and validate the scoring systems and the suggested PAIs and classifications. Currently, there is a scientific debate whether aggregation should be performed and how it should be performed, and how often herds should be assessed to safeguard animal welfare during the entire life of the animals (10, 11). Finally, a level of investigation is based on an interview with the farmer, and the answers could be biased by the farmer's background, education, and experience (49). However, notwithstanding the aforementioned limitations and the fact that the protocol needs future applications and refinements, this is a first step; the current study indeed proposes a tool that has the benefit of using a standardized protocol in animal welfare assessment (42). However, it is worth highlighting that the introduced protocol is not intended solely for research purposes but to encourage governmental organizations and expert advisors to start assessing the welfare of camels with a standardized protocol, as only when there will be a harmonized way in welfare data collection, there may be enough data and research-based evidence to propose welfare standards for camels.

5 Conclusion

This theoretical paper describes the process of how a new tool to assess welfare in dromedary camels kept under nomadic conditions was developed. Several indicators were selected at two levels of investigations based on literature and expert knowledge and the reasons beyond their selection and scores and their limitations were discussed. However, the presented protocol signifies the initial phase of an extensive process starting with its application in different regions and countries, the refinement and validation of the proposed indicators, and the identification of thresholds for their acceptability. Further studies are therefore needed to apply the present protocol on several herds kept under pastoralism; data will be firstly collected on a high number of animals (at least 1,000) kept in different herds (at least 50) in a specific area of a country, and then similar data collections will be carried out in different countries until there will be data enough to propose minimal welfare standards. The protocol could also be refined and applied to assess the welfare of Bactrian camels under pastoralism, but more studies should be carried out to test its feasibility.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

BP: Writing – original draft, Resources, Funding acquisition, Data curation, Conceptualization. LM: Writing – review & editing, Data curation, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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True uterus didelphys in she-camel: a case report and review of literature

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Background: Uterus didelphys is a rare congenital anomaly of the female reproductive tract characterized by a divided uterine cervix and body. It occurs due to abnormal development of the paramesonephric (Müllerian) duct. Different forms of uterus didelphys have been reported in several animal species, including bovine, equine, ewe, goat, swine, and bitch. However, there is no previous report that has documented a completely divided female genital tract in she-camel. Moreover, there is a lack of literature regarding this anomaly in animals. Therefore, the present study reports, for the first time, a rare case of a completely divided female genital tract in a she-camel. In addition, the existing relevant literature on uterus didelphys in different animal species is reviewed.

Case presentation: A female reproductive tract of she-camel, approximately 10 years old, with a history of previous successful pregnancy, was brought to the anatomy department following the slaughtering of the animal. Initial examination revealed a normal reproductive tract consisting of two ovaries, two fallopian tubes, a uterus, and a vagina. A closer examination revealed a completely divided vagina, with an external os opened into each part of the vagina, as well as a divided uterine body and cervix. Intrauterine infusion of saline through one external os confirmed complete separation of uterine body and cervix.

Conclusion: To the authors' knowledge, this is the first reported case of a completely divided female genital tract in a she-camel. This review summarizes the previous reports about uterus didelphys in farm animals.

KEYWORDS

congenital abnormalities, Müllerian duct anomalies, she-camel, uterus didelphys, case report

Introduction

Uterus didelphys is a rare congenital anatomical defect of the female reproductive tract of different animal species, including bovine, equine, sheep, goat, and pig (1–6). It happens due to the failure of fusion of the two paramesonephric (Müllerian) ducts, the primordium of the female reproductive tract. This congenital anomaly varies according to the degree of fusion failure of the two Müllerian ducts (7). A complete fusion failure of the two ducts results in uterus didelphys (true uterus didelphys), which is characterized by a completely divided genital tract including a double uterine body, two separated crevices, and a longitudinal vaginal septum (7–9). Partial fusion failure results in varying degrees of division affecting either the caudal part of the uterine body, cervix, or vagina, according to the area affected (7).

Abusineina (10) classified cervical abnormalities into four main types: uterus didelphys, complete double cervix, incomplete double cervix, and double external uterine orifices.

Uterus didelphys

Uterus didelphys (completely divided female genital tract) is caused by the persistence of the median walls of the Müllerian ducts along their entire length, resulting in two cervices and two separate uterine bodies.

Complete double cervix

It is caused by the persistence of the median walls of the Müllerian ducts along the whole length of the cervix, resulting in two cervices and one uterine body.

Incomplete double cervix

It is caused by the persistence of the median walls of the Müllerian ducts at the posterior part of the cervix, resulting in one cervical canal cranially and two cervical canals caudally.

Double external uterine orifices

It is caused by the persistence of the median walls of the Müllerian ducts at the external uterine orifice, resulting in one cervix with a band of tissue at the external os.

Different types of cervical abnormalities reported in different animal species are listed in Table 1. Clinical studies reported that congenital anomalies due to fusion failure of the Müllerian ducts are associated with reproductive difficulties (35). Ishiyama (36) reported that severe cases of incomplete fusion, such as double external os with blind diverticulum, complete double cervix with blind diverticulum, and uterus didelphys, are associated with infertility in cattle, while double cervix and external cervical os increase the incidence of dystocia in animals (33).

Although different forms of uterus didelphys have been reported in several animal species, only a case of double cervix and divided vagina has been reported in she-camel (11). There is no previous report that has documented a case of true uterus didelphys (completely divided female genital tract) in she-camel. Moreover, there is a lack of literature regarding this anomaly in animals. Therefore, the present study reports, for the first time, a rare case of a completely divided female genital tract in a she-camel. In addition to reviewing the existing relevant literature on uterus didelphys in different animal species, the current paper provides a brief summary of the anatomy,

TABLE 1 Different reported types of cervical abnormalities in different animal species.

Anomaly	Species	Anatomical feature	Reference
- Double cervix and double vagina	She-Camel	- Double cervix and divided vagina	(11)
- Uterus didelphys	Cow	- Two cervices and two separate uterine bodies	(10, 12)
		- Two cervices and two uterine bodies with divided cranial vagina	(2, 13)
- Complete double cervix		- Two cervices and one uterine body	(10, 14–17)
- Incomplete double cervix		- One cervix with a band of tissue divides the external os	(10)
- Double external uterine orifices		- Double cervix and divided vagina with normal uterus	(10)
- Double cervix and double vagina		- Double cervix and divided vagina with normal uterus	(18)
- Uterus didelphys	Buffalo	- Two cervices and two separate uterine bodies	(4, 19)
- Double cervix		- Double cervix, each cervix opens directly in the uterine horn with an absent uterine body and intercornual ligaments	(20, 21)
- Uterus didelphys	Ewe	- Two cervices and two uterine bodies with divided anterior vagina	(22, 23)
- Complete double cervix		- Two cervices and one uterine body	(24)
- Double cervix and double uterine body		- Two uterine bodies and two cervices with one cervix open into the common vestibule.	(25)
- Uterus didelphys	Goat	- Double cervix and divided uterine body	(26)
		- Two cervices and two uterine bodies with divided anterior vagina	(3)
- Double cervix and double vagina		- Double vagina and cervices.	(26)
- Double cervix	Mare	- Two cervical canals	(27)
- Double cervix and divided cranial vagina		- Double cervix with divided anterior vagina and common uterine body.	(5)
- Double cervix and double uterine body		- Double cervix with divided uterine body	(28–30)
- Double cervix	Sow	- Completely divided cervix	(31)
- Triple cervices and double uterine body		- Three cervical canals open at the divided uterine body with two cervical canals open into the right side and one canal into the left side.	(6)
- Double cervix and double vagina	Bitch	- Separate uterine horns, double cervix, and two vaginal canals.	(32)
- Double cervix and divided cranial vagina		- Double cervix with divided cranial vagina and common uterine body.	(33)
- Double vagina		- Two vaginal canals and one vestibule	(34)

physiology, and development of the female reproductive tract in she-camels.

This condition is thought to be hereditary and associated with a recessive gene of unknown etiology. Cases of cervical duplication are detected incidentally at the time of breeding (33). The absence of Müllerian inhibiting substance (MIS) induces the differentiation of the Müllerian duct, forming the female reproductive tract (37). The cranial part that runs parallel to the mesonephric ducts and the transverse part that crosses the mesonephric duct develop, forming the epithelium of the fallopian tubes. The medial walls of the caudal fused part degenerate, forming a single canal (38) which develops into the epithelium of the uterus and cranial vagina (39). The species-specific morphological characteristics of the uterus (either simplex, bicornuate, or duplex) depend on the degree of fusion of Müllerian ducts being either complete, partial, or incomplete (38). The differentiation of Müllerian ducts is regulated by the members of Hoxa genes, specifically Hoxa9, Hoxa10, Hoxa11, and Hoxa13 (8, 37). The morphological diversity in the uterus among different animal species is due to different expression levels of Hoxa13 and Hoxd13gene (38). Other genes involved in the development of the Müllerian ducts include Emx2, Pax2, Lim1, and members of Wnt family 4 (Wnt4, Wnt5a, and Wnt7a) (8, 37, 40, 41).

Case presentation

A female reproductive tract of she-camel was brought to the anatomy department, Faculty of Veterinary Medicine, King Salman International University, Egypt, following slaughter at an abattoir in Giza governorate. Consultation with the owner revealed that the animal's weight was approximately 270 kg and aged approximately 10 years old, with a history of previous successful pregnancy. Initial gross examination revealed a normal reproductive tract consisting of two ovaries and two flexuous fallopian tubes; each tube opened into a uterine horn. The two ovaries were normal and functional, as evidenced by the presence of some growing follicles on the left ovary and corpus luteum on the right one. The two horns were attached to a uterine body, followed by a cervix and vagina with no oblivious marks externally. The vulva and the caudal part of the vagina had been cut away. Closer examination of the reproductive tract revealed the presence of a completely divided vagina. Each division of the vagina had an external os opened into it (Figure 1). Morphometric measurements of the different parts of the female reproductive tract in this case were taken using a caliper. The data are listed in Table 2.

Intrauterine infusion of saline solution through the left external os revealed complete separation of uterine body and cervix. The uterus

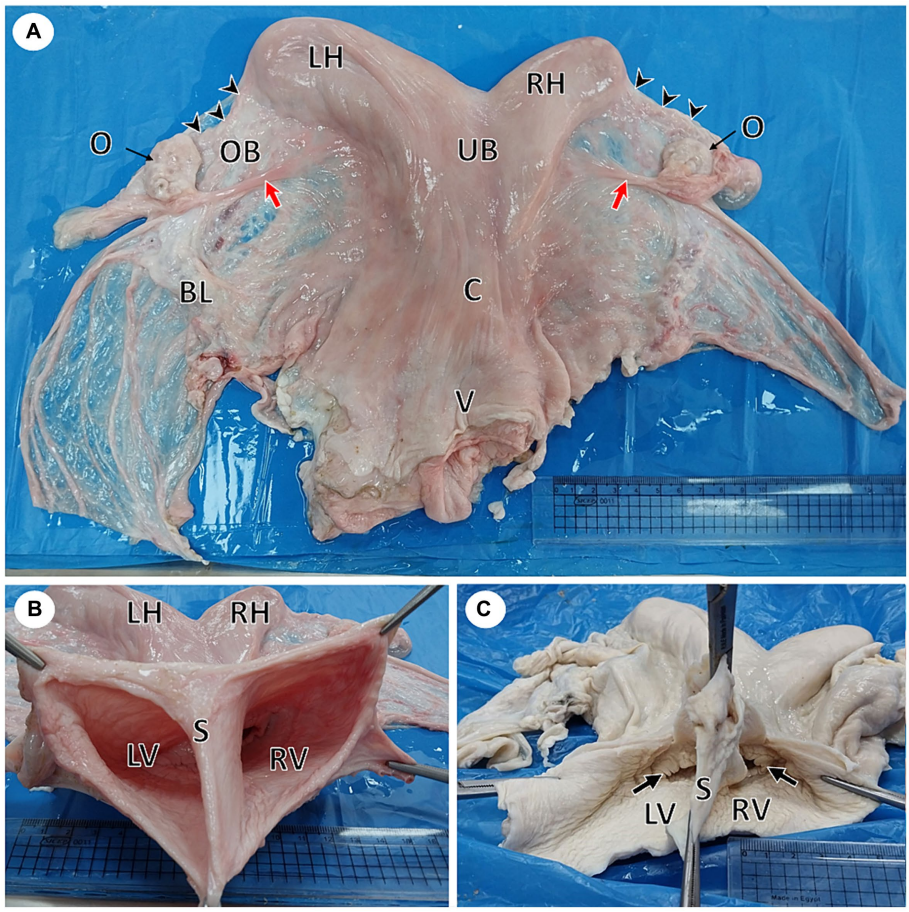

FIGURE 1
Gross photographs of female reproductive system of a she-camel show: **(A)** Apparently normal reproductive system consisted of right and left ovaries (O), each ovary was located inside an ovarian bursa (OB), the ovary attached to the uterine horn by the round ligament (red arrow), fallopian tubes (arrowheads), right (RH) and left (LH) horns, uterine body (UB), cervix (C), and vagina (V). Note the broad ligament of the uterus (BL). **(B)** Per vaginal view shows a completely divided vagina into the left (LV) and right (RV) vagina by a median septum (S). **(C)** The dorsal wall of the vagina was opened, showing each vagina had its external os (arrow).

TABLE 2 Morphometric measurements of the female reproductive tract of the reported case.

Dimensions	Measurements (mm)
Right ovary: –Length	26
–Width	22
Left ovary:–Length.	36
–Width	22
Right Fallopian tube length	144
Left Fallopian tube length.	161
Right uterine horn: –Length	68
–Width at the upper third	26
–Width at the middle third	38
–Width at the lower third	46
Left uterine horn:–Length	112
– Width at the upper third	33
–Width at the middle third	46
– Width at the lower third	58
Width of the uterine body	78
Right vagina width	62
Left vagina width	75

was then preserved in 10% neutrally buffered formalin. Later, a longitudinal incision was performed through each uterine horn, passing through the uterine body, cervix, and vagina. A complete longitudinal septum extending from the fundus to the vagina was observed. Each horn was connected to a separate uterine body that had its own internal and external os (Figure 2).

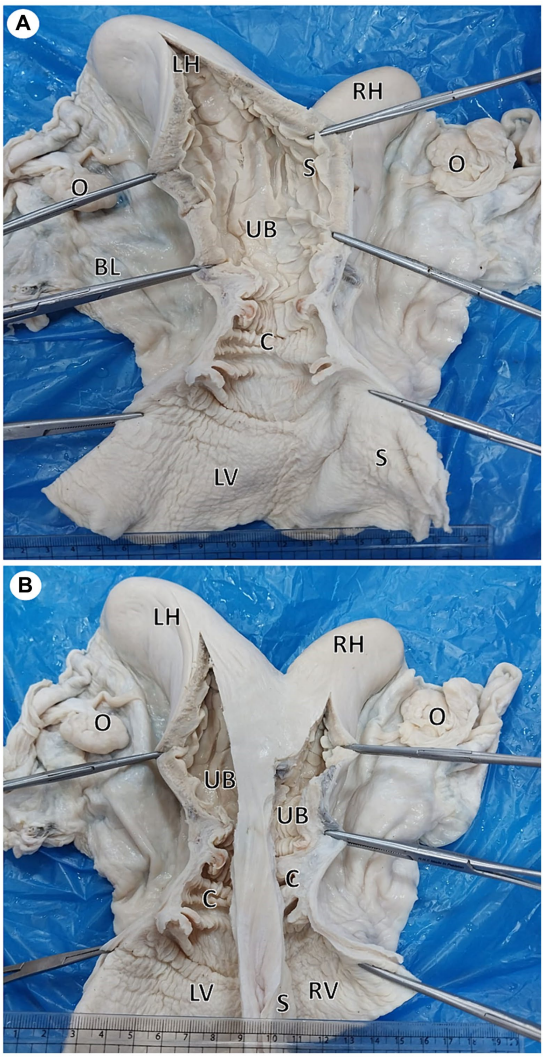

FIGURE 2
Gross photographs showing a dorsal view of uterus didelphys: **(A)** An incision was performed in the left horn (LH), body (UB), cervix (C), and vagina (LV). Note the complete septum extending from the fundus to the vagina. **(B)** Incised right (RH) and left (LH) horns show each horn connected to a separate uterine body that had its own internal and external os.

Discussion

The female reproductive tract develops from the paramesonephric (Müllerian) ducts. The Müllerian duct appears as a longitudinal invagination of the genital ridge lateral to the mesonephric (Wolffian) ducts. This invagination deepens and then separates from the peritoneal lining, forming a solid cord, which canalizes later (37, 39). The Müllerian ducts run lateral and parallel to the Wolffian ducts, with their cranial ends opening into the coelomic cavity with a funnel-like structure. The ducts pass caudomedially and ventrally, crossing the mesonephric ducts to fuse, forming the uterovaginal duct, with its caudal end projecting into the urogenital sinus, forming the Müllerian tubercle (8, 39).

Uterus didelphys is a rare congenital anomaly of the female reproductive tract that has been reported in different animal species (3, 4, 19, 20, 24, 25). Abusineina (10) classified cervical abnormalities into four main types: uterus didelphys, complete double cervix, incomplete double cervix, and double external uterine orifices. In the present case, the uterus was completely divided by a longitudinal septum, resulting in two uterine bodies, two separated cervices with their own internal and external os, and a completely divided vagina, indicating a case of uterus didelphys.

The condition has been attributed to the failure of fusion of the two paramesonephric (Müllerian) ducts. This congenital anomaly varies according to the degree of fusion failure of the two ducts (7). A complete fusion failure of the two ducts results in a double uterine body, the uterus didelphys. In this case, each uterine horn opens into a separate uterine body that leads to two separate crevices with a longitudinal vaginal septum (7–9).

The present case had functional ovaries, as indicated by the presence of growing follicles on the left ovary and a corpus luteum on the right one, which indicated normal cyclic activity of the ovary. Clinical studies have reported that congenital anomalies due to the fusion failure of the Müllerian ducts are associated with reproductive difficulties (35). Ishiyama, Nakamura (36) reported that severe cases of incomplete fusion, such as double external os with a blind diverticulum, complete double cervix with blind diverticulum, and uterus didelphys, are associated with infertility in cattle, while the double cervix and external cervical os increase the incidence of dystocia in animals (9, 33). Uterine anomalies restrict uterine space, which either alters or decreases the efficiency

of the functional placenta, causing fetal growth deficiency (42). Moreover, artificial insemination is considered a challenge as the semen might be deposited in the cervix on the side opposite to the ovary from which ovulation has occurred. However, a cow with uterus didelphys had a normal birth after semen was introduced into each cervical canal (14). The present case had a history of previous successful pregnancies following natural mating and normal parturition. In accordance with the present data, reports claim that animals with uterus didelphys undergo a normal conception rate following natural mating (9, 20). Moreover, pregnancy has been reported in a cow (12) and an ewe (24) with uterus didelphys. Chethan and Singh (20) claimed that the condition is hereditary and associated with recessive genes of unknown etiology. Therefore, such animals should be excluded from breeding after diagnosis.

Uterus didelphys can be diagnosed during the pre-breeding examination, through a variety of methods, including physical examination using vaginoscopy, endoscopy, transrectal ultrasonography, and intrauterine injection of saline through one cervix to visualize the two separate uterine bodies with the median septum in between (28).

Molecular (DNA) marker-assisted selection and cytogenetic analyses linked to genes of interest with significant impacts on reproduction, such as WNT and HOXA genes, can be reinforced for the early selection of breeding females. This would aid in the development of cytogenetic profiles and molecular markers for diagnosing reproductive diseases with a genetic or physiological origin. This, in turn, would allow for the detection of animals with reproductive disorders and enable culling at an early stage (43).

In conclusion, the present study reported, for the first time, a rare case of uterus didelphys in a she-camel. The uterine body, cervix, and vagina were divided completely by a longitudinal septum extended from the fundus to the vagina. This rare case may have an educational role, either for the students studying the congenital abnormalities of the reproductive tract or for the practitioner/technician in the field, who should be alert for the pre-breeding diagnosis of conditions that may hinder female fertility. Moreover, the current study provides an overview of uterus didelphys in farm animals.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the study was done on slaughterhouse materials, so no need for ethical approval. Written informed consent was obtained from the owners of the animals for the publication of this case report.

Author contributions

MM: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. MN: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Development and application of a 1K functional liquid chip for lactation performance in Bactrian camels

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Introduction: The advancement of high-throughput, high-quality, flexible, and cost-effective genotyping platforms is crucial for the progress of dairy breeding in Bactrian camels. This study focuses on developing and evaluating a 1K functional liquid single nucleotide polymorphism (SNP) array specifically designed for milk performance in Bactrian camels.

Methods: We utilized RNA sequencing data from 125 lactating camels to identify and select 1,002 loci associated with milk production traits for inclusion in the SNP array. The array's performance was then assessed using 24 randomly selected camels. Additionally, the array was employed to genotype 398 individuals, which allowed for population validation to assess the polymorphism of SNP sites.

Results: The SNP array demonstrated high overall SNP call rates (> 99%) and a remarkable 100% consistency in genotyping. Population validation results indicate that camels from six breeding areas in Northwest China share a similar genetic background regarding lactation functionality.

Discussion: This study highlights the potential of the SNP array to accelerate the breeding process of lactating Bactrian camels and provides a robust technical foundation for improving lactation performance.

KEYWORDS

lactation performance, SNP markers, RNA-seq, liquid chip, Bactrian camel

1 Introduction

The versatile applications of Bactrian camels, such as their provision of milk, meat, and down, in addition to their adaptability to various ecological conditions, render them a crucial species in both contemporary and conventional agricultural practices. Notably, their milk is not only a dietary staple but also renowned for its medicinal properties, making it a crucial resource for both nutritional and therapeutic purposes (1–3). However, the full potential of

Bactrian camel milk production is yet to be harnessed, often limited by the lack of advanced breeding strategies that focus on lactation performance traits.

Single nucleotide polymorphism (SNP) chips are transforming animal breeding; low cost "assay-by-sequencing" methodologies and high quality reference genome sequences provide the opportunity for further significant improvement in both breeding and management (4). Functional SNPs are generally defined as SNPs from genome sequences that affect structure, expression, or function of a gene. SNPs, which were located within expressed genes, are especially important because they have the potential to change the function of a protein (5). RNA sequences are exclusively transcribed from exonic regions of the genome, making them ideal candidates for developing markers specific to these genic regions. Such markers are particularly valuable in domestic animal breeding. Several studies used RNA sequencing to identify markers in human (6) and animals (7–14). However, the genetic underpinnings of lactation traits in Bactrian camel remain insufficiently explored.

Single nucleotide polymorphism (SNP) arrays represent a high-quality and user-friendly platform for genotyping (15, 16). Utilizing a SNP array enables the simultaneous detection of tens of thousands of SNPs per sample, thereby facilitating high-throughput and efficient methodologies in genetic research and breeding programs. More recently, advances have led to the development of a liquid SNP chip panel, leveraging Genotyping By Targeted Sequencing (GBTS) technology (17). This innovation aims to further reduce costs and enhance the accuracy of genomic selection. When compared to traditional SNP chips that utilize magnetic beads, liquid-phase chips offer several advantages, including reduced cost and increased flexibility (17, 18).

However, despite the widespread application of various high-throughput methods in the genetic study of dairy cattle, poultry, and aquatic animals, their utilization remains limited in Bactrian camel research. Based on high-quality SNPs selected from transcribed regions, GBTS was utilized to develop a 1K functional SNP liquid array, named "CamelBell No. 1," for Bactrian camels. The genotyping performance and prediction accuracy of the 1K SNP liquid array were validated. This array will become a valuable tool for enhancing Bactrian camel milk performance, attributable to its stable genotyping capabilities and its robust correlation with lactation traits in Bactrian camels.

2 Methods

2.1 Samples collection

Phenotypic data, along with milk and blood samples, were meticulously collected from 125 lactating Alxa Desert Bactrian camels located at the Alashan League, Inner Mongolia. All camels were in a consistent lactation period, with a parity ranging from 2 to 5, and were maintained in optimal body condition within a uniform environment. They were fed the same diet: dry clover supplemented with 2 kg of grain concentrate (68% corn +12% wheat bran +20% soybean cake after oil extraction) and 30 g of table salt for each animal daily. Blood and milk samples were collected from 32 and 83 Bactrian camels at approximately 30 and 270 days postpartum, respectively. Daily milk production was estimated based on the average milk yield over three consecutive days. For compositional analysis, 25 mL of milk was sampled to determine the concentrations of key constituents such as fat, protein, and lactose using mid-infrared spectrometry (MilkoScan Minor, Foss Analytics, Hillerød, Denmark). Additionally, 10 mL of whole blood was collected from the jugular vein of each camel for genetic analysis. Blood samples were treated with TRIzol reagent (TaKaRa, United States) and stored at −80°C until RNA extraction could be performed.

Genomic DNA was collected from 398 Bactrian camels used for chip genotyping. These camels were from six key breeding regions in China, which are major areas for Bactrian camel breeding. Specifically, samples were obtained from Subei County, Gansu (GSB; 7 individuals), Alxa Right Banner, Inner Mongolia (NAY; 82 individuals), Alxa Left Banner, Inner Mongolia (NAZ; 257 individuals), Sunit Right Banner, Inner Mongolia (NSNT; 22 individuals), Siziwang Banner, Inner Mongolia (NSZW; 10 individuals), and Urad Rear Banner, Inner Mongolia (NWLT; 20 individuals). The dataset comprises three breeds of Bactrian camels: Alxa Desert Camel ($n = 343$), Alxa Gobi Camel ($n = 22$), and Sunit Bactrian Camel ($n = 33$). Alxa Desert Camels were sampled from GSB, NAY, and NAZ, while Alxa Gobi Camels were exclusively sampled from NWLT, and Sunit Bactrian Camels were sampled from NSNT and NSZW. The methodologies used in this study were approved by the Institutional Animal Care and Use Committee of Inner Mongolia Agricultural University, Hohhot, China.

2.2 RNA extraction and sequencing

We isolated total RNA from 125 blood samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The blood samples were homogenized in TRIzol reagent and chloroform, followed by precipitation using isopropanol. Total RNA from each sample was treated for genomic DNA contamination using the RNase-free DNase set (QIAGEN, Crawley, West Sussex, United Kingdom) and purified using the RNeasy mini kit according to the supplied guidelines (QIAGEN, Crawley, West Sussex, United Kingdom). RNA sample quality was assessed using the NanoPhotometer® spectrophotometer (IMPLEN, CA, United States) and the Agilent Bioanalyzer 2100 system. RNA samples exhibited 28 S/18 S ratios ranging from 1.8 to 2.0 and RNA integrity number values between 8.0 and 10.0.

For mRNA cDNA library preparation, 1.0 μg of total RNA was utilized from each sample using the TruSeq RNA Library Preparation kit v2 (Illumina, San Diego, CA, United States). Poly A-containing mRNA was enriched from the total RNA using poly-T oligo attached beads and fragmented for first-strand cDNA synthesis, followed by second-strand synthesis. The ends were repaired, and 3′ end adenylation and adapter ligation were performed for each library. Subsequently, libraries were polymerase chain reaction (PCR) amplified, validated using the Bioanalyzer (Agilent Technologies Inc., Cedar Creek, TX, United States), and finally normalized and pooled. Clustering of the index-coded samples was conducted on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. Following cluster generation, the library preparations were sequenced on the Illumina NovaSeq 6000 high-throughput sequencing platform, generating 150 bp paired-end reads.

2.3 SNP detection and selection

The identification of SNPs was executed following the workflow depicted in Figure 1, with the comprehensive SNP

calling pipeline script provided in Supplementary file S4. Fastp v0.23.4 (19) was employed to assess the quality of sequence reads, targeting the identification of sequencing read artifacts such as low-quality Phred scores, duplicated reads, uncalled bases (N sequences), and potential contamination. Subsequently, the filtered reads from each sample underwent individual alignment to the Bactrian camel reference genome (Ca_bactrianus_MBC_1.0) using Hisat2 v2.1.0 software (20). The aligned results were exclusively utilized for Single Nucleotide Polymorphism (SNP) calling, while insertions and deletions (indels) were excluded from the analysis due to challenges associated with accurate indel calling. Variant calling was performed for each read merging method using the "mpileup" and "call" commands from BCFtools v1.9–77-gd0cf724+ (21). Only those SNP variants were retained where the alternative allele manifested in all samples, accompanied by a Phred quality score of at least 25 and a minimum read depth of 10. We calculated the minor allele frequencies (MAFs) and missing rates using PLINK v1.90 (22), and the SNPs with MAF <0.05 were excluded. We tested the Hardy–Weinberg equilibrium (HWE) using VCFtools v0.1.11 (23) with the -hwe option and removed SNPs that severely departed from HWE ($p < 0.01$).

2.4 Development of the SNP panel

2.4.1 SNP identification with camel milk traits

To identify SNPs crucial to Bactrian camel lactation and to develop functional chips essential for enhancing their breeding, we conducted Exon-Wide Selection Signature (EWSS) and Genome-Wide Association Study (GWAS) algorithm (24) analyses on carefully evaluated SNPs. Initially, EWSS is an SNP screening method based on transcription level by detecting the F_{ST} index of population differentiation. Thus, from 83 Bactrian camels at 270 days postpartum, we selected 30 individuals each, based on traits such as milk yield, milk protein, lactose, and milk fat, and conducted F_{ST} analysis on these extreme groups using VCFtools v0.1.11 (23). The single-locus F_{ST} for each SNP in transcriptomic regions was calculated, and those with an F_{ST} greater than 0.2 were retained. The Genome-Wide Association Study (GWAS) analysis was conducted utilizing the Genomic Association and Prediction Integrated Tool (GAPIT) within the R programming environment (25). The association analysis examined individual markers among 19,177 single nucleotide polymorphisms (SNPs) in relation to the best linear unbiased estimate (BLUE) value of each accession for each trait. The BLUE values provide unbiased estimates of phenotypic traits, incorporating fixed effects such as

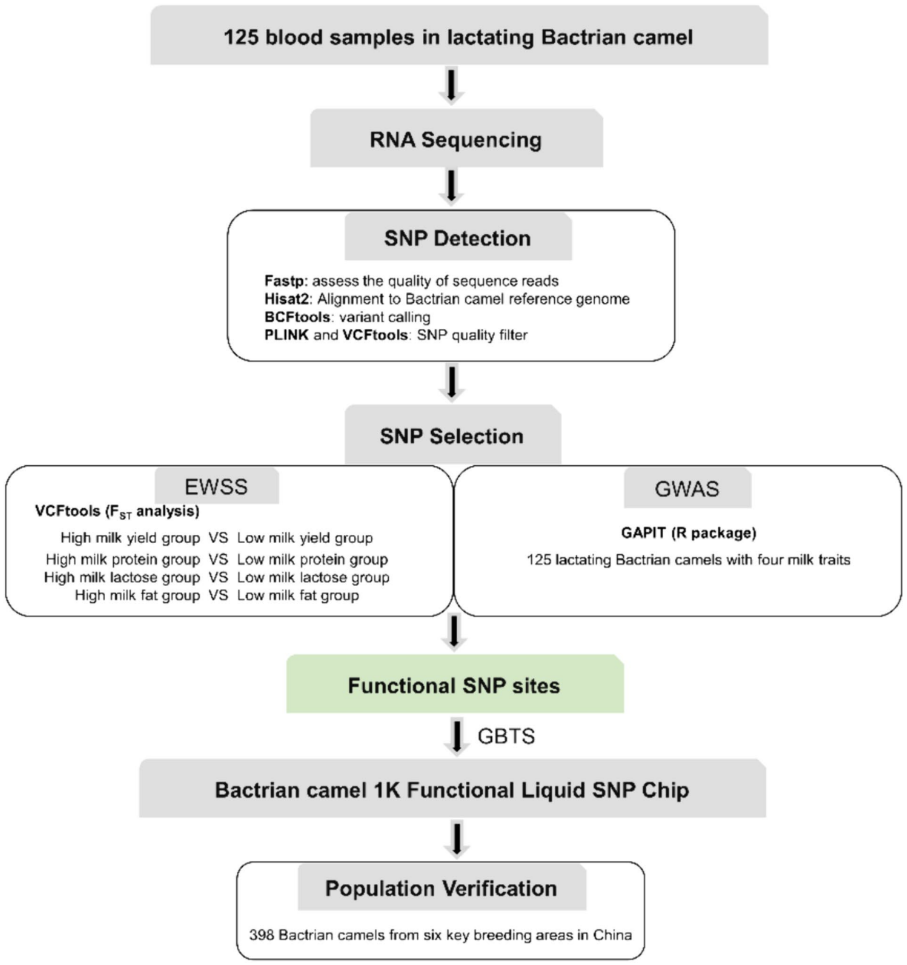

FIGURE 1
The workflow used for SNP chip design procedure.

lactation stage and parity within the model. For population correction and stratification within the mixed linear model (MLM), both a kinship matrix and principal component analysis (PCA) were computed. The kinship matrix accounts for the genetic relatedness among individuals, while PCA addresses population structure, thereby mitigating the risk of false positives attributable to population stratification. *p*-values were subsequently adjusted at a 5% false discovery rate (FDR) to ascertain significant associations. To determine the relevance of the applied model for GWAS, quantile–quantile (QQ) plot was derived among the observed and expected log10(*p*) value. The workflow used for SNP chip design procedure is summarized in Figure 1.

2.4.2 Variants annotation and enrichment analysis

Single nucleotide polymorphism (SNP) annotations, categorized by functional class (such as genic or intergenic), along with their genomic distributions, were performed using custom Perl scripts. The annotation process utilized the Generic Feature Format (GFF) file[1] of the Bactrian camel genome reference (Ca_bactrianus_MBC_1.0). This file provided the necessary information to determine the genomic context of each SNP. A SNP was classified as genic if it was located within the start and end positions of an mRNA transcript, which includes coding sequences (CDS), 5′ untranslated regions (5′UTR), or 3′ untranslated regions (3′UTR). Conversely, SNPs that did not fall within these mRNA boundaries were designated as intergenic.

For the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, we utilized the g:Profiler[2] web interface (26). This platform facilitated the identification of significant biological processes, cellular components, and molecular functions (as classified by GO), as well as the pathways (as cataloged in KEGG) that are overrepresented in our gene set. To account for the issue of multiple comparisons, which can lead to false positives, we employed Bonferroni for multiple testing correction. Only results with an adjusted *p*-value below 0.05 were considered statistically significant.

2.4.3 Design and synthesis of the liquid chip

The liquid chip utilizes GBTS technology (18, 27, 28), which operates on the principle of target capture through the complementary pairing of probes with the target sequences. In this study, we initially assessed and scored the selected candidate loci using the Compass probe design system (Compass Biotechnology, Guilin, China). The probe design considered the complexity and GC content of the sequences upstream and downstream of the target loci to ensure accuracy and efficiency. Priority was given to positioning the target loci in the middle of the probes, each designed to be 120 bp in length. After verification, these loci were submitted to Compass for probe synthesis, with each probe modified with a biotin group at the 5′ end to facilitate subsequent experimental steps.

2.4.4 DNA extraction and library construction

DNA was extracted from the 398 samples using a magnetic bead method, known for its efficiency and ability to yield high-quality DNA. The extracted DNA was then fragmented to align with the PE150 sequencing strategy, targeting a main fragment size range of 200–300 bp. This fragmentation was followed by selection, end-repair, and A-tailing, preparing the DNA for sequencing library construction. The library construction process involved the ligation of sequencing adapters and PCR amplification to enrich the target fragments, ensuring comprehensive coverage. Subsequently, the libraries were quantified using the dsDNA HS Assay Kit for Qubit. Electrophoresis was employed to confirm that their main peak sizes fell within the 350–450 bp range, a step crucial for ensuring sequencing quality and accuracy.

For hybrid capture, pooled libraries, comprising the whole-genome DNA from each sample, were prepared, totaling 4 μg per hybrid capture library. These pooled libraries were then concentrated and subjected to probe hybridization, specifically targeting fragments from the whole-genome library. Following hybridization, excess probes, reagents, and other components were removed during the elution process. Post-hybridization, PCR amplification was performed to further enrich the target regions, culminating in the final library ready for sequencing. These libraries were quantified and subsequently sequenced using the MGI-T7 sequencer.

2.5 SNP array performance evaluation

To assess the stability and reliability of the "CamelBell No. 1" SNP chip, we performed genotyping on 24 lactating camels randomly selected from the 398 DNA samples. The sample set included three pairs of duplicate samples to evaluate reproducibility. Next, the sequencing reads were first subjected to quality control using FastQC v0.11.5 (29) to assess the quality of the raw data. Low-quality reads and adapter sequences were trimmed using Trimmomatic v0.39 (30). The resulting data were aligned to the Bactrian camel reference genome (Ca_bactrianus_MBC_1.0) using BWA v0.7.17 (31). Post-alignment, the alignment rate was calculated to assess sequencing efficiency and accuracy. The alignment files were processed using SAMtools v1.17 (21) to convert, sort, and index the aligned reads. Alignment quality was further assessed by examining metrics such as the percentage of mapped reads, coverage depth, and uniformity of coverage across the genome. To ensure the chip's precision and reliability in genotyping, we meticulously analyzed the genotype concordance rate among the duplicate samples. This analysis is vital for validating the chip's capability to produce consistent and reproducible results. Additionally, we quantified the detection rates of all test samples across various site coverage depths, providing a comprehensive evaluation of the chip's performance under different genomic conditions.

2.6 Analysis of genetic diversity and population structure

To evaluate the performance of the SNP array in detecting population genetic diversity and structure, we genotyped and analyzed 398 DNA samples. VCFtools v0.1.11 (23) was employed to filter the SNPs and individuals. Specifically, individuals and SNPs with a detection rate lower than 95% were excluded. SNPs with minor allele frequencies lower than 0.05 were excluded. SNPs with significant deviation from Hardy–Weinberg equilibrium ($p < 0.01$) in any population were

1 https://www.ncbi.nlm.nih.gov/datasets/taxonomy/9837/

2 https://biit.cs.ut.ee/gprofiler/gost

excluded. The genotyping data were extracted and converted into PLINK. bed and .fam formats, and then imported into PLINK v1.90 (22) and ADMIXTURE v1.3.0 (32) software for analysis. Using PLINK, PCA is conducted to identify major sources of genetic variation across the six breeding areas. ADMIXTURE is used to infer population structure and assign individuals to genetic clusters. It provides estimates of the proportion of an individual's genome that originates from each of K ancestral populations. The tested *K* was set from 2 to 9.

2.7 Genomic prediction

The GBLUP model is used to calculate GEBV as follows:

$$y = 1\mu + X\beta + Zu + e$$

where y is the vector of corrected phenotypic values. These are the phenotypic measurements (e.g., milk yield, milk protein, milk lactose milk fat) that have been adjusted for fixed effects (e.g., breed, parity, lactation period). 1 is a vector of ones, which ensures that the overall mean ($\propto$) is added to each observation, with μ being the overall mean of the phenotypic values across all individuals in the study. $X\beta$ represents the fixed effects, where X is the incidence matrix for these effects, and β is the vector of fixed effects coefficients. Z is the incidence matrix that relates the additive genetic values (u) to the phenotypic values (y), with each row of Z corresponding to an individual and each column to a genetic effect. u is the vector of additive genetic values (or genomic breeding values, GEBVs). These values represent the genetic contribution of each individual to the trait. It is assumed that $u \sim N(0, G\sigma_u^2)$, where G is the relationship matrix built with the HIBLUP v1.4.0 software (33). This matrix represents the genetic relationships between individuals based on SNP marker data. σ_u^2 represents the additive genetic variance. e is the vector of random residual effects, representing the variation in the phenotypic values not explained by the model. It is assumed that $e \sim N(0, I\sigma_e^2)$, where I is an identity matrix and σ_e^2 is the residual variance.

Cross-validation (CV) is usually used to obtain a reliable and stable model, and to evaluate the quality of the model (34). To evaluate the accuracy of genomic prediction, we utilized five-fold cross-validation. The dataset was divided into five approximately equal-sized groups. In each iteration of the cross-validation, four groups were used as the training set to estimate model parameters, while the remaining group was used as the validation set to test the model's predictive performance. Prediction accuracy was calculated as the correlation between the predicted estimated breeding values (EBVs) and the actual phenotypes in the validation set, divided by the square root of the heritability estimated in the validation population. The standard error (SE) was calculated as the standard deviation of the five calculated reliability values from the five-fold cross-validation, divided by the square root of five.

3 Results

3.1 Variant calling from transcribed region sequences

A total of 19,177 confident SNPs were detected from the RNA-seq data of lactating Bactrian camels. Initially, transcriptomes of peripheral blood from 125 selectively chosen individuals, exhibiting consistent lactation period out of 1,243 Bactrian camels, were sequenced. This sequencing yielded approximately 3,575 million paired-end reads, averaging 29 million paired-end reads per individual sample. After quality control, the base effectiveness rate stood at 97.88%. The Q30 ratio surpassed 93.14%, and the GC content exceeded 52.08%. Remarkably, about 91.72% of the reads were accurately mapped to the Bactrian camel genome (Ca_bactrianus_MBC_1.0), with nearly 80.54% of the reads from each individual uniquely aligning with the camel genome. Detailed alignment information for each sample is tabulated in Supplementary Table S1. Subsequently, a comprehensive SNP calling was conducted in the reference genome from 125 transcriptome datasets, unveiling 9,790,170 SNPs. Only those SNP variants where the alternative allele appeared in all samples and had a Phred quality score of at least 25 and a minimum read depth of 10 were retained. Minor allele frequencies (MAFs) and missing rates were calculated, with SNPs having MAF <0.05 excluded. Additionally, we tested for Hardy–Weinberg equilibrium (HWE) using the -hwe option and removed SNPs that significantly deviated from HWE ($p < 0.01$). Among these, 19,177 SNPs were confidently ascertained across the entire cohort.

3.2 Identification of SNPs associated with milk traits

For the lactation performance traits of milk yield, milk protein, milk lactose, and milk fat, both Exon Wide Selection Signature (EWSS) and Genome Wide Association Study (GWAS) methodologies were employed to generate a comprehensive SNP set. Initially, the calculation of F_{ST} was applied to 19,177 SNPs to discern associations with extreme phenotypic milk traits. Groupings according to extreme values of milk traits are shown in Table 1. The statistical analysis of other milk components in each extreme value group is shown in Tables 2–5. Using a threshold of $F_{ST} > 0.2$, we identified 178 loci as outliers associated with various milk traits. Specifically, 138 loci were related to milk yield, 28 to milk protein, 30 to milk lactose, and 6 to milk fat. These loci are depicted in Figure 2. These identified loci serve as a partial reference for the selection of SNPs associated with lactation traits. Simultaneously, leveraging the 19,177 SNPs identified within the transcribed regions, we also employed GWAS to associate them with four continuous lactation performance traits across 125 subjects, thereby providing an additional reference set for the screening of pertinent loci. Therefore, significant associations were found with 923 SNPs for milk yield, 980 SNPs for milk protein, 955 SNPs for lactose, and 756 SNPs for milk fat traits (FDR <0.05), shown in Figure 3. During the analysis, we calculated the genomic inflation factor (λ) to assess the potential inflation of test statistics due to population structure or cryptic relatedness. The results showed a genomic inflation factor of 0.945, indicating minimal inflation and suggesting that the test statistics were not significantly affected by population structure or cryptic relatedness (Figure 4A). The overlap of SNPs associated with different traits is shown in Figures 4B,C. Overall, the amalgamation of both methods furnished us with a reference set comprising 2,960 SNP loci, which are associated with four lactation traits and dispersed within the transcribed regions of 1,395 genes, shown in Supplementary Table S2. A total of 81 overlapping sites were identified between F_{ST} and GWAS analyses. The functional enrichment

TABLE 1 The phenotype group of 4 milk traits.

Groups	Traits	Sample size	Mean	Min	Max	SD
HMY	High milk yield (kg)	15	2.28	2.05	2.70	0.10
LMY	Low milk yield (kg)	15	0.56	0.32	0.81	0.18
HMP	High milk protein (%)	15	4.11	4.01	4.26	0.08
LMP	Low milk protein (%)	15	2.82	2.25	3.33	0.29
HML	High milk lactose (%)	15	5.57	5.45	5.84	0.14
LML	Low milk lactose (%)	15	3.73	3.04	4.19	0.31
HMF	High milk fat (%)	15	8.38	7.53	10.00	0.84
LMF	Low milk fat (%)	15	2.49	1.04	3.58	0.95

TABLE 2 Statistical analysis of milk components in extreme milk yield group.

Milk component	High milk yield group	Low milk yield group	*p*-value
Milk yield, kg/day	2.28 ± 0.10	0.56 ± 0.18	<0.01
Milk protein, %	3.67 ± 0.06	3.7 ± 0.15	0.79
Milk lactose, %	5.05 ± 0.14	5.06 ± 0.32	0.95
Milk fat, %	5.80 ± 0.58	5.96 ± 0.33	0.56

TABLE 3 Statistical analysis of milk components in extreme milk protein group.

Milk component	High milk protein group	Low milk protein group	*p*-value
Milk yield, kg/day	1.08 ± 0.64	1.16 ± 0.0.83	0.75
Milk protein, %	4.11 ± 0.08	2.82 ± 0.29	<0.01
Milk lactose, %	5.53 ± 0.02	4.59 ± 0.21	<0.01
Milk fat, %	5.07 ± 0.27	4.76 ± 0.23	0.25

TABLE 4 Statistical analysis of milk components in extreme milk lactose group.

Milk component	High milk lactose group	Low milk lactose group	*p*-value
Milk yield, kg/day	1.29 ± 0.40	1.44 ± 0.47	0.49
Milk protein, %	4.03 ± 0.01	2.96 ± 0.12	<0.01
Milk lactose, %	5.57 ± 0.14	3.73 ± 0.31	<0.01
Milk fat, %	5.02 ± 1.77	4.96 ± 0.80	0.83

TABLE 5 Statistical analysis of milk components in extreme milk fat group.

Milk component	High milk yield group	Low milk yield group	*p*-value
Milk yield, kg/day	1.12 ± 0.20	1.41 ± 0.46	0.12
Milk protein, %	3.98 ± 0.02	3.51 ± 0.14	0.64
Milk lactose, %	5.44 ± 0.03	4.83 ± 0.31	0.37
Milk fat, %	8.38 ± 0.84	2.49 ± 0.95	<0.01

Values are presented as mean ± standard deviation (SD).

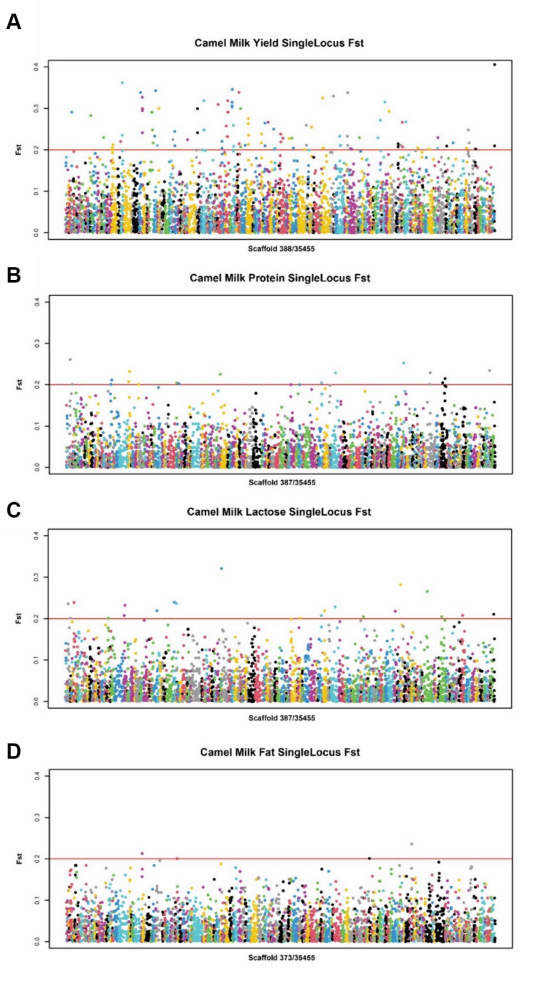

FIGURE 2
Manhattan plot of SNPs for milk traits by EWSS. **(A)** Manhattan plot of SNPs between high and low milk yield group. **(B)** Manhattan plot of SNPs between high and low milk protein group. **(C)** Manhattan plot of SNPs between high and low milk lactose group. **(D)** Manhattan plot of SNPs between high and low milk fat group.

analysis conducted on the identified genes indicated a significant overrepresentation in several biological processes (BP), particularly in areas related to the immune system, organonitrogen compound metabolism, and regulation of metabolic processes. Additional processes such as vesicle-mediated transport, positive regulation of

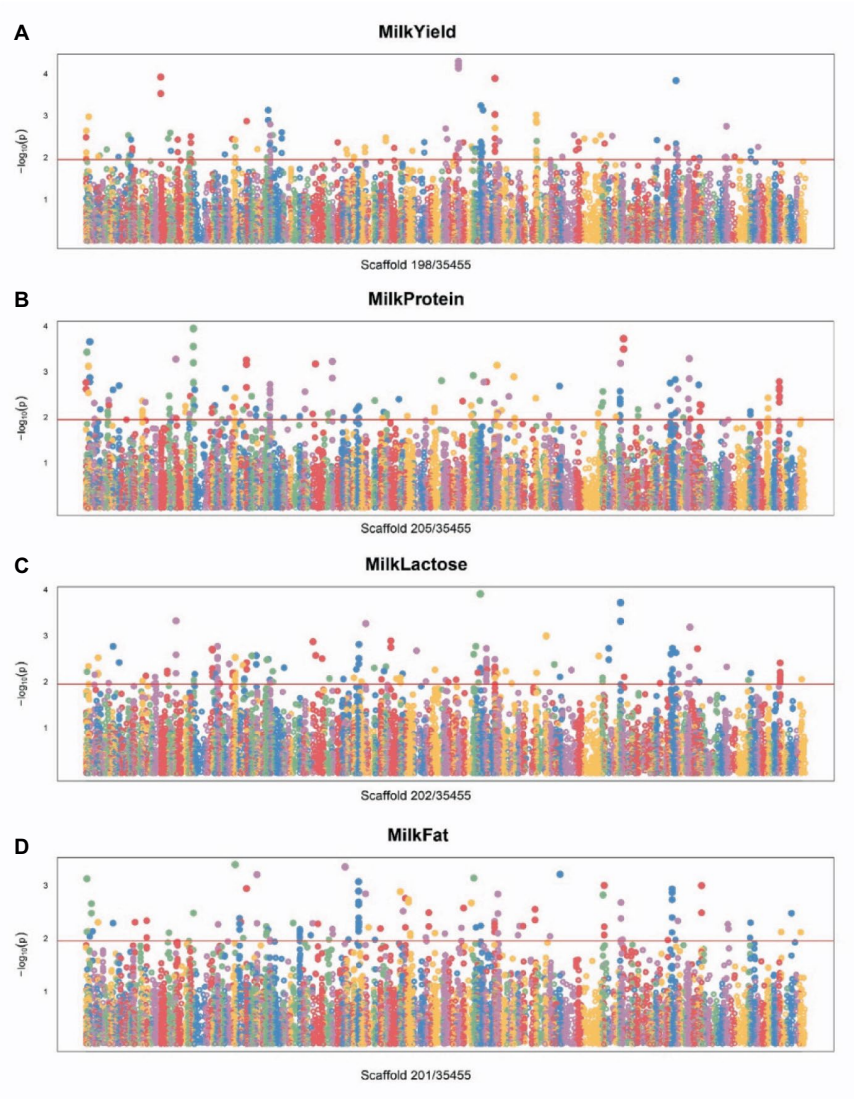

FIGURE 3
Manhattan plot of SNPs for milk traits by GWAS. **(A)** Manhattan plot of SNPs associated with milk yield traits. **(B)** Manhattan plot of SNPs associated with milk protein traits. **(C)** Manhattan plot of SNPs associated with milk lactose traits. **(D)** Manhattan plot of SNPs associated with milk fat traits.

biological processes, protein metabolism, and cellular localization establishment were also notably enriched. Furthermore, genes involved in catabolic processes were highlighted as part of the biological process category. To provide a comprehensive overview, the top 10 items from each category—Biological Processes (BP), Cellular Components (CC), Molecular Functions (MF), and Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) were visually summarized in Figure 4D.

3.3 SNPs for the "CamelBell No. 1" array

To explore the variation in SNP density across different chromosomes, a box plot was generated (Figure 5A). The median distances and the ranges vary significantly, with some scaffold showing a higher concentration of closely spaced SNPs. To mitigate the incidence of false positives in genotyping, SNPs were judiciously selected for inclusion on the chip, guided by criteria such as SNP quality, polymorphism status, SNP density, and the extent of association with specific traits. In brief, we submitted 2,960 previously identified candidate SNPs associated with lactation traits to the Targeted Capture Sequencing Probe Design System, and 2,681 SNPs passed evaluation. Next, considering the location and function of these SNPs, we eliminated one of the two sites within less than 100 bp from each other, focusing on retaining sites supported by existing literature. Thus, 1,002 SNPs were finally manually selected for the "CamelBell No. 1" array. For those SNP site, there were 460 SNPs located in exon region, 54 located in intron region and 10 located in intergenic region, shown in Figure 5B, and the details are in Supplementary Table S3. The association of these SNPs with lactation traits is visually represented in Figure 5C, with 201 SNPs being correlated with two or more lactation traits.

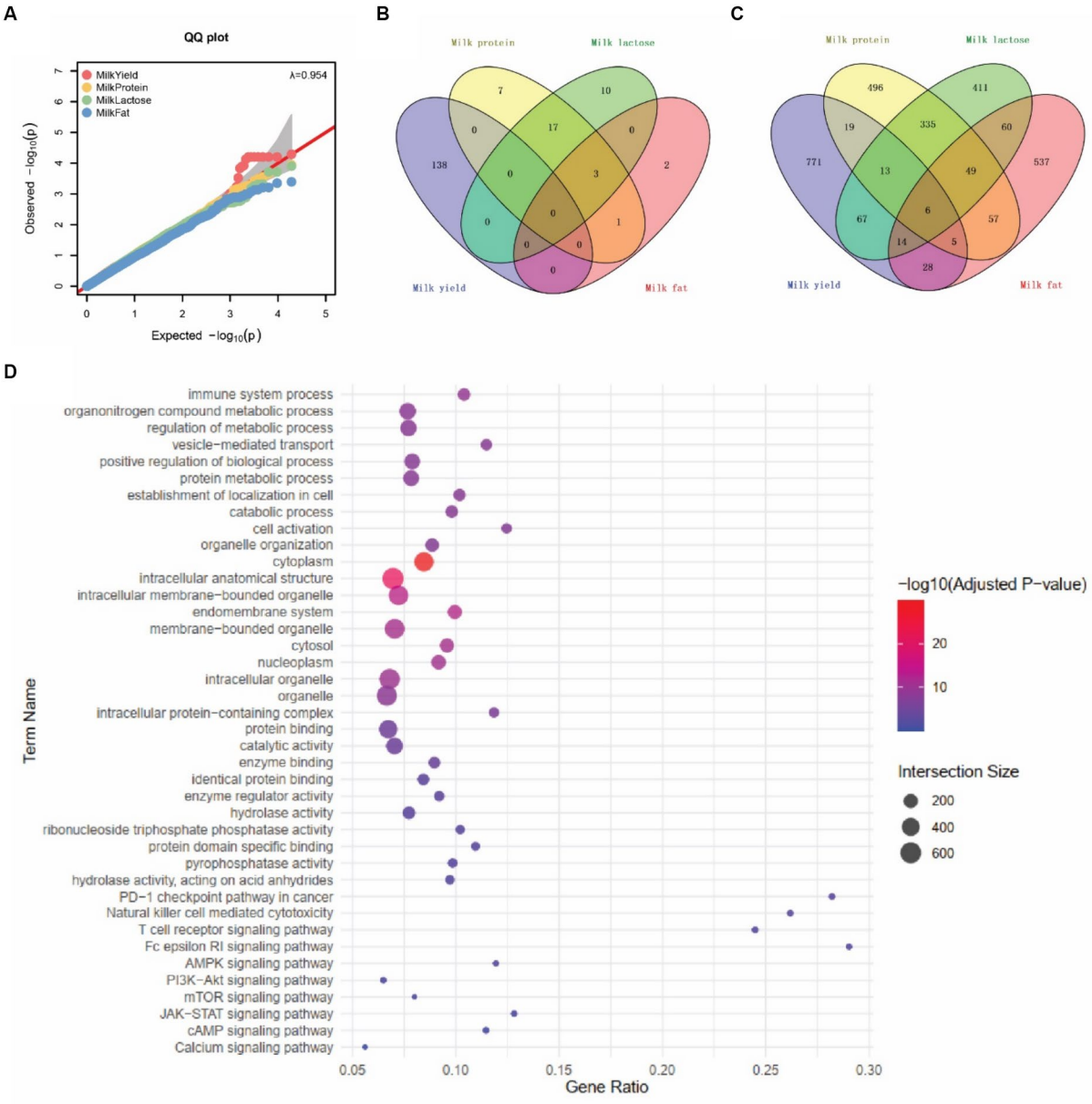

FIGURE 4
SNPs associated with four milk traits. **(A)** Quantile−quantile (QQ) plots represent the negative logarithms of the expected *p*-values (*x*-axis) and observed *p*-values (*y*-axis) (right panel). **(B)** SNPs associated with four milk traits by EWSS. **(C)** SNPs associated with four milk traits by GWAS. **(D)** Top 10 Gene Ontology (GO) and KEGG pathway terms associated with milk trait-related snp genes.

3.4 Genotyping performance of SNP array

The efficacy of the SNP array was assessed through the genotyping of 24 DNA samples from Alashan League, Inner Mongolia. Inclusion of three pairs of replicate samples facilitated the evaluation of the chip's detection performance. A genome alignment rate exceeding 99% was achieved for all samples, culminating in an average alignment rate of 99.77%, shown in Figure 5D. Separate testing of three duplicate samples yielded a typing consistency of 100% for each pair, underscoring the chip's detection stability (Figure 5E). Comprehensive statistics pertaining to the detection rates across all test samples revealed that with a target site coverage depth exceeding 5×, an average site detection rate of 99.72% was maintained. Remarkably, even at a coverage depth surpassing 20×, the detection rate remained robust at 99.07%, shown in Figure 5F.

3.5 Genetic diversity of the core breeding populations

To assess the genetic diversity of the core breeding populations in of dairy Bactrian camels in northwest China, we collected 398 camels from six representative breeding areas, and conducted SNP genotyping using the SNP array. Firstly, we calculated the minor allele frequency (MAF) distribution of the population and plotted the histogram, as shown in Figure 6A. The MAF data from SNP array genotyping

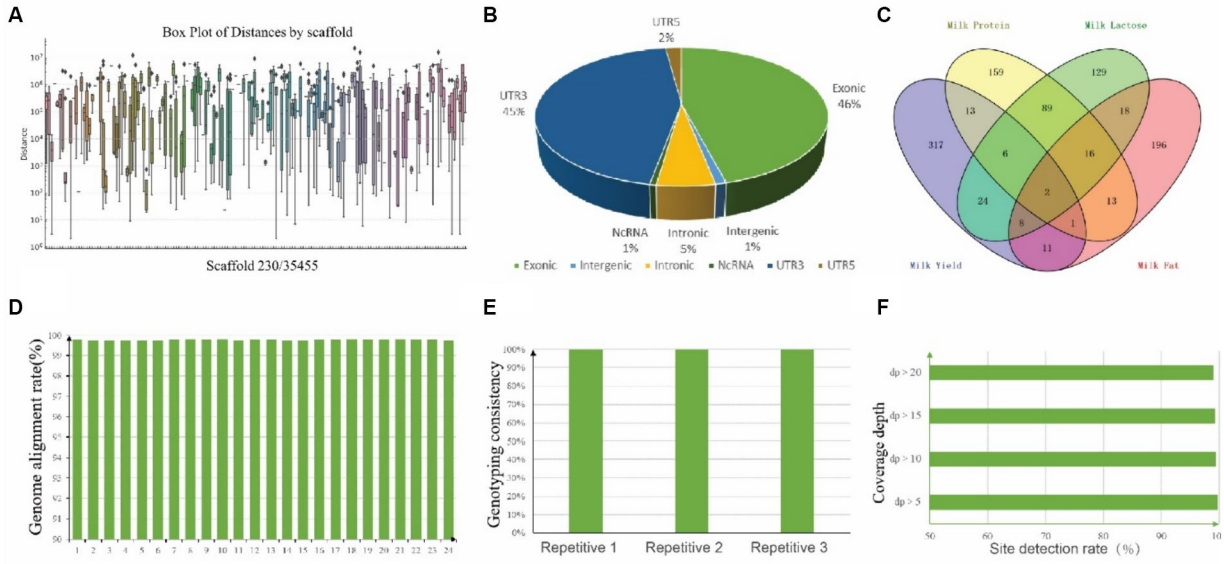

FIGURE 5
Annotation of 1,002 SNPs and Quality assessment of CamelBell No. 1 SNP array. **(A)** Box plot of SNP distances by 230 scaffolds. The *x*-axis lists the chromosomes, and the *y*-axis represents the distance between adjacent SNPs on a logarithmic scale. **(B)** Pie chart depicting the distribution of SNP positions across the genome. **(C)** Venn diagram illustrating the overlap of SNPs associated with various lactation traits. **(D)** Genome alignment rates achieved using the CamelBell No. 1 SNP array. **(E)** Genotyping consistency of duplicate samples analyzed with the CamelBell No. 1 SNP Array. **(F)** Site detection rates at varying coverage depths using the CamelBell No. 1 SNP array.

revealed that 95% of SNPs had a minor allele frequency greater than 0.1. Subsequently, we estimated the genetic structure and distance, observing minimal genetic divergence among samples from different breeding areas (Figures 6B,C). These results indicate that camels from six breeding areas share a similar genetic background regarding lactation functionality.

3.6 The prediction accuracy of CamelBell No. 1 SNP array

Genomic prediction (GBLUP) accuracy for the four milk traits was assessed using genotype information from 914 markers that passed the quality control filters (MAF >0.01, Call Rate >0.9). A total of 398 camels were randomly divided into training (80%) and validation (20%) sets for cross-validation, and this process was repeated five times to ensure robust evaluation. The box plot of prediction accuracy values is shown in Figure 7. The genomic prediction accuracies for the four milk traits vary, with milk lactose showing the highest and most consistent accuracy, followed by milk yield and milk fat, while milk protein exhibits the lowest and most variable accuracy. The mean Pearson correlation coefficients of the four milk traits were 0.27, 0.30, 0.34, and 0.44, respectively, indicating modest linear relationships between the predicted Genomic Estimated Breeding Values (GEBVs) and the actual phenotypes. Among these traits, milk fat exhibited the strongest correlation. The standard error (SE) values of the estimated correlation coefficients for all traits were less than 0.10. The mean regression coefficients for the four milk traits were 0.88, 0.76, 1.14, and 0.69, respectively. These values suggest that while predictions for milk yield are relatively unbiased, predictions for milk protein and milkfat tend to underestimate the actual genetic values, and predictions for milk lactose tend to overestimate them. The SE values of the estimated regression coefficients for all traits were less than 0.06.

4 Discussion

In this study, we developed a new SNP array (CamelBell No. 1) in Bactrian camel based on genotyping by target sequencing. To our knowledge, this is the inaugural liquid chip specifically designed for dairy Bactrian camel breeding. After collecting lactation performance and phenotype data from thousands of lactating Bactrian camels in Northwest China, we handpicked 523 to establish a core breeding group. From this pool, we further selected 125 camels that shared the same peak lactation period and identical feeding conditions, aiming to identify single nucleotide polymorphisms (SNPs) linked to lactation traits. Utilizing these 1,002 SNPs, we advanced the genetic enhancement of dairy Bactrian camel breeding. When SNPs are identified using RNA-seq data, a limited number of individuals can effectively pinpoint loci exhibiting significant polymorphism (35). Given that lactation traits are quantitative and influenced by a multitude of genes and environmental factors, a larger sample size is instrumental in deciphering the genetic architecture of these complex traits. Consequently, in this study, RNA-seq data from the 125 lactating Bactrian camels were chosen as the reference dataset for SNP discovery, providing a comprehensive basis for understanding the genetic determinants of lactation characteristics. One advantage of SNP discovery using RNA-seq is the versatility of the sequenced data, which extends beyond initial research objectives. This data can be repurposed for further investigative queries, including the exploration of how organisms adapt to varying environmental conditions. This multifaceted utility enhances the value of RNA-seq in genetic research (36). This method, widely adopted in genomic

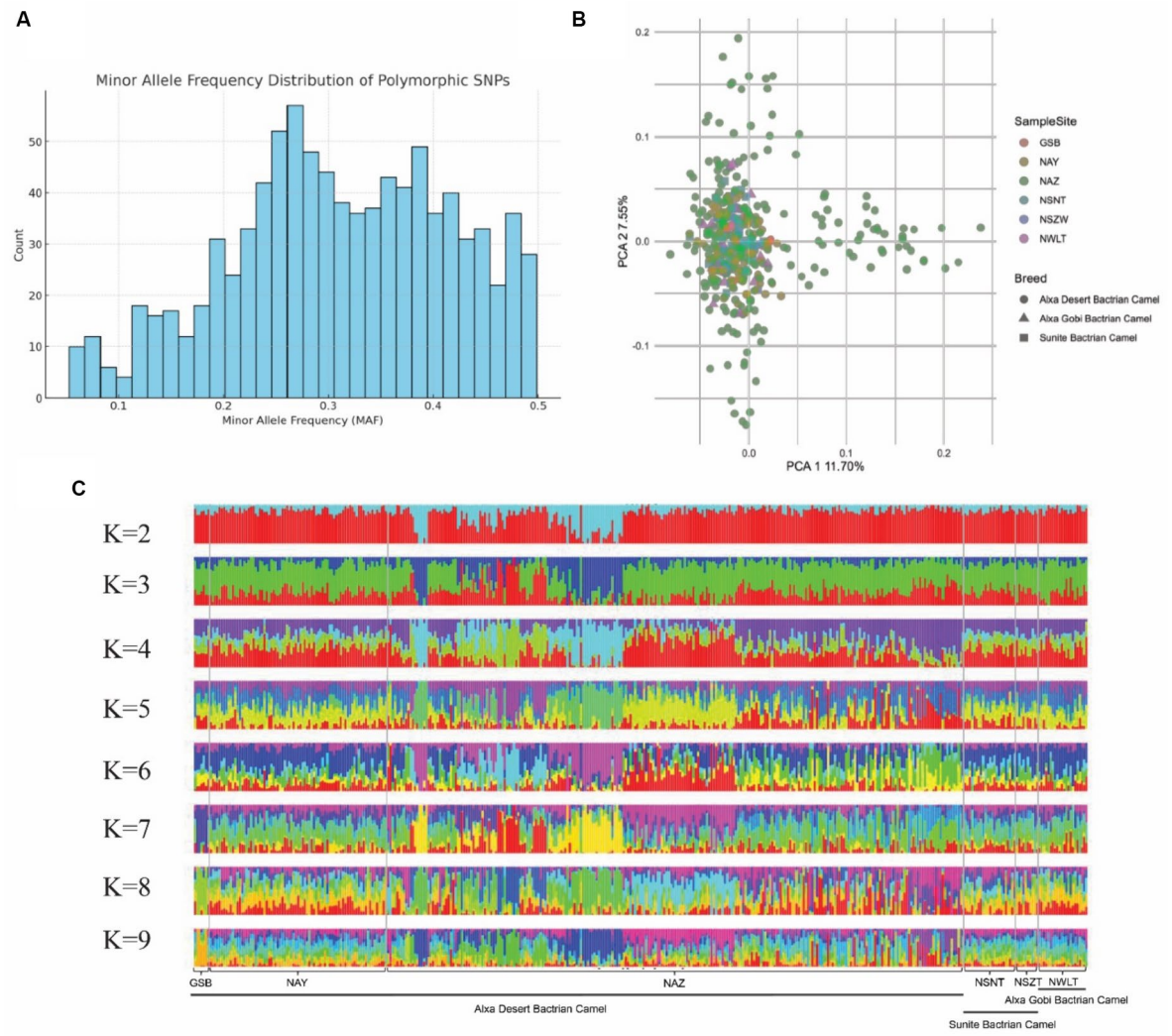

FIGURE 6
Genetic structure of camel core breeding population. **(A)** Bar plot of minor allele frequency (MAF) distribution. **(B)** PCA plot of the first and second PCs showing genetic distances among samples. Samples from different locations are represented in different colors. **(C)** Admixture plot ($K = 2–9$) for the 398 large camel individuals. Each individual is shown as vertical bar divided into K colors.

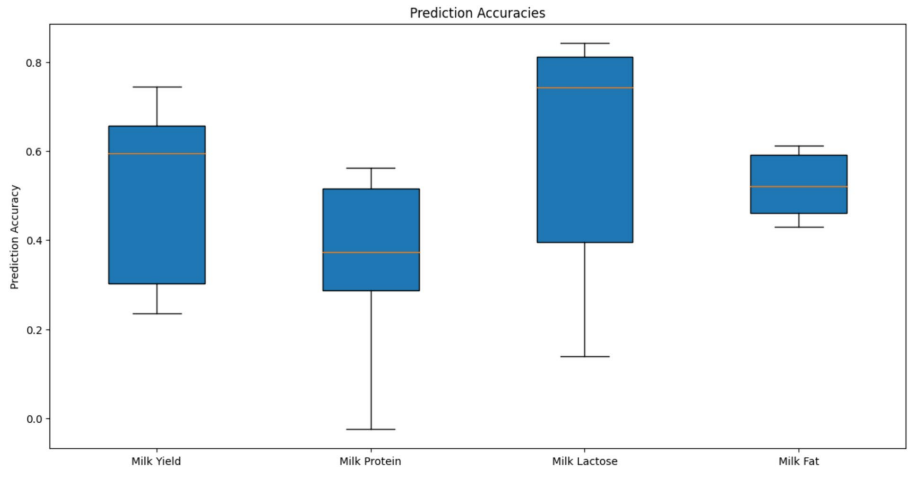

FIGURE 7
Prediction accuracies of genomic estimated breeding values for different milk traits using GBLUP in camel.

research, has proven effective in identifying SNPs associated with traits such as muscle yield and quality in fish (37), as well as thermo-resistant in oysters (38). Additionally, it has been instrumental in unraveling the genetic mechanisms behind tail fat deposition in sheep (9) and lactation performance in dairy cows (7), highlighting its versatility and importance in the field of animal genetics.

In our research, we employed Extended Window Sum Statistic (EWSS) to calculate fixation indices (F_{ST}) (39, 40) for populations exhibiting significant variations in lactation performance. This approach allows us to screen for SNP variants in extreme phenotypic groups after controlling for environmental variables. Concurrently, we adopted the principles and methodologies of Genome-Wide Association Studies (GWAS) (41, 42) to investigate the correlation between lactation performance and polymorphic sites in transcription regions of the Bactrian camel. By integrating these two methods, we were able to screen for the most pertinent functional loci in Bactrian camels, providing a foundational set of functional loci crucial for chip design. However, despite our efforts to control for various confounding factors in our analysis, several potential confounding factors may still influence our results. These include environmental variation, physiological and health status, and genetic background. Specifically, although we standardized diet and housing conditions, other environmental factors such as microclimatic conditions, handling practices, and feed intake were not explicitly controlled. Variations in the health status or physiological conditions of the camels, such as subclinical infections, stress levels, and hormonal imbalances, were not measured or controlled. While principal component analysis (PCA) was used to account for major population structure, subtle genetic stratification or cryptic relatedness within the population may still exist. These unaccounted confounding factors may introduce biases or residual confounding in our GWAS results, potentially affecting the generalizability and validity of our findings. Future research should aim to measure and control for these variables more comprehensively to ensure more accurate and reliable genetic association results.

Compared to traditional silicon-based SNP panels, Genotyping By Targeted Sequencing (GBTS) (43) panels offer enhanced flexibility in handling varying sample sizes for genotyping. The GBTS marker system offers the flexibility to create multiple marker panels from a single master panel. It allows researchers to select a specific number of markers tailored to their unique research goals. While the breeding of dairy camels, including *Camelus dromedarius* and *Camelus bactrianus*, is progressing in various countries (44–46), conventional breeding methods still predominate. This study lies in its contribution to the breeding of camels with desirable milk production traits, such as milk yield, protein content, fat content, and lactose content, bolstered by advancements in Bactrian camel genome research. The development of CamellBell No. 1 provides technical support for the large-scale promotion of dairy breeding in Bactrian camel.

Our evaluation of the array's typing performance revealed high levels of consistency and stability. Remarkably, even at a coverage depth exceeding 20×, the detection rate remained robust at 99.07%. This reliability is crucial for the chip's application in further genetic studies. We used the chip to sequence DNA from individuals in core breeding groups across six regions to analyze their population structure. The analysis of 398 individuals did not show complete separation in terms of lactation function, suggesting a common ancestral origin or an early stage in the selection process. The breeding values were estimated using the GBLUP model, and the accuracy of trait prediction was evaluated using five-fold cross-validation. We found that although the model for the lactose trait had the risk of overfitting, its prediction accuracy was the highest. This high accuracy may be attributed to the genetic stability of the lactose trait. Lactose percent has been reported to be highly heritable (0.53), according to a study in Holstein cows from Michigan (47). The statistical model, marker density, and sample size influenced on selection accuracy (48). Due to the low samples size in this research, there may be bias in the prediction. Additional animals are required to fully evaluate the array for genomic selection (GS), and we are working to increase the sample sizes of the reference and candidate populations for genotyping with the SNP array. Given that large-scale breeding of Bactrian camels is not as established as in cows and other animals, and considering the challenges in sampling, low-cost and highly flexible liquid phase chips like CamelBell No. 1 play a vital role in accelerating the breeding of dairy Bactrian camels.

5 Conclusion

In summary, this study is the first try to report a functional liquid chip of Bactrian camel, CamelBell No. 1 SNP array, and conduct a comprehensive evaluation of the chip's typing performance, with a view to providing tools and carriers for dairy Bactrian camels breeding.

Data availability statement

The data presented in the study are deposited in the SRA repository, accession number PRJNA1127254.

Ethics statement

The animal studies were approved by Inner Mongolia Agricultural University Laboratory Animal Welfare and Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LG: Methodology, Visualization, Writing – original draft. LD: Writing – review & editing, Writing – original draft. BL: Software, Validation, Writing – review & editing. JW: Validation, Writing – review & editing. ZL: Formal analysis, Writing – review & editing. FM: Investigation, Writing – review & editing. BM: Data curation, Writing – review & editing. CC: Investigation, Writing – review & editing. YB: Data curation, Writing – review & editing. YG: Data curation, Writing – original draft. CS: Supervision, Writing – original draft. JC: Conceptualization, Project administration, Writing – review & editing. WZ: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing.

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Conflict of interest

BL was employed by Inner Mongolia Bionew Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1359923/full#supplementary-material

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Genomic signatures of positive selection in Awarik dromedary camels from southwestern of Saudi Arabia

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Introduction: The Awarik camel population in southwestern Saudi Arabia exhibits unique genetic and phenotypic traits compared to other domestic camel populations. This study aims to explore the genomic signatures of positive selection in Awarik camels to understand their evolutionary history and identify genetic adaptations potentially shared with East African camel populations.

Methods: Whole genome sequencing data from nine Awarik camels were analyzed using two robust intra-population haplotype-based statistical methods: integrated haplotype score (iHS) and number of segregating sites by length (nSL). These analyses were conducted to identify candidate regions under positive selection within the Awarik camel genome.

Results and discussion: These analyses identified 66 and 53 candidate selection regions, encompassing 185 and 123 genes, respectively. The iHS analysis revealed significant selection signals on chromosomes 15 and 16, including a robust overlap on chromosome 15 (10 regions) involving the TRNAI-AAU gene, suggesting its critical role in adaptive processes. Additionally, chromosome 3 exhibited the highest number of candidate regions totaling 10. The nSL analysis highlighted statistically significant regions on chromosomes 2 and 7, as well as a high concentration of candidate regions on chromosome 14, totaling five regions. Notably, large candidate regions were also identified on chromosome 11 (200 kb: 51.750–51.950 kb) and chromosome 9 (325 kb: 45.825–46.150 kb). Functional annotation of these genes revealed involvement in diverse biological processes including olfactory activity, immune regulation, metabolism, insulin secretion, reproductive performance, kidney function, and cellular signaling, with specific genes like BAG5, septin 7, SLC13A1, PCED1B, BMPR1B, ZAR1, JAKMIP2, and NOTCH2 highlighted. These findings contribute to our understanding of the adaptive mechanisms of Awarik camels and have important implications for breeding and conservation strategies. Further research on these genetic adaptations, particularly those affecting immune response, is crucial to mitigate the impacts of climate change on camel populations.

KEYWORDS

dromedary, positive selection, evolutionary adaptation, haplotype-based statistics, camels

1 Introduction

The Awarik camel population in Saudi Arabia is distinguished by its unique geographical distribution, phenotypic traits, and genetic characteristics. Studies have consistently demonstrated that the Awarik camels, particularly those from the western and southwestern regions, possess distinct genetic traits when compared to other camel populations, particularly those in the northern and central regions of the country (1).

The uniqueness. The uniqueness of the Awarik camel population has been highlighted in various studies, indicating that these camels represent a genetically distinct group within Saudi Arabia (2–5). Recent research, including the study by Bahbahani et al. (6) which analyzed the whole genomes of 40 dromedary camels from across the Arabian Peninsula, has provided pivotal insights into the geographical genetic distinctions and regions under positive selection in dromedaries. This research has identified specific regions and haplotype blocks associated with adaptive physiological traits that are crucial for conservation. Building upon these comprehensive findings, this study focuses on exploring the unique genomic signatures of positive selection in the Awarik population. By examining these specific genetic adaptations, w aim to further understand the evolutionary pressures and adaptations that have uniquely shaped the Awarik camels, emphasizing the necessity for focused research on this distinct population.

The consistency of the Awarik camel population is evidenced by both genetic and demographic data. Genetically, the Awarik camels exhibit significant differentiation from other regional populations, reflecting a high degree of genetic homogeneity and stability within this group. This is supported by specific alleles and haplotypes that are consistent across generations, indicative of strong selective pressures and limited gene flow with other populations. Demographically, the population size has been relatively stable, as suggested by historical and recent surveys. This stability is crucial for preserving genetic integrity and ensures the reliability of evolutionary and adaptive studies focused on this unique group. Together, these genetic and demographic aspects confirm the Awarik population's consistency, underscoring its suitability for detailed genomic and adaptive analyses.

These one-humped dromedary camels are primarily located in the southwestern region of the country, which features a semi-arid climate with variable humidity levels, particularly high along the western coasts and mountains. Historically, Awarik camels share genetic ties with camel populations from the Horn of Africa, including Kenya, Somalia, and Sudan, regions that collectively host the largest camel populations in Africa.

Awarik camels have adapted to thrive in the harsh desert environment and humid conditions of the Red Sea coastal areas. Traditionally bred by local tribes for their milk and meat, these camels are highly valued for their resilience, endurance, and adaptability to coastal and mountainous terrains. They graze on the arak plant (*Salvadora persica*), which is abundant in their natural habitat. With unique physiological and behavioral traits, Awarik camels exhibit efficient temperature regulation and heat tolerance, enabling them to flourish in their coastal and mountainous environments (2).

Named after the arak plant, a significant part of their diet, the Awarik camel population is primarily found near the Red Sea coast of Saudi Arabia and is colloquially known as "Beach camels." Predominantly concentrated in the Jazan region, these camels are characterized by a light brown coat, almost white, and short hair. They typically have a well-developed udder, medium neck circumference, pointed ears, and a hump positioned toward the hind of the back, giving them a shorter stature compared to other desert camels (7, 18). Awarik camels exhibit moderate milk production, with total lactation yields averaging 1,047.5 ± 11 liters (8).

This study aims to analyze the genomic signatures of positive selection in Awarik camels, seeking to uncover their evolutionary history and identify the genetic factors associated with their adaptation. Insights from this research can inform camel breeding programs, enhance conservation efforts, and develop strategies to mitigate the impact of climate change on camel populations.

2 Materials and methods

2.1 Sample collection and whole genome sequencing

Blood samples were obtained from nine unrelated female Awarik camels residing in the southwestern regions (Jazan) of Saudi Arabia, selected based on stringent phenotypic criteria. Genomic DNA was extracted from these samples using the Puregene® Blood Core Kit C (Qiagen) according to the manufacturer's instructions. Subsequently, the extracted DNA underwent sequencing on the Illumina NovaSeq 6000 platform, employing a 150 bp paired-end approach. This sequencing was conducted at the Beijing Genomics Institute in China. The sequences data were deposited at European Nucleotide Archive Bioproject number: PRJEB47650.

2.2 Whole genome sequence read processing and variant calling

Whole genome sequences from the Awarik camels were aligned to the African dromedary reference genome (CamDro3) (9) using the *bwa-mem* algorithm of Burrows-Wheeler Aligner version 0.7.17 (10). Post alignment, reads were organized by coordinates using the *SortSam* and PCR duplicates were removed using the *MarkDuplicates* tool with (*REMOVE_DUPLICATES*=true) from Picard tools version 3.0.0 (http://broadinstitute.github.io/picard/index.html). SNPs calling was performed on Awarik autosomes using the *HaplotypeCaller* algorithm in GVCF mode of Genome Analysis Toolkit (GATK) version 4.2.5.0 (19). Autosomal SNPs were then filtered based on the criteria outlined by Bahbahani et al. (6), retaining those with a depth of coverage within ten reads and three standard deviations from the mean across all samples, for subsequent selection signature analyses.

2.3 Single nucleotide polymorphism quality control and pruning

A total of 5,720,174 autosomal SNPs were subjected to quality control pruning using PLINK v1.9 (11). SNPs were excluded based on the following criteria: a call rate below 100% of the genotyped

TABLE 1 Quality control criteria and the number of excluded and remaining SNPs for the signatures of selection analysis.

Quality control criteria	Number of excluded SNPs	Remaining SNPs
Raw autosomal SNPs	–	**5,720,174**
Genotypic Call Rate <100%	−81,638	5,638,536
MAF ≤5%	−2,273,655	3,364,881
HWE (*P*-value < 1 × 10^{-6})	0	3,364,881
Final number of SNPs	–	**3,364,881**

samples, deviation from Hardy-Weinberg equilibrium ($P < 1 \times 10^{-6}$), or a minor allele frequency (MAF) of 5% or less. After filtering, 3,364,881 SNPs remained for selection signature analysis (Table 1). Samples were also evaluated and would be excluded for a genotyping call rate under 100% or a maximum pairwise identity-by-state (IBS) of 95% or greater; however, no samples met these exclusion criteria.

2.4 Signatures of selection analysis

Signatures of selection analyses were conducted on nine Awarik dromedary camels from southwestern Saudi Arabia using two intra-population haplotype-based statistics, integrated haplotype score (iHS) (12) and number of segregating sites by length (nSL) (13). These statistics were converted to *P*-values based on fractional ranks using the *stat_to_pvalue* based on the fractional ranks. These *P*-values were further transformed into rank-based values using two-tailed tests. Windows displaying–log10 (*P*-values) ≥ 4 (equivalent to $P \leq 0.0001$) were defined as candidate windows with signatures of selection.

2.5 Functional annotation and enrichment analysis

The coordinates of the candidate regions were cross-referenced against the dromedary camel reference genome assembly CamDro3 gene list using the *GenomicRanges* package (14) in R. Functional profiling of the overlapping genes was conducted using the g: GOSt function of gProfiler (15), which identified functionally enriched terms for gene ontology biological processes and molecular functions. The gProfiler g: SCS algorithm was employed to compute multiple testing corrections for *P*-values from the gene ontology and pathway enrichment analyses. All identified genes were also analyzed using the functional annotation tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics resource version 6.7 (16, 17) to determine enriched functional terms. An enrichment score of 1.3, equivalent to a Fisher exact test *P*-value of 0.05, was used as the threshold to define significantly enriched functional terms compared to the dromedary reference genome background. The genes were then cross-referenced with the literature to evaluate their relevance to the dromedaries' environmental adaptations and physiological traits.

3 Results

3.1 Summary statistics of the mapped sequence reads

The depth of coverage for the mapped sequence reads among the dromedary samples ranged from 19X to 22X, with an mean of 21X. On average, 99.7% of the sequence reads were mapped to the dromedary reference genome, and 95% of these were properly paired. The mapped reads covered ∼94.7% of the reference genome.

3.2 Signatures of selection analysis of the two analysis

In the iHS analysis, the most significant regions were identified on chromosomes 15 and 16 as depicted in Figure 1. These regions displayed the strongest signals of selection, indicating that they may play a crucial role in the genetic adaptation of Awarik camels. Notably, a region on chromosome 15 was identified by both the iHS and nSL analyses, with the TRNAI-AAU gene located within this overlapping region, indicating a robust selection signal that may be significant for adaptive processes in camels. Additionally, chromosome 3 exhibited the highest number of candidate regions, with a total of 10 identified (see Supplementary Tables S1, S5).

Conversely, the nSL analysis, chromosomes 2 and 7 exhibited the most statistically significant selection signals as shown in Figure 2. Although these regions were not the largest in terms of selection signal size, their high statistical significance makes them noteworthy. In particular, chromosome 2 showed a pronounced peak that warrants further investigation to identify potential candidate genes related to adaptive traits in Awarik camels. The nSL analysis also revealed a high concentration of candidate regions on chromosome 14, totaling five regions. Furthermore, the largest candidate regions were located on chromosome 11 (spanning 200 kb: 51.750–51.950 kb) and chromosome 9 (spanning 325 kb: 45.825–46.150 kb) in the iHS and nSL analyses, respectively (see Supplementary Tables S1, S5).

3.3 Functional annotation of candidate regions and enrichment analysis

3.3.1 Integrated haplotype score (iHS)

The iHS analysis identified 185 genes within the 66 candidate selection regions (Supplementary Table S1). Functional profiling of these genes revealed several enriched molecular and biological processes, including the Wnt signaling pathway (see selected results in Table 1, Supplementary Tables S1, S2). However, none of these processes were significantly enriched. DAVID analysis identified six functional clusters, showing enrichment for functions related to olfactory activity (enrichment score = 4.65), immunoglobulin subtype (enrichment score = 0.65), ATP binding (enrichment score = 0.41), basic and acidic residues (enrichment score = 0.24), and zinc finger C2H2-type/integrase DNA-binding domain (enrichment score = 0.13). Literature review highlighted candidate

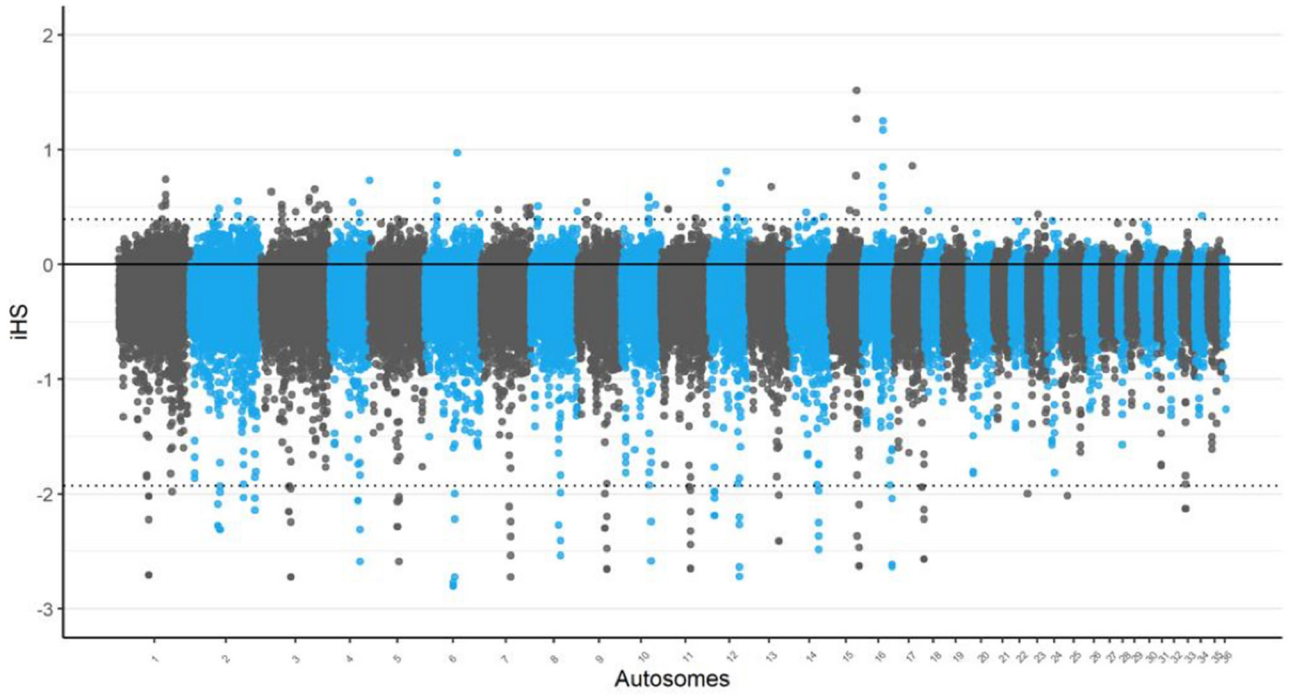

FIGURE 1
Signatures of selection analysis on autosomes of Awarik dromedary camels. Manhattan plots of genome-wide iHS analysis with a two-tailed Z-test applied and the significance threshold is set at−log10 (two-tailed *P*-value) = 4.

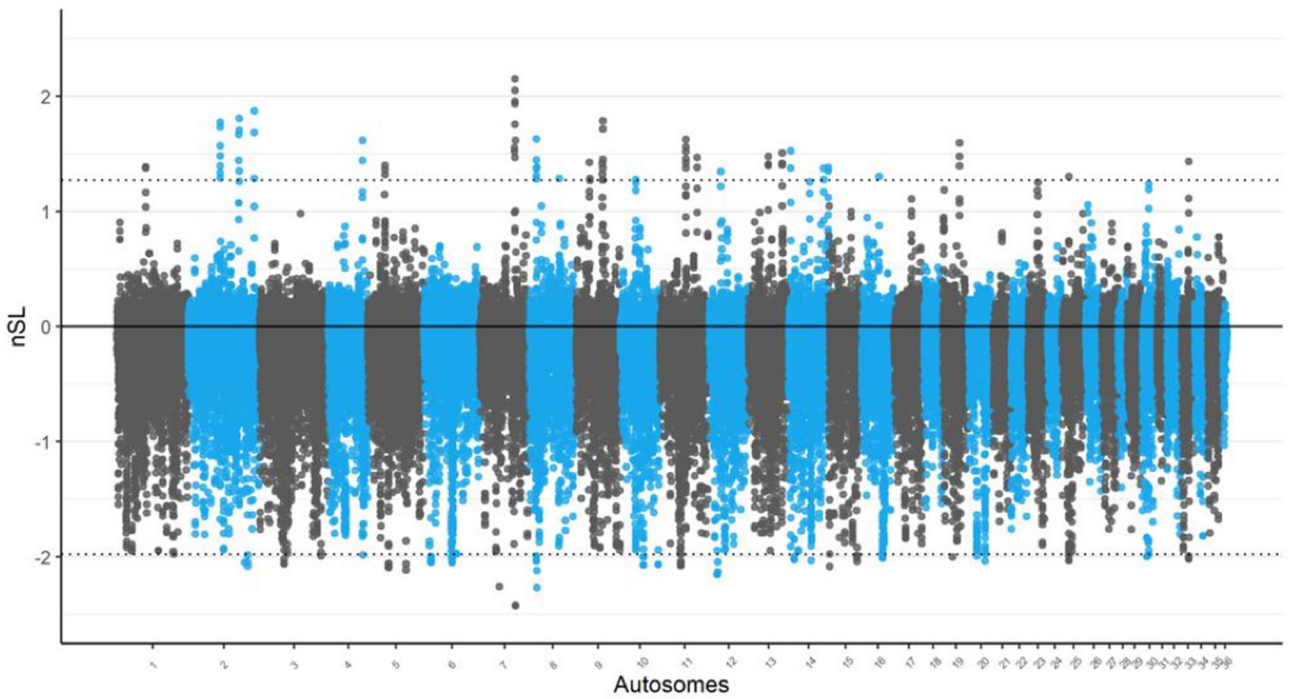

FIGURE 2
Signatures of selection analysis on autosomes of Awarik dromedary camels. Manhattan plots of genome-wide nSL analysis with a two-tailed Z-test applied and the significance threshold is set at−log10 (two-tailed *P*-value) = 4.

TABLE 2 Biological functions of candidate genes under selection using iHS analysis on Awarik dromedary camel autosomes.

Functional category	Gene ID	Candidate region (chromosome: start-stop)
Immune regulation and inflammatory responses	BAG5	6: 95.53–95.54 Mb
	septin 7	7: 48.49–48.58 Mb
	N4BP1	9: 51.77–51.86 Mb
	DOCK9	14: 60.02–60.28 Mb
	HTR7	11: 51.89–51.97 Mb
Translation and protein synthesis	TRNAT-GGU	4: 58.64–58.64 Mb
	TRNAI-AAU	15: 48.61–48.61 Mb
	TRNAT-UGU	16: 35.97–35.97 Mb
	EIF3F	10: 39.45–39.46 Mb
Cell/receptors signaling and regulation	BMPR1B	2: 55.21–55.56 Mb
	JAKMIP2	3: 10.73–10.74 Mb
	NOTCH2	9: 23.18–23.33 Mb
	GNAO1	9: 45.71–45.88 Mb
	WARS2	9: 22.64–22.73 Mb
	CES5A	9: 45.88–46.11 Mb
	CES1	9: 46.13–46.16 Mb
	ATOH7	11: 35.45–35.48 Mb
	YAP1	10: 65.71–65.82 Mb
	CRK	16: 35.70–35.73 Mb
	CDK8	14: 34.98–35.83 Mb
	MYO1C	16: 35.73–35.76 Mb
	WASF3	14: 36.64–37.41 Mb
	YWHAE	16: 35.65–35.69 Mb
	INPP5K	16: 35.76–35.78 Mb
	TMX4	19: 28.99–29.10 Mb
	PLCB1	19: 29.14–29.81 Mb
	RIMS2	25: 11.59–12.10 Mb
	DPYS	25: 12.21–12.29 Mb
	TMPRSS13	33: 12.25–12.28 Mb
	5-hydroxytryptamine receptor 3C-like	1: 51.80–51.81 Mb
	5-hydroxytryptamine receptor 3E	1: 51.84–51.85 Mb
	CASC4	6: 20.59–20.69 Mb
	PTPRN2	7: 85.74–86.12 Mb
	NT5DC3	12: 28.86–28.92 Mb
	5-hydroxytryptamine receptor 3C-like	1: 51.82–51.82 Mb
Metabolism	SLC13A1	7: 60.76–60.83 Mb
	PCED1B	12: 14.12–14.26 Mb
	CLYBL	14: 60.65–60.86 Mb
	PIGB	6: 11.95–11.98 Mb
	PIGBOS1	6: 11.98–11.98 Mb
	FXYD6	33: 12.30–12.33 Mb
	FXYD2	33: 12.34–12.35 Mb
	Creatine kinase (CKB)	6: 95.50–95.51 Mb
	ER-resident protein 44 (ERp44)	4: 39.21–39.28 Mb
	GLT8D2	12: 28.71–28.75 Mb
	Wdr78	13: 51.72–51.80 Mb
Cellular/membrane transport and vesicle trafficking	SLC10A4	2: 87.91–87.91 Mb
	SLC2A13	12: 19.47–19.83 Mb
	RAB27A	4: 58.58–58.60 Mb
	DOC2B	16: 35.61–35.64 Mb
Cytoskeleton and cellular structure	SLAIN2	2: 87.95–88.02 Mb
	RBMS1	5: 35.33–35.53 Mb
	TBX15	9: 22.50–22.61 Mb
	ST6GALNAC3	13: 58.80–59.34 Mb
Protein synthesis and modification	EIF2B5	1: 51.86–51.87 Mb
	RPL24	1: 81.72–81.72 Mb
	60S ribosomal protein L17 pseudogene	2: 48.65–48.66 Mb
	60S ribosomal protein L34 pseudogene	3: 94.63–94.64 Mb
	MRPS27	3: 48.51–48.61 Mb
	PTCD2	3: 48.61–48.64 Mb
	SENP7	1: 81.79–81.92 Mb
	UBE2E3	5: 51.84–51.92 Mb
Enzymes and catalytic activity	ALG3	1: 51.94–51.95 Mb
	ACSL6	3: 94.68–94.74 Mb
	Dram2	9: 16.02–16.04 Mb
	CTDSPL2	6: 20.52–20.59 Mb
	PRKG1	11: 47.54–48.65 Mb
	MSH2	15: 34.92–34.99 Mb
Transporters and Ion channels	SLC39A8	2: 50.04–50.11 Mb
	SLC35D1	13: 51.87–51.92 Mb
	ZFP62	3: 11.76–11.76 Mb
Nuclear and transcriptional regulation	TAF1D	10: 60.09–60.10 Mb
	NR2C2	17: 48.56–48.64 Mb
	UROC1	17: 51.78–51.81 Mb
	ZXDC	17: 51.81–51.84 Mb
	Tdg	12: 28.73–28.77 Mb

TABLE 2 (Continued)

Functional category	Gene ID	Candidate region (chromosome: start-stop)
Cellular structure and motility	LAMTOR3	2: 51.83–51.85 Mb
	DAPP1	2: 51.85–51.90 Mb
	ESyts 2	7: 86.33–86.40 Mb
	WDR60	7: 86.42–86.47 Mb
Neuronal development and function	ASTN2	4: 51.26–52.06 Mb
	EpCAM	15: 34.90–34.92 Mb
	CNTNAP2	7: 77.81–79.82 Mb
	VSTM5	10: 60.15–60.17 Mb
	Cntn1	12: 18.76–19.05 Mb

Functional categories encompass examples of candidate genes and their respective candidate regions.

genes involved in immune regulation and inflammatory responses, metabolism, cell signaling and receptor regulation, neuronal development and function, and cytoskeleton and cellular structure (Table 2).

3.3.2 Number of segregating sites by length (nSL)

The nSL analysis identified 123 genes within the 53 candidate selection regions (Supplementary Tables S4, S5). Functional profiling of these genes revealed several enriched molecular and biological processes, including the Wnt signaling pathway (see selected results in Table 2, Supplementary Tables S4, S5). However, none of these processes were significantly enriched. DAVID analysis identified five functional clusters, showing enrichment for functions related to transmembrane helices (enrichment score = 0.52), olfactory and transducer activity (enrichment score = 0.39), immunoglobulin-like domains (enrichment score = 0.38), cell and plasma membranes (enrichment score = 0.38), and ion and zinc binding (enrichment score = 0.20). Literature review highlighted several candidate genes associated with key biological processes such as reproductive performance, immune response, neuroplasticity, insulin secretion and signaling, as well as kidney absorption and reabsorption (Table 3, Supplementary Table S6). These genes are of particular interest due to their roles in physiological adaptations that are critical for the survival and reproductive success of Awarik camels in challenging environments.

4 Discussion

This study provides a comprehensive genomic analysis the Awarik camel population, indigenous to the southwestern region of Saudi Arabia, offering significant insights into the genetic mechanisms underlying their adaptation to challenging environments (2, 3). Through high-depth whole genome sequencing, we identified key genomic regions under positive selection, shedding light on the evolutionary strategies that have enabled these camels to thrive in semi-arid and coastal habitats (6). The identification of significant selection regions on chromosomes 2 and 7 in the nSL analysis, and on chromosomes 15 and 16

TABLE 3 Biological functions of candidate genes under selection using nSL analysis on Awarik dromedary camel autosomes.

Functional category	Gene ID	Candidate region (chromosome: start-stop)
Immune response	JAKMIP2	03: 10.73–10.74 Mb
	NOTCH2	09: 23.18–23.33 Mb
Metabolism/enzymes	PBLD	11: 35.41–35.45 Mb
	CDK8	14: 34.98–35.83 Mb
	INPP5K	16: 35.76–35.78 Mb
	PITPNA	16: 35.78–35.81 Mb
	CLYBL	14: 60.65–60.86 Mb
	DPYS	25: 12.21–12.29 Mb
	TMPRSS13	33: 12.25–12.28 Mb
	FXYD6	33: 12.30–12.33 Mb
	INPP5K	16: 35.76–35.78 Mb
	PITPNA	16: 35.78–35.81 Mb
	TLCD2	16: 35.92–35.92 MB
Insulin secretion and signaling	DOC2B (105088125)	16: 35.61– 35.64 MB
	MYO1C (105088122)	16: 35.73– 35.78 MB
Cellular components/ structural genes	RUFY2	11: 35.36–35.40 Mb
	PBLD	11: 35.41–35.45 Mb
	PCED1B	12: 14.12–14.26 Mb
	AMIGO2	12: 14.26–14.26 Mb
	ZSWIM5	13: 34.00–34.13 Mb
	ST6GALNAC3	13: 58.80–59.34 Mb
	CRK	16: 35.70–35.73 Mb
	MYO1C	16: 35.73–35.76 Mb
	WDR81	16: 35.93–35.94 Mb
DNA/RNA binding/ transcription/ translation	EIF3F	10: 39.45–39.46 Mb
	HNRNPH3	11: 35.40–35.41 Mb
	TRNAI-AAU	15: 48.61–48.61 Mb
	DOC2B	16: 35.61–35.64 Mb
	RPA1	16: 36.00–36.05 Mb
	TMX4	19: 28.99–29.10 Mb
Signal transduction/ cell communication	SLC13A1	07: 60.76–60.83 MB
	TUB	10: 39.35–39.43 Mb
	YAP1	10: 65.71–65.82 Mb
	YWHAE	16: 35.65–35.69 Mb
	MYO1C	16: 35.73–35.76 Mb
	CRK	16: 35.70–35.73 Mb
Membrane transport	SLC2A13	12: 19.47–19.83 Mb
	WASF3	14: 36.64–37.41 Mb
	PITPNA	16: 35.78–35.81 Mb
Cell cycle/ development	ATOH7	11: 35.45–35.48 Mb
	MYPN	11: 35.48–35.57 Mb

(Continued)

TABLE 3 (Continued)

Functional category	Gene ID	Candidate region (chromosome: start-stop)
	WDR81	16: 35.93–35.94 Mb
	DSCAML1	33: 12.38–12.68 Mb

Functional categories encompass examples of candidate genes and their respective candidate regions.

in the iHS analysis, emphasizes the complexity of the selection landscape in Awarik camels. The statistically significant signals on chromosomes 2 and 7 suggest that these regions may harbor genes of adaptive significance, potentially influencing traits that are critical for survival in the harsh environments inhabited by these camels. In particular, the overlap identified on chromosome 15, where the TRNAI-AAU gene was detected in both the iHS and nSL analyses, strengthens the case for importance of this gene. which likely plays a pivotal role in the genetic adaptation of Awarik camels. Further functional analysis is needed to elucidate its specific role in camel physiology and adaptation.

Our analysis highlighted significant selection signatures on chromosomes 3, 14, 11, and 9, each pointing to different aspects of genetic adaptation. Chromosomes 3 and 14, which showed the highest number of candidate regions in the iHS and nSL analyses, respectively, are particularly important as they likely host genes conferring adaptive advantages in response to the specific selection pressures faced by the Awarik population (Supplementary Tables S1, S5). This pattern suggests a tailored response to the unique selection pressures faced by the Awarik population, potentially reflecting localized adaptations not as pronounced in other regional camel populations.

Moreover, the notable presence of large candidate regions on chromosomes 9 and 11 underscores the importance of these loci in the adaptation of the Awarik camels. Previous studies such as those by Bahbahani et al. (6) and Al Abri et al. (5) have reported similar high-frequency haplotypes on these chromosomes in mixed groups of dromedaries, including Awarik camels. The consistency of these findings across studies supports the hypothesis that these genomic regions may hold unique significance for the Awarik population, necessitating further research to fully understand their biological relevance.

Functional annotation of these regions revealed involvement in diverse biological processes critical to survival in extreme environments, such as immune regulation, metabolism, and reproductive performance. Although no significant enrichment was found in pathways like Wnt signaling, the recurring identification of genes associated with this pathway across different analyses suggest its potential role in developmental and adaptive processes (15). The DAVID analysis further supported this by identifying functional clusters related to sensory functions, immune responses, and metabolic processes, all of which are vital for thriving in the variable climates of the Red Sea coastal areas (16, 17). These findings imply a complex and multifaceted genetic basis for the adaptation Awarik camel, involving various physiological and cellular processes.

The genes uncovered in this study, particularly those involved in olfactory activity, immune response, and kidney function, highlight the Awarik camel's genetic specialization for their diet and environmental stressors. These adaptations appear to be deeply embedded in the genetic fabric of the population, suggesting a complex evolutionary history that has finely tuned these animals to their specific ecological niches (5).

Furthermore, the implications of these genomic insights extend beyond academic understanding to practical applications in breeding and conservation. The detailed genetic markers identified here can guide selective breeding programs aimed at enhancing desirable traits like resilience to climate variability and disease resistance. Additionally, the genetic diversity revealed through this study underscores the importance of conservation strategies that preserve these unique genetic resources, which are invaluable for the Awarik camel's continued adaptation and survival (1).

Future research should build on on these findings by increasing the sample sizes and incorporating comparative genomic studies with other camel populations, including those from East Africa with whom the Awarik camels share historical ties (9). Such studies could illuminate both shared and unique adaptations, providing a broader understanding of camel evolution across different environments. Integrating environmental and phenotypic data could refine the connections between genetic adaptations and specific environmental challenges, enhancing the predictive power of genomic studies in conservation and management practices.

In conclusion, the genomic signatures of positive selection identified in the Awarik camels not only deepen our understanding of their unique adaptations but also provide a foundational knowledge base for developing targeted interventions in breeding and conservation. These interventions are critical for sustaining the Awarik camel population amid the escalating pressures of climate change and habitat loss, ensuring their resilience and productivity for future generations.

Data availability statement

The data presented in the study are deposited at European Nucleotide Archive Bioproject number: PRJEB47650.

Ethics statement

The animal studies were approved by Ethics Research Committee approval at the King Faisal University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

FA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1443748/full#supplementary-material

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Morphological and molecular identification of *Eimeria rajasthani* (coccidia: Eimeriidae) in the dromedary camel (*Camelus dromedarius*) in Riyadh, Saudi Arabia

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Introduction: Coccidiosis is a serious parasitic disease in camels caused by an intestinal protozoan parasite of the genus *Eimeria*, which is linked to significant causes of reduced milk and meat production. In Saudi Arabia, scare literature focused on the coprological investigation of dromedary camels (*Camelus dromedarius*). To determine the taxonomic status of camel parasite species, we performed morphological characterization of oocysts and genetic analysis (*18S rRNA* and *ITS-1* gene regions) of *Eimeria* species collected from camels in Riyadh, Saudi Arabia.

Methods: A total of 150 faecal samples were obtained from camels at the old camel market. These samples were tested for the presence of *Eimeria* oocysts using the conventional floatation technique before being sporulated in a 2.5% potassium dichromate solution. *Eimeria* oocysts were morphologically and molecularly examined and identified, and the infection rate of parasitic infections was determined.

Results: Our findings revealed that the overall frequency of oocysts was 30%. The identified species was *Eimeria rajasthani*, which had a typical ellipsoidal oocyst shape. Oocystic polar granule, micropyle, micropylar cap, and oocyst residuum are not visible. Sporocysts are oval with stieda body. Sporocyst residuum contains many granules and sporozoites with refractile bodies and nuclei. Genetic analyses of the sequence data from the partial *18S rRNA* and *ITS-1* gene regions revealed that the sequences obtained from *E. rajasthani* oocysts are related to DNA sequences reported from *E. lamae* from the Alpaca from China, particularly the *18S rRNA* sequences.

Conclusion: This study emphasized the need to use molecular phylogenetic tools to describe camel intestinal coccidian parasites with traditional morphology-based approaches to better understand their biology. For camel husbandry and disease control, more studies should be conducted to better understand the epidemiology of these protozoan parasites.

KEYWORDS

dromedary camels, coccidiosis, prevalence, taxonomy, morphology, genetic analysis

Introduction

The dromedary camel, *Camelus dromedarius* (Order: Artiodactyla), is the most prevalent Camelidae species. Camels have been an essential animal in desert locations for ages due to their ability to tolerate severe conditions (high temperatures and drought), supply milk and meat, and serve as a means of transportation (1, 2). Camels are found in 35 countries around the world, 18 of which are African. According to recent official statistics, Saudi Arabia is home to approximately 1.8 million camels. Camels are prone to a variety of diseases, especially due to the lack of sufficient veterinary services (3). Gastrointestinal parasites are one of the most common challenges facing the global camel population (4), causing not only nutritional and immune deficiencies but also stunted growth and delayed development (5, 6). These parasitic infections affect camel production and the quality of their meat and milk (7–9).

Eimeria species are gut-dwelling intracellular coccidian parasites that spread by the fecal–oral pathway; non-sporulated oocysts are discharged in feces of infected animals (10). Sporulation of oocysts occurs over 2–7 days, depending on coccidian species and environmental factors (e.g., oxygen, temperature, and moisture) (11). Five *Eimeria* species are thought to have the capability of infecting the camel's intestine (5). *Eimeria cameli* (12) and *Eimeria dromedarii* (13) are the most widely distributed species of camelid *Eimeria*, while others [*Eimeria bactriani* (14); *Eimeria rajasthani* (15); and *Eimeria pellerdyi* (16)] are found in specific geographical zones. Coccidiosis is most commonly reported in young animals, but adults are resistant due to an immunological response to previous *Eimeria* exposure (17–19). Camels with severe *Eimeria* infections exhibit symptoms such as hemorrhagic enteritis and diarrhea, loss of appetite, dehydration, and increasing weight loss (20). Furthermore, the free movement of camels across borders could lead to the spread of parasitic diseases (21–23).

Eimeria species have been identified using the shape of the sporulated oocysts and sporocysts (24). *Eimeria* species were identified using morphological features such as size, shape, color, sporulation time, texture of oocyst wall, presence or absence of micropyle, and micropylar cap, as well as (25) taxonomy keys. However, only a few *Eimeria* species have morphological resemblance with one another. Molecular analysis is required to reliably define *Eimeria* species and establish evolutionary relationships between them (26). Few studies have focused on the ability to use the internal transcribed spacer (ITS) region to identify camelid's *Eimeria* species (27, 28). The previous studies in Saudi Arabia had addressed the phylogenetic relationships of coccidian species based on the ability of the use target genetic regions, including the small subunit ribosomal RNA (*18S rRNA*), internal transcribed spacer (*ITS*)-1, and mitochondrial cytochrome c oxidase I (*COI*) genes in identification and taxonomy of *Eimeria* species, which parasitize rodents (29), rabbits (30), sheep (31), broiler chicken (32), and domestic pigeons (33).

Several investigations on camelid coccidian infection have been conducted in Saudi Arabia (17–19, 34–36). Three protozoan parasites, namely, *Eimeria dromedarii, E. rajasthani*, and *E. cameli*, were detected in the dromedary camel in Saudi Arabia. The pathology of the three species has been evaluated, and they are pathogenic in young camels causing enteritis as a result of the intestinal mucosa destruction whereas older camels did not show clinical signs (17).

Similarly, to control coccidiosis in camels successfully and economically, an extensive understanding of the *Eimeria* species implicated is required. Therefore, the purpose of this study was to morphologically identify camelid *Eimeria* species and molecularly corroborate their classification.

Materials and methods

Fecal sample collection

A total of 150 fecal samples (10 g/animal) were collected, between January and April 2024, from dromedary camels in the old camel market in Riyadh (Saudi Arabia). These samples were obtained directly from the rectum using disposable gloves, placed into screw-capped plastic containers, and labeled with epidemiological data. The samples were then transported in an icebox to the Laboratory of Parasitology Research (Department of Zoology, College of Science, King Saud University) for further analysis.

Coprological examination

All fecal samples were subjected to a floatation technique using a saturated saline solution (Sheather's solution, specific gravity = 1.28) as reported by Soulsby (37). In brief, 3 g of fecal material from each sample was weighed, mixed with 15 mL of saturated sucrose solution, and homogenized. The fecal suspension was then centrifuged at 1,500 rpm for 3 min at room temperature (RT). The samples were examined using a light microscope (Olympus Corporation, Tokyo, Japan). To identify the species, positive samples with *Eimeria* oocysts were cultivated in Petri dishes containing 2.5% (w/v) potassium dichromate (Sigma-Aldrich) and incubated at 26 ± 2°C for 2–7 days until sporulation was achieved (38). After sporulation, the oocysts were washed three times in 1× phosphate-buffered saline (PBS) and kept at 4°C for further investigation. Photographs of oocysts (non-sporulated and sporulated) were acquired with a Leica DM 2500 microscope (NIS ELEMENTS software, version 3.8). The size and shape index of oocysts and sporocysts were calculated using ImageJ 1.53e software (Wayne Rasband and contributors, National Institute of Health, United States). The length, width, and shape index of the oocysts and sporocysts were measured for parasite species. Data were presented in micrometers (μm) as the mean, with the range in parentheses.

Molecular analysis

DNA was isolated from *Eimeria* oocysts via a commercial QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of the genetic sample were evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, United States). PCR was performed under conditions that targeted the partial *18S rRNA* and *ITS-1* gene regions. Amplification was carried out utilizing the genus-specific primers as follows: for the *18S rRNA* gene region was 5′-TAC CCA ATG AAA ACA GTT T-3′ and 5′-CAG GAG AAG CCA AGG TAG G-3′ (39), and the *ITS-1* gene region was

5′-GCA AAA GTC GTA ACA CGG TTT CCG-3′ and 5′-CTG CAA TTC ACA ATG CGT ATC GC-3′ (40). The reaction conditions were as follows: initial denaturation at 94°C for 2 min, then denaturation at 94°C for 50 s, annealing at 50°C (*18S rRNA*), and 52°C (*ITS-1*) for 30 s, and extension at 72°C for 30s in 35 cycles. PCRs were carried out using a Multigene™ thermocycler (Labnet International, Inc., NJ, United States). Amplified products were electrophoretically analyzed using a 1.5% (w/v) agarose gel (Sigma-Aldrich, United States) in 1 × Tris–boric acid–EDTA (TBE) and stained with SYBR Safe DNA gel dye (Thermo Fischer Scientific, Canada) and using Easy Ladder 1 (100 bp to 2000 bp) from Bioline, United Kingdom, as a molecular weight marker, indicating the size of the PCR products resulted from using these primers. Products were visualized using a gel documentation system (Image Analyzer, United Kingdom). The PCR products were sequenced using the Sanger dideoxy method available from Macrogen® (Seoul, South Korea). Both *18S rRNA* and *ITS-1* regions were selected for easy comparison with related sequences in GenBank. Sequences were deposited at a public sequence database, GenBank of NCBI.[1] The sequence homology was analyzed in GenBank using the BLASTn search.[2] Data were aligned using CLUSTAL-X software (41). MEGA X software (42) was used to conduct maximum likelihood (ML) and neighbor-joining (NJ) analyses with the best-fit substitution models. Statistical support for each node was evaluated using a non-parametric bootstrap test with 1,000 replicates. Trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Results

Out of the 150 examined fecal samples, 45 (30%) were infected with eimerian parasites. The recovered parasite possesses a unique taxonomic affinity for the genus *Eimeria*, particularly for *E. rajasthani*, as detailed below. Figure 1 depicts the oocysts of *Eimeria* species recovered from camels during the current study. Table 1 summarizes the morphometric parameters of the recovered *Eimeria* species.

Description

Non-sporulated oocysts are ellipsoidal, measuring 25.64–35.39 (32.15) in length and 20.20–25.65 (23.66) in width (Figure 1A). The oocyst wall is double-layered, with the outer one being thicker and inner one being membranous (Figure 1A). The micropyle is visible; the micropylar end has a dome-shaped micropylar cap, measuring 1.78–2.82 (1.99) in height and 7.63–10.53 (8.41) in width (Figure 1A). The sporont (zygote) is cylindrical, measuring 17.41–20.54 (20.31) µm × 17.44–20.84 (19.65) (Figure 1A). Sporulation took approximately 7 days at 27°C.

Sporulated oocysts are ellipsoidal, measuring 27.86–37.42 (33.71) in length and 21.19–27.86 (25.61) in width. Micropylar cap measures 7.63–10.53 (8.41) in width, whereas oocystic polar granule and oocyst residuum are absent (Figure 1B). Each oocyst was tetrasporozoic (Figure 1B). Sporocysts are oval, measuring 12.13–15.46 (13.97) in length and 8.64–11.58 (10.12) in width. They have a single-layered wall and Stieda body at the narrower end (Figure 1B). Sporocyst residuum exists between the two sporozoites (Figure 1B). Each sporocyst is dizoic. Sporozoites are elongated, lying longitudinally head to tail in the sporocysts, 10.84–12.83 (11.96) µm × 3.12–4.85 (4.10) µm, with one end broad and the other narrower but pointed and having two or more conspicuous globules (Figure 1B). Each sporozoite has one refractile body at the wider end (Figure 1B).

Molecular analysis

The amplification of both *18S rRNA* and *ITS-1* gene regions for *E. rajasthani* was successful using primers that were used in the present study. The expected PCR products of ~613 bp and ~380 bp were obtained and sequenced for the *18S rRNA* and *ITS-1* gene regions, respectively. Four sequences were obtained from the *18S rRNA* region and deposited in GenBank and were given the accession numbers PP965651 to PP965654. Two sequences were obtained from the *ITS-1* region and were also deposited in GenBank and were given the accession numbers PP965655 and PP965656.

The *18S rRNA* sequences showed two haplotypes with one sequence (PP965653) with a mutation (C/T) at position 238 on the alignment. Sequences showed 99% identity to sequence MT337428 isolated from the feces of Alpaca (*Vicugna pacos*) from China. The sequence from Alpaca showed one to two mutations when compared with sequences from *E. rajasthani* obtained in the present study. There is another sequence from the Alpaca MT337427 which was shorter than MT337428, which also showed identity to sequences obtained in the present study. Although the sequence did not cover the whole region studied, it has shown differences in three bases at positions 243, 245, and 280 of the alignment. The closest match for the sequences obtained in the present study other than MT337427 and MT337428 from the Alpaca was a sequence (MK170375) from *Eimeria mayeri* from Reindeer (*Rangifer tarandus tarandus*) from Norway with 97.8% identity. Phylogenetic analysis of the *18S rRNA* sequence data, resulting from neighbor-joining (NJ) and maximum-likelihood (ML) analyses, revealed that sequences from *E. rajasthani* and *Eimeria* sp. from the Alpaca shared a common ancestor and formed a monophyletic group (Figure 2). Sequences from the present study have shown 97% identity to several other eimerian sequences from carnivores and birds. Furthermore, it showed the same values from some isospora sequences from birds as it has been shown in Figure 2. Taxa used in the analysis are presented in Table 2.

The *ITS-1* sequences (PP965655 and PP965656) from *E. rajasthani* have shown 94–95% to *E. lamae* (GQ330537 {USA}, MW838990 {China}, MW838989 {China}), the only three sequences available in GenBank. The two sequences obtained in the present study showed two haplotypes with a mutation at position 136 (C/G) on the alignment.

Discussion

Infection with coccidian intestinal parasites has a significant economic impact because of losses due to enteritis, diarrhea, and

1 https://www.ncbi.nlm.nih.gov/

2 https://blast.ncbi.nlm.nih.gov/Blast.cgi

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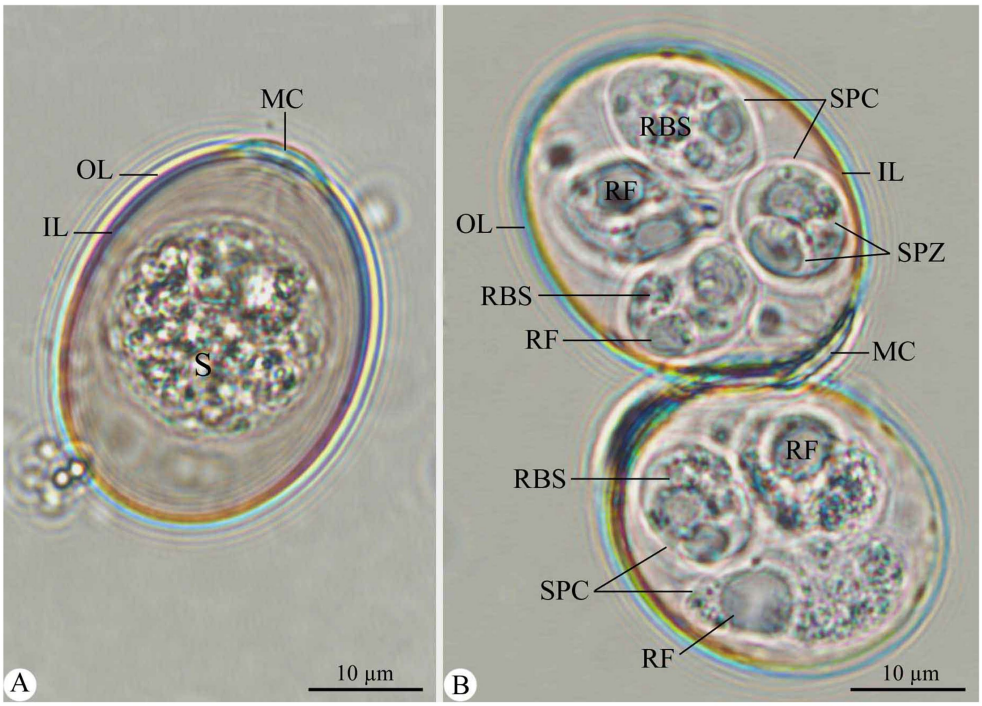

FIGURE 1
Morphological features of *E. rajasthani* oocysts infecting camels. (A) Non-sporulated *Eimeria* oocyst. (B) Sporulated *Eimeria* oocysts. OL, outer layer; IL, inner layer; S, sporont; MC, micropylar cap; SPC, sporocyst; SPZ, sporozoite; RF, refractile body; RBS, sporocyst residuum. Scale bar = 10 µm.

TABLE 1 Morphological characteristics of sporulated oocysts for *E. rajasthani* from *Camelus dromedarius*.

References of *E. rajasthani*	Oocyst size				Oocyst shape	Sporocyst size			Locality
	Size	Micropyle	Polar granule	Oocyst residuum		Size	Sporocyst residuum	Stieda body	
Dubey and Pande (15)	34–39 (36) × 25–27 (25)	+ capped	–	–	Ellipsoidal	14–15 (15) × 8–11 (11)	+	+	India
Yagoub (46)	34–39.5 (35.6) × 26–29 (26.5)	+ capped	–	–	Ellipsoidal	12–16 (13.5) × 9–11 (9.9)	+	+	Eastern Region of Sudan
Mahran (47)	39.5 × 26	+ capped	–	–	?	?	?	?	Red Sea Governorate, Egypt
Metwally et al. (36)	25–30 × 21–24	+ capped	–	–	Oval	12–14 (13) × 9–10 (9.5)	–	?	Riyadh and Al-Qassim, Saudi Arabia
Present study	**27.86–37.42 (33.71) × 21.19–27.86 (25.61)**	**+ capped**	**–**	**–**	**Ellipsoidal**	**12.13–15.46 (13.97) × 8.64–11.58 (10.12)**	+	+	**Riyadh, Saudi Arabia**

+ = present; – = absent; ? = not specified.

decreased body weight in camels, which also affects meat yield and quality (43). There is little information available on the epidemiology of coccidian intestinal parasites in dromedary camels in Saudi Arabia. As a result, the purpose of this study was to conduct coprological and molecular investigations of camelids *Eimeria* species to provide additional information about these parasites in the Riyadh region, Saudi Arabia. In the current study, the infection rate with *Eimeria* species in dromedary camels was 30%. Several studies have revealed infection rates in various camel-rearing regions across the world. In earlier studies, Kawasmah and El Bihari (35), Kasim et al. (18), and Hussein et al. (17) discovered one or more species (*E. rajasthani*, *E. cameli*, and *E. dromedarii*) with an overall prevalence of 14, 41.6, and 40% in Saudi Arabian camels, respectively. Mahmoud et al. (19) found a mean infection rate of 15.7% for adult camels and 10.2% for

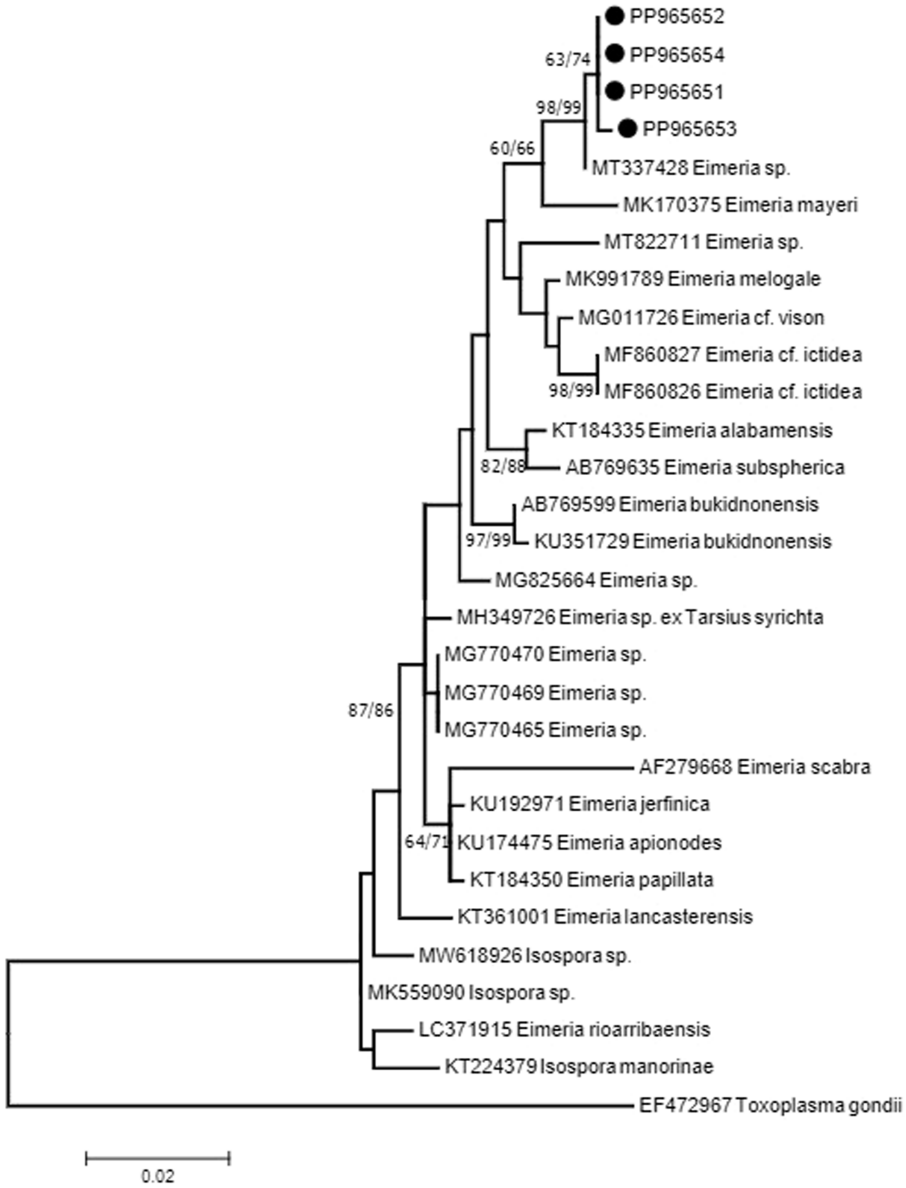

FIGURE 2
Consensus phylogenetic tree constructed with maximum-likelihood (ML) and neighbor-joining (NJ) methods, showing phylogenetic relationships between *E. rajasthani* (PP965651 to PP965654; shown on solid circles) and 25 related taxa obtained from NCBI GenBank with *Toxoplasma gondii* as an out-group. Numbers indicated at branch nodes are bootstrap values (ML/NJ). Only bootstraps >60% are shown.

camel calves in Saudi Arabia's central region. Metwally et al. (36) investigated coccidiosis in camels in Saudi Arabia and discovered that the prevalence of *Eimeria* oocysts in Riyadh was 33.89% and in Al-Qassim 38.46%. According to Sazmand et al. (44), changes in the prevalence of coccidian infections in camels are likely due to environmental and host-related factors.

Different diagnostic methods for *Eimeria* species are currently available, with varying degrees of specificity and sensitivity, including morphological examination and DNA molecular tools (45). There are five recognized old-world camelid eimerian species (including *E. cameli*, *E. dromedarii*, *E. bactriani*, *E. rajasthani*, and *E. pellerdyi*). Based on the morphological findings, the species detected in the camel in Riyadh (Saudi Arabia) is related to *E. rajasthani*. The main criteria for identifying recovered *E. rajasthani* were the ellipsoidal shape of oocysts and the presence of a dome-shaped micropylar cap. Our descriptions of the sporulated oocysts of *E. rajasthani* were similar to those of Dubey and Pande (15), Yagoub (46), Mahran (47), and Metwally et al. (36). Although Metwally et al. (36) described the oocysts of *E. rajasthani* as oval, they did not demonstrate the oocyst residuum and the oocyst Stieda body.

Five eimerian species have also been described from the New World camelids which are as follows: *E. macusaniensis, E. lamae, E. alpacae, E. punoensis, and E. ivitaensis* (48). *E. rajasthani* showed close similarity in measurements with *E. lamae* from the Alpaca (*V. pacos*). However, the shape of the micropylar cap is different between the two organisms. There was no association between Alpacas and the dromedary camel in the present study; therefore, it is unlikely that the species of *Eimeria* detected in the present study could

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TABLE 2 Taxa and their *18S rRNA* sequences GenBank accession numbers, their hosts, and their origin were used in the present analyses.

Accession number	Host	Country
PP965651 *Eimeria rajasthani*	**Camel (*Camelus dromedarius*)**	**Saudi Arabia**
PP965652 *Eimeria rajasthani*	**Camel (*Camelus dromedarius*)**	**Saudi Arabia**
PP965653 *Eimeria rajasthani*	**Camel (*Camelus dromedarius*)**	**Saudi Arabia**
PP965654 *Eimeria rajasthani*	**Camel (*Camelus dromedarius*)**	**Saudi Arabia**
MT337428_*Eimeria*_sp.	Alpaca (*Vicugna pacos*)	China
MK170375_*Eimeria_mayeri*	Reindeer (*Rangifer tarandus tarandus*)	Norway
MF860827_*Eimeria_cf._ictidea*	Black-footed Ferrets (*Mustela nigripes*)	Canada
MF860826_*Eimeria_cf._ictidea*	Black-footed Ferrets (*Mustela nigripes*)	Canada
MK991789_*Eimeria_melogale*	Javan ferret-badger (*Melogale orientalis*)	Czech Republic
MG011726_*Eimeria_cf._vison*	American Mink (*Neovison vison*)	Australia
KT184335_*Eimeria_alabamensis*	Cow (*Bos taurus*)	Canada
MG825664_*Eimeria*_sp.	Capercaillie grouse (*Tetrao urogallus*)	Poland
MT822711_*Eimeria*_sp.	Père David's deer (*Elaphurus davidianus*)	China
MG770470_*Eimeria*_sp.	Shrew (*Crocidura* sp.)	Romania
MG770469_*Eimeria*_sp.	Shrew (*Crocidura* sp.)	Bulgaria
MG770465_*Eimeria*_sp.	Shrew (*Crocidura suaveolens*)	Czech Republic
KU192971_*Eimeria_jerfinica*	Striped Field Mouse (*Apodemus agrarius*)	Czech Republic
MH349726_*Eimeria_sp._ex_Tarsius_syrichta*	Philippine tarsier (*Carlito syrichta*)	Czech Republic
MW618926_*Isospora*_sp.	Northern Flicker (*Colaptes auratus luteus*)	Canada
MK559090_*Isospora*_sp.	Eurasian Wren (*Troglodytes troglodytes*)	Czech Republic
LC371915_*Eimeria_rioarribaensis*	Northern Bat (*Eptesicus nilssonii*)	Japan
AF279668_*Eimeria_scabra*	Pig (*Sus domesticus*)	Germany
KT224379_*Isospora_manorinae*	Yellow-throated miner (*Manorina flavigula wayensis*)	Australia
KT361001_*Eimeria_lancasterensis*	Eastern Gray Squirrel (*Sciurus carolinensis*)	Czech Republic
AB769635_*Eimeria_subspherica*	Cow (*Bos taurus*)	Japan
KU174475_*Eimeria_apionodes*	Yellow-necked mouse (*Apodemus flavicollis*)	Czech Republic
KT184350_*Eimeria_papillata*	House Mouse (*Mus musculus*)	Canada
AB769599_*Eimeria_bukidnonensis*	Cow (*Bos taurus*)	Japan
KU351729_*Eimeria_bukidnonensis*	Cow (*Bos taurus*)	Turkey
EF472967_*Toxoplasma_gondii*	RH Strain	China

Sequences from *E. rajasthani* reported in the present study are shown in bold.

be *E. lamae*. Furthermore, *E. lamae* has never been reported from Saudi Arabia.

According to Ipczynski (49), Hussein et al. (17), and Dia et al. (50), *E. dromedarii*, *E. rajasthani*, and *E. cameli* are more pathogenic species to young camel calves; thus, the presence of these three pathogenic *Eimeria* species indicated that coccidiosis could be contributing to enteric syndromes in camels. Yagoub (46) described a clear identification of *E. dromedarii* and *E. cameli*, which may be utilized to distinguish the recovered *E. rajasthani* from them. In this study, the oocysts of *E. rajasthani* are distinct from *E. cameli* on account of the shape of the oocyst (vs. truncate ovoid in *E. cameli*), sporocyst (vs. elongated in *E. cameli*), and sporozoites (vs. comma-shaped in *E. cameli*), the smaller size of both oocyst (vs. 86.6 × 66.2 µm in *E. cameli*) and sporocyst (vs. 37.4 × 18.61 µm in *E. cameli*), bilayered oocyst wall (vs. three-layered in *E. cameli*), the presence of micropyle with 17.3–26.0 µm in width as well as polar granule in *E. cameli*, and 7 days for sporulation (vs. 12–15 days in *E. cameli*). The eimerian oocysts from the present study differ from those of *E. dromedarii* due to the larger size of both oocysts (vs. 28.1 × 23.4 µm in *E. dromedarii*) and sporocysts (vs. 9.0 × 7.3 µm in *E. dromedarii*), their different oocyst shape (subspherical to ovoid shape in *E. dromedarii*) and sporozoites (vs. ovoid in *E. dromedarii*), the presence of micropyle as well as Stieda body in *E. dromedarii*, and the absence of sporocyst residual in *E. dromedarii*.

Furthermore, Prasad (16) provided a detailed description of *E. pellerdyi*, which was utilized to compare with the recovered *E. rajasthani*. The recovered *E. rajasthani* oocysts differ from *E. pellerdyi* in terms of oocyst shape (vs. ovoidal in *E. pellerdyi*) and sporozoites (vs. club-shaped in *E. pellerdyi*), the smaller size of its oocyst (vs. 22.5–24 × 12–13.5 µm in *E. pellerdyi*) and sporocyst (vs. 4.5–6 × 9–10.5 µm in *E. pellerdyi*), the absence of a micropylar cap, and 7 days for sporulation (vs. 5 days in *E. pellerdyi*). Furthermore, Utebaeva et al. (10) described *E. bactriani* in detail, and their data were used to compare it

to the recovered *E. rajasthani*. *Eimeria* oocysts differ from *E. bactriani* in the shape of oocyst (vs. spherical in *E. bactriani*), sporocyst (vs. lemon-shaped in *E. bactriani*), and sporozoites (vs. pear-shaped in *E. bactriani*), larger oocyst size (vs. 29.1 × 26.6 µm in *E. bactriani*), and indistinct micropyle (vs. observed in *E. bactriani* with 5–7 µm width).

Our findings are regarded as a re-description of the discovered camelid's *E. rajasthani* parasite in Saudi Arabia, with adequate morphological and morphometric data. Molecular characterization has recently gained popularity for assuring accurate *Eimeria* species identification, especially when morphological differentiation is problematic due to shape and size similarities (32).

The *18S rRNA* sequences obtained from oocysts of *E. rajasthani* showed 99% sequence similarity to those from *Eimeria* sp. from the Alpaca (MT337428) from China, which was later described as *E. lamae* by Gao et al. (51). The phylogenetic tree generated from the *18S rRNA* sequence data indicated that both *E. lamae* and *E. rajasthani* shared a common ancestor. Another sequence from *E. lamae* (MT337427) reported by Gao et al. (51) was shorter; however, it showed identity to sequences from *E. rajasthani* and MT337428. The similarity of *E. rajasthani* and those from reindeer and carnivores raises a question about the origin and evolution of *E. rajasthani*.

ITS-1 sequences reported from *E. rajasthani* have shown 94–95% identity to sequences from *E. lamae* (51). There were no available sequences for the same region at GenBank; therefore, it was not possible to generate a phylogenetic tree from the available data.

It was proposed by Hnida and Duszynski (52) that eimerian parasites from rodents with a sequence of ≤5% at the *ITS-1* region could support conspecific types which are morphologically similar, whereas differences of >5% in the same region may be used to resolve separate species of *Eimeria*. This suggestion was further supported by Motriuk-Smith et al. (53) who studied genetic variation in squirrels (*Sciurus niger*). It has also been added that the *ITS-1* marker must be used cautiously, and it must be supplemented by other markers together with morphometric data (52, 54).

Morphological and morphometric data of *E. rajasthani* detected from the dromedary camel indicated a close resemblance to *E. lamae* from the New World camelid, the Alpaca from China. In addition, molecular data from the 18S rRNA sequences from *E. rajasthani* showed the identity of 99% to those of *E. lamae* as well; however, there was 95% identity to sequences from the *ITS-1* region of both sequences. The identity of the organism we are dealing with from the dromedary camel is certainly *E. rajasthani*, as there is no possibility that it has been acquired from another species other than the dromedary camel and there is no mixture between the dromedary camel and Alpacas in Saudi Arabia. From the present results, in particular the *ITS-1* data results, it is tempting to suggest that *E. rajasthani* and *E. lame* are conspecific. However, further study is required on different genes, particularly a mitochondrial gene such as cytochrome oxidase I (*COI*), to support this assumption and resolve the taxonomic status of each of *E. rajasthani* and *E. lamae*.

Conclusion

This study provides further understanding regarding *E. rajasthani* oocysts infecting its type host (*C. dromedarius*) from Riyadh (Saudi Arabia) by combining a morphological description of oocysts and a genetic analysis. Furthermore, the GenBank database currently includes unique genetic sequences for the target gene regions retrieved from this coccidian species. Further studies are recommended to incorporate preventative and control approaches to combat *E. rajasthani* infection in the dromedary camel in Saudi Arabia.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PP965651, PP965652, PP96563, PP965654, PP965655, PP965656.

Ethics statement

The animal studies were approved by Research Ethics Committee (REC) at King Saud University (Saudi Arabia). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EA-S: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SO: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RA-G: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. OM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Welfare assessment of dromedary camels kept under pastoralism in Pakistan

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Standardized welfare assessment protocols are crucial to enhance animal welfare; up to date, there is no data on the level of welfare of camels kept under pastoralism. A tailored protocol for measuring welfare in dromedary camels kept under nomadic pastoralist conditions was recently developed, drawing from the currently available welfare protocol for dromedary camels kept in intensive systems. This study, therefore, aimed to apply the newly developed tailored protocol and assess the welfare of dromedary camels kept under pastoralism in the Southern Punjab Province of Pakistan. A total of 44 welfare indicators (animal-, resource, and management-based measures) aligning with animal welfare principles ("Good Feeding", "Good Housing", "Good Health", and "Appropriate Behavior") were gathered into two assessment levels: "Caretaker-Herd level" and "Animal level". Data were collected in 2023 in the Cholistan desert in the southern Punjab province. Fifty-four herds were evaluated for a total population of 1,186 camels, of which 510 (495 females and 15 males; average age: 5–6 years old) were assessed at the animal level. The indicators were scored and aggregated to obtain Principle Aggregated Indexes (PAIs) and a total Welfare Index (TWI). Using the PAIs classification, 4 herds were categorized as excellent, 42 satisfactory, and 8 unsatisfactory. Total Welfare Index (TWI) varied from 55.7 to 82.2, and the thresholds for classification into tertiles were 65.4 and 70.6. Good feeding and Good housing were the most problematic PAIs, with Good feeding as the most influential variable for classification into welfare categories. As expected, camels kept under pastoralism had a higher level of welfare than those reported in the literature for intensive systems, especially concerning the Appropriate Behavior principle. Our findings are a first step in proposing welfare standards for dromedary in Pakistan and worldwide.

KEYWORDS

animal-level, camels, Good feeding, Good housing, Good health, Good behavior, herd-level

1 Introduction

Assessing animal welfare requires a multidimensional approach which may vary depending on the husbandry system (1, 2). Assessing the welfare of livestock in extensive systems requires a comprehensive understanding of the heterogeneous extensive environment, the behavior of the animal, human-animal relationships (stockmanship), health factors, and

observing the animals during routine husbandry practices (2). Dromedaries or one-humped camels (*Camelus dromedarius*) have long been integral to the livelihoods and cultural identity of many societies across arid and semi-arid regions of the world (3, 4). Renowned for their adaptability to harsh environmental conditions, dromedaries are mostly prized for their utility in transportation, milk production, and meat provision (3). Their significance extends beyond mere economic considerations, as they often serve as symbols of resilience and endurance in the face of adversity (5). Currently, in different regions of the globe, there's a growing body of research focused on milk quality, production, health, reproduction, nutrition, calf management, and management systems of dromedary camel. However, there remains a notable scarcity of research addressing the welfare of dromedary camels in diverse management systems (6).

The global landscape of dromedary camel rearing and production is undergoing significant transformation, driven by varying socio-economic interests and evolving husbandry practices. Increasingly, dromedaries are integrating into intensive and semi-intensive production systems, reflecting shifts in consumer demand and agricultural trends (7, 8). Recognizing the inherent relationship between animal welfare and production outcomes (i.e., improved productivity and product quality), efforts have been made to develop assessment tools for evaluating the welfare of dromedary camels within intensive and semi-intensive regimes (9). However, it is important to note that the vast majority of the global dromedary population is raised under nomadic, traditional pastoralist conditions in the arid and semi-arid ecosystems of Africa and Asia (4, 10). Nomadic pastoralism represents a traditional husbandry system where livestock are allowed to graze freely over vast expanses of rangeland. This lifestyle is intimately intertwined with the ecological and socio-cultural dynamics of arid regions, where sedentary agriculture may be impractical (11).

Despite the prevalence of nomadic pastoralism in dromedary camel husbandry, animal welfare assessment in these extensive production systems has received limited attention in research (6, 12). This discrepancy is partly attributable to the perception that animals in open ranges enjoy natural lives, thus minimizing welfare risks (13). While it is true that animals at pasture can exhibit a broader range of behaviors and enjoy environmental enrichment, the challenges associated with outdoor living, such as thermoregulation, the presence of predators, and nutritional inadequacy, necessitate careful consideration (2, 14). Moreover, the traditional knowledge and practices governing camel husbandry may not always align with modern standards of animal welfare (15). In response to these challenges, a new protocol tailored specifically to the unique conditions of dromedary camels reared under nomadic pastoralist conditions has recently been proposed but has not yet been applied (16). Within the Asian continent, Pakistan emerges as a prominent hub of dromedary camel pastoralism (17, 18). Despite the cultural and economic significance of dromedaries in this country, there remains a dearth of scientific research elucidating the welfare implications associated with nomadic pastoralist systems. Understanding the welfare challenges and opportunities inherent in these production systems is crucial for devising targeted interventions aimed at improving the lives of both animals and their human caretakers. Moreover, such insights can apprise policy decisions aimed at promoting sustainable and ethically sound livestock production practices. We hypothesized that dromedary camels under pastoralism could live in their natural environment and express natural behaviors, having an acceptable welfare level, but with the applied protocol, we could identify some challenges, such as limited feed and water due to environmental conditions.

In light of these considerations, employing the newly developed welfare assessment protocol tailored to this specific context (16), this study aimed to assess the welfare status of dromedary camels kept under pastoralism in the Southern Punjab Province of Pakistan.

2 Materials and methods

This study was approved by the Bahauddin Zakariya University Animal Ethics Committee (Protocol number DLPP/272/27-11-2023). A native speaker research team member (ARA) facilitated the meeting between the assessor and the herd manager or owner. During this meeting, the objectives and methods of the welfare protocol were explained, and permission to carry out the assessment protocol was obtained.

2.1 Study location and sampling

A prospective field study was performed in the southern Punjab province, Pakistan, from 29th September to the 7th October 2023. Mean ambient temperature and relative humidity were 33.4°C (min-max: 21.3°C–40.9°C) and 48.1% (min-max: 20.9–88%), respectively. Fifty-four dromedary camel pastoralist herds inhabiting eight different localities within the neighborhood of Multan and the Cholistan desert (Lohari Gate ($n = 11$), Kotla Pul ($n = 10$), Channan Pir ($n = 9$), Gelewala ($n = 7$), Nag Shah ($n = 5$), Naubahar Pul ($n = 5$), Gograan ($n = 4$), and Chak 97 ($n = 3$)) were visited for welfare assessment (Figure 1).

One of the authors (Dr Ali Raza Abbasi) contacted local community leaders to facilitate and ease the communication with herd managers/ pastoralists. Subsequently, herd managers/ pastoralists were approached in collaboration with these leaders and their networks, ensuring their participation remained entirely voluntary. According to the previous schedule, each herd was visited only once to respect pastoralists' routines. Herd size ranged from 5 to 63 animals, with an average size of 43 individuals. The most popular breeds in the area were Barela and Marecha. The management practices across all herds were broadly similar. The camels were permitted to roam and feed in the nearby cultivated areas throughout most of the day and sometimes provided supplemental feed sourced from grass collected from agricultural fields. Milk production and camel rearing for sacrificial purposes were the primary breeding purposes of all the herds involved in the present study. Females were milked twice daily (morning and afternoon), with an average milk production of 9.5 liters/day. The collected milk was partly used for household and sold out to urban vendors to help generate income for the pastoralists. Most male calves were usually sold around 1 year of age during fairs/ festivals, mainly for meat production (sacrificial purposes). Following the protocol (16), only adult animals (>3 years old) were assessed.

At each herd, the number of dromedary camels for assessment at the Animal level was selected randomly following the general statistical rules reported by Padalino and Menchetti (9). As required by the protocol, animals under treatment for severe or acute pathological conditions such as chronic and acute mastitis, abscess, chronic wounds, and chronic skin infections were excluded from this calculation (Supplementary Figure S1).

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FIGURE 1
Examples of different camel herds assessed in different locations.

In total, 510 adult dromedary camels (495 females and 15 males; average age: 5–6 years old) were assessed. Based on physiological status, females were differentiated into 3 different categories: lactating ($n = 234$), pregnant ($n = 162$), and non-lactating/non-pregnant ($n = 99$). It is worth mentioning that females were naturally mated, and pregnancy diagnosis was carried out by observing an erect and coiled tail in the pregnant animal when approached by a male camel ("tail cocking") (19). As the pregnancy status could not double-checked using ultrasonography, the previous classification could be inaccurate, and it was not further used. None of the assessed herds practiced male castration. Few herds had a breeding male within the herd, but most used to introduce a breeding bull during the reproductive season. These breeding male were kept in different locations and were not assessed in the current study.

2.2 Welfare assessment protocol

The welfare assessment was performed following the protocol by Padalino and Menchetti (16). It contained 44 welfare indicators (animal, resource, and management-based measures) aligning with animal welfare principles ("Good Feeding," "Good Housing," "Good Health," and "Appropriate Behavior"). Indicators were gathered into two assessment levels: "Caretaker-Herd level" and "Animal level". The assessment at the caretaker herd level was performed by the native speaker research team members (FA, TNA), while the Animal level was conducted by the lead researcher (BP). A three-point scale was used to score the indicators: 0 denoting good welfare, 1 indicating compromised welfare, and 2 reflecting unacceptable welfare. For indicators with binary responses (e.g., presence/ absence), only scores of 0 (good welfare) and 2 (unacceptable welfare) were taken into account.

The "Caretaker-Herd level" consists of a face-to-face interview with the herd manager or owner. This interview included 16 unique closed-ended questions that probed various aspects such as feeding and healthcare practices, housing conditions, and the handling experience. At the end of the interview, the assessors watched from a distance as caretakers (those who handle, feed, and water the animals) interacted with the camels. The caretakers' mood and techniques were observed, as well as the use of any equipment (such as a stick) and how it was used, and this observation was taken into

consideration to score the caretaker's attitudes toward animal handling.

The "Animal level" included 27 distinct welfare-related indicators that always aligned with the four welfare principles. The selected indicators were applied to assess feeding and watering practices, thermal and resting comfort, monitor health conditions, and observe the behavior of the animals in relation to the environment, their conspecific, and humans. At this assessment level, after randomly picking the camels to be assessed, a 3-min behavioral observation was carried out without disturbing the animal. This observation records parameters for "Good Housing" (such as access to shaded places, risk of harm, and voluntary resting behavior), "Good Feeding" (including food and water availability), and "Appropriate Behavior" principles. The "Appropriate Behavior" principle entails an approaching test as in previous studies (12, 20). Briefly, the assessor approaches the camel slowly from the side, extending their arm and hand in a non-threatening manner. The test was completed if the camel demonstrated avoidance or hostile behavior or if the assessor successfully approached and placed a hand near the camel's nose. The camel's response could be negative, neutral, or positive, representing protective or anxious behavior, calmness, or interest and involvement, respectively.

After the behavioral test, a visual inspection was performed to determine the camel's Body Condition Score (BCS) and other indicators of "Good Housing" (such as the presence of ectoparasites, cleanliness, and physical restraint), as well as to look for clinical signs, pain-induced practices, and injuries listed in the "Good Health" principle. The camel's ribs, ischial and coxal tuberosities, flank hollow, and recto-genital zone are used to assess body condition on a 0–5 scale (21). The presence of bleeding and open wounds was also noticed, with bleeding denoting obvious blood flow and open wounds, including damaged skin that exposes underlying tissues. Furthermore, if a dromedary camel remained resting during the assessment, it was gently coaxed to stand and move a few steps to determine lameness. This enabled the evaluation of the camel's gait to establish whether it could carry weight evenly and if there were any interruptions in its movement. In contrast, if the camel needed assistance to stand or could not bear weight on one leg, or exhibited a relieving posture, assessing its gait in motion was not necessary to confirm lameness. Assessed camels were marked to avoid reassessment, as freely moving camels can be challenging to track. The welfare assessment data were documented manually on paper utilizing the protocol's example recording sheets (16). Once fieldwork was completed, data were transferred into an Excel recording sheet for further analysis.

2.3 Calculation of partial and aggregate welfare indices

The indices of welfare were calculated following Menchetti et al. (22) and as reported in full in Padalino and Menchetti (12). Briefly, the measures' scores were initially summed into 8 partial indices (PIs) corresponding to the 4 welfare principles ("Good Feeding," "Good Housing," "Good Health," and "Appropriate Behavior") evaluated at the two levels ("Caretaker-Herd" and "Animal level"). In these PIs, the initial 0–2 scale was translated into a 0–100 scale, with 0 representing the lowest (i.e., unacceptable welfare) value and 100 representing the highest (i.e., optimal welfare).

Subsequently, PIs were merged into weighted sums, resulting in aggregated indices for each welfare principle, known as Principle Aggregate Indices (PAIs; Good Feeding Index, Good Housing Index, Good Health Index, and Appropriate Behavior Index). In the PAIs, a lower weight (20%) was assigned to the PAIs obtained at the "Caretaker-Herd level" as it mainly includes indicators self-reported by the farmer from memory, hence susceptible to potential "questionnaire bias". Finally, the Total welfare index (TWI) was calculated as a weighted sum of the 4 PAIs (12, 22).

2.4 Classification of herds

Various welfare classes were established to categorize the herds based on the four PAIs (i.e., "welfare profiles system"). Each herd was classified using a mixed rule system that involved comparing the PAI scores of the herd with predefined reference profiles. According to Menchetti et al. (22), herds were classified as "Excellent" if all PAIs were over 60 and two were over 80; "Satisfactory" if all PAIs were over 30 and three were over 60; "Unsatisfactory" if all PAIs were over 20 and three were over 30; and "Unacceptable" if they failed to meet the abovementioned criteria.

Furthermore, an additional type of classification was used (22) based on the tertiles of the TWI (i.e., "traffic light system"); herds could thus have a "green light" if their TWI belongs into the third tertiles, an "orange light" if the TWI belongs to the second tertiles, or a "red light" if their TWI belongs to the first tertiles.

2.5 Statistical analysis

Descriptive statistics was performed and data presented as absolute and relative frequencies, median (Mdn), maximum (Max) and minimum (Min) values, first and third quartiles, and interquartile range (IQR). For the indicators at the "Caretaker-Herd level", frequencies were presented relative to the total number of assessed herds ($n = 54$). At "Animal-level", frequencies were expressed in relation to the total number of assessed dromedary camels ($n = 510$). One sample binomial or Chi-square tests were used to compare the observed distributions with the expected probability distributions (each assuming all categories equal). Differences between medians of partial or aggregated indices were analyzed using related-samples Friedman tests with Bonferroni correction for multiple comparisons, while Wilcoxon signed tests were used to compare the scores obtained at Caretaker-Herd and Animal levels. Finally, discriminant analyses (DAs) were used to define the relative importance of each welfare principle in classifying the herds. In the DAs, the welfare categories (i.e., Excellent, Satisfactory, and Unsatisfactory for the "welfare profiles system"; Green, Orange, and Red Light for the "traffic light system") were included as grouping variables while the four PAIs scores were included as independent variables. The standardized coefficients of the discriminant functions indicated the relative importance of each PAI in classifying the herds. The centroids (i.e., mean discriminant scores of the discriminant functions) were used to establish the cutting point for classifying herds (23, 24). Statistical analysis was performed using SPSS Statistics for Windows, version 25.0, IBM Corp. (2017), while GraphPad Prism, version 7.0 (GraphPad Software, San Diego, CA,

United States) was used for the data visualization. p values <0.05 were considered statistically significant.

3 Results

3.1 Welfare assessment at the Caretaker-Herd level

The scores obtained from 54 caretakers-herd level assessments were presented in Table 1. The majority of caretakers, 36/54 (66.7%; $p < 0.001$), depend only on grazing for feeding their animals, while only a small fraction, 10/54 (18.5%), stated they provided supplementation in addition to grazing. All caretakers said their animals had access to water more than once a day. However, most caretakers 47/54 (87%; $p < 0.001$) revealed that their camels lacked shaded areas, and 21/54 (38.9%; $p = 0.134$) stated their camels lacked overnight resting places. Although all caretakers claimed their animals' received vaccinations, only a few 6/54 (11.1%; $p < 0.001$) stated they used a veterinarian to treat sick animals. In terms of experience, all caretakers had over 10 years of camel rearing experience, with a significant portion 42/54 (77.8%; $p < 0.001$) showing gentle handling practices with their camels.

3.2 Welfare assessment at the animal level

The assessment findings of the 510 dromedary camels assessed at the Animal level are summarized in Table 2. Regarding the Good feeding principle, 69% (352/510) of animals had access to food, but only 89/510 (17.5%) had access to water during the inspection ($p < 0.001$; Supplementary Figures S2A,B). Body condition scores (BSC) evaluation indicated that 267/510 (52.4%) had moderate and 35.3% (180/510) had good body conditions ($p < 0.001$; Supplementary Figure S2C). Concerning the Good housing principle, 469/510 (92%) camels have no shade available at the time of evaluation ($p < 0.001$; Supplementary Figure S2D). The most frequent health issues detected during the evaluation were skin disorders, 181/510 (35.5%), discharge 140/510 (27.5%), and injuries 51/510 (10%) (Supplementary Figures S2E,F). Despite overall good health, 152/510 (29.8%) of the camels had experienced pain-induced management practices (i.e., cauterization, branding, nose pag, mutilation; Supplementary Figure S2G). Behavioral observations revealed a minimal incidence of animals showing stereotypy (2/510; 0.2%) and aggressive interactions (10/510; 2%). At the approaching test, the majority of the camels showed a positive response (272/510, 53.3%), while a third of them (151/510, 29.6%) were neutral, and only 87/510 (17.1%) showed negative interactions ($p < 0.001$; Supplementary Figure S2H).

3.3 Partial and aggregate welfare indices

The partial indices (PIs) statistics are presented in Figure 2 and Supplementary Tables S1, S2. At the Caretaker-Herd level, the partial indices (PIs) of Appropriate Behavior had the highest median, followed by Good Feeding and Good Housing whereas Good Health obtained the lowest median ($p < 0.001$). At the Animal level, the highest score was found for Good Health, followed by Appropriate Behavior and Good housing, while Good feeding obtained the lowest median ($p < 0.001$).

The comparison between assessment levels (Supplementary Figure S3) showed that the PIs of Good Housing and Good Health were higher at the Animal level than at the Caretaker-Herd level, while the PI of Appropriate Behavior was higher at the Caretaker-Herd level (both $p < 0.001$). No difference was found instead for the Good Feeding principle ($p = 0.423$).

The statistics of the principal aggregate indices (PAIs) corresponding to the four welfare principles are shown in Figure 3. The PAIs of Good health and Appropriate behavior had the highest median, followed by Good Housing and finally by Good feeding ($p < 0.001$). The Good feeding principle also showed the greatest variability between herds (range: 23.33–83.94).

3.4 Classification of herds

The herds were classified into welfare categories based on the computed PAIs ("welfare profiles system") and TWI ("traffic light system"). The classifications of each herd based on PAIs were presented in Supplementary Table S3. In summary, four herds obtained excellent, forty-two satisfactory, eight unsatisfactory and none of the herds obtained unacceptable category (Table 3). Furthermore, using TWI, the herds were categorized into three tertiles, and the classifications of each herd based on TWI were presented in Supplementary Table S4. Total Welfare Index (TWI) ranged from 55.7 to 82.2. Herds ($n = 12$) that fell into the third tertile (i.e., green light) had a TWI score > 70.6, those in the second tertile (i.e., orange light; $n = 22$) had a score between 65.4 and 70.6, while those in the first tertile (i.e., red light: $n = 20$) had a score ≤ 65.3.

Figure 4 shows the discriminant scores of the DAs carried out to identify the most important variables in the classification according to the "welfare profiles system" (Panel A) and "light traffic system" (Panel B). The first functions extracted explained more than 90% of the variance for both systems, and it had the highest coefficients for the Good Feeding variable. This suggests that this PAI was the most influential variable in classifying herds. The coefficients of the Good Feeding variable were positive, as well as the centroids for both the Excellent and Green light categories (Supplementary Table S5), indicating that herds having high scores for Good Feeding PAI could achieve the highest levels of welfare.

4 Discussion

The present study employed a recently developed protocol to assess the welfare of dromedary camels under nomadic pastoralist conditions (16), adapted from existing welfare protocols for dromedary camels in intensive and semi-intensive systems (9). Our study facilitated a better understanding and recognition of key welfare concerns among dromedary camels under pastoralism in Pakistan. Through this initiative, our goal was not only to enhance scientific knowledge but also to foster informed decision-making and policy formulation, promoting sustainable and ethical management of dromedary camel populations in Pakistan and globally. To the best of our knowledge, this could be the first welfare assessment of dromedary camels managed under a nomadic pastoralist production system

TABLE 1 Frequency table of the scores of the welfare indicators obtained at the Caretaker-Herd level corresponding to the four welfare principles (i.e., Good feeding, Good housing, Good health, and Appropriate behavior) collected from 54 herd caretakers/owners in Pakistan in 2023.

Welfare principle	Question/welfare indicator	Answer/observation	Scoring scale	Number	Percentage	*p* value
Good feeding	How often do you feed the camels?	Grazing for around 10–12 h per day + supplementation	0	10	18.5	<0.001
		Only grazing for 10–12 h per day	1	36	66.7	
		Only grazing for less than 6–8 h per day	2	8	14.8	
	How often do you water the camels?	Always available	0	0	0	–
		Available more than once daily	1	54	100	
		Available less than once daily	2	0	0	
Good housing	Do camels have a resting place overnight?	Yes	0	21	38.9	0.134
		No	2	33	61.1	
	How many adult animals do you have in your herd?	<30 camels (*Small size*)	0	46	85.2	<0.001
		>30 camels (*Large size*)	2	8	14.8	
	Do the camels have access to shaded areas?	Free access during the whole day	0	0	0	<0.001
		For a short time period of time per day	1	7	13	
		Never	2	47	87	
	Do you practice any type of predator control?	Yes	0	54	100	–
		No	2	0	0	

(Continued)

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TABLE 1 (Continued)

Welfare principle	Question/welfare indicator	Answer/observation	Scoring scale	Number	Percentage	*p* value
Good health	Who routinely assess the camel's health?	A veterinarian	0	6	11.1	<0.001
		A non-veterinarian	1	48	88.9	
		Not conducted	2	0	0	
	Who treats the camels when they are sick?	A veterinarian	0	6	11.1	<0.001
		A non-veterinarian	1	48	88.9	
		Not conducted	2	0	0	
	Are vaccinations routinely conducted?	Yes	0	54	100	–
		No	2	0	0	
	Is deworming routinely conducted?	Yes	0	6	11.1	<0.001
		No	2	48	88.9	
	What is the 1-year-old calf mortality rate?	Below 10%	0	0	0	–
		Over 10%	1	0	0	
		Records not available	2	54	100	
	Do you identify your animals?	Yes, using non-invasive methods	0	21	38.9	<0.001
		Yes, using pain-induced practices	1	33	61.1	
		No	2	0	0	
	Do your animals have the possibility to have contact with other livestock herds (commingling)?	No	0	0	0	–
		Rarely	1	0	0	
		Yes	2	54	100	
Appropriate behavior	Do you have any aggressive/dangerous animals in your herd?	No	0	32	59.3	<0.001
		Yes, but only during the breeding season	1	0	0	
		Yes	2	22	40.7	
	How many years of experience in handling camels do you have?	More than 10	0	54	100	–
		Between 5 and 10	1	0	0	
		< 5 years	2	0	0	
	What is the ratio between the number of caretakers and the number of animals kept in the herd?	Ratio ≥ 0.05 (1/20)	0	54	100	–
		Ratio < 0.05 (1/20)	2	0	0	
	Caretaker attitudes during animal handling	Speaks, touches and/or whistles softly/quietly	0	42	77.8	<0.001
		Speaks, touches and/or whistles harshly/loudly	1	6	11.1	
		Speaking/shouting impatiently, forceful use of stick/hand	2	6	11.1	

– means that the *p* value was not calculated as the distribution of the answers was constant.

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TABLE 2 Frequency table of the scores of the welfare indicators obtained at the animal level corresponding to the four welfare principles (i.e., Good feeding, Good housing, Good health and Appropriate behavior) collected from 510 camels in Pakistan in 2023.

Welfare principle	Welfare indicator	Observation	Scoring scale	Number	Percentage	*p* value
Good feeding	Food availability	Yes	0	352	69	<0.001
		No	2	158	31	
	Water availability	Yes	0	89	17.5	<0.001
		No	2	421	82.5	
	Body condition score	BCS = 3 (good body condition)	0	180	35.3	<0.001
		BCS = 2 or BCS = 4 (moderate body condition)	1	267	52.4	
		BCS = 0–1 or BCS = 5 (cachexia or obesity)	2	63	12.4	
Good housing	Currently available shade	Yes	0	41	8	<0.001
		No	2	469	92	
	Risk of injury	No	0	445	87.3	<0.001
		Yes	2	65	12.7	
	Presence of ectoparasites	No	0	391	76.7	<0.001
		Yes	2	119	23.3	
	Camel coat cleanliness	Clean	0	375	73.5	<0.001
		Partially clean	1	112	22	
		Dirty	2	23	4.5	
	Tethered	No	0	488	95.7	<0.001
		Yes	2	22	4.3	
	Restrained into two/three legs	No	0	365	71.6	<0.001
		Yes	2	145	28.4	
	Hobbled	No	0	486	95.3	<0.001
		Yes	2	24	4.7	
	Voluntary resting behavior	Yes	0	143	28	<0.001
		No	2	367	72	

(Continued)

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TABLE 2 (Continued)

Welfare principle	Welfare indicator	Observation	Scoring scale	Number	Percentage	*p* value
Good health	Presence of bleeding	No	0	477	93.5	<0.001
		Yes	2	33	6.5	
	Presence of injury (open wounds)	No	0	459	90	<0.001
		Yes	2	51	10	
	Presence of swollen joints	No	0	495	97.1	<0.001
		Yes	2	15	2.9	
	Presence of lameness	No	0	507	99.4	<0.001
		Yes	2	3	0.6	
	Presence of skin disorders	No	0	329	64.5	<0.001
		Yes	2	181	35.5	
	Presence of discharge (nose, eye, vulva)	No	0	370	72.5	<0.001
		Yes	2	140	27.5	
	Presence of diarrhoea	No	0	499	97.8	<0.001
		Yes	2	11	2.2	
	Presence of respiratory disorders	No	0	507	99.4	<0.001
		Yes	2	3	0.6	
	Presence of other health disorders	No	0	425	83.3	<0.001
		Yes	2	85	16.7	
	Presence of pain-induced management practices (cauterization, branding, nose pag, mutilation)	No	0	358	70.2	<0.001
		Yes	2	152	29.8	
	Evident pain	No	0	493	96.7	<0.001
		Yes	2	17	3.3	
Appropriate behavior	Positive social camel-camel interactions (cow-calf contact, allogrooming, sniffing)	Yes	0	339	66.5	<0.001
		No	2	171	33.5	
	Aggressive camel-camel interactions	No	0	500	98	<0.001
		Yes	2	10	2	
	Stereotypies	No	0	508	99.6	<0.001
		Yes	2	2	0.4	
	Feeding or rumination	Yes	0	207	40.6	<0.001
		No	2	303	59.4	
	Approaching test	Positive	0	272	53.3	<0.001
		Neutral	1	151	29.6	
		Negative	2	87	17.1	

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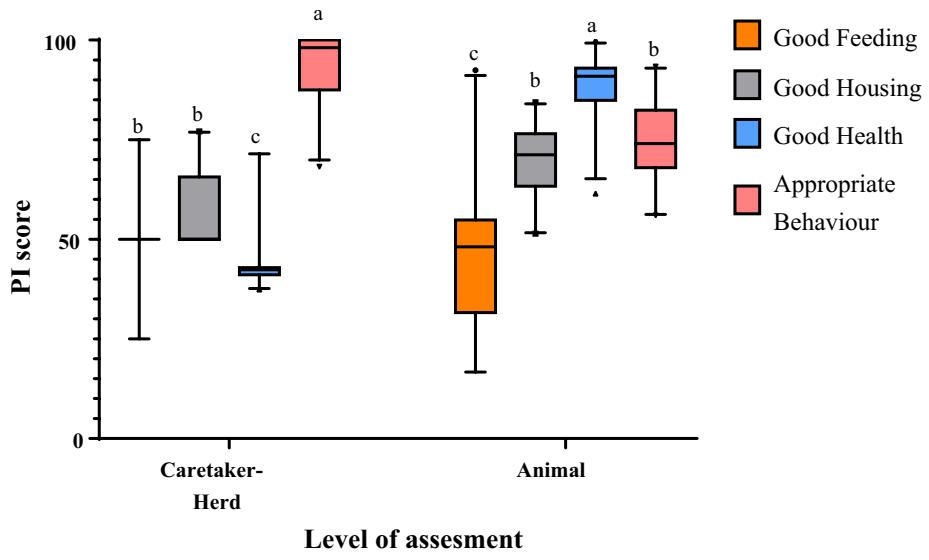

FIGURE 2
Boxplot for partial indices (PIs) at Caretaker-Herd and Animal levels corresponding to the four welfare principles (i.e., Good feeding, Good housing, Good health, and Appropriate Behavior). The whiskers on the plot define the range from the 2.5 to the 97.5 percentile, while the dots show the outliers (that fall below or above this range). Boxes not sharing any superscript (a,b,c) for each level of assessment are significantly different at $p < 0.05$.

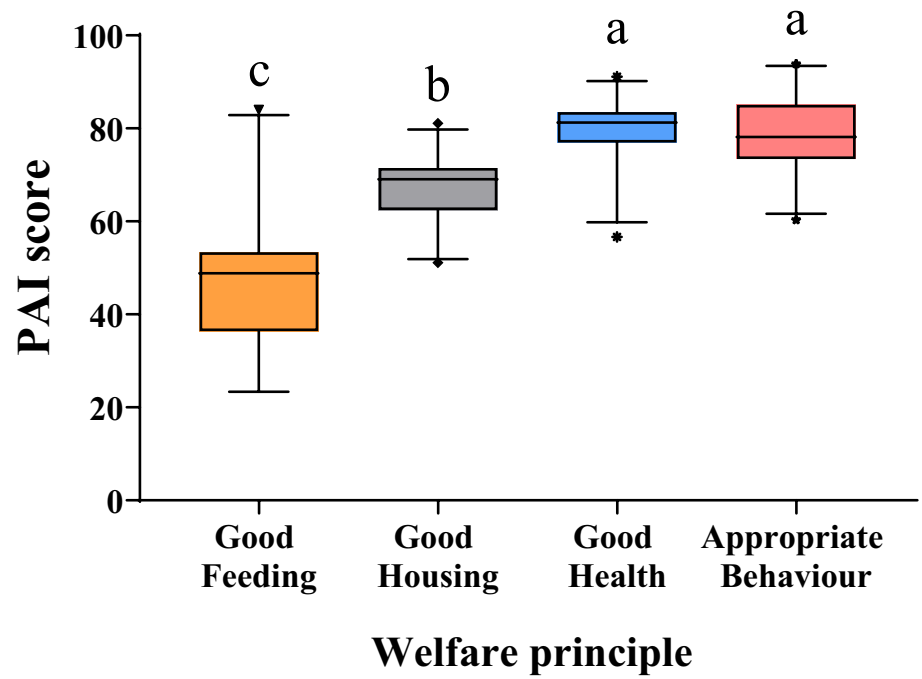

FIGURE 3
General boxplot for partial aggregate indices (PAIs) corresponding to the four welfare principles. The whiskers on the plot define the range from the 2.5 to the 97.5 percentile while the dots show the outliers (that fall below or above this range). Boxes not sharing any superscript (a,b,c) are significantly different at $p < 0.05$.

using a standardized protocol (16), which also reports the classification of the herds. The application of a standard protocol allows the possibility to compare different herds and animals kept in different countries, develop benchmarking, and propose minimal welfare standards.

The initial stage of our assessment targeted the caretakers or herd keepers, who typically were the owners of the camels in most of the cases in Pakistan. This was expected as camel breeding under pastoralism generally and in this country is mainly based on the family tradition; each kid heredities one or more camels, and then he/

TABLE 3 The summary of the classification of the welfare category of the herds based on the principal aggregate indices (PAIs; "welfare profiles system").

Welfare category	Criteria	Number of herds
Excellent	>60 for each PAI and > 80 for at least two PAIs	4
Satisfactory	>30 for each PAI and > 60 for at least three PAIs	42
Unsatisfactory	>20 for each PAI and > 30 for at least three PAIs	8
Unacceptable	Failure to meet the abovementioned criteria	0

she can start his/her own camel herd when grows up (25, 26). Caretakers primarily play an important role in caring, treating, and gently handling their animals; their experience and awareness are important factors as they may lead to fear and other negative emotions in the managed animals (27), and information gathered from them helps to determine the welfare status of their animals (9, 27). The pastoralists/ herders in the study area, specifically the "Cholistan" desert, move their animals seasonally in search of grazing pasture and water for their animals (18). The majority of the caretakers indicated grazing as the sole feeding practice. The availability of grazing vegetation in pastoral areas depends on the amount of rainfall, and it may become a welfare concern. There are no problems when the rainfall is high (more than 250 mm or more), as it results in expansive grazing areas covered by vegetation for the animals (28), but during dry periods, this is not the case. Moreover, Raziq et al. (18) stated that pastoralists faced a shortage in grazing land due to the increased mechanized land cultivation for wheat and cotton production in the study area. As a matter of fact, the Good Feeding score at the Caretaker-Herd level showed considerable variability between herds, suggesting that the supply of feed and water is highly variable depending on their availability in the environment and the keeper. Even though the herd caretakers claimed that their animals get water more than once a day, the availability of water depends on the season, and the available source of water in the area is primarily ponds, either natural or man-made, called "Tobas" and underground water (17, 18, 28). In agreement with our findings, Faraz et al. (17) reported that herders claimed they provide water 2–3 times a day to their camels. Appropriate feeding and watering practices are also important for production and reproduction; indeed, puberty depends on the skeletal maturity and body condition score (BCS) of the animals (29, 30). Integrating the diet when the pasture quality is low may be a best practice not only for the welfare of the animals but also to enhance sexual maturity and start breeding careers earlier.

Almost all camels lacked shaded areas during the assessment, and almost half of the caretakers stated their camels had no overnight resting places. Our findings are in line with the literature (31). Faraz et al. (17) reported similar findings for other areas of Pakistan where most camels were reared in open and semi-open facilities. However, regarding shade, Faraz et al. (17) reported that the camels were managed with the availability of many trees during hot days, and herders made semi-open shade using bamboos and "Sirki." This disagrees with our findings, as in most cases, there were no trees or "Sirki" during our assessments. Furthermore, the literature has tested that ensuring livestock resting areas at night serves crucial purposes, safeguarding them from potential dangers such as predator attacks and protecting them from extreme environmental stressors (4). While conducting our research, we observed a few herds at an assembly point called "Channan Pir rest house." This assembly/resting point was constructed by the government. It had water facilities, water tanks, and shaded resting areas for camels and animals, and it was an ideal place where animals could be fenced overnight. Similar 50 watering points were also constructed in the desert area of Cholistan. This initiative should be replicated and extended to other isolated areas to improve the well-being of the camels.

According to most caretakers, a non-veterinarian treated sick camels. The lack of veterinary structure and services in the far-flung area of the desert, coupled with the lack of trust by pastoralists in the service provided by the veterinarian, leads the pastoralists to prefer traditional practices and ethnoveterinary medicine to treat their animals (18). In fact, in a study in Qatar (32) and Egypt (33) camel markets, caretakers treat and self-administer the camels. Faraz et al. (17) reported herders use traditional and ethnoveterinary medicine to treat their animals in the Bhakkar district in the province of Punjab, Pakistan. In many pastoralist and camel-rearing communities, the widespread practice of traditional and ethnoveterinary medicine for treating camels is evident (17, 34–36). While the use of traditional and ethnoveterinary medicines has some potential benefits, it also has several risks to the health and welfare of the camels. Therefore, strategies to improve the pastoralists' attitude toward using modern veterinary services should be realized. A detailed investigation of the indigenous traditional practices and ethnoveterinary medicine knowledge should be conducted to improve and use it in a better way that does not degrade the welfare of the camels. Moreover, the lack of animal identification methods and proper record-keeping of health could pose a challenge for pastoralists, making it difficult to enhance the overall health status of the camels. In many countries, camel identification is not mandatory, which impairs any possible disease prevention/eradication. Many camels around the world are moved from area to area and from country to country. All those movements should be recorded to minimize the risk of spreading infectious diseases. A campaign on camel identification for proper tracking of the animal is suggested not only to improve camel health and welfare but also human safety. The limited recourse to veterinary care, the use of pain-induced management practices, and the lack of health monitoring and record practices can explain the low score of the partial index (PI) of Good Health at the Caretaker-Herd level. A similar finding has been reported by Lamuka et al. (34) in Kenya, where pastoralist use of veterinary services is very low, and absence of treatment or health records of camels. Globally, in pastoralist communities, the use of modern veterinary services is scarce due to low availability and also low awareness of the use of modern veterinary services (18, 37). In addition, the distribution of diseases is high in most pastoral management systems, making camel production challenging by affecting their production and well-being (38–40). Stakeholders bear a great responsibility in implementing necessary policies to improve and facilitate the availability of veterinary services and provide adequate training to instill confidence in pastoralists in modern veterinary services. In addition, the government needs to ensure the availability of veterinarians and community animal health workers who are selected from the community, and train and work within the community. This approach will help to enhance the

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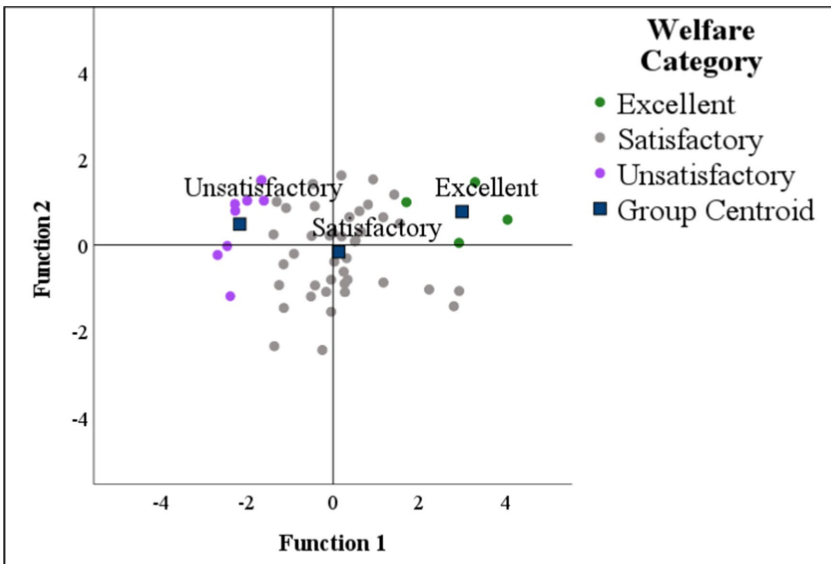
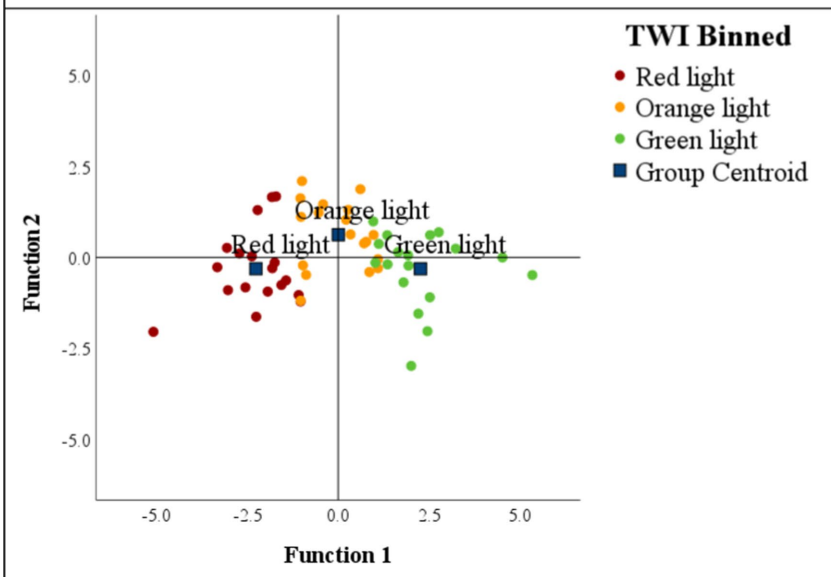


A

PAI	Function	
	1	2
Good Feeding	0.934	-0.255
Good Housing	-0.041	0.591
Good Health	0.071	0.161
Appropriate Behavior	0.287	0.790
Variance explained (%)	93.3	6.7



B

PAI	Function	
	1	2
Good Feeding	0.531	-0.794
Good Housing	0.259	0.486
Good Health	0.283	0.514
Appropriate Behavior	0.216	0.431
Variance explained (%)	94.6	5.4

FIGURE 4
Scatterplots of discriminant scores for Function 1 vs. Function 2 and structure matrix indicating the standardized coefficients of the discriminant functions. (A) Presents the results of the discriminant analysis of the "welfare profiles system," while (B) presents the results of the discriminant analysis of the "light traffic system" (B).

accessibility of veterinary services within the communities and the superior welfare of the camel.

In the current study, most caretakers had long experience in camel handling, and they used to treat their animals gently. The good caretaker-camel relationship was also supported by the results of the approaching tests, during which most camels responded positively or neutrally. Herders' experience and knowledge in appropriate handling greatly impact the welfare and health of camels (36). The principle of Appropriate behavior, in fact, received a good score at both the Caretaker-Herd and Animal levels. Our data have, therefore, supported the findings of previous researchers, as it was found that the experience in handling and care provided by caretakers and herd keepers significantly influenced the response to the approaching test (41). Education on animal handling based on learning theory has been suggested to improve animal welfare in other species (42, 43); increasing knowledge on animal behavior and how to assess animal welfare have been proven to be useful in enhancing animal welfare (33). Thus, workshops on these topics should be implemented in countries where the cruel handling of camels and poor knowledge of animal welfare have been reported in the media (44).

The assessment at the Animal level was performed on 510 camels, randomly selected from the 54 herds. The number of animals assessed in some herds, unfortunately, was lower than that one suggested, and this was due to logistic reasons (e.g., the animals had to move for grazing, and we did not want to cause further delay). Nevertheless, the total number of camels assessed is still a significant sample size of the total population. During the Animal level assessment, more than half of the camels had food available as they were grazing. It is worth noticing that the examined area, particularly the "Cholistan" desert, has a wide variety of vegetation that sustains the food needs of the camels (18). However, it is still a desert, so the scanty and rugged vegetation, coupled with the harsh environment, is uniquely suited to be utilized by camels, which are more resilient than other livestock. Except for brief wet periods, camels primarily depend on woody and bushy vegetation for sustenance (18). Hence, during dry periods, it's crucial to have alternative food sources accessible, and district government and responsible bodies should educate pastoralists on methods for conserving food. Body condition score (BSC) was the other ABM of Good Feeding and confirmed the Caretaker-Herd level findings; over three-fourths of the evaluated camels were indeed classified as having good or moderate body condition, and less than a quarter were classified as cachexia or obese. During our study in the field, we encountered a lot of diverse wastes, including plastics, which could be ingested by the camels, leading to digestive issues and subsequently resulting in weight loss. Thus, it is crucial to emphasize waste management in the study localities, particularly concerning plastics, and caretakers should be educated about the potential hazards of foreign objects ingestion by camels. Another important indicator of Good Feeding is the availability of water. Water availability at the Animal level was very limited. This disagrees with what was reported by the caretakers, stating that they provide water more than once daily. During the assessment, there were few visible watering points for the camels in a few locations. Except for the observation of the surrounding environment, we had no better indicator for the water availability test, like the bucket test (9), which was not feasible, and there was a lack of particular indicators for prolonged thirst. In times of drought, the ponds containing water may dry up, leaving no water source for the camels. As a result, pastoralists often migrate to cultivated irrigated areas, causing conflicts on many occasions (18). In arid regions, access to drinking water for livestock is frequently restricted, as watering points are typically located several days' journey away from grazing areas. Consequently, all livestock, especially during the dry season, routinely experience thirst (4). Even though, due to the anatomical and histological particularities of their kidneys, camels can withstand water deprivation (45), their ability to withstand water depends on many factors, including breed, climatic factors, quality and quantity of grazing vegetation, and its water content, purpose and the type of drought work they are involved in (46). Therefore, water should be available to camels, especially during the dry season and for the lactating camels, which require more water to produce milk, so that these camels do not suffer from prolonged thirst. Prolonged thirst could be a common welfare consequence for camels, mainly based on the belief that they do not need water, as they can survive without water longer than other animals. Similar findings have been reported, indeed, where camels were kept in a market in Doha, where the lack of a watering point was identified as a critical point that hindered the welfare of the camels (22). Since almost all the camels depended totally on grazing as a food source, and there was a critical lack of water in the area, it is not surprising that the Good Feeding PAI scored the lowest and was the most variable among the herds.

Under the assessment of Good Housing, the absence of shade was one of the most important critical points, where almost all assessed camels had no shade to prevent themselves from strong sun/ heat during the daytime. Zappaterra et al. (47) explored the preference of camels regarding shade. Despite the belief that camels, as desertic animals, do not need shade, the authors concluded that camels tend to prefer shaded areas during hot sunny days. Underscoring shade is, therefore, paramount in improving camels' welfare. Animal welfare is indeed not only what animals need but also what animals want (48), so it is important to test their preference and provide what they prefer to ensure their welfare. As temperatures and humidity rise, livestock exhibit a preference for shade; the provision of shade significantly influences their behavior and contributes to enhancing their welfare while also aiding in minimizing thermal stress (49–52). On the other hand, most of the animals showed a clean coat and enjoyed freedom of movement. These animal-based measures compensated for the critical issues at the Caretaker-Herd level and could increase the final score of the Good Housing PAI. As mentioned before, however, it would be good if resting and sheltered points could be built along the common pastoralism ways so that camels could be watered, shaded, and rested not only at night but also when temperatures are above their thermal neutral zone (53).

Regarding Good Health, skin disorders, discharge, and injuries were mostly observed abnormalities and clinical signs in the examined camels during the animal-level assessment. However, it is worth noticing that despite these findings, most of the camels observed were so healthy that this welfare principle received one of the highest PAI scores. Ashraf et al. (54) reported trypanosomiasis, pneumonia, mange, and anthrax in the Cholistan desert, Raziq et al. (55) reported mange, Orf, camelpox, trypanosomosis, and contagious skin necrosis in Suleiman Mountainous Region in Pakistan, showing the extent of the incidence of camel disease in Pakistan. Our findings were expected as most of the clinical signs and abnormalities we reported are in line with those caused by the most common camel disease. However, it is worth noticing that it is not the scope of a welfare assessment to reach a diagnosis, so we do not know the real etiology of the clinical signs noticed. Throughout the globe, where camels are managed under nomadic pastoralists, semi-nomadic pastoralists, and semi-intensive systems, health problems are the main challenge in camel production, which is an obstacle to the welfare of these animals (32, 39, 56, 57). The pastoralist communities mostly move in search of food and water for their animals, and they tend to spend lots of time in remote locations where veterinary service is not easily accessible (18). Some of the disease conditions observed at the animal level could be of zoonotic importance, so awareness-creating training, including a detailed investigation of the diseases, should be conducted, and, as mentioned before, the veterinary service could be improved along the pastoralism ways. Another important indicator of good health is pain-inducing management procedures, which are practiced by pastoralists either to retrain or control the camels (nose rings/pag) (4) or as a traditional treatment method for different diseases (cauterization) (36). Despite overall good health, more than a quarter of our camels had experienced pain-induced management practices. These practices, either as control, restraining, or treatment procedures, should not cause prolonged pain to the camels. Restraining methods, like nose

pag, are based on creating pain and discomfort, and they may affect the welfare of animals if used for prolonged periods (4).

As expected in camels kept under pastoralism, the behavioral needs were met, and the PAI for Appropriate Behavior was among the highest compared to the other PAIs. As expected, behavioral observations revealed minimal incidence of stereotypies and aggressive interactions in the examined animals. Oral and locomotory stereotypy were described in camel bulls kept in captivity when they were housed for 24 h in individual boxes. Restricting space allowance and limited social contact were revealed to be the causes of stereotypies in camels (58). Stereotypy frequency decreased when the bulls could be housed freely in a paddock and spend time close to females (59–61). Oral stereotypy was strongly dependent on the time of feeding in camels kept in intensive farming with rationed feeding regimes (59), so it is not surprising that our camels showed no oral stereotypy as they had all access to pasture and could meet their needs for grazing and browsing (62, 63). The observed camels showed many indicators of positive welfare, such as positive social interactions; many she-camels were seen taking care of their calves, and often camels were seen resting and ruminating close to each other, and some calves were also playing together. The fact that these animals live in a habitat closer to their natural one justifies their behavioral repertoire (63). This could also be the reason why, despite the belief that camels are aggressive animals, no aggressive interactions were recorded, and no aggressivity was reported as a behavioral problem by the caretakers. In the study conducted by Menchetti et al. (41), caretakers reported a higher incidence of behavioral problems in a camel market. However, in the latter study, camels were confined in their pens all day long with limited space in crowded conditions. The fact that our camels had the possibility to express their natural behavior and they were also managed gently by their owners reflected in the fact that the majority of them reacted neutrally or positively when a stranger tried to approach them, even though they were free to move and consequently to run away. Our findings may be useful in suggesting better standards for camels housed in intensive and semi-intensive systems.

The classifications of the herds were conducted using Principal Aggregate Indices (PAIs) and Total Welfare Index (TWI). Based on the PAI classification, none of the herds obtained an unacceptable level of classification, and four herds obtained excellent levels, indicating that the camels were managed well. These findings, moreover, suggest a better condition compared to intensively managed camels. Indeed, Menchetti et al. (22) have found in a camel market that no pens achieved the "excellent" category, and some pens had an "unacceptable" welfare level. Similarly, in intensively managed Dutch dairy cattle herds, "unacceptable" classification was also reported with no "excellent" classification (64). Based on the TWI, employing a traffic light classification, we were able to produce a threshold for this population (65.4 for the separation between Red and Orange light categories, 70.6 for the distinction between Orange and Green light categories). These thresholds were higher than those identified in intensive farming by Menchetti et al. (i.e., 56.0 and 62.0, respectively) (22). This confirms that camels managed under nomadic pastoralism in Pakistan have better welfare levels compared to intensively managed farm animals. The score of Appropriate Behavior PAI was the highest, probably contributing to the better result of herd classification compared to the previous study in the market. Animals managed under extensive systems tend to enjoy the expression of their natural behavior, have relative freedom (65), and have social contact with other camels and humans, making the animals more friendly (66). The critical areas that needed improvement were Good feeding, Good health, and Good housing. In particular, the Discriminant analysis showed that Good Feeding was the most influential variable in classifying and determining discrimination between different levels of welfare. These are expected results because continuity in food and water supply, shelter, and veterinary care are the major welfare concerns of all extensive systems (14, 67). To improve in these areas, particular attention should be given to the availability of water, the provision of shade, natural or manmade, to protect the camels from extreme heat, the availability of nighttime resting places and modern veterinary services to improve the well-being of the camels in the study localities.

Our data must be interpreted cautiously, as the study has several limitations. The initial limitation starts from the fact that the protocol was being applied for the first time and needs to be refined and validated by further studies. In particular, the validation of certain indicators within the pastoralist management production system is needed. The evaluation of the quality of the feed and water, when available, could not be also conducted. The presence of multiple individuals during the assessment in the open desert setting could potentially influence the outcome, as complete prevention of human presence was not possible. In addition, during observation the caretaker's mood and techniques of handling the camels could be influenced due to the presence of the assessor in nearby distance, and could affect the outcome of the assessment. The other limitation is that in some of the herds, a limited number of animals were assessed due to logistic problems. Finally, it is important to highlight that this is a single welfare assessment conducted in a particular season (i.e., autumn), so the welfare classifications of the heard cannot be generalized for the full year, and multiple welfare assessments should be performed, as usually happen in every welfare scheme in other species. Notwithstanding these limitations, this study is the first to report the welfare levels of camels kept under pastoralism with an objective protocol. Our data may be useful to suggest recommendations tailored for the camels kept in Pakistan or similar conditions.

5 Conclusion

This study investigated the welfare of dromedary camels managed under a nomadic pastoral using a tailored protocol. The examined camels had better welfare compared to those in intensive systems, but critical welfare issues were identified. Key welfare risks included poor feeding, limited water availability, lack of shade, and insufficient overnight shelter. Health risks were also noted, with non-veterinarians treating camels and poor record-keeping. Stakeholders and the government should take action to address those issues. Our findings are a first step in proposing best practices for dromedary camels, not only in Pakistan but worldwide. They also are the groundwork for future research, providing valuable insight into the main welfare issues likely to be encountered in camels under the nomadic pastoral management system. Therefore, this work's findings could help improve the welfare of the dromedary camels. The responsible stakeholders and government policymakers could benefit from this study's findings in proposing and implementing appropriate policies and taking corrective action.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were approved by Dr. Abdul Waheed, Department of Livestock and Poultry Production, Bahauddin Zakariya University, Multan, Pakistan; Dr. Asim Faraz, Department of Livestock and Poultry Production, Bahauddin Zakariya University, Multan, Pakistan; Dr. Hafiz Muhammad Ishaq, Department of Livestock and Poultry Production, Bahauddin Zakariya University, Multan, Pakistan. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AF: Data curation, Investigation, Methodology, Validation, Visualization, Writing – review & editing. NM: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. AW: Investigation, Validation, Writing – review & editing. HI: Investigation, Writing – review & editing. NT: Investigation, Writing – review & editing. AA: Investigation, Writing – review & editing. LM: Data curation, Formal analysis, Methodology, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1442628/full#supplementary-material

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The effects of camel milk in systemic inflammation and oxidative stress of cigarette smoke-induced chronic obstructive pulmonary disease model in rat

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Background: The effects of camel milk in inflammation and systemic oxidative stress of cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD) associated with small airway inflammation in rats were investigated.

Methods: 35 male Wistar rats were randomly divided into five groups: (a) control, (b) CS-exposed rats, c and (d) CS-exposed rats treated with the 4 and 8 mL/kg camel milk, and (e) CS-exposed rats treated with 1 mg/kg dexamethasone.

Results: Total and differential WBC counts, serum level of TNF-α and malondialdehyde (MDA) level in serum and homogenized tissues of the heart, kidney, liver, and testicle were significantly increased, but catalase (CAT), superoxide dismutase (SOD) and thiol levels were significantly decreased in CS-exposed rats ($p < 0.01$ to $p < 0.001$). Treatment with dexamethasone and both doses of camel milk improved all measured variables compared to the COPD group ($p < 0.05$ to $p < 0.001$). The improvements of most variables in the treated group with high dose of camel milk were higher than the effect of dexamethasone ($p < 0.05$ to $p < 0.001$). These findings suggest that camel milk has a therapeutic potential for treating systemic oxidative stress and inflammatory induced by CS.

Conclusion: Therefore, camel milk might be effective in attenuating the effects of CS-induced systemic inflammation and oxidative stress.

KEYWORDS

camel milk, COPD, cytokine, oxidative stress, pulmonary disease

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the incurable and common lung diseases that can turn into diseases such as cardiopulmonary disease and respiratory failure (1). Cough, shortness of breath, and increased sputum along with slow development of airflow limitation are the characteristics of this disease. In patients with COPD, increased anxiety and depression, weight loss, anorexia, sleep disorders, and daytime sleepiness were also observed (2). The history of COPD varies among individuals with different COPD phenotypes according

to the onset, early stages, and progression of the disease (3). Genetics and environment are two critical factors in the development of COPD as well as other influenced factors include age, gender (4), particle exposure, lung development and growth, socioeconomic status, asthma, infections, and chronic bronchitis (1).

The characteristics of COPD are lung emphysema, fibrosis, and chronic inflammation of small airways (5, 6). COPD may not resolve even with cessation of exposure to cigarette smoke (CS) in the presence of some endogenous factors such as persistent infection or autoimmunity (6). The development and progression of pathogenic mechanisms involved in COPD can occur due to oxidative stress. So exogenous oxidants such as CS and air pollution, endogenous oxidants such as superoxide anions and mitochondrial oxidants, and the reduction of antioxidants such as superoxide dismutase (SOD) and thioredoxin can increase lung oxidant stress in COPD (6).

For physiological cell function, the control of reactive oxygen species (ROS) production is necessary, because an excessive increase in the level of ROS compared to the antioxidant capacity of a cell leads to oxidative stress. Antioxidant enzymes including SOD, catalase (CAT), and glutathione peroxidase (Gpx) neutralize ROS inside cells (7). Thus, considering the influential role of oxidative stress in the pathogenesis of COPD, basic mechanisms in neutralizing oxidative stress are essential and necessary for a more effective treatment of COPD (8).

High concentrations of bioactive compounds including lactoferrin and various immunoglobulins (IgG, IgA, IgM, IgD) are found in camel milk, which has turned camel milk into a substance with extraordinary medicinal properties (9). The presence of high levels of these compounds along with various vitamins (C, B1, B2, E, A) (10), lysozymes, insulin-like molecules and lactoperoxidase cause the therapeutic potential of camel milk in many diseases such as asthma, hepatitis, autism, cancer, diabetes, jaundice, tuberculosis, food allergies and diarrhea. Therefore, the high concentrations of whey proteins, lactoferrin, casein, lactic acid bacteria (LAB) and vitamin C in camel milk may be the main factor in reducing oxidative stress and thus its antioxidant effects (11). The expression of the genes carried out by the peptides isolated from camel milk has increased significantly, indicating the clearing of free radicals and antioxidant properties of camel milk and as a result neutralization of stress and apoptosis (12).

Therefore, in the present study, the effects of camel milk on systemic inflammation and oxidative stress of CS-induced COPD in rats were investigated.

Materials and methods

Animal groups and the CS-induced COPD protocol

From the animal house of Mashhad University of Medical Sciences, 35 Wistar rats (male, 200 ± 250 g) were purchased and kept in standard conditions (free access to food and water, 12 h light/dark cycle, 22 ± 2°C and humidity 54 ± 2%). This study was approved by the ethics committee of Mashhad University of Medical Sciences in animal experiments (Code 981778).

The animals were randomly allocated to the following groups (n = 7 in each group):

(A) Control (Ctrl) group
(B) CS-exposed (COPD) group
(C) CS-exposed and treated with the 4 mL/kg camel milk (CM-L) group (13).
(D) CS-exposed and treated with the 8 mL/kg of camel milk (CM-H) group (13)
(E) CS-exposed and treated with 1 mg/kg dexamethasone (Dexa) group (13).

Based on the model by Mehtaj et al. (14), rats were exposed to the CS for three consecutive months, except the control group. To acclimate the rats to the CS, the number of cigarettes was gradually increased over the first 4 days: from one cigarette on the first day to two on the fifth day, three on the ninth day, four on the thirteenth day, and five cigarettes on the seventeenth day. Following this 20-days initial period, each rat was then exposed to five cigarettes per week for the remaining duration of the 3 months (13, 14), (Figure 1).

Preparation of camel milk

Camel milk was purchased from the Asaish Company in Gonbad kavos, Iran. The camel's milk in the Turkmen Sahara region contains 2.9% protein, 83–90% water, 4.52% lactose, 12.38% total solids, 4.19% fat, and 0.77% ash (15). Using the lyophilization method (16), camel milk was prepared in powder form and mixed with drinking water (0.2 g/mL). Different concentrations of the resulting solution (4 and 8 mL/kg) were administered to rats by gavage.

Blood collection, measurement of total and differential WBC and biochemical analysis

Rats were anesthetized by intraperitoneal injection of 50 mg/kg ketamine at the end of the experimental period (3 months) and sacrificed and 5 mL blood was taken from the heart. Then, 1 ml of the blood was added to a coagulated tube for counting white blood cells (WBC) and 4 mL was centrifuged at 3000 rpm for 15 min and the serum was stored at 4°C for measuring oxidative stress markers and cytokines (17).

The total and differential number of WBC were measured using a Neubauer chamber and Wright-Giemsa staining of a smear of the blood, respectively, (18).

The level of SOD, CAT, thiol and malondialdehyde (MDA) were measured in the serum, homogenized tissues of the heart, kidney, liver, and testicle tissues based on previous studies. SOD activity was assessed by measuring superoxide generation through the auto-oxidation of pyrogallol, and its activity was indirectly evaluated at 570 nm (19). The rate at which hydrogen peroxide (H_2O_2) decomposes by CAT was assessed using Aebi's method, and its activity was measured with a spectrophotometer at 240 nm (20). Total thiol

Abbreviations: CAT, Catalase; COPD, Chronic obstructive pulmonary disease; CS, Cigarette smoke; CYP, Cyclophosphamide; ELISA, Enzyme-linked immunosorbent assay; Gpx, Glutathione peroxidase; LAB, Lactic acid bacteria; MDA, Malondialdehyde; ROS, Reactive oxygen species; SEM, Standard error of the mean; SOD, Superoxide dismutase; TNF-α, Tumor necrosis factor-alpha; WBC, White blood cells.

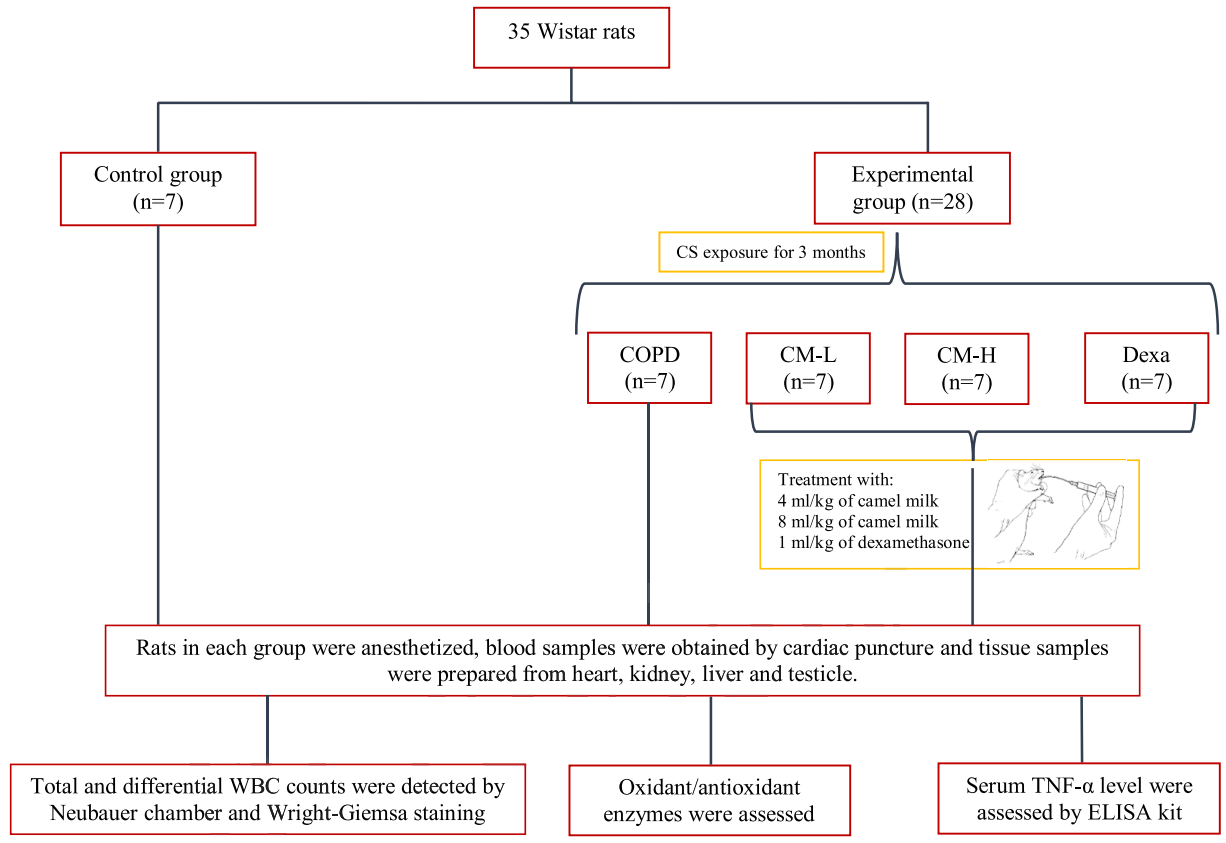

FIGURE 1
Flowchart of the study design of the animal model.

content was assessed by generating a yellow complex from SH groups, which exhibits an absorbance peak at 412 nm (21). The reaction of MDA with thiobarbituric acid (TBA) results in a pink solution, which has an absorbance peak at 535 nm (22). Also, using a double enzyme-linked immunosorbent assay (ELISA) kit (Carmania Pars, Kerman, Iran), the concentration of tumor necrosis factor-alpha (TNF-α) was measured in the serum (23, 24).

Statistical analysis

The mean ± standard error of the mean (SEM) of the data was presented. Statistical data comparison was done using one-way analysis of variance (ANOVA) with Tukey–Kramer post-test using Instat software and $p < 0.05$ was considered as significance criteria.

Results

Effect of camel milk on total and differential WBC count in the serum

In CS-exposed rats, total and differential WBC, neutrophil, monocyte, lymphocyte and eosinophil counts in the blood were significantly increased compared to the control rats ($p < 0.05$ for eosinophil and $p < 0.001$ for other cases, Figure 2).

Total and differential WBC counts in the blood in CS-exposed rats treated with two doses of camel milk and dexamethasone were significantly reduced compared to the COPD group ($p < 0.05$ to $p < 0.001$, Figure 2).

The effects of a high dose of camel milk on total and differential WBC were higher than its low dose except for eosinophil ($p < 0.05$ for lymphocyte and $p < 0.01$ for other cases, Figure 2). In CS-expo rats treated with two doses of camel milk total and differential WBC were lower than the dexamethasone treated group except monocyte in those treated with a lower dose and eosinophil in those treated with both doses of camel milk ($p < 0.05$ to $p < 0.001$, Figure 2).

In CS-exposed rats treated with a high dose of camel milk, total and differential WBC were lower than its low dose except for eosinophil ($p < 0.05$ to $p < 0.01$, Figure 2).

Effect of camel milk on TNF-α level in the serum

The level of TNF-α in the serum of CS-exposed rats was significantly increased compared to the control rats ($p < 0.001$, Figure 3).

The serum level of TNF-α in CS-exposed rats treated with two doses of camel milk was significantly reduced compared to the non-treated CS-exposed rats ($p < 0.01$ for low dose and $p < 0.001$ for high dose of camel milk, Figure 3). In the CS-exposed rats treated

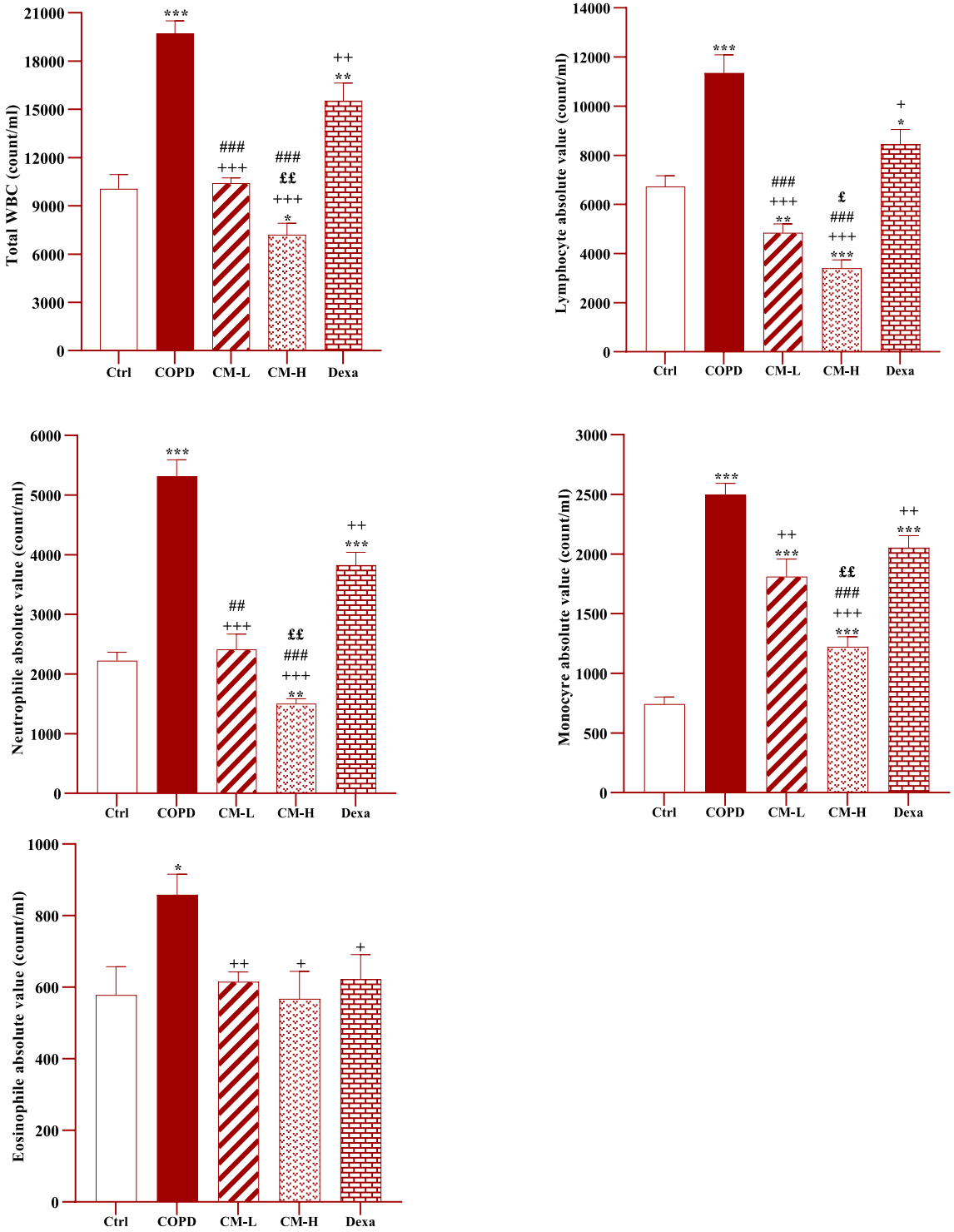

FIGURE 2
Effects of camel milk on improving total and differential WBC counts in the blood of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). *p < 0.05, **p < 0.01 and ***p < 0.001; compared to the control group. +p < 0.05, ++p < 0.01 and +++p < 0.001; compared to untreated COPD group. £p < 0.05 and ££p < 0.01; compared to COPD group-treated with 4 mL/kg camel milk. ##p < 0.01 and ###p < 0.001; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

with a low dose of camel milk and dexamethasone, the level of TNF-α in the serum was lower than in the control rats (p < 0.01 for a low dose of camel milk and p < 0.001 for dexamethasone, Figure 3).

The level of TNF-α in the serum of CS-exposed rats treated with a high dose of camel milk was significantly lower than its low dose (p < 0.05, Figure 3).

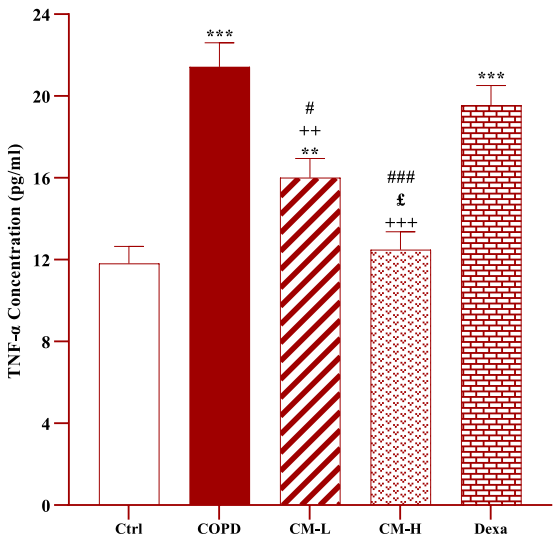

FIGURE 3
Effect of camel milk on improving TNF-α level in serum of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). **p < 0.01 and ***p < 0.001; compared to the control group. ++p < 0.01 and +++p < 0.001; compared to untreated COPD group. £p < 0.05; compared to COPD group-treated with 4 mL/kg camel milk. #p < 0.05 and ###p < 0.001; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

The effects of both doses of camel milk on serum TNF-α level were significantly higher than dexamethasone ($p < 0.05$ and $p < 0.001$ for low and high dose of camel milk respectively, Figure 3).

Effect of camel milk on oxidative stress markers

In the serum and homogenized tissues of the heart, kidney, liver, and testicle, SOD and CAT activities and thiol level in the CS-exposed rats were significantly decreased but the MDA level was significantly increased compared to the control group ($p < 0.001$ in all cases, Figures 4–8).

In CS-exposed rats treated with two doses of camel milk and dexamethasone, in the serum, homogenized heart, kidney, liver, and testicle tissues, SOD and CAT activities and thiol level were significantly increased but the MDA level was significantly decreased compared to the non-treated CS-exposed rats except some cases in the treated group with a low dose of camel milk and only one case in the dexamethasone treated group ($p < 0.05$ to $p < 0.001$, Figures 4–8). In CS-exposed rats treated with two doses of camel milk and dexamethasone, oxidative stress markers the improvement in some cases were significantly lower compared to the control rats ($p < 0.05$ to $p < 0.001$, Figures 4–8).

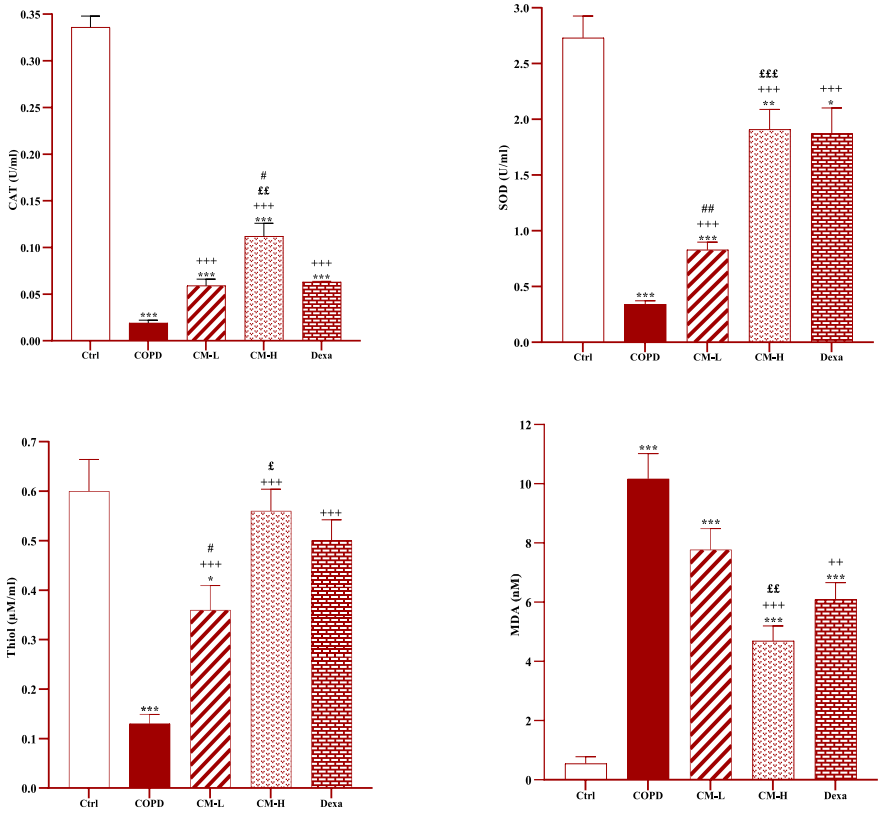

FIGURE 4
Effects of camel milk on improving CAT, SOD, thiol and MDA levels in serum of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). *p < 0.05, **p < 0.01 and ***p < 0.001; compared to the control group. ++p < 0.01 and +++p < 0.001; compared to untreated COPD group. £p < 0.05, ££p < 0.01 and £££p < 0.001; compared to COPD group-treated with 4 mL/kg camel milk. #p < 0.05 and ##p < 0.01; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

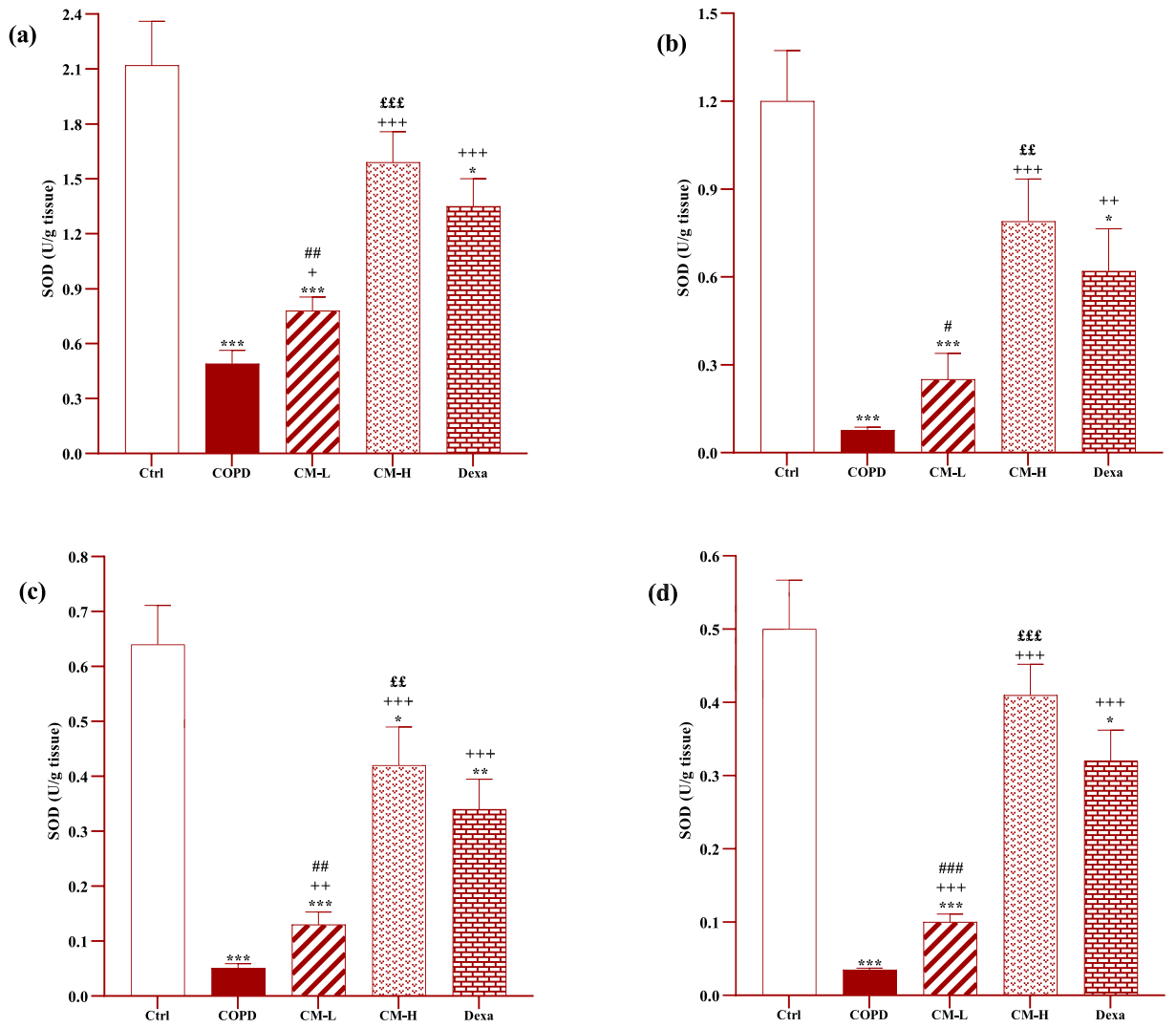

FIGURE 5
Effects of camel milk on improving SOD levels in the heart **(A)**, kidney **(B)**, liver **(C)**, and testicle tissues **(D)** of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). *p < 0.05, **p < 0.01 and ***p < 0.001; compared to the control group. +p < 0.05, ++p < 0.01 and +++p < 0.001; compared to untreated COPD group. ££p < 0.01 and £££p < 0.001; compared to COPD group-treated with 4 mL/kg camel milk. #p < 0.05, ##p < 0.01 and ###p < 0.001; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

The improvement in oxidative stress markers in the serum, and homogenized heart, kidney, liver and testicle tissues in the treated group with a high dose of camel milk, in most cases were higher than its low dose and, in some cases, than dexamethasone. However, the effects of a low dose of camel milk on some cases of oxidative stress markers were lower than dexamethasone ($p < 0.05$ to $p < 0.001$, Figures 4–8).

Discussion

In this study, rats were exposed to CS for three months to induce an experimental model of COPD and the effect of treatment with two doses of camel milk and dexamethasone during the exposure period to CS was examined. The findings revealed increased total and differential WBC and TNF-α level in the serum and level of MDA in the serum and homogenized tissues of heart, kidney, liver, and testicle, but decreased levels of CAT, SOD and thiol in the COPD group. In all experimental groups except the control group, exposure to cigarettes resulted in weight loss, a persistent cough, a fatigued appearance, decreased mobility, inadequate feeding, dull fur, yellowish urine, and dry stools. However, these symptoms showed improvement in the treatment groups. In addition, the same methodology for induction of COPD was applied in our previous studies (18, 25). Also, in another study we investigated the lung pathological changes caused by cigarette smoke in a rat model of COPD which indicated that cigarette smoke effectively induces COPD in rats (13).

Camel milk (4 and 8 mL/kg) and dexamethasone reduced total and differential WBC counts in the blood, the serum level of TNF-α, and the level of MDA in the serum and homogenized tissues of the heart, kidney, liver, and testicle, but significantly increased the levels of antioxidant enzymes and thiol level.

In the peripheral blood of COPD patients, an increase in the number of white blood cells and systemic inflammation have been

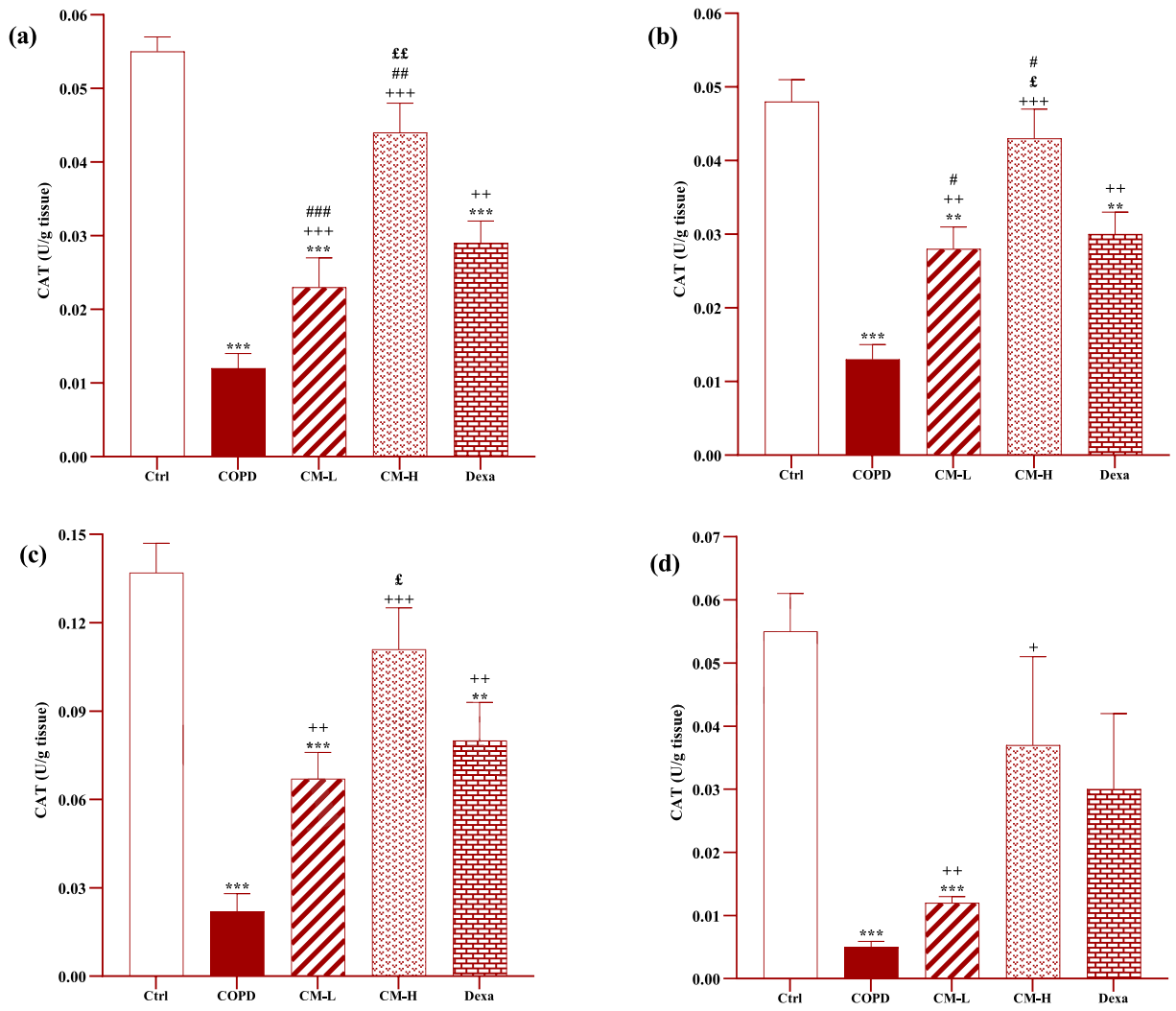

FIGURE 6
Effects of camel milk on improving CAT levels in the heart **(A)**, kidney **(B)**, liver **(C)**, and testicle **(D)** tissues of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). $^{**}p < 0.01$ and $^{***}p < 0.001$; compared to the control group. $+p < 0.05$, $++p < 0.01$ and $+++p < 0.001$; compared to untreated COPD group. £$p < 0.05$ and ££$p < 0.01$; compared to COPD group-treated with 4 mL/kg camel milk. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

reported (26). Smoking can influence the correlation between WBC count and lung function. One of the potential markers in COPD patients is WBC count, which can predict the severity of COPD together with the chest examination and lung function tests (27). The other clinical indicators of acute exacerbation of COPD is blood eosinophil count. Considering the activation of various immune cells including monocytes and lymphocytes in the lungs and the prominent role of neutrophils in the pathogenesis of inflammation, the counting of differential WBC is an indicator of COPD severity (27, 28). These evidences support the changes in rats exposed to CS in this study.

Camel milk is a potent protector against cyclophosphamide (CYP)-induced toxicity that prevents structural changes in leukocytes of leukopenia rats (29). Camel milk is a rich source of biological proteins, iron and B vitamins, which are essential for red blood cells. As a result, the consumption of camel milk increased the number of red blood cells and the concentration of fetal hemoglobin in sickle cell anemia. So, camel milk can prevent anemia crises and manage sickle cell diseases (30). In mice with immunosuppression caused by CYP, the number of WBC, lymphocyte, and neutrophil were significantly decreased but treatment with camel milk increased the concentration of immunoglobulins and antioxidant enzymes and inhibited oxidative stress (31).

In rats with COPD caused by CS exposure, serum and pulmonary tissue TNF-α levels were increased (32, 33). The increase of TNF-α in the serum of stable COPD patients can be a biomarker reflecting the systemic inflammatory response (34). The systemic inflammatory response in COPD can be justified through systemic hypoxia, which stimulates the local expression of inflammatory cytokines, including TNF-a and IL-6 (34, 35). Also an increase in the serum TNF-α level, which could be associated with bronchial patency disorder in COPD was observed (36). The effect of some cytokines, including TNF-α, on the development of pathophysiological mechanisms in COPD may be due to the correlation between serum level of TNF-α and lung function parameters (37).

In the present study, the serum level of TNF-α significantly decreased due to the administration of camel milk. Since TNF-α is

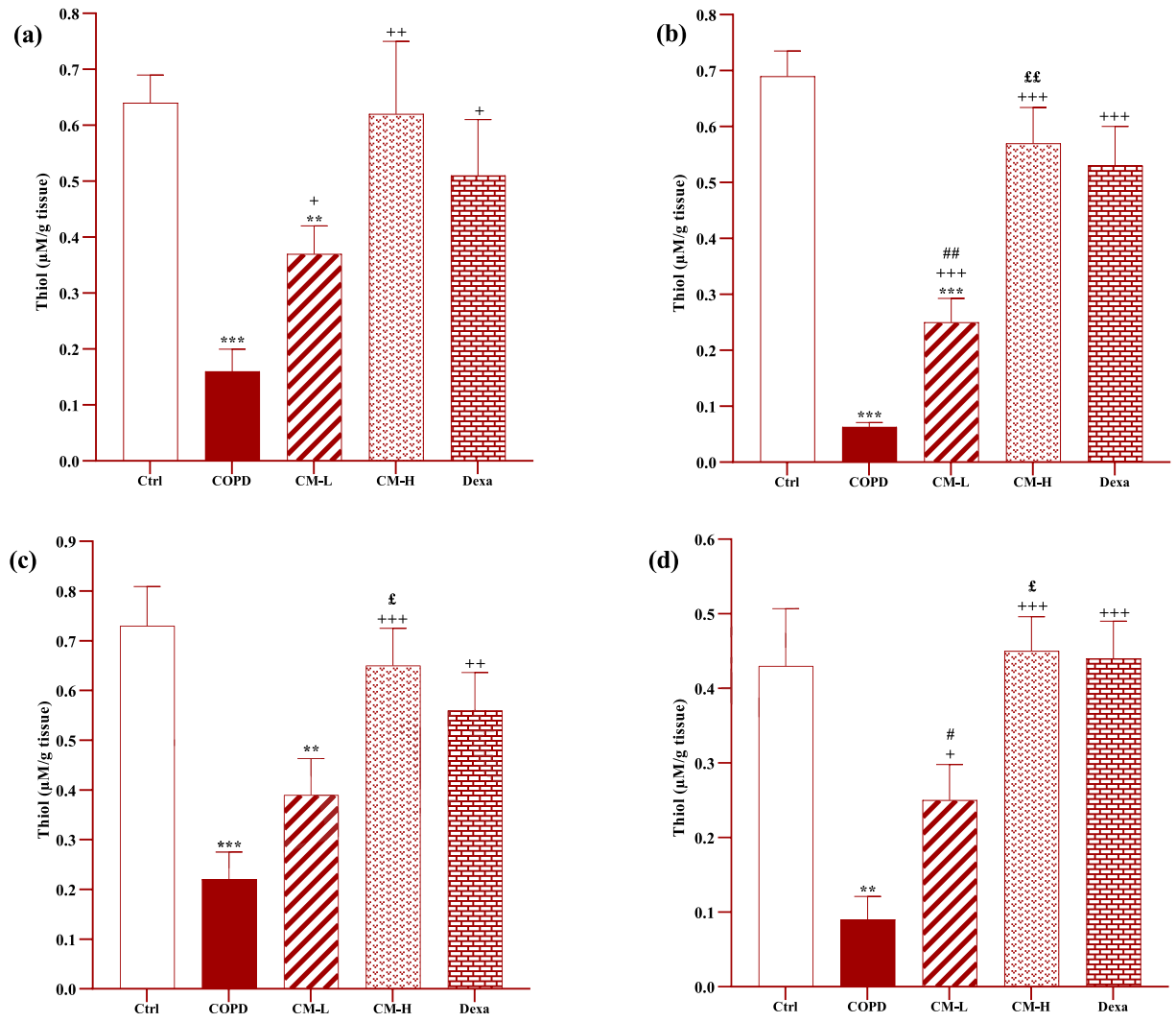

FIGURE 7
Effects of camel milk on improving thiol levels in the heart **(A)**, kidney **(B)**, liver **(C)**, and testicle **(D)** tissues of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). $^{**}p < 0.01$ and $^{***}p < 0.001$; compared to the control group. $+p < 0.05$, $++p < 0.01$ and $+++p < 0.001$; compared to untreated COPD group. £$p < 0.05$ and ££$p < 0.01$; compared to COPD group-treated with 4 mL/kg camel milk. #$p < 0.05$ and ##$p < 0.01$; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

mainly produced by macrophages and it is the main regulator of inflammatory responses, camel milk can inhibit inflammation by reducing TNF-α. In a clinical study of 64 children with autism, milk reduced neuro-inflammation and gastrointestinal symptoms (38). Administration of six camel milk LAB strains in rats with lipopolysaccharide/galactosamine-induced acute liver damage, reduced the level of TNF-α in the liver (39).

Increased total and differential WBC and TNF-α level in the serum and MDA levels in the serum and homogenized tissues of the heart, kidney, liver, and testicle, but decreased levels of CAT, SOD and thiol in the COPD group were seen in the present study. Cell damage caused by oxidative stress is inhibited by antioxidants as the most essential defense mechanism, antioxidant enzymes can have potential therapeutic effects against inflammatory diseases, rheumatoid arthritis, cancer and neurological diseases (40). According to previous studies, an increase in the serum level of MDA in COPD patients was reported (41). The results of a double-blind randomized clinical trial showed decreased levels of SOD, CAT and thiol in patients with COPD (42).

Treatment with camel milk, reduced total and differential WBC counts in the blood, the serum levels of TNF-α, and the level of MDA in the serum and homogenized tissues of the heart, kidney, liver, and testicle, but increased the levels of antioxidant enzymes and thiol level in the present study. The activities of SOD and CAT in rats poisoned with ammonium chloride were significantly increased by camel milk. Camel milk is rich in zinc, magnesium, vitamins C, A, B2 and E (43), which can protect against cadmium-induced anemia by reducing the production of free radicals in red blood cells (44). High levels of α-lactalbumin, β-casein and vitamin C have made camel milk an antioxidant that can prevent the production of superoxide anions, free radicals and ROS (11). Camel milk protein hydrolysates can increase SOD and CAT levels in streptozotocin-induced diabetic rats and decrease MDA level. Camel milk with antioxidant properties, improves the symptoms of hyperglycemia and hyperlipidemia (45). Due to the immune system strengthening and anti-toxic properties of camel milk, in mice with leukopenia caused by CYP, hepatic SOD and CAT were increased compared to the untreated group (29).

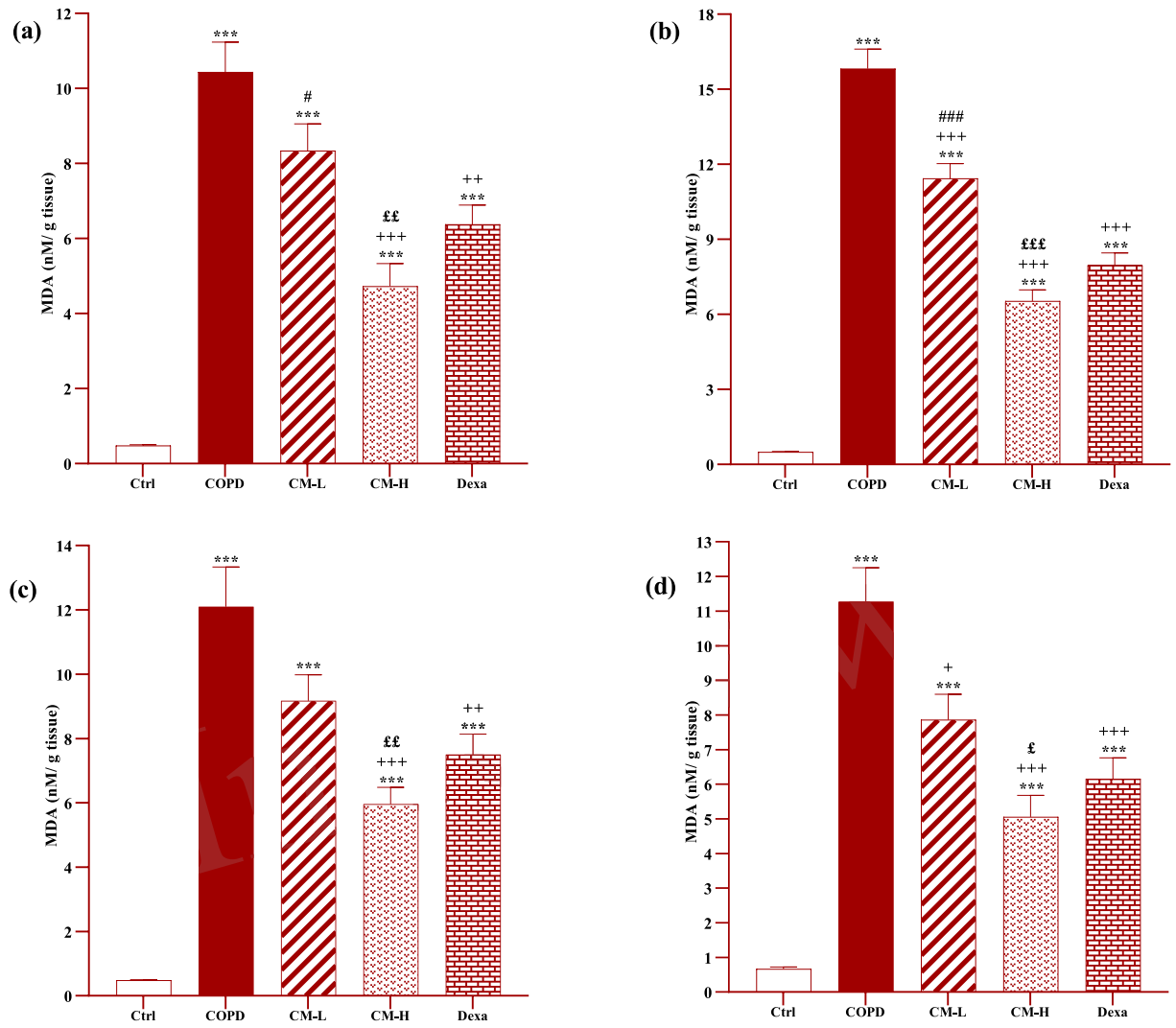

FIGURE 8
Effects of camel milk on improving MDA levels in the heart **(A)**, kidney **(B)**, liver **(C)**, and testicle **(D)** tissues of CS-exposed rats. Data are presented as mean ± SEM (n = 7 in each group). ***p < 0.001; compared to the control group. +p < 0.05, ++p < 0.01 and +++p < 0.001; compared to untreated COPD group. £p < 0.05, ££p < 0.01 and £££p < 0.001; compared to COPD group-treated with 4 mL/kg camel milk. #p < 0.05 and ###p < 0.001; compared to Dexa group. ANOVA and Tukey–Kramer test were used for statistical analysis.

In the present study, the pulmonary function tests of experimental groups and oxygen saturation results were not provided which should be performed in further studies. Also, it is suggested that in future studies a third group treated with higher camel milk be investigated to drive a causal regression analysis. The camel milk may contain residue-free, especially about the chemicals affecting pulmonary function or the studied parameters. However, the results of the current study indicated beneficial effects on all measured parameters. Considering the presence of residue-free, affecting pulmonary function or the studied parameters in the camel milk, the measured parameters should be deteriorated or at least do not improved. In away in further studies, the appropriate methods should be taken to ensure regarding residue-free, of the camel milk.

The results showed systemic antioxidant and anti-inflammatory properties of camel milk in a rats model of COPD caused by CS exposure, which are more effective compared to dexamethasone. Therefore, camel milk could be considered for the treatment of inflammatory diseases including asthma and COPD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the ethics committee of Mashhad University of Medical Sciences in animal experiments (Code 981778) on February 28, 2021. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SB: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft. MM: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft. HS: Writing – review & editing. MB: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Vacuum level for opening the streak canal and measurement of machine-induced changes in teat tissue during milking of dairy camels (*Camelus dromedarius*)

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This research aims to study some of the teat characteristics involved in milkability of dairy camels, including the relationship between teat anatomy and the vacuum needed to open the teat sphincter (VLOTS). It also investigates short-term machine milking-induced changes in teat tissue thickness and teat anatomical characteristics, as well as their implications for udder health in dairy camels. To study VLOTS, 10 dairy camels in mid-lactation (weight: 516.6 ± 19 kg; age: 13.4 ± 3.8 years; parity: 5 ± 1.8; average milk yield: 7.3 ± 0.8 L/day) were used in Experiment 1. VLOTS was measured 4 h after morning milking for all four teats using an apparatus called a vacuumeter, without a liner or pulsation. Measurements were repeated three times at 2-day intervals and considered as repetition. Teat canal length (TCL), teat wall thickness (TWT), teat cistern separation wall thickness (TSWT), teat cistern diameter (TCD), and teat length were measured using ultrasound. Experiment 2 was performed on six dairy camels in late lactation (weight: 460 ± 55.15 kg; age: 10.2 ± 2.4 years; parity: 3.5 ± 1.0; average milk yield: 3.6 ± 0.7 L/day). External teat measurements (length, barrel, and apex diameters) were recorded with a caliper. Pre-and post-milking teat-end thickness (TET) were evaluated with a cutimeter at 2 cm from the teat end. Ultrasound imaging was performed pre-and post-milking to determine TCL, TWT, TCD, and teat apex diameter (TAD). Milk ejection time and total milking duration were recorded. Residual milk volume was estimated after an injection of 10 IU oxytocin. Milk samples were taken for somatic cell count (SCC). The results showed that only 50 teats out of 120 observations exhibited milk flow at a vacuum up to 70 kPa. Teats were divided into three groups: Group 1 included easy-opening teats that opened at a vacuum level of less than 30 kPa; group 2 included teats that were hard to open, requiring a higher vacuum (31 up to 69 kPa); and group 3 included teats that did not show milk flow at a vacuum higher than 70 kPa. The mean VLOTS for groups 1 and 2 were 19.39 ± 0.66 kPa and 47.13 ± 2.14 kPa, respectively. VLOTS was positively and highly correlated with TCL, TWT, and TSWT (r = 0.71, r = 0.62, and r = 0.51, respectively, $p < 0.001$) and negatively correlated with teat length and diameter (r = −0.50 and r = −0.30, respectively, $p < 0.01$). Observation of teats immediately after cluster removal

revealed a 15.4% decrease in TET. TCL and TWT increased by 20.3 and 40.5%, respectively, while TCD and TAD decreased by 40.3 and 19.9%, respectively, after milking. This suggests the stretching of the teat extremity and congestion of the teat barrel wall. The mean SCC recorded in this study was 149.6 10^3 cells/mL, varying from 37.5 10^3 cells/mL to 287.5 10^3 cells/mL. This study confirms the need for a high vacuum level to overcome the sphincter barrier in dromedary camels. However, it suggests the deleterious effect of large camel teats in a cow's liner.

KEYWORDS

machine milking, vacuum level, teat sphincter, cutimeter, diagnostic imaging, camels

1 Introduction

Efficient and sustainable milk withdrawal from camels' (*Camelus dromedarius*) udders remains a cornerstone for optimizing machine milking and enhancing the economic viability of dairy camel farming. Understanding the animal/machine interaction is critical for adapting machine milking to the various situations encountered worldwide and to the specific needs of different animals (1). Unlike bovine species, the anatomy of the camel's mammary gland and the dynamics of milking remain underexplored (2–4). The streak canal acts as the primary barrier against microbial invasion while facilitating milk emission during milking. Its functionality is influenced by various factors, including vacuum levels applied during milking. Insufficient or excessive vacuum pressure can either increase the probability of liner slips because the adhesion of the liner to the teat is reduced or cause tissue damage, compromising udder health (5, 6). Therefore, determining the optimal vacuum level for opening the streak canal is important for developing milking protocols that balance milk yield, efficiency, and animal welfare (7, 8). Furthermore, the response of teat-end thickness to used parameters or to the liner of milking equipment is also a good tool for the study of milkability and the measurement of machine-induced changes in teat tissue during milking in dairy animals. The impact of machine milking on the teat tissue is inevitable and can be sorted into short-, medium- and long-term effects (9–11). Short-term reversible teat changes including color changes, firmness, thickness, and openness of the teat orifice are commune (12). However, only repeated or prolonged exposure to improper milking conditions causing excessive load on the teat can lead to long-term alterations in teat tissue structure that become hyperkeratosis and potentially increasing susceptibility to mastitis (13–15). Differences in short-term implications for milking efficiency or udder health have been attributed to the variation in anatomical dimensions and structures among teats with different teat-end shapes (9, 16). Thus, understanding the teat's functional anatomy, particularly the streak canal and its interaction with the milking machine, is crucial for improving milking efficiency while maintaining udder health and animal welfare (15). In dairy species, considerable research has been conducted on the effects of the milking liner on the teat tissue integrity (9, 10, 17, 18). However, in dairy camels, such insights remain scarce, necessitating targeted studies to fill this knowledge gap. This research aims to investigate the vacuum levels required to open the streak canal effectively in dairy camels and explore the machine-induced short-term changes in teat tissue during milking.

2 Materials and methods

2.1 Animals, housing, and management

The trial was carried out in the experimental farm of the Arid Regions Institute (IRA, Chenchou, Tunisia). Clinically healthy Maghrebi dromedary camels free from clinical mastitis and have an udder without abnormalities such as non-lactating quarters or teat injuries, and edema were used for the experiments. Animals were housed in a free-stall barn. For the first experiment, camels received/head 5 kg of fresh lucerne (dry matter, DM 17.2%; crude protein, CP, 17.9%; neutral detergent fiber, NDF, 39.6.0%; net energy for lactation, NEL, 1.53 Mcal/kg; on a DM basis), 7 kg of oat hay (DM, 84.6%; CP, 8.4%; NDF, 68.2%; NEL, 0.71 Mcal/kg; on DM basis), 3 kg of lucerne pellets (DM,90.4%; CP, 19.6%; NDF, 64.2%; NEL, 1.25 Mcal/kg on DM basis), and 2 kg of commercial concentrate (DM 91.0%; CP 17.0%; NDF 22.2%; NEL 1.53 Mcal/kg; on a DM basis).

Camels in the second experiment were fed (/head) with a forage mixture of 5 kg of lucerne hay (DM, 96.6%; CP, 17.1%; NDF, 47.3%; NEL, 1.42 Mcal/kg; on a DM basis) and 7 kg of oat hay (DM 96.0%; CP 9.7%; NDF 70.3%; NEL 0.58 Mcal/kg; on a DM basis), supplemented with 2 kg of a commercial concentrate (DM 91.0%; CP 17.0%; NDF 22.2%; NEL 1.53 Mcal/kg; on a DM basis). Diets were calculated based on milk production and lactation stage of camels and available feed in the farm at the time of the experiment. All animals had free access to water.

2.2 Milking

Camels were milked in a 2×3 herringbone milking parlor (FLACO, Spain) once a day at the time of the experiment. The milking unit was equipped with a FLACO milking cluster (weight: 2.1 kg; claw volume: 240 cc). The milking liner was rubber liner type ULTRAmilk, JD317 (mouthpiece bore diameter, 22.5 mm; mouthpiece depth, 27 mm; barrel shape, concave; barrel length, 155 mm). The machine was set at a 60 ppm pulsation rate with a 60:40 pulsation ratio and 48-kPa vacuum level. The pre-milking (cleaning, fore-stripping, and manual teat stimulation) lasted for 30 s; then clusters were attached. Cluster removal was manual when milk flow ceased and visually confirmed.

2.2.1 Experiment 1: vacuum level to open teat sphincter

Ten dairy camels at mid-lactation (weight: 516.6 ± 19 kg; Age: 13.4 ± 3.8 years, parity: 5 ± 1.8; average daily milk yield was

7.3 ± 0.8 L/day) were used for the first experiment. The vacuum needed to open the teat sphincter (VLOTS) was determined with a prototype apparatus called a "vacuumeter," a transparent teat cup without liner nor pulsation (19). The vacuum was then gradually increased from zero until milk flow started or to a maximum of 70 kPa. The teat sphincter was considered open when the first drop of milk emerged from the teat. VLOTS was recorded 4 h after morning milking for all four teats to prevent spontaneous milk ejection and limit the effect of high intra-mammary pressure. Measurements were repeated three times at 2-day intervals and considered as repetition. Teats' characteristics were evaluated by ultrasound. After VLOTS evaluation, teats were divided into three groups where group 1 included easy-opening teats that opened at a vacuum level of less than 30 kPa, group 2 included teats hard to open that needed vacuum ranging between 31 and 69 kPa, and group 3 included teats that did not show milk flow at vacuum higher than 70 kPa.

2.2.2 Experiment 2: milking-induced changes in the teat

Six multiparous Maghrebi camels (weight: 460 ± 55.15 kg; age: 10.2 ± 2.4 years; parity: 3.5 ± 1.0) well-trained to machine milking were selected for this trial. Camels were at late lactation with an average daily milk yield of 3.6 ± 0.7 L. The average teat length was 4.1 ± 0.1 cm for front teats and 4.6 ± 0.1 cm for rear teats. The teat barrel diameter for the front and rear teats was on average 3.3 ± 0.1 cm and 3.6 ± 0.1 cm, respectively. Teats' shapes were cylindrical (68%), conical (17%), and sometimes irregular (15%). Teat external measurements (length, barrel, and apex diameters) were recorded with a caliper. Internal teat measurements were taken before and immediately after milking. Teat-end tissue thickness (TET) was measured before and after milking with a cutimeter at 2 cm of teat end. The cutimeter measurements were made with a spring-loaded caliper as described by Hamann et al. (20) and Marnet et al. (21). These measurements were performed after the ultrasound scans, so it would not affect the images. In this experiment, the jaws of the cutimeter were placed approximately 2 cm above the teat tip. Internal teat measurements were measured by ultrasonography as presented in the following section. Milking-induced changes were calculated as follows:

$$Milking\ induced\ changes\% = \frac{\left(\begin{array}{l}\text{post milking value} - \\ \text{premilking value}\end{array}\right)}{\text{premilking value}} \times 100$$

All measurements were taken immediately before and after morning milking by a trained researcher.

Milk ejection time and milking duration were recorded. Residual milk volume was estimated after an injection of 10 IU oxytocin, and samples of machine and residual milk fractions were taken for somatic cell count (SCC).

2.3 Ultrasound scanning

An ultrasound unit (Aquila Pro, e-saote piemedical, The Netherlands) equipped with a 6-MHz linear probe was used to measure the teat internal measurements and changes in the teat's tissue. The ultrasound was performed in the milking parlor on both the front and rear teats on the operator's side. The water bath method was used as described by Gleeson et al. (22) to prevent deformation and ensure the complete presentation of the teat. In brief, the teat was immersed in a plastic container containing warm water. The probe was coated with a film of lubricant gel and applied to the external surface of the container. A vertical cross-section of each teat was scanned in triplicates before VLOTS recording (*Experiment 1*), during pre-milking preparation, and immediately after cluster removal (*Experiment 2*). Longitudinal cross-sectional images were used for measurements (Figure 1) by means of image treatment software (Image Tool 3.00).

2.4 Somatic cell count

Milk SCC was counted using the direct microscopic method. In brief, an amount of 10 µL milk sample was spread over the Malassez slide. The smear was stained with methylene blue for 10 min, followed by somatic cell count via direct microscopic examination.

2.5 Statistical analysis

All analyses were performed using Mixed Procedures of SAS version 9.3 (SAS Institute Inc., Cary, NC). For the first trial, the models included the general mean, the random effect of the animal (1–10), the fixed effect of the teat position (front and rear), and the fixed effect of the VLOTS class (1–3) and the random error. For the second, the model included the general mean, the random effect of the animal (1–6), the fixed effect of the treatments (before and after milking), teat position (front and rear), and the random error. Differences between means were tested by the PDIFF test. The level of statistical significance was set at a p-value of <0.05, unless otherwise stated. All data are presented as means ± SEM.

The SCC showed a non-normal distribution and was transformed using a logarithm function. We used the formula proposed by Ali and Shook (23) as follows:

$$logSCC = \log 2\left(SCC / 100\,000\right) + 3$$

logSCC was then analyzed using the PROC mixed model. Pearson's correlation was calculated to show possible relationships between teats' characteristics and VLOST in the first experiment and teat characteristics, residual milk, milking times, and SCC in the second experiment.

3 Results

3.1 Vacuum level to open teat sphincter

The vacuum level of the milking machine required to open the teat sphincter (VLOTS) in camels varied widely both inter- and intra-animal. The continuously increasing vacuum (no liner, no pulsation) caused milk flow in only 50 out of 120 teats up to a vacuum of 70 kPa during this experiment. Teats were classed into three groups according to VLOTS level, where group 1 referred to easy-opening teats needing an

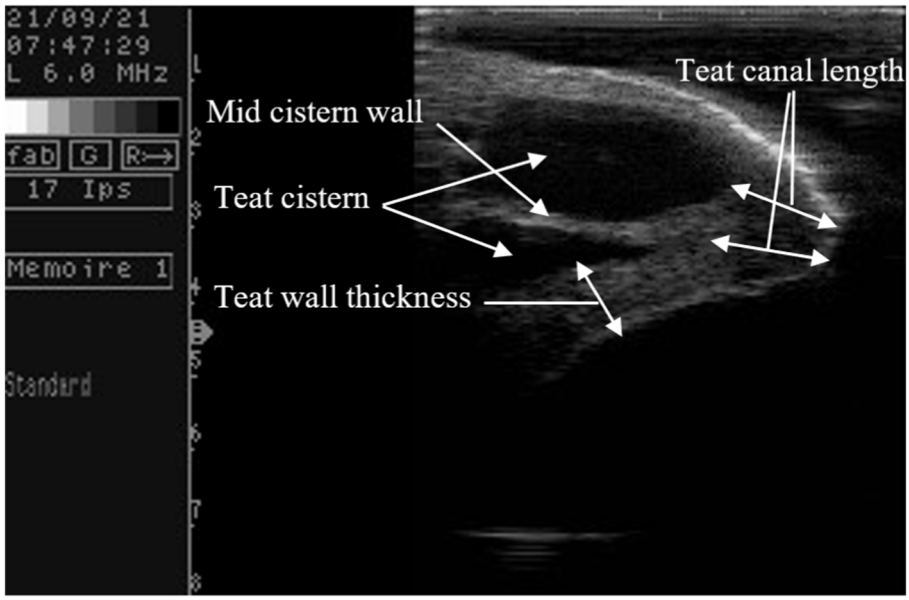

FIGURE 1
Measurements taken on camel teat ultrasound scan.

average of 19.39 ± 0.66 kPa, group 2 required a mean of 47.13 ± 2.14 to observe the first milk drop, while in group 3, the teat sphincter did not open even when vacuum exceeded 70 kPa. Internal teat measurements according to VLOTS group are presented in Table 1. TCL, TWT, and TSWT were significantly shorter, while TCD and TL were significantly higher in easy-opening teats. Interestingly, camels with harder opening teats had significantly higher milk yield ($p < 0.01$). The effect of parity and teat position was tested, and no significant effect was detected.

Pearson's correlation coefficient (Table 2) among internal teat measurements, milk yields, and VLOTS was strong and positive, except for the TCD and TL which showed a strong negative correlation with VLOTS. Teat internal traits showed a strong and positive correlation between TCL, TWT, and TSWT and were negatively correlated with TL. Only TCL and TSWT were correlated with milk yields, while TCD was correlated with only the daily milk yield.

TABLE 1 Overall means of vacuum level to open teat sphincter, evaluated internal teat measures, and milk yield according to levels of teat sphincter opening.

	Group 1	Group 2	Group 3
VLOTS, kPa	19.39 ± 0.66	47.13 ± 2.14	≥ 70
TCL, cm	0.71 ± 0.06^{b}	1.43 ± 0.05^{a}	1.46 ± 0.03^{a}
TWT, cm	0.64 ± 0.04^{b}	0.98 ± 0.05^{a}	1.03 ± 0.03^{a}
TAD, cm	2.16 ± 0.06	2.22 ± 0.07	2.09 ± 0.04
TCD, cm	4.03 ± 0.14^{a}	3.89 ± 0.17^{ab}	3.65 ± 0.07^{b}
TL, cm	6.16 ± 0.14^{a}	5.59 ± 0.21^{b}	5.33 ± 0.10^{b}
TSWT, cm	0.42 ± 0.02^{b}	0.66 ± 0.04^{a}	0.63 ± 0.04^{a}
MMY, kg	4.01 ± 0.03^{c}	5.07 ± 0.08^{b}	5.77 ± 0.12^{a}
DMY, kg	6.74 ± 0.09^{c}	7.50 ± 0.08^{b}	8.61 ± 0.23^{a}

TCL, teat canal length; TWT, teat wall thickness; TAD, teat apex diameter; TCD, teat cistern diameter; TL, teat length; TSWT, teat cistern separation wall thickness; MMY, morning milk yield; DMY, daily milk yield; VLOTS, vacuum level to open teat sphincter. ^{a,b,c}Means within a line with different superscripts are significantly different ($p < 0.05$).

3.2 Changes in the teat tissue parameters immediately after milking

Average teat internal measurements before and immediately after milking are presented in Table 3, while the relative changes induced by milking according to the teat position are illustrated in Figure 2. TWT increased by 40.5 ± 4.3%, TCL increased by 20.3 ± 5.3%, while TCD and TAD decreased by 40.3 ± 8.2% and 19.9 ± 7.8%, respectively. Teat position affected only TCL and TCD with a significantly stronger effect ($p < 0.001$) observed in front teats. TET decreases by 15.4 ± 1.1% with no difference according to teat position.

Pearson's coefficients calculated between internal and external teat measurements (Table 4) showed negative correlations between TET measured by cutimeter and TWT and TCL with $r = -0.21$, $p < 0.05$ and $r = -0.36$, $p < 0.0001$, respectively. Similarly, TCD was strongly and negatively correlated with TWT and TCL ($r = -0.50$; $p < 0.0001$ and $r = -0.45$; $p < 0.0001$, respectively). Furthermore, TET was positively correlated with all external teat measurements, whereas TCL was negatively correlated with external teat diameter measured at different levels (apex, barrel, and basal levels) and positively correlated with TL. Moreover, the mean SCC recorded in this study was 149.6 10^3 cells/mL and was positively correlated with TCL ($r = 0.21$; $p < 0.01$) and negatively correlated with TAD ($r = 0.20$; $p < 0.05$). Residual milk, time to milk ejection, and total milking duration were not correlated with the teat characteristics, except for TL, which was positively correlated with milk ejection time and total milking duration ($r = 0.35$; $p < 0.0001$ and $r = 0.22$; $p < 0.01$, respectively) (Table 5).

4 Discussion

A high inter- and intra-variability between she-camels regarding VLOTS was observed. Only 30% of the tested teats required a low

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TABLE 2 Correlation coefficient between studied internal teat's traits, milk yields, and VLOTS.

	TCL	TWT	TAD	TSWT	TCD	TL	MMY	DMY
TCL	1							
TWT	0.69***							
TAD	0.01	0.16						
TSWT	0.56***	0.53***	0.08					
TCD	−0.13	0.03	0.72***	0.05				
TL	−0.29*	−0.20*	0.64***	−0.15	0.76***			
MMY	0.30**	0.18	0.14	0.26*	0.17	0.10		
DMY	0.28*	0.15	0.20	0.26*	0.27*	0.17	0.96***	
VLOTS	0.71***	0.62***	−0.19	0.51***	−0.30**	−0.50***	0.40***	0.33**

TCL, teat canal length; TWT, teat wall thickness; TAD, teat apex diameter; TCD, teat cistern diameter; TL, teat length; TSWT, teat cistern separation wall thickness; MMY, morning milk yield; DMY, daily milk yield; VLOTS, vacuum level to open teat sphincter. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

TABLE 3 Effect of machine milking on average teat tissue measurements (cm) determined by ultrasonography and cutimeter before and immediately after milking.

	Before milking	After milking
Teat wall thickness	1.00 ± 0.26[b]	1.41 ± 0.36[a]
Teat canal length	1.29 ± 0.62[b]	1.56 ± 0.51[a]
Teat cistern diameter	2.71 ± 0.67[a]	1.62 ± 0.61[b]
Teat apex diameter	2.70 ± 0.31[a]	2.17 ± 0.24[b]
Teat-end thickness	1.3 ± 0.26[a]	1.1 ± 0.10[b]

[a,b]Means within a line with different superscripts are significantly different ($p < 0.05$).

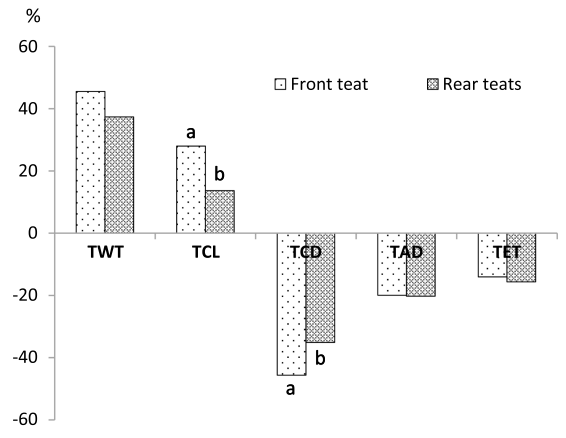

FIGURE 2
Average milking-induced change (%) in front and rear teats. TWT, teat wall thickness; TCL, teat canal length; TCD, teat cistern diameter; TAD, teat apex diameter; TET, teat-end thickness. [a,b]Columns with different superscripts are significantly different ($p < 0.05$).

vacuum level to overcome the teat sphincter. For these loose teats, VLOTS was within the range reported for most tested species. Indeed, Sinapsis et al. (7) recorded an average of 16.59 ± 0.7 kPa of VLOTS in Greek Boutsiko ewes. Skapetas et al. (8) found that indigenous Greek goats required an average of 23.57 kPa to open the teat sphincter. In dairy cows, Le Du et al. (19) reported that the VLOTS was 17.0 kPa and 14.5 kPa for front and rear teats, respectively, while Weiss et al. (24) reported levels ranging between 17.8 and 21.0 kPa according to the teat position. Group 2, characterized by firm teat sphincters, accounted for 15% of the tested teats and required an average pressure of 47.13 ± 2.14 kPa to overcome the sphincter barrier, while 55% of the teats did not open even when the vacuum exceeded 70 kPa. Similar observations were reported in buffaloes (25). In this experiment, a continuously increasing vacuum (without a liner or pulsation) induced milk flow in only 40% of tested teats at a vacuum level of up to 45 kPa, while the remaining 60% exhibited no milk flow. The aforementioned studies reported a wide variation in VLOTS across species, suggesting that genetic factors may partially explain this variability. Except for the study on VLOTS in buffaloes (25), the authors suggested reducing the milking vacuum for machine milking to improve teat conditions and reduce the residual milk (7). Weiss et al. (24) reported that the initial force required for the start of the milk flow was greater than the force needed to keep the teat sphincter open during milking. In our study, most teats did not open under excessive vacuum levels, even though milking settings recommended for similar conditions were set at 48 kPa (26). This suggests that the combined force of external vacuum and increased internal pressure from milk ejection facilitates teat sphincter opening in camels. Therefore, it is important to stimulate milk ejection before applying the milking liner to prevent excessive vacuum pressure on the teats. Parity and teat position had no effect on VLOTS in our study, consistent with the findings of Sinapis et al. (7) and Skapetas et al. (8), who reported that parity did not influence VLOTS in ewes and goats. Moreover, Weiss et al. (24) recorded that the vacuum needed to open the teat canal at the start and at the cessation of the milk flow did not differ significantly between teat positions. Nevertheless, Le Du et al. (19) reported that rear teats required less vacuum to open the sphincter and were easier to milk in cows (Table 5).

Longitudinal ultrasound scans of camel teats revealed an outer layer of homogeneous hyperechoic tissue at the periphery, representing the teat epidermis, followed by an echogenic inner layer corresponding to the teat wall (Figure 1). Between the two teat walls lies an anechoic region representing the teat cistern, which is divided into two compartments by the teat cistern wall. These two halves of the teat cistern are drained externally through two or sometimes three teat canals, which may appear in the same layout or, in some cases, only one is visible in the scanned section. In the first investigation, the easiest teats to milk had a thin wall and a shorter teat canal as observed

TABLE 4 Pearson correlation coefficients between teat internal and external measurements.

	TWT	TCL	TCD	TET	TAD	TBsD	TBrD
TCL	0.29**						
TCD	−0.50***	−0.45***					
TET	−0.21*	−0.36***	0.31**				
TAD	−0.30**	−0.17	0.59***	0.41***			
TBsD	0.04	−0.55***	0.21*	0.29**	−0.01		
TBrD	0.28**	−0.46***	0.20*	0.27**	−0.01	0.61***	
TL	0.09	0.30**	−0.08	0.34**	0.20*	0.44***	0.22**

TWT, teat wall thickness; TCL, teat canal length; TCD, teat cistern diameter; TET, teat-end thickness; TAD, teat apex diameter; TBsD, teat basal diameter; TBrD, teat barrel diameter; TL, teat length. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

TABLE 5 Pearson's correlation coefficients between teat measurements, residual milk, milk ejection time, total milking time, and somatic cell counts.

	TWT	TCL	TCD	TET	TAD	TBsD	TBrD	TL
RM	−0.01	−0.02	−0.01	−0.03	0.18	−0.17	−0.12	0.02
TE	0.05	0.02	0.01	0.17	0.08	0.08	0.06	0.35***
TMT	−0.03	−0.05	0.04	0.07	−0.03	0.03	0.01	0.22**
SCC	0.01	0.21**	−0.16	−0.20*	−0.21*	−0.12	−0.02	−0.07

TWT, teat wall thickness; TCL, teat canal length; TCD, teat cistern diameter; TET, teat-end thickness; TAD, teat apex diameter; TBsD, teat basal diameter; TBrD, teat barrel diameter; TL, teat length; RM, residual milk; TE, milk ejection time; TMT, total milking time; SCC, somatic cell count. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

in dairy cows (19). Unexpectedly, VLOTS was strongly correlated with milk yield ($r = 0.4$; $p < 0.0001$ and $r = 0.33$; $p < 0.01$, respectively, for morning and daily milk yield), and camel with hard opening teats produced significantly more milk. This contrasts with findings in small ruminants (7, 8) and dairy cows (19, 24). However, these studies reported increased stripping milk with increased VLOTS, suggesting a selection of animals that require lower VLOTS. Further studies are needed in camels to evaluate the relationship between VLOTS and milk flow parameters to better understand milking efficiency and optimize dairy management practices.

Different methods have been tested for measuring the teats' reaction to machine milking by several researchers. Although ultrasonography with warm water bath method has proven to be an efficient, non-invasive technique detecting minimal changes in the teat tissue in several species (9, 11, 17, 27, 28), the cutimeter method has been recommended for measuring changes in teat thickness and has been tested in most dairy species including cows (11, 20, 29), ewes (7, 21, 27), and goats (8, 30, 31). To the best of our knowledge, this is the first study to tackle machine-induced changes in the teat tissue of the dromedary camels using both methods. Ultrasound teat scanning in a water bath prevented tissue deformation, as reported in previous studies (17, 24, 28). The results have shown that the milking machine caused an increase in the teat wall thickness (TWT) and in the teat canal length (TCL), against a decrease in teat cistern diameter (TCD) and teat apex diameter (TAD) observed by ultrasound. Teat-end thickness (TET), also called teat tissue density, measured by cutimeter decreased significantly after milking in our dairy camels. Using an ultrasound technique, similar results have been reported in small ruminants (30) and Holstein cows (32, 33).

Indeed, Gašparík et al. (9) reported that the milking-induced changes in teat tissue have a complex interaction with the teat measurements in dairy cows. They revealed that a thick teat barrel and thick teat apex decreased in thickness during milking, whereas a thin teat barrel and thin teat apex thickness increased. In this case, the liner encloses the teat, acting as a physical boundary that either restricts or facilitates the expansion of teat tissue. Similarly, in our study, the pre-milking dimensions of camels' teats were within the range of thick teat barrels (>26,5 mm). The length of the teat canal increased, while the diameter of the teat apex and teat-end thickness decreased, indicating that the teat tip endured longitudinal stretching due to the vacuum suction and the weight of the milking claw attached to the teats. We hypothesize that the physical response observed in the camels' teats under the applied milking conditions suggests that the teats were drawn into the liner under the effect of high vacuum (48 kPa) up to the point of closure, which varies from one teat to another depending on its morphology and measurements. This resulted in two main effects: (i) the formation of compression rings at the teat base, which also varied according to the teat length and diameter of the teat, and (ii) the stretching of the teat tip, which was pulled into the pulsation chamber. This led to an elongation of the teat canal and a reduction in the apex diameter, as observed via ultrasound, and consequently, a decrease in teat tip tissue density, as measured by the cutimeter. Incidentally, ultrasound scans clearly show that the accumulation of lymphatic or blood-derived fluid and the formation of edema occur above the contact line between the teat and the liner (Figure 3), which causes the formation of compression rings at the attachment point of the liner to the teat (Figure 4). Ayadi et al. (34) also reported that teat length increased after machine milking at high vacuum level (50 kPa) approximately 10.3 to 14.7% indicating that camels' teats tend to stretch after milking. This contrasts with observations in cattle, where fluid accumulation is typically concentrated at the teat tip (11, 28).

Although the milking liner affected significantly the teat's dimensions, no correlation was recorded between residual milk and teat measurements. Residual milk averaged at 0.57 ± 0.1 L and did not exceed 15% of total milk production. This suggests that the milking equipment and settings used in this experiment emptied efficiently and evenly the udder of camels with different teat dimensions and

shapes (2). In this study, only teat length was positively correlated with time to milk ejection and total milking duration. This implies that longer teats need more stimulation time, consequently, more time to finish the milking procedure. In a previous study, teat length was associated with the massaging efficiency of the liner, which could be not sufficient on the teat (2) since the animal was only under the stimulation of the milking liner. This suggests that long teats penetrate too deeply into the pulsation chamber, reducing their exposure to the massaging effect of pulsation. As reported by Gleeson et al. (22), liner design plays a crucial role in teat tissue response and milking efficiency, with improper liner fit leading to inadequate milking and potential teat tissue damage. Similarly, Kaskous and Pfaffl (35) emphasize the importance of matching liner design to teat morphology to prevent issues such as teat congestion and compromised blood flow. These observations indicate that the liner used in this study may not be optimally suited for the teat conformation of these camels, potentially affecting milk ejection efficiency.

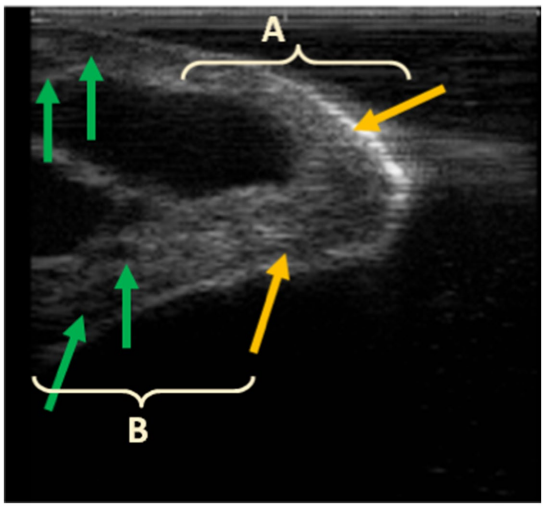

FIGURE 3
Post-milking ultrasound of the teat apex (A) and barrel (B); hyperechoic area at the tip of the teat (yellow arrows) and fluid accumulation in the teat barrel, seen as small hypoechoic areas in the teat wall (green arrows).

The teat plays a crucial role as a barrier between the animal's internal environment, which must be protected, and the external environment, where potential contamination can occur. It is continuously exposed to various stressors, including the mechanical effects of milking. Maintaining teat integrity is essential as it directly influences milk quality and contributes to reducing the risk of elevated somatic cell counts and subsequent mastitis development. The increase in thickness may affect the teat defense mechanisms, raising the likelihood of new intramammary infections (11). In the current study, we found an average somatic cell count (SCC) of 149.6 10^3 cells/mL, ranging between 37.5 10^3 cells/mL and 287.5 10^3 cells/mL. These values reflect a good mammary health status in the camels used throughout this study (36–38). However, a positive correlation between SCC, TET, and TCL was noted. Moreover, teat ring formation was positively associated with SCC ($r = 0.33$, $p < 0.01$). This implies the potential effects of machine milking on camel's udder health, particularly those with thicker teat ends.

The findings of this study indicate that in dromedary camels, the teat sphincter response to milking vacuum requires careful consideration. As most camels require high vacuum to open the teat sphincter, the need for pre-stimulation seems more crucial in this species. The observed teat morphology, including a potentially thicker teat-end wall and a more resistant sphincter, may influence the effectiveness of machine milking. This suggests the need to reassess the applied vacuum levels to optimize milking efficiency and ensure proper teat opening. Furthermore, the impact of overmilking should be investigated as prolonged exposure to vacuum can exacerbate teat tissue stress and increase the risk of teat-end congestion. Given the strong relationship between vacuum level, teat tissue characteristics, and residual milk, further research is necessary to establish

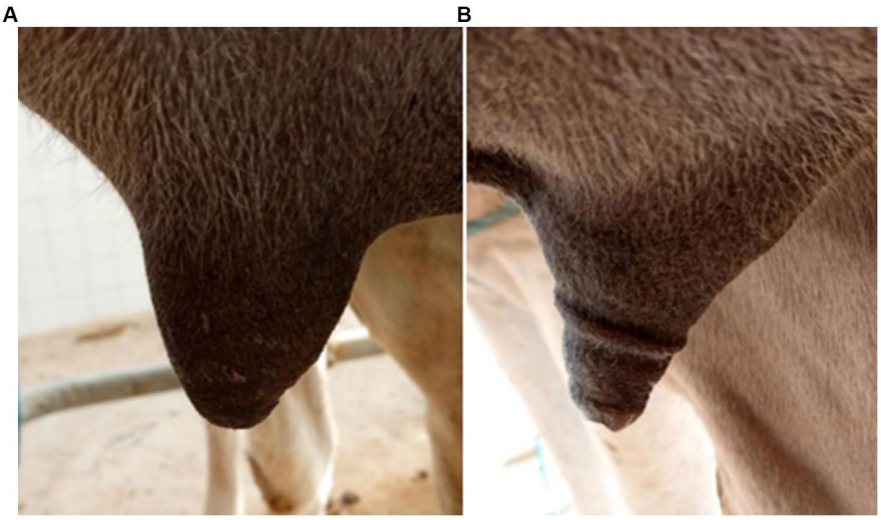

FIGURE 4
Visual appearance of camel teats before **(A)** and immediately after milking **(B)**.

species-specific milking parameters that improve milk removal efficiency while preserving udder health in camels.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Ethics Committee of Animal Experimentation at the National School of Veterinary Medicine, AEC/IACUC, ENMV-Sidi Thabet, Tunisia (CEEA-ENMV 81/24). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft. MB: Formal analysis, Investigation, Writing – original draft. MS: Formal analysis, Writing – review & editing. FA: Funding acquisition, Validation, Writing – review & editing. KD: Data curation, Formal analysis, Investigation, Writing – original draft. WS: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. MH: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. P-GM: Conceptualization, Methodology, Project administration, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

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Milk production profile of *Barela* camel (*Camelus dromedarius*) supplemented with postbiotics in a semi-intensive management system: pilot study

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This pilot study investigated the effects of postbiotics supplementation on both milk yield and composition within a semi-intensive management system in *Barela* camels. The key indicators included daily milk yield, fats, protein, solid not fats, and lactose levels. A total of 12 dairy camels from early to mid-lactation stages (second to fourth parity) were divided into four groups to obtain similar milk production among groups. Prior to the study, all camels were dewormed and confirmed for good health status. The first group served as the control and was permitted to graze for only 8 h per day without any supplementation. The second group received an additional 3 kg of concentrate feed alongside the same grazing schedule. The third and fourth groups were supplemented with 3 kg of concentrate feed plus 6 grams and 15 grams of extra pure metabolites (postbiotics - XPM), respectively, while maintaining the daily grazing duration of 8 h. The trial spanned 45 days, with an initial adaptation period of 15 days. Milk yield was recorded at four intervals: days 0, 16, 30, and 45. Milk composition analysis occurred on days 0 and 45 to establish baseline and final metrics. A complete randomized design was used, and one-way ANOVA was applied for statistical analysis at a significance level of 5%. The least significant difference test facilitated comparisons among treatment means. Results indicated significant differences in milk production across groups ($p < 0.005$), with the highest yield observed in the fourth group (8.93 $\pm$ 0.74 kg) compared to the control group (4.64 $\pm$ 0.32 kg) ($p = 0.0010$). In terms of milk composition, there was a notable effect on fat percentage among treatment groups, with the fourth group exhibiting the highest fat content (3.40 $\pm$ 0.05%) and the control group showing the lowest (2.82 $\pm$ 0.05%) ($p = 0.0450$). However, variations in protein, lactose, and solids-not-fat levels were not statistically significant. In short, postbiotics significantly enhance fat content in *Barela* dromedary camels, highlighting their potential as a valuable dairy breed within semi-intensive management systems. This will serve as a pilot study for the field of camel science, which could be used for further detailed studies about camel semi-intensive and intensive feeding management systems.

KEYWORDS

camel, dromedary, postbiotics, milk, semi-intensive

1 Introduction

More than 50 distinct breeds of camels, totaling 38.5 million (Food and Agriculture Organization Corporate Statistical Database) (1, 2) are found globally and approximately 27 breeds are present in Africa, Pakistan, and Saudi Arabia (2–4). The dromedary camels (*Camelus dromedarius*) hold significant importance in sustaining life (5), as they are adapted to thrive in harsh environments, and limited water availability (6), such as *Cholistan* and *Thal*. This adaptability allows them to utilize poor-quality forage, making them indispensable for pastoral communities. The climatic factors and demographical situations influence the environment, feed and water availability, and feed quality, hence directly affecting camels' milk (7) and meat production. Pakistan has more than 1.2 million camels (8), and the *Barela* breed is famous for its dairy potential (70). Generally, the milk yield in dromedaries varies between 3.5 and 20 liters, depending on geographical regions (9).

Camel milk is a primary product derived from camels, serving as a crucial dietary component in arid and semi-arid regions (10). The yield and composition of milk are influenced by various factors, such as breed, nutritional intake, physiological status, milking practices, milking frequency, calf suckling behavior, and feed and water availability (6, 11, 12). These variables collectively determine the quantity and quality of milk produced, making camel milk production a complex interplay of environmental, genetic, and management factors. The presence of immunoglobulins and other bioactive compounds in camel milk has great potential for health benefits, as anti-inflammatory (13, 14, 59, 61). However, the milk profile is closely linked to age, sex, and reproductive status. The milk composition of *Barela* typically contains higher fats and protein compared to cows, with average fat 4.26%, protein 3.62%, solid non-fat (9.02%), and total solids (13.28%) (15, 16). The camels' milk has lower lactose levels (4.84%), making it suitable for individuals with lactose intolerance (7). *Barela* camel milk exceeds the nutritional standards set by other dromedary breeds globally (11). *Barela* can produce an average of 6.0-liter milk/day, with a 586-day lactation period and a lactation curve peaking up to the fourth month (6). This pattern is consistent across different breeds and management systems, indicating that dromedaries can sustain high levels of milk production under optimal conditions. Seasonal variations also play a crucial role in influencing milk production throughout the year (17). Thus we need to adopt management strategies to consider seasonal dynamics and plan feeding regimens.

Despite the recognized potential for camel milk production, several challenges impede optimal productivity. Limited research on camel management in Pakistan has resulted in a lack of standardized protocols for feeding and milking (16). Furthermore, factors as drought can significantly impact forage availability, affecting the camels' health and productivity. Mustafa et al. (6) emphasized improved management practices for improvement in reproductive efficiency and productivity in dromedary camels, which can be achieved by regular health checks, vaccinations, breeding programs, and ensuring welfare.

Postbiotics were proposed in 2019 as "preparation of inanimate microorganisms and/or their components for improving animal's health" by the International Scientific Association of Probiotics and Prebiotics (18). Their supplementation (19) has emerged as a promising strategy to enhance both milk yield and composition in animals. They promote a healthy gut environment, improve the nutrient digestibility and absorption (20), modulate the gut microbiota and inhibit pathogens, possibly via quorum sensing (cell–cell communication) and adhesion (21). Hence, leading to increased dry matter intake and efficient functioning of the gastrointestinal tract. Camel α-lactalbumin utilizes bioactivities for reactive oxygen species scavenging and anti-inflammation for treating specific metabolic disorders (22). These benefits translate to higher milk production, with a significant increase in milk yield and milk composition (fat, protein, and lactose) (23). Postbiotics enhance the colostrum quality for fresh lactating dairy camels and reduce the presence of undesirable compounds such as urea and ammonia-N production. They also reduce the greenhouse gas production, thus utilizing energy to improve the animal's productivity.

Postbiotics have been proven to improve animal performance, but there are limited data on specific postbiotics for enhancing camels' milk yield. Certain probiotics in camel milk produce postbiotics (24). *Lactobacillus brevis* strains synthesize *gamma-aminobutyric acid* (GABA), which can regulate blood glucose and reduce blood lipid levels (25), influencing the gut–brain axis and systemic metabolic health (26). Furthermore, some probiotic bacteria in camel milk produce exo-polysaccharides (EPSs). Certain lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Weissella* species, are present in raw camel milk, and *Lactobacillus paracasei*, in fermented camel milk products (27), have antagonistic properties (28). Though these findings are promising, more studies are needed to determine whether postbiotics can directly influence milk yield in camels.

Camels are often raised in marginalized communities, and their welfare standards as good feeding, are often compromised, and animals with low body condition scores are fed only on pasture (62). Consequently, the hypothesis supports that supplementation with postbiotics would increase milk quantity and quality, with improvement in camel welfare. This study aimed to compare the effects of an integration of specially formulated concentrate feed (Dairylac camel feed) and postbiotics (XPM) on the milk quantity and quality in camels kept under a semi-intensive management system.

2 Materials and methods

2.1 Animal ethics

This study was approved by the Department of Livestock and Poultry Production, Bahauddin Zakariya University Animal Ethics Committee (Protocol number 279/15-10-2024).

2.2 Methodology

This study was conducted at the camel facility in Jalalpur Pirwala, located in Chak 81 M, south Punjab, adjacent to the *Cholistan*, recognized as a significant hub for camel milk production and export to Gulf countries and China. The trial involved 12 female dairy camels, selected based on their parity (second to fourth) and lactation stage (early to mid) during November to December 2024. The camels were divided into four groups, with three replicates in each, having the same average milk (20 liters/group) production. The camels were kept at an average ambient temperature (AAT) between 24.6°C and 8°C during the day and night, average relative humidity (ARH) of 26%, and average day length (ADL) of 10 h and 18 min during the year's shortest days, with sunrise at 6:52 a.m. and sunset at 5:26 p.m. (PKT). Thereby ensuring exposure to natural climatic variations that may affect milk production and quality.

All camels received treatment for endo and ecto-parasites before the trial to ensure their good health status. An adaptation period of 15 days preceded a 45-day trial where milk yield and composition were measured. The camels were provided with clean water bi-daily, commercial ration (Figure 1; Table 1) (29) and grazed for 8–10 h daily under consistent conditions. The feeding regimen varied by group: Group 1: Grazing only; Group 2: Grazing + 3 kg of concentrate; Group 3: Grazing + 3 kg of concentrate + 6 grams of XPM; Group 4: Grazing + 3 kg of concentrate + 15 grams of XPM (Procured from Bioaugment research laboratory, Faisalabad - Table 2).

2.3 Parameters

Milk yield was recorded at days 0, 16, 30, and 45; two times in the morning (6 a.m. and 8 a.m.) and evening (6 p.m.) (Figure 1). Milk composition was analyzed only at days 0 and 45 with Lactoscan equipment (Milkotronic Ltd., China). Daily AAT, ARH, ADL, and grazing patterns were documented, considering environmental factors affecting milk production. The concentrate feed for each group was measured and provided daily to guarantee precision in supplementing, and the XPM dosages were adjusted to align with experimental specifications, with rigorous compliance with feeding schedules. This analysis aimed to provide insights into cost-effective feeding strategies for camel milk producers targeting export markets. The parameters studied are reflected in Table 3, while Figure 1 shows different research activities during the trial.

2.4 Statistical analysis

The data collected were analyzed statistically on SPSS software, the design used was a completely randomized design, one-way ANOVA with repeated measures analysis with independent factors as (feeding, concentrate, and XPM supplementation), and dependent factors as (milk yield and milk composition) was applied. Least significant differences were used as a post-hoc test to compare the differences among the treatment means.

3 Results

3.1 Milk yield

The data of 12 dairy camels from early to mid-lactation stages (second to fourth parity) based on 04 groups for obtaining the same range of milk production in all groups have been mentioned in Tables 4, 5. Table 4 illustrates milk yield trends for four groups of camels during the 45-day trial period. Group 1 (control) exhibited steady reductions in milk yield, decreasing from 6.70 kg on the first day of the experiment to 4.64 kg on the last day. The average milk of the control group during this period decreased by a drastic 6.16 kg as the animals were shifted from extensive settings to control (semi-intensive settings). Conversely, Groups 2, 3, and 4 demonstrated incremental improvements in production, with Group 4 attaining its peak yield of 8.93 ± 0.74 kg on day 45. The G4 showed a 6.5 kg increase in milk yield on

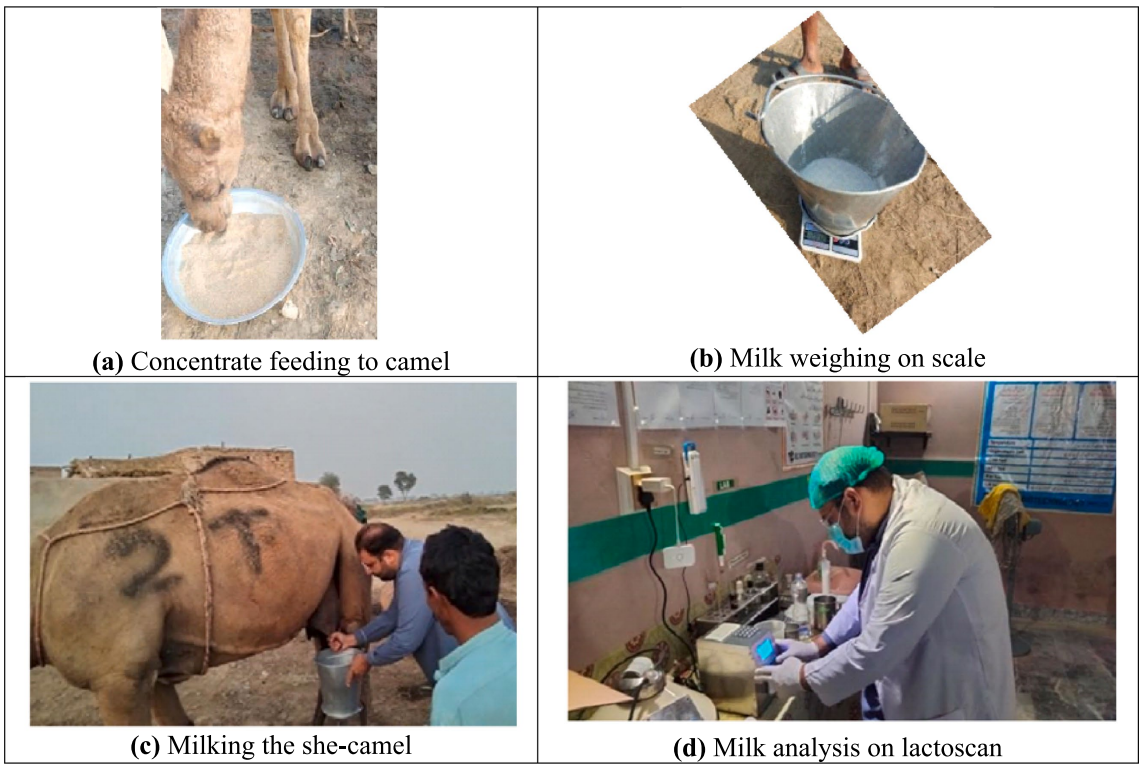

FIGURE 1
Different research activities during the experiment **(a)** concentrate feeding to the camel, **(b)** milk weighing on the scale, **(c)** milking of the she-camel, and **(d)** milk analysis on lactoscan.

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TABLE 1 Chemical composition of the commercial ration offered to experimental camels.

No.	Parameters	Percentage
1	Dry matter	88%
2	Crude protein	16%
3	Crude fat	5.5%
4	Crude fiber	6.5%
5	Ash	9%
6	ME	12 MJ/Kg

TABLE 2 Chemical composition of XPM offered to experimental camels.

S. No.	Parameters	Percentage
1	Crude protein	15–16%
2	Crude fat	1–2%
3	Crude fiber	22–25%
4	Ash	7–9%
5	Saccharomyces yeast and the media consisting of soyhulls, wheat bran, and cane molasses	

TABLE 3 Parameter procedures to be studied in the trial.

S. No.	Parameters	Procedure
1	Milk yield morning	Recorded in kg on the weighing scale at 2 time intervals
2	Milk yield evening	Recorded in kg on the weighing scale at one time
3	Milk fat %	Lactoscan by Milkotronic
4	Milk protein %	
5	Milk lactose %	
6	Milk solids not fats	

TABLE 4 Effects of different diets on camel groups' milk production at four different times.

Treatments	Day 0	Day 16	Day 30	Day 45
G1 (Control)	6.70 ± 0.32^{a}	5.17 ± 0.32^{c}	4.84 ± 0.32^{b}	4.64 ± 0.32^{b}
G2	6.79 ± 0.11^{a}	7.11 ± 0.11^{b}	7.74 ± 0.11^{a}	8.12 ± 0.11^{a}
G3	6.72 ± 0.33^{a}	7.29 ± 0.33^{ab}	7.97 ± 0.33^{a}	8.52 ± 0.33^{a}
G4	6.77 ± 0.74^{a}	7.85 ± 0.74^{a}	8.62 ± 0.74^{a}	8.93 ± 0.74^{a}
P-value	NS	0.0001	0.0002	0.0010

*All pairwise comparisons were performed at a 5% level of significance. Means that having different superscripts are significantly different; NS: non-significant.

average. The difference in the net change in the milk yield between groups across the trial period was statistically significant from days 16 to 45 (p-values < 0.05). However, no significant differences were observed on day 0 ($p = 0.9968$). The findings indicate that feed supplementation and feeding practices in Groups 2, 3, and 4 enhanced milk production relative to the control group. The results particularly highlight that dietary supplements positively influence milk yield in groups receiving additional feed and postbiotics.

3.2 Milk composition

Table 5 shows the milk composition (fat, protein, lactose, and solids-not-fat) for four feeding treatments (G1, G2, G3, and G4) in camels at days 0 and 45. Non-significant differences were observed between all groups in milk compositions, and they were the same.

Fat percentage showed divergent trends among the groups, with G1 and G2 showing a slight decrease at day 45 (2.90 ± 0.07 to 2.82 ± 0.05 and 3.04 ± 0.07 to 2.85 ± 0.05, respectively), but G3 and G4 with postbiotic supplementation showed an increase, with G4 attaining the highest fat percentage (3.40 ± 0.05) at day 45. Fat increased during the experiment, showing statistically significant differences. The protein content showed consistency across all groups, with slight variations, and the variations across groups were not statistically significant ($p = 0.3898$). G2 exhibited a minor increase (from 3.46 ± 0.04 to 3.54 ± 0.07), but G1 underwent a decrease from 3.41 ± 0.04 to 3.18 ± 0.07. The changes in fat content at day 45 were statistically significant ($p = 0.045$), suggesting an influence from diet or management practices. The amounts of lactose and SNF exhibited fluctuation between days 0 and 45. Group 1 demonstrated slight decreases in lactose (from 4.79 ± 0.03 to 4.74 ± 0.04) and solids-not-fat (SNF) (from 8.84 ± 0.05 to 8.61 ± 0.14), but Group 2 revealed increases in both metrics, with lactose escalating from 4.92 ± 0.03 to 5.12 ± 0.04 and SNF rising from 8.86 ± 0.05 to 9.27 ± 0.14. Notably, G3 and G4 demonstrated steady or marginally variable values for these parameters, with G4 presenting the highest day 0 lactose level (5.02 ± 0.03), which decreased by day 45 (4.83 ± 0.04). The variations in lactose and SNF levels were not statistically significant, since the p-values exceeded 0.05. The data indicate changes in milk composition impacted by feeding regimes, with G4 exhibiting greater fat percentage at the trial's conclusion, while G2 showed an increase in protein and lactose levels. These findings underscore the possibility of targeted dietary measures to improve milk quality components.

4 Discussion

In the desert area with less vegetation, camel feeding has greatly shifted toward reliance on supplementary feeding for meeting the animal's nutritional requirements. While grazing ecosystems are the primary source of sustenance for camels, hence grazing alone is inadequate to fulfill the complete nutrient demands, particularly the mineral needs, of lactating and pregnant camels (30). This highlights the critical role of supplemental feeding in fulfilling nutritional deficiencies, ensuring optimal health, and enhancing milk production in semi-intensive camel management. Semi-intensive systems strike a balance between natural grazing and supplementary feeding, enhancing nutritional balance, productivity, and cost efficiency. All this is dependent on the providence of supplementation to animals and good managerial practices.

In the current scenario of a semi-intensive system, camel feeding becomes progressively dependent on supplements for providing the required nutrients. The wide variation in the constituents of camel milk yield and composition can be attributed to factors such as parity, season, physiological state, geography, and feed (7, 63). However, Abdelgadir et al. (31) showed the non-significant effect of parity on milk constituents. Thus, the inclusion of concentrate and postbiotics likely enhanced the nutritional intake of the camels, leading to

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TABLE 5 Milk composition (%) of *Barela* camel (*Camelus dromedarius*) with concentrate and postbiotics.

Treatments	Fat		Protein		Lactose		SNF	
	Day 0	Day 45	Day 0	Day 45	Day 0	Day 45	Day 0	Day 45
G1	2.90 ± 0.07	2.82 ± 0.05^{b}	3.41 ± 0.04	3.18 ± 0.07	4.79 ± 0.03	4.74 ± 0.04	8.84 ± 0.05	8.61 ± 0.14
G2	3.04 ± 0.07	2.85 ± 0.05^{b}	3.46 ± 0.04	3.54 ± 0.07	4.92 ± 0.03	5.12 ± 0.04	8.86 ± 0.05	9.27 ± 0.14
G3	3.09 ± 0.07	3.18 ± 0.05^{ab}	3.22 ± 0.04	3.34 ± 0.07	4.89 ± 0.03	4.92 ± 0.04	8.89 ± 0.05	8.97 ± 0.14
G4	2.80 ± 0.07	3.40 ± 0.05^{a}	3.48 ± 0.04	3.39 ± 0.07	5.02 ± 0.03	4.83 ± 0.04	9.22 ± 0.05	8.33 ± 0.14
P-value	NS	0.0450	NS	NS	NS	NS	NS	NS

*All pairwise comparisons were performed at a 5% level of significance. Means having different superscripts are significantly different; NS: non-significant.

improved energy availability for milk production. Camels generally cope with feed scarcity, lactation, and pregnancy by deposition and mobilization as a physiological strategy. Normally, the she-camels utilize their body fats after calving for milk production due to the low feed intake. The camels' body fat reserves improve in 2–3 months beyond the peak lactation (32).

Regarding seasonal variation, Musaad et al. (67) reported the lowest fat content in July (2.29%), which is lower than our results (2.90%). Similar observations were reported by Igbal (33), which may be due to the dilution effect linked to the production increase (34). Furthermore, the season is a great factor in their variation. While their protein content was lower (2.76%) during October, while our results showed 3.34%. The lactose content in our study was higher (4.90%) in October than in Musaad et al. (67), which was 3.83%. With the advancement of the experiment toward winter, the protein% did not change, but the lactose % gradually decreased.

In our study, the camel parities were second to fourth and were in mid-stage lactation. Normally, the body condition score (BCS) of animals decreases with the parities if the nutritional requirements of the animals are not met properly and as per standards. Hence, it will decrease the camel milk yield and milk composition. Although research has shown that lower BCS of animals at calving did not affect the milk yield, it can lower the milk fat content. Camels that consumed concentrate supplementation may have good body reserves and health status (35). The stage of lactation significantly impacts the protein content in camel milk and is lower at the beginning of lactation (36) but increases significantly during the first 4 months of lactation. The non-significant variation in the milk composition could be due to parity and camels' body condition scoring. The postbiotics act as antimicrobials and modulate the microbiota and immunity to help the animals toward various metabolic and physiological functions (19). Concentrates and nutrients in XPM provide extra energy to camels by boosting their digestive fermentation, hence producing the building blocks for milk fats in the form of fatty acids. That is why the milk fat content is more flexible and responds quickly to extra energy from supplementation. Along with this, second and third-parity camels usually have a more developed digestive tract for enhanced metabolism, thus can use these supplements more efficiently toward enhancing milk fats. Thus, the provision of concentrate and postbiotics supports higher milk fat without changing the milk protein levels, giving an insight into how camels' bodies manage milk production in a balanced way. The stability of milk is due to the protein, which supports the animal's protein needs and strengthens immunity.

Camel milk yield and composition are influenced by a complex interplay of interrelated factors such as parity, management, and lactation stage (37). Our research showed a range of milk yield from 4.84 to 8.93 liters/ day, which is similar to that reported by Faraz et al. (15) that dromedaries can yield approximately 7.38 liters of milk/day under traditional management conditions. Research indicates that adequate nutrition maximizes lactation performance in camels, and supplementing postbiotics may improve the animals' health, average daily gain, final weight, and milk production (64). Studies have shown that probiotics and their metabolites can positively influence the milk yield by enhancing.

Postbiotics enhance nutrient digestibility and utilization, particularly during periods of high nutritional demand, thereby supporting the critical nutrients needed for production, growth, and absorption. This improved nutrient uptake optimizes feed transformation efficiency in animals (38). Postbiotics also promote gut health and digestive efficiency (39), which further leads to better nutrient utilization. Moreover, postbiotics modulate the intestinal microbiota, fostering the growth of beneficial bacteria such as Lactobacillus and *Bifidobacterium*, while reducing populations of harmful bacteria such as *E. coli* and *Clostridium* spp. (40).

The statistically significant differences in milk yield ($p < 0.05$ from days 16–45) underscore the critical role of strategic dietary supplementation in camel milk production. Our results parallel investigations by Iqbal et al. (65), which demonstrated that precise nutritional management can substantially modify milk production parameters in dromedary camels. Studies have shown that milk yield generally increases from the first to fifth parities, with the highest yields typically observed in the third to fifth parities (41). The remarkable 6.5 liters increase in milk yield in Group 4, with the highest level of XPM supplementation, suggests a potential synergistic effect between concentrate feed and postbiotic interventions. Faraz et al. (16) reported a daily milk yield of 6 liters in first parity camels, increasing to 8.8 liters in the second parity. These results are quite important for sustainable camel farming practices, especially in regions with challenging environmental conditions (68). While Raziq et al. (42) and Ahmad et al. (37) reported the mean daily milk yield in camels to be 8.17 liters in Pakistan. However, as per a study in Sudan, the average daily yield is 4.4 liters/day (43). Comparative analyses of the existing literature reveal the complexity of nutritional interventions in camel milk production. Our study shows clear improvements in milk yield through supplementation. The differential responses observed in Groups 2, 3, and 4 suggest that optimal supplementation strategies may vary depending on the camels' specific nutritional requirements and environment.

Our studies showed a range of 2.8 to 3.4% fat percentage after supplementing with postbiotics, which are similar to the fat percentage (38.2 ± 10.8 g/L) in whole camel milk; as reported by Konuspayeva et al. (7) and Mati et al. (44). Furthermore, the milk fats remained in

both groups at the trial start and day 45. The change in milk fats within all groups remained statistically non-significant. While the milk fat % of 2.5–2.8% has been reported in the Kohi white dromedary camel by Raziq et al. (42), which is lower than that found in the current study. The fat content in camels' milk can vary between 1.2 and 4.5% (45). However, Park and Haenlein (46) reported that the fat percentage may reach up to 6.4% with the presence of unsaturated and long-chain fatty acids, which helps in lowering the lipid levels in human serum. The same results have been shown by Iqbal et al. (33), with a fat percentage of 3.47–3.68% under Pakistani management conditions. The parity can also affect the fat content of camels. Research showed an increase or no statistically significant differences in fat content with parities, while others showed a decrease in fat content from 5.25% in second parity to 4.69% in third parity in camels (47). Proper management practices during the trial may have reduced stress levels among the camels, contributing to higher productivity. The initial adaptation period allowed the camels to adjust to their feeding regimens, which could explain the significant increases in milk yield observed after this phase.

The results showed a non-significant improvement in milk composition during 45 days' trial. Results of milk components showed the same range in both groups at days 0 and 45, and could be due to various interrelated factors. One of the main reasons could be that camels were in different parities, and the experimental period is one of the feed scarcities. Another reason could be the non-availability of commercial or balanced rations in remote areas or no fodder or forage availability during winter seasons in desert areas. The feed scarcity due to extreme temperatures can cause animals to be deprived of their basic feed requirements. Hence, the animal's whole maintenance and productivity are disturbed, and upon supplementation, the animal's preference is to fulfill their basic maintenance requirements. The supplementation of concentrates provides readily fermentable carbohydrates for enhancing the gut microbial activity and production of volatile fatty acids as acetate and butyrate, the precursors for milk fat synthesis. While the XPM postbiotics improve the gut health and fermentation efficiency, improving the nutrient absorption and energy availability in the camels' stomach. However, further studies are suggested on a larger number of camels for a longer period of time for validation of these changes.

As per our results, the milk fat as 3.20%, protein 3.43%, lactose 4.93%, and SNF as 8.77%. While Faraz et al. (48) also reported milk fat, protein, lactose, and total solids percentages of 3.88–4.70%, 2.66–4.02%, 3.67–5.04%, and 12.22–14.65% in the Barela camel milk in desert conditions (16). The inclusion of concentrates and postbiotics likely provided essential nutrients that improved milk quality parameters such as fat and protein content (16). This aligns with findings that adequate nutrition is crucial for maximizing lactation performance in camels. The statistically significant changes in fat content ($p = 0.045$) suggest that strategic nutritional interventions can effectively modulate milk biochemical characteristics, potentially offering opportunities for targeted milk production optimization (42). Furthermore, the total solids and SNF may increase with the advancement of lactation, thus fluctuating the milk density throughout the lactation period (47). Additionally, environmental variables, breeds, and analytical procedures contribute to the variability in camel milk constituents as well (49, 50).

As per Park and Haenlein (46) the total protein of camels' milk varies from 2.15 to 4.90%, while in our research it lies in the same range of 3.18 to 3.54%. The percent of camels' milk casein to whey protein ratio is relatively lower in camels' milk than in cows, thus affecting a softer coagulum. Cossins (51) also reported that camels can produce 2.7 times the protein at only 1.3 times the dry matter as required by cattle. The presence of beneficial compounds from postbiotics can influence metabolic pathways associated with milk synthesis. The adaptation phase allowed camels to adjust to new feeding regimens, potentially enhancing their ability to utilize nutrients effectively for milk production (33). Research have shown that protein content increases with parity (47). A study in Sudan showed a reduction in protein content in camel milk from traditional pasture (43). Another study showed a lower protein percentage in second parity camels (3.60%) than in third parity (3.46%) (43).

The lactose percentage in our study was observed to be in the range of 4.74–5.12%, which is similar to the values reported by 5.15% (37). As per Devendra et al. (45), lactose content is stable and ranges from 3.5 to 4.5%. Mustafa et al. (47) reported that lactose varies with season and decreases with increasing parity. Lactose is the main carbohydrate promoting the formation of *a Bifidobacterium* environment for the development of the nervous system (46). Postbiotics may enhance gut health and nutrient absorption, leading to improved milk composition (52). Variations in environmental conditions can also affect milk composition; however, controlled management practices during the trial helped mitigate these effects.

This probiotic approach demonstrates potential for improving the nutrient digestibility of crude protein and total digestible nutrients, promoting gut health, and boosting the resistance to infections in camels (53). This enhancement resulted in elevated dry matter intake and voluntary water consumption, facilitating improved nutrition absorption and hydration, particularly during extended working circumstances (52). Postbiotics are characterized as non-viable microbial metabolites or metabolic byproducts that provide health advantages to the host. They are essential for boosting gut health and optimizing metabolic activities, hence significantly affecting growth performance through improved nutrient absorption and metabolic health indices (69). This study emphasizes the potential of utilizing lactic acid bacteria from camels as probiotics, which may enhance health by supporting the camels' microbiome (54).

The incorporation of postbiotics into the dietary regimen of dromedary camels confers numerous health advantages. Increased gut microbial diversity correlates with enhanced digestion and nutrient absorption, essential for sustaining good health in semi-intensive feeding regimes. Postbiotic supplementation may also influence immunological responses, potentially decreasing the prevalence of illnesses typically seen in intensively managed herds (55). Moreover, research has demonstrated that postbiotics can mitigate oxidative stress and enhance metabolic profiles in animals subjected to intense feeding protocols (66). The composition of camel milk, specifically its protein, fat, and total solid content, seems to be influenced by the use of postbiotics and rigorous feeding methods. However, more studies are needed for confirmation of changes in milk composition and yield. Research indicates that augmenting camels' diets with supplementary protein can result in elevated amounts of both milk fat and protein. This may enhance the nutritional quality of camel milk for human consumption, which is advantageous for places that depend significantly on it (56).

The comparative analyses with existing literature underscore the complexity of camel milk composition modifications and variations depending on production systems, locations (60), availability of water

and differences in feed types. While our study showed relatively stable protein content across all treatments, and relatively improved with parities. Mati et al. (44) reported that protein can vary more under different nutritional interventions. The fat percentage in camels' milk normally increases beyond peak lactation by 2–3 months, and by supplementation. The observed increases in lactose and solids-not-fat (SNF) in Group G2, coupled with the marginal changes in other groups, indicate that specific nutritional combinations can differentially impact milk composition. These findings contribute to the growing body of research demonstrating the potential for precision nutrition in camel dairy systems, offering insights that could be valuable for producers targeting specialized markets, particularly in regions such as the Gulf and China (57, 58). As this is a short study, more treatment groups, an increased number of animals, a longer experimental period, and various seasons can confirm these preliminary results and also reveal more interesting outputs.

This is a pilot study, and before testing something new on many animals it is important to do a pilot. Hence, the study was limited by the small number of animals, in different parities, and the fact that the animals were grazing in a desert area. Notwithstanding the limitations, it contributes valuable knowledge for enhancing sustainable camel farming practices while also addressing the growing global demand for camel milk. Thus, further studies are needed as there are some limitations as a low number of animals available for study, confinement to a particular pasture because of the difficulty of performing a field study in camels, the study's duration, and to check different weather effects. Notwithstanding these limitations, this pilot study has increased the knowledge of enhancing camel milk production and welfare. Future research should focus on comprehensive economic assessments to clarify the financial implications and investigate the long-term effects of these nutritional strategies on camel productivity and welfare perspective that is very important. Expanding research to include various breeds, larger sample sizes, and diverse environmental conditions could provide deeper insights into improvement in camel milk yield and quality.

5 Conclusion

This pilot study tested feed integration of grazing systems in dairy camels and revealed that dietary supplementation, particularly with higher levels of XPM, led to a significant increase in milk yield, with one group improving milk production up to a 6.5-liter increase. While milk fat content ranged from 2.8 to 3.4% after supplementation and was improved significantly by postbiotics and supplementation, milk protein remained the same. However, lactose and solids-not-fat contents did show increases in specific groups. These improvements are attributed to postbiotics enhancing gut health, nutrient absorption, and metabolic activities in camels. Our findings suggest that supplementation with postbiotics can improve milk production and quality, promoting the animal's wellbeing. Further studies are needed to confirm our preliminary results.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Bahauddin Zakariya University Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AF: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. HB: Data curation, Investigation, Methodology, Project administration, Writing – original draft. AW: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. SH: Writing – original draft, Writing – review & editing. SR: Conceptualization, Methodology, Project administration, Writing – review & editing. MB: Conceptualization, Methodology, Project administration, Writing – review & editing. BP: Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that they (Asim Faraz and Barbara Padalino) were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision. Furthermore, the Dairylac Pvt. Ltd. provided concentrate for the camels and Bio-Augment Research Laboratories provided XPM for testing on camels, the author(s) (Muhammad Mukarram Bashir and Sajjad ur Rahman) are working with these companies and by anyway there is no impact of this.

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Characterization of the vitrification parameters for oviduct aggregates in *Camelus dromedarius*

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Introduction: Camel oviductal isthmus aggregates provide a novel and promising model for studying sperm attachment and longevity, offering a potential alternative for short-to mid-term sperm preservation and transport under non-cryogenic conditions. Effective cryopreservation of the aggregates for later use can contribute to addressing challenges associated with camel semen preservation by potentially extending sperm lifespan and facilitating semen transport to remote areas without cryogenic facilities. Challenges in preserving the structural integrity and viability of camel oviductal aggregates remain a key critical gap during cryopreservation. This study evaluated the efficiency of vitrification protocols for camel oviductal isthmus aggregates, focusing on the effects of aggregate size, cryoprotectants (CPA), cryodevices, post-thaw viability, and sperm-binding capacity.

Methods: Aggregates retrieved from the oviductal isthmus were classified into four size groups (50, 100, 150, and 200 μm) and vitrified to determine the influence of size on post-thaw outcomes. CPA concentrations (3, 5, and 7 M) of DMSO and EG in a 1:1 ratio were tested for their impact on structural integrity and viability. The performance of cryodevices, including cryovials, 0.5 mL straws, and 0.25 mL straws, was also assessed.

Results and discussion: The results indicated that aggregates sized 150 μm and 200 μm demonstrated superior post-thaw viability, with intactness rates of 78 ± 2.0% and 83 ± 2.8%, respectively. Among the tested CPA concentrations, 7 M showed the highest post-vitrification viability (69 ± 1.9%). Additionally, 0.25 mL straw cryodevice achieved significantly better post-thaw viability (67 ± 2.7%) compared to 0.5 mL straws (32 ± 2.1%) and cryovials (10 ± 1.1%). Regarding sperm-binding capacity post-thaw, aggregates treated with 5 M (69 sperm) and 7 M (74) CPAs showed the highest binding rates, with no significant difference between these concentrations. Further studies are required to optimize vitrification protocols to enhance the aggregate's post-vitrification viability and structural integrity.

KEYWORDS

isthmus, cryoprotectant, cryodevice, camel, reproductive biotechnology

1 Introduction

The dromedary camel (*Camelus dromedarius*) holds significant cultural, economic, and recreational value in the Middle East, playing vital roles in milk and meat production, racing, and show competitions (1). Reproductive performance in this species remains a significant challenge, with fertility issues affecting both males and females. Low conception rates and male infertility are widespread concerns, limiting overall reproductive efficiency (2). One of the major limitations in advancing camel reproductive management is the incomplete understanding of the physiological mechanisms underlying fertility, particularly in the context of gamete interaction and embryo development (3). These challenges are further complicated by the difficulty of preserving camel semen, which is an essential component of assisted reproductive technologies. Maintaining semen viability and fertilizing capacity during extended storage remains a major obstacle, hindering the broader implementation of artificial insemination programs (4). Given these reproductive challenges, understanding the microenvironment where fertilization occurs is essential.

The oviduct, recognized as the site of fertilization, plays a critical role in ensuring reproductive success across various animal species. The oviduct exhibits a remarkable capacity to support sperm survival, particularly within the isthmus, or "sperm nest." Sperm can be stored within the oviduct of swine and cattle for approximately 24 to 48 h, while in other vertebrate and invertebrate species, sperm storage may extend to several days or even months (5).

Oviductal cell aggregates have been adopted as an *in vitro* model to closely mimic the oviduct's physiological environment and allow investigation of sperm behavior under conditions that resemble those *in vivo* (6). These aggregates are highly comparable to the *in vivo* oviduct in their three-dimensional architecture and microenvironmental features, including cell–cell interactions, secretory activity, and spatial organization. Such characteristics make them an ideal platform for exploring sperm attachment, motility, and survival (7). Supporting this approach, Dutta et al. (8) demonstrated that co-incubation of bovine sperm with bovine oviductal cell aggregates *in vitro* significantly extended sperm lifespan, emphasizing the potential of this model to preserve sperm viability.

However, despite these advances, a critical knowledge gap persists regarding the effective cryopreservation of camel oviductal isthmus aggregates, particularly how to preserve their structural integrity and biological functionality post-thaw to maintain their utility as a model for sperm preservation and fertility studies. To address this gap, the preservation of these cell aggregates has become an essential focus in cellular biology. Techniques adapted from stem cell and oncology research, such as the freezing of spheroids, have been employed to ensure the availability and readiness of these models for experimentation (9). By maintaining their three-dimensional structure and biological functionality post-preservation, these aggregates serve as a versatile and reliable tool for studying cellular behavior and advancing reproductive biology research.

In this context, the application and refinement of cryopreservation methodologies are essential to achieving successful preservation outcomes, particularly in the field of reproductive biotechnology. Cryopreservation is a cornerstone technique in reproductive and cellular biology, enabling the long-term storage and availability of biological materials such as cells, tissues, and aggregates for research and clinical applications (10). Various methods, including conventional freezing, programmable slow freezing, and vitrification, have been developed to preserve cellular integrity and functionality. Vitrification has emerged as a superior technique due to its ability to achieve higher cell survival rates (11). Among cryopreservation methods, vitrification has gained prominence due to its rapid cooling and improved cell survival. Given its advantages, vitrification represents a particularly promising approach for the cryopreservation of camel oviductal aggregates, providing a methodological basis for the current investigation on the success of cryopreservation.

Vitrification is an ultra-rapid cooling method that prevents ice crystal formation by solidifying cells into a glass-like state through direct exposure to liquid nitrogen and high concentrations of cryoprotectants (12). Unlike slow freezing, vitrification maintains cellular integrity by avoiding intracellular ice, which is particularly damaging to the delicate structure of mammalian oocytes (13). Its effectiveness is linked to the ability of high CPA concentrations to induce osmotic dehydration, minimizing cryo-injury during cooling and warming. Moreover, vitrification has become the method of choice in many species due to improved post-warming morphology and developmental potential.

The success of vitrification, however, depends on several critical factors that must be carefully optimized. These factors include the type, concentration, and permeability of the cryoprotectant agent (CPA) used, such as dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, and propylene glycol, as well as the type of cryodevice (cryovials, straws, and cryotop) employed during the process (14). In addition to these parameters, the size and structural integrity of the aggregates are critical factors influencing cryosurvival, with careful handling and gentle pipetting significantly enhancing post-thaw recovery (15). Furthermore, the efficiency of a freezing protocol can be assessed through various methods, such as evaluating cell viability, membrane integrity, and mitochondrial function. Yet, beyond these immediate assessments, the ultimate measure of success lies in the ability of the frozen cells to resume their normal physiological capabilities after thawing, with minimal damage to their structure and function (16). These factors collectively determine the preservation of structural integrity and viability post-thaw.

Therefore, this study aimed to assess the efficacy of a vitrification protocol for camel oviductal isthmus aggregates by optimizing cryoprotectant concentration, evaluating the suitability of cryodevices, and determining the impact of aggregate size on post-thaw outcomes such as viability, structural integrity, and sperm-binding capacity, thereby supporting future research on sperm-oviduct interactions in camels.

2 Materials and methods

2.1 Ethical considerations

This study was conducted in accordance with ethical guidelines and approved by the Research Ethics and Integrity Committee at the Higher Colleges of Technology, under Approval Number REIC2024-FAC35. All experimental procedures adhered to institutional and international standards for the ethical use of biological materials in research and animal welfare.

2.2 Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

2.3 Animals and sample collection

The study was conducted from September to November 2024. The samples for this study were collected from a local slaughterhouse in the Al Ain region, Abu Dhabi. Testes and their attached epididymides were obtained from 23 mature dromedary camels, aged between 5 and 10 years, at a local slaughterhouse. A total of 46 testes were collected over eight separate collections. Only testes that appeared normal in size, color, and consistency, with no visible lesions or abnormalities, were included in the study. Immediately following slaughter, the samples were placed in phosphate-buffered saline containing antibiotics (penicillin and streptomycin) and transported to the laboratory in a temperature-controlled container maintained at 4°C within 1 to 2 h.

2.4 Experimental design

This study was composed of five sequential experiments designed to systematically optimize key parameters influencing the cryopreservation of camel oviduct explant aggregates. The effects of aggregate size, cryoprotectant concentration, and cryodevice type on post-thaw viability, structural integrity, and reproductive functionality were evaluated. Each experiment built upon the findings of the previous one to enable stepwise refinement of vitrification conditions. Experiment 1 evaluated the impact of aggregate size on viability; Experiments 2 and 3 assessed the effects of cryoprotectant concentration on structural integrity and viability, respectively; Experiment 4 compared different cryodevices; and Experiment 5 examined the sperm-binding ability of thawed aggregates.

2.4.1 Experiment 1: effect of aggregate size on post-thaw viability

This experiment aimed to identify the most suitable aggregate size for successful cryopreservation, hypothesizing that aggregate size influences cryosurvival due to factors such as cryoprotectant penetration and ice formation. Aggregates were categorized into four size groups: 50 µm, 100 µm, 150 µm, and 200 µm in diameter. Vitrification was performed using 0.25 mL straws. Post-thaw viability was assessed using SYBR14/PI dual staining, allowing differentiation between live and dead cells.

2.4.2 Experiment 2: effect of CPA concentration on structural integrity

This experiment was conducted after determining the optimal aggregate size, focusing on optimizing CPA concentration to minimize cryo-injury and preserve the three-dimensional structure critical for aggregate function. The objective of this experiment was to evaluate the impact of varying CPA concentrations on the structural preservation of vitrified aggregates. Aggregates were exposed to three CPA concentrations—3 M, 5 M, and 7 M—consisting of dimethyl sulfoxide (DMSO) and ethylene glycol (EG) in a 1:1 ratio. Post-thaw structural integrity was analyzed to identify the concentration that best maintained aggregate morphology and cohesion.

2.4.3 Experiment 3: effect of CPA concentration on post-thaw viability

This experiment aimed to establish the relationship between CPA concentration and cellular survival, determining the most suitable concentration for optimal viability. By evaluating viability alongside structural integrity, this step refined the CPA concentration choice to balance toxicity and cryoprotection. This experiment investigated the influence of CPA concentration (3 M, 5 M, and 7 M) on aggregate viability post-thaw. SYBR14/PI dual staining was performed to assess live and dead cell populations.

2.4.4 Experiment 4: influence of cryodevice type on post-thaw viability

The fourth experiment was designed to compare the performance of different cryodevices in maintaining post-thaw aggregate viability. Three cryodevice types—cryovials, 0.5 mL straws, and 0.25 mL straws—were tested under identical vitrification and warming conditions. Post-thaw viability was evaluated to determine which cryodevice offered the most effective cryopreservation outcomes.

2.4.5 Experiment 5: effect of CPA concentration on post-thaw sperm-binding ability

Based on the vitrification parameters identified in the previous experiments (the aggregate size, the CPA concentration, and the cryodevice), this experiment aimed to evaluate the impact of the CPA concentration on the capacity of oviduct aggregates to bind to sperm after thawing. Thawed aggregates were incubated with epididymal sperm, and the binding ability was assessed by quantifying the number of sperm bound to the aggregates. This experiment provided insights into how varying CPA concentrations influence the sperm-binding competence of vitrified aggregates post-thaw.

2.5 Retrieval of the epithelial cells from the oviduct

Oviducts were collected from apparently healthy pubertal female camels aged 4–8 years old ($n = 10$) slaughtered at a local abattoir and transported on ice to the laboratory at Higher Colleges of Technology, Abu Dhabi. The oviduct epithelial cells were collected and prepared at the laboratory according to El-Sokary et al. (7). Isolation of oviductal epithelial sheets from the isthmus involved gently squeezing them out with a glass slide angled at 45 degrees. The collected sheets were then transferred to a 15 mL conical tube and pelleted via centrifugation at 100 × g for 30 s. The supernatant was decanted, and the pellet was resuspended in 1 mL of capacitating TALP medium. Disaggregation was initiated by aspirating and expelling the suspension 10 times through a 1 mL pipette tip. The partially separated cells were then washed with 5 mL of fresh TALP and re-centrifuged at 100 × g for 1 min. After discarding the supernatant, the cells were re-suspended in an additional 1 mL of TALP. Complete disaggregation was achieved by passing the suspension through a 23-gauge needle 10 times. The total volume was then brought to 9 mL with TALP and equally aliquoted

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(3 mL each) into three 100-mm petri dishes and incubated at 39°C for 90 min to allow cells to re-aggregate.

2.6 Aggregate selection and size measurement

After retrieval of oviduct tissue, aggregates were produced as described in the previous section. The resulting aggregates were washed three times by gentle pipetting in pre-warmed TALP medium to ensure their structural stability and viability during handling. Aggregates were then visually inspected under a stereo microscope (magnification: ×40–100) to identify and select those with clear, intact morphology (17). Aggregates were categorized into four size groups: 50 µm, 100 µm, 150 µm, and 200 µm in diameter (Figure 1B). To ensure accurate size measurement, aggregates were imaged using a digital microscope with calibrated imaging software (ImageJ). Diameters were measured across the widest part of the aggregates, and only those within the defined size ranges were included in the experiments. A total of 30 aggregates per size group were selected for observation in each experimental replicate.

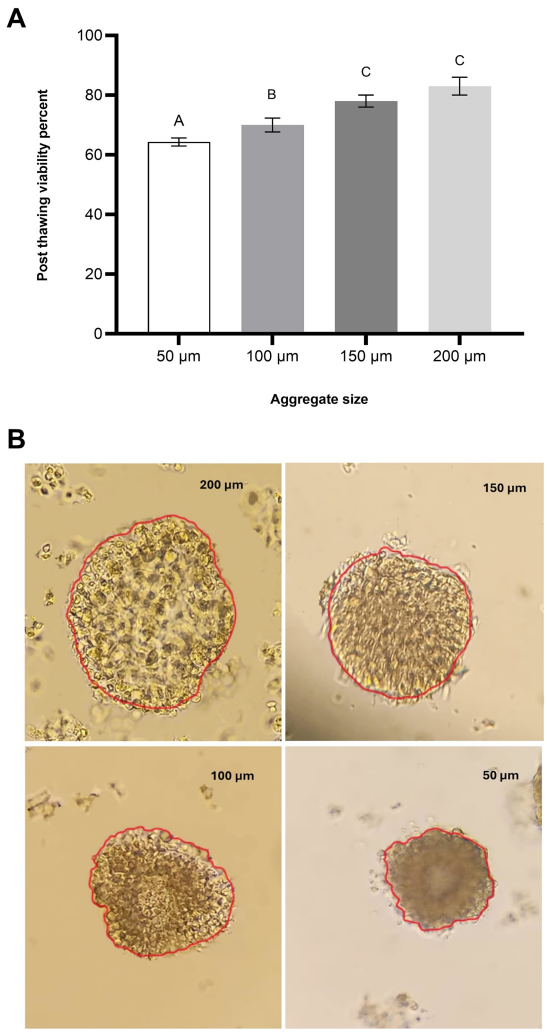

FIGURE 1
Effect of aggregate size on post-thaw viability of oviduct aggregates. **(A)** Column graph illustrating the post-thaw viability (%) of oviduct aggregates of varying sizes: 50 µm, 100 µm, 150 µm, and 200 µm. Columns with different letters denote statistically significant differences among the groups ($p < 0.05$). **(B)** Representative microscopic images of oviduct aggregates of different diameters (50 µm, 100 µm, 150 µm, and 200 µm). Scale bar = 50 µm.

2.7 Vitrification and warming by straw and cryovial methods

Oviduct aggregates were exposed to two-step cryoprotectant treatments at varying concentrations as illustrated in Table 1. The holding medium used during vitrification contained TCM 199 supplemented with 2.5 mM HEPES and 20% fetal calf serum. For cryovial vitrification, oviduct aggregates ($n = 5–10$) were transferred into 1.5 mL cryovials (Nunc, Rochester, MN, United States) and directly plunged into liquid nitrogen (LN2) (11). In the case of straw vitrification, the aggregates were immediately loaded into 0.25 mL or 0.5 mL straws, placed in the middle column of VS2, and separated by air bubbles. The straws were sealed with straw plugs and pre-cooled by exposure to liquid nitrogen (LN2) vapor at approximately 4 cm above the LN2 surface for at least 60 s before being vertically immersed in LN2 for storage for 2 weeks. Straws were transferred rapidly in less than 5 s through the air to avoid structural damage (18) and then placed in a water bath at 35–37°C for 20 s for warming. Following warming, the expelled oviduct aggregates were equilibrated for 5 min in a 0.5 M galactose solution (19) in TCM 199 to dilute and remove cryoprotectants. Oviduct aggregates were then washed 4–5 times in a fresh washing medium and cultured in a warm TCM medium for 2 h.

2.8 Assessment of the structural integrity of aggregates following vitrification

The structural integrity of the aggregates was evaluated post-vitrification and warming using phase-contrast microscopy (×100–200 magnification). Aggregates were assessed for compactness, shape, surface characteristics, and overall cohesion to determine their quality (20). The evaluation of the aggregates is categorized as shown in Table 2.

2.9 Evaluation of viability in vitrified oviduct aggregates

To evaluate aggregate viability, SYBR-14 (1 µM) was added to the aggregate suspension and incubated for 10 min in a dark room to allow sufficient staining of the viable cells. Following this incubation, propidium iodide (PI, 1 µM) was introduced to the suspension and incubated for an additional 5 min. PI is a vital stain that can only penetrate the membranes of non-viable aggregates and emits a red fluorescence. In contrast, SYBR-14 stains the DNA of viable aggregates, producing a green fluorescence (21). The stained aggregates were then examined under a fluorescence microscope to visually differentiate between viable and non-viable aggregates based on the distinct green and red fluorescence patterns (Figures 2C,I–IV).

TABLE 1 Two-step cryoprotectant treatments applied to oviduct aggregates at varying concentrations.

CPA concentration	Step 1 (VS1)	Exposure time	Step 2 (VS2)	Exposure time
3 M	0.75 M EG + 0.75 M DMSO	45 s	1.5 M EG + 1.5 M DMSO	25 s
5 M	1.25 M EG + 1.25 M DMSO	45 s	2.5 M EG + 2.5 M DMSO	25 s
7 M	1.75 M EG + 1.75 M DMSO	45 s	3.5 M EG + 3.5 M DMSO	25 s

TABLE 2 Morphological grading criteria for post-thaw oviductal cell aggregates.

Grade	Description
Grade A (high quality)	Aggregates in this category exhibited a compact and rigid structure with no loose cells in the surrounding medium, reflecting strong cell adhesion and cohesion. The surface was smooth and intact, indicating healthy cellular connections. Morphologically, they maintained a spherical and uniform shape with consistent size, presenting a well-defined three-dimensional (3D) architecture that closely mimicked natural cellular interactions (Figures 4II,A)
Grade B (moderate quality)	Aggregates were generally compact and retained their spherical shape; however, minor surface irregularities and occasional deviations in size were observed. A few loose cells were present in the surrounding medium, indicating moderate cell cohesion and a slight reduction in overall quality (Figures 4II,B)
Grade C (low quality)	Aggregates displayed noticeable looseness with partially detached cells and irregular, inconsistent shapes. Variability in size and a lack of surface uniformity were also noted, reflecting compromised cell cohesion and structural integrity (Figures 4II,C)
Grade D (poor quality)	Aggregates exhibited severe structural disintegration, characterized by significant cell detachment and highly irregular, non-spherical shapes. The surface appeared rough and damaged, and the 3D architecture was severely disrupted, making them unsuitable for further analysis (Figures 4II,D)

2.10 Epididymal sperm processing and preparation

Testes with epididymis were carefully separated and transported to the laboratory on chilled (approximately 4°C) physiological saline solution (0.9% NaCl) to minimize potential damage during transport. Upon arrival at the laboratory, the testes and epididymis were meticulously separated using sterile dissection techniques. The epididymides were then thoroughly rinsed with a sterile 0.9% saline solution to remove any residual blood or debris. Subsequently, the epididymal tissue underwent a brief dip in 70% ethyl alcohol for surface disinfection. The epididymis was incised to allow for the collection of sperm, which was subsequently examined [modified from Rashad et al. (22)]. The epididymal sperm was evaluated for motility and viability, and aliquoted for further use.

2.11 Assay of sperm binding to oviduct epithelial cells

The sperm-binding assay was carried out according to the methodology described by Winters et al. (17), with minor modifications as reported by El-Sokary et al. (7). Briefly, 20 oviduct cell explants were combined with 20 µL sperm droplets (1.6×10^6 cells/mL) and the assay was performed in triplicate. Before incubation, sperm were stained with MitoTracker Green (1 µM) and allowed to stand for 10 min at room temperature in the dark room to ensure optimal staining. The stained sperm were then incubated with thawed oviduct cell aggregates at 39°C for 30 min. The experiment was independently replicated 3 times to ensure reproducibility. Each experimental condition utilized 30 oviductal aggregates, and following the incubation, unattached and loosely bound spermatozoa were carefully removed by washing with TALP medium. The number of spermatozoa bound to the periphery of each oviduct aggregate was subsequently quantified to estimate the binding capacity (Figures 3B,I,II).

2.12 Statistical analysis

Statistical analysis was performed separately for each experiment using one-way ANOVA to evaluate the effect of a single independent factor within each experiment (as in the experimental design). Tukey's post-hoc test was used for multiple comparisons in each analysis, using IBM SPSS Statistics (version 22). A p-value of less than 0.05 was considered statistically significant. All experiments were independently repeated three times to confirm reproducibility. Data distribution was assessed for normality before analysis. Results are expressed as the mean ± standard error of the mean (SEM).

3 Results

3.1 Experiment 1: effect of aggregate size on post-thaw viability

The post-thaw viability of oviduct cell aggregates was significantly influenced by aggregate size. Aggregates with a diameter of 50 µm exhibited the lowest viability, with a mean value of 64.31 ± 0.33%. In the 100 µm group, viability increased to 70 ± 2.3% ($p < 0.05$). A further increase in aggregate size to 150 µm resulted in a higher viability of 78 ± 2.0% ($p < 0.05$), while the 200 µm group achieved the highest viability at 83 ± 2.8% ($p < 0.05$). No statistically significant difference was observed between the 150 µm and 200 µm groups ($p > 0.05$), indicating a plateau in post-thaw viability at these larger sizes (Figure 1A). However, both the 150 µm and 200 µm groups demonstrated significantly higher viability compared to the 100 µm

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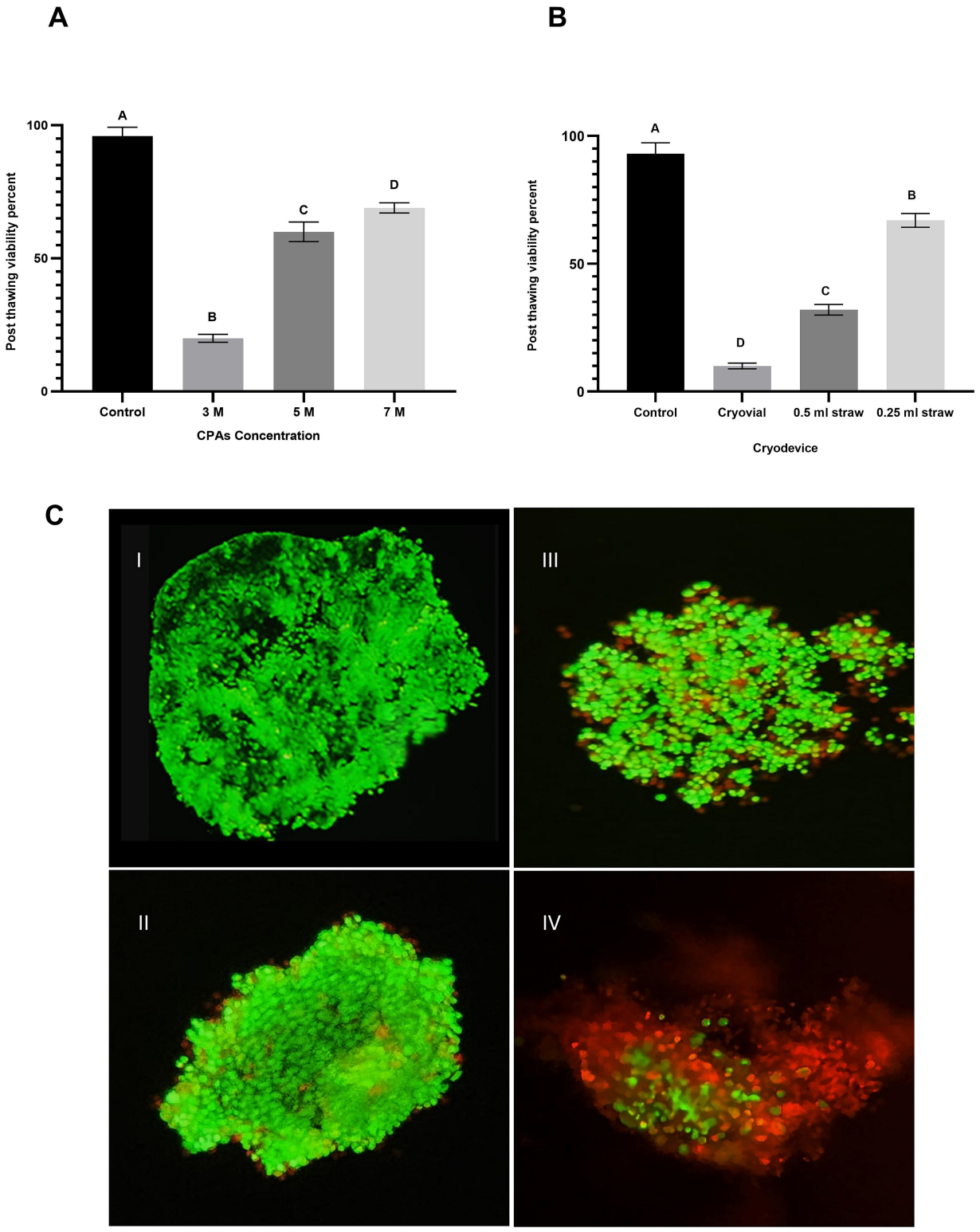

FIGURE 2
Impact of cryoprotectant (CPA) concentration and cryodevice type on post-thaw viability of oviduct aggregates. **(A)** Column graph illustrating the effect of CPA concentrations (3 M, 5 M, and 7 M) on post-thaw viability percentage of oviduct aggregates. Columns with different letters denote statistically significant differences among the groups ($p < 0.05$). **(B)** Column graph demonstrates the influence of cryodevice type (cryovials, midi-straws, and mini-straws) on post-thaw viability percentage. Columns with different letters denote statistically significant differences among the groups ($p < 0.05$). **(C)** Fluorescence microscopy images evaluating aggregate viability using SYBR-14 (green; live cells) and PI (red; dead cells) staining. **(C,I)** Control group. **(C,II)** 7 M CPA. **(C,III)** 5 M CPA. **(C,IV)** 3 M CPA. Scale bar = 50 μm.

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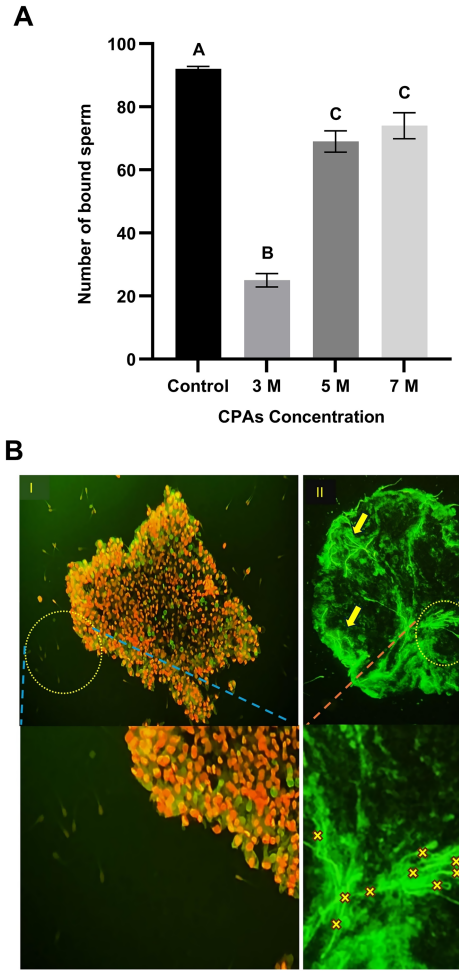

FIGURE 3
Effect of cryoprotectant (CPA) concentrations on sperm binding to post-thaw aggregates. **(A)** Number of sperm bound to oviduct aggregates following post-thawing across CPA concentrations (3 M, 5 M, and 7 M). Columns with different letters denote statistically significant differences ($p < 0.05$). **(B)** Fluorescence microscopy images illustrating sperm binding to oviduct aggregates. **(B,I)** Viable and intact aggregate post-thawing with sperm stained using MitoTracker Green. Yellow arrows and "X" indicate attached sperm. **(B,II)** Disintegrated aggregate post-thawing, with most cells stained with PI (red; dead) while few cells were stained with SYBR-14 (green; live), indicating extensive post-thaw damage and the absence of sperm binding. Scale bar = 50 µm.

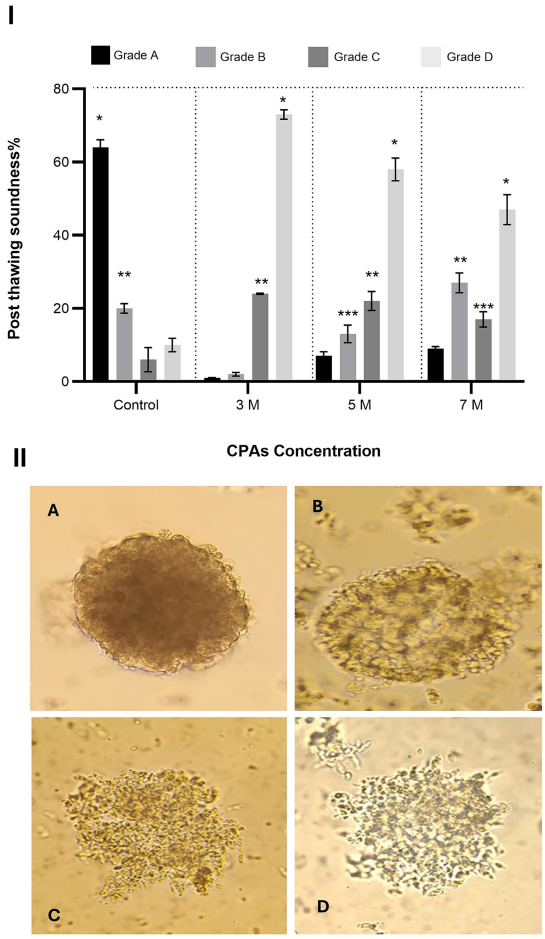

FIGURE 4
Effect of cryoprotectant (CPA) concentration on post-thaw structural integrity of oviduct aggregates. **(I)** Column graph showing the post-thaw soundness (%) of oviduct aggregates treated with CPA concentrations of 3 M, 5 M, and 7 M. Columns with asterisks denote statistically significant differences ($p < 0.05$). **(II)** Representative microscopic images illustrating different grades of aggregate structural integrity (Grades A to D). Scale bar = 50 µm.

group ($p < 0.05$), which itself was significantly higher than the 50 µm group ($p < 0.05$).

3.2 Experiment 2: effect of CPA concentration on structural integrity

The results of Experiment 2 are presented in Figure 4I. The concentration of cryoprotectant agents (CPA) significantly affected the post-thaw structural integrity of oviduct cell aggregates, as indicated by the distribution of Grades A, B, C, and D.

In the control group, Grade A aggregates were the most abundant (64 ± 2.1%), significantly higher than Grade B (20 ± 1.3%), Grade C (6 ± 3.3%), and Grade D (10 ± 1.8%) ($p < 0.05$). Grade B was also significantly higher than Grade C and Grade D ($p < 0.05$). When exposed to 3 M CPA, Grade A aggregates dropped sharply to 1 ± 0.13%, while Grade D aggregates increased substantially to 73 ± 1.3%, becoming the most dominant. Grades B and C were 2 ± 0.5% and 24 ± 0.19%, respectively. Grade D was significantly higher than Grade C, and Grade C was significantly higher than Grades A and B ($p < 0.05$).

At 5 M CPA, Grade A aggregates increased slightly to 7 ± 1.2%, while Grade D decreased to 58 ± 3.1%. Grades B and C were 13 ± 2.4% and 22 ± 2.6%, respectively. Grade D was significantly higher than all other grades. Grade C was significantly higher than Grade B, and Grade B was significantly higher than Grade A ($p < 0.05$). At 7 M CPA, Grade A increased further to 9 ± 0.57%, and Grade D decreased to 47 ± 4.1%. Grades C and B were 27 ± 2.7% and 17 ± 2.1%, respectively. Grade D remained significantly higher than all other grades. Grade C was significantly higher than Grade B, and Grade B was significantly higher than Grade A ($p < 0.05$).

3.3 Experiment 3: effect of CPA concentration on post-thaw viability

The results of Experiment 3 are presented in Figure 2A. The effect of CPA concentration on the post-thaw viability of oviduct cell aggregates was evaluated across four experimental groups: control, 3 M, 5 M, and 7 M. The 3 M group showed a dramatic reduction in viability to 20 ± 1.5% ($p < 0.05$ compared to all other groups. Viability significantly improved in the 5 M group, with 60 ± 3.7% of aggregates retaining their viability post-thaw ($p < 0.05$) compared to 3 M) demonstrating a marked enhancement with an increased CPA concentration. The 7 M group exhibited further significant improvement, reaching 69 ± 1.9% viability ($p < 0.05$) compared to 5 M.

3.4 Experiment 4: influence of cryodevice type on post-thaw viability

The results of Experiment 3 are shown in Figure 2B. Post-thaw viability of oviduct aggregates was significantly affected by the type of cryodevice used. In the control group, viability was high at 93 ± 4.3% ($p < 0.05$). Among the cryopreserved groups, aggregates frozen in cryovials had the lowest viability at 10 ± 1.1% ($p < 0.05$). Viability improved in the 0.5 mL straw group, reaching 32 ± 2.1%, which was significantly higher than in cryovials ($p < 0.05$). The highest viability among cryopreserved samples was observed in the 0.25 mL straw group, with 67 ± 2.7%, which was significantly greater than both the cryovial and 0.5 mL straw groups ($p < 0.05$).

3.5 Experiment 5: effect of CPA concentration on post-thawing sperm binding ability to oviduct aggregates

The effect of CPA concentration on post-thaw sperm-binding ability was evaluated across four experimental groups: control, 3 M, 5 M, and 7 M. In the control group, the number of sperm bound to the oviduct aggregates was highest, with an average of 92 bound sperm, significantly higher than all CPA-treated groups ($p < 0.05$). Among the CPA-treated groups, the 3 M concentration resulted in the lowest sperm-binding ability, with an average of 25 sperm bound per aggregate ($p < 0.05$). In contrast, the 5 M and 7 M concentrations demonstrated significantly higher sperm-binding abilities (compared to 3 M group), with averages of 69 and 74 bound sperm, respectively. However, no significant difference was observed between the 5 M and 7 M groups. Statistical analysis confirmed that both 5 M and 7 M concentrations were significantly higher than the 3 M group. These findings suggest that CPA concentration affects sperm-binding ability, with higher concentrations (5 M and 7 M) maintaining better binding efficiency compared to the 3 M group (Figure 3A).

4 Discussion

This study evaluated the effects of aggregate size, cryoprotectant concentration, and cryodevice type on the structural integrity, post-thaw viability, and sperm-aggregate binding ability of oviduct aggregates, contributing to a better understanding of factors influencing cryopreservation outcomes. The results demonstrated a significant influence of aggregate size on post-thaw viability, with viability improving as aggregate size increased up to 150 µm. However, the plateau observed between the 150 µm and 200 µm groups suggests that additional increases in size do not further enhance viability, potentially due to diffusion limitations of cryoprotectants or increased thermal gradients.

Furthermore, CPA concentration played a pivotal role in maintaining structural integrity post-thaw. As CPA concentration increased from 3 M to 7 M, a gradual improvement in the proportion of Grade A aggregates was observed. This indicates that higher CPA concentrations can mitigate osmotic and ice crystal damage by stabilizing cellular membranes and intracellular structures (23). However, the concurrent increase in Grade D aggregates across all CPA-treated groups highlights the potential cytotoxicity associated with cryoprotectants at suboptimal concentrations.

Cryoprotectants (CPAs), particularly at the high concentrations used in vitrification, can impact mammalian cells by affecting membrane integrity and cellular metabolism. Agents like dimethyl sulfoxide (DMSO) and ethylene glycol effectively prevent ice formation but may cause osmotic stress and oxidative damage if exposure is prolonged or not well controlled. Moreover, the evaluation of sperm-binding ability revealed a concentration-dependent effect of CPAs. The reduced binding observed at 3 M suggests compromised aggregate functionality. The improved viability observed at the 150 µm aggregate size underscores its importance in cryosurvival. These findings align with previous studies emphasizing the role of aggregate structure in preserving cellular integrity during cryopreservation (15).

The observed increase in Grade A aggregates with higher CPA concentrations, despite some cytotoxic effects, supports previous insights into cryoprotectant dynamics. These results are consistent with earlier findings on the balance between cryoprotective efficacy and cytotoxic effects of CPAs (24). Increasing CPA concentration from 3 M to 7 M improved post-thaw viability, reflecting better protection against ice crystallization and osmotic shock (25). Although viability in the 7 M group was slightly lower than controls, this is likely due to mild residual CPA toxicity rather than substantial harm. This underscores the delicate balance between achieving cryoprotection and minimizing cytotoxicity, highlighting the importance of optimizing CPA concentration and exposure duration to maintain cell survival and function (26). In this study, the combination of EG and DMSO appeared beneficial in improving post-thaw outcomes. However, previous studies reported mixed findings. Yadav et al. (13) found no advantage in combining DMSO with EG, and (27) reported similar results when using either 20% EG or 20% DMSO individually.

The superior viability associated with the 0.25 mL straws highlights how cryodevice choice shapes post-thaw outcomes. The higher viability observed in 0.25 mL straws may be attributed to their ability to facilitate faster and more uniform cooling and warming rates, which are critical for minimizing intracellular ice formation and osmotic stress (28). Conversely, the lower viability in cryovials (1.5 mL) is likely due to their larger volume and slower cooling rates, which can exacerbate cryo-induced damage (29). The findings emphasize the importance of cryodevice selection in cryopreservation protocols, particularly for sensitive biological materials like oviduct aggregates. However, vitrification in 0.25-ml straws causes a delay in heat loss from the solutions, possibly leading to devitrification, i.e., intracellular recrystallization during warming (30). This apparent contradiction may be due to differences in sample composition, cryoprotectant penetration, or technical variations in the handling and warming steps.

The CPA concentration-dependent improvement in sperm binding reinforces the link between structural integrity and functionality. This is consistent with Parnpai et al. (16), who reported that a strong indicator of cryopreservation success is the ability of thawed cells to restore normal physiological function with minimal structural damage. The reduced binding rate is likely due to structural and membrane damage or alterations in the surface receptors essential for sperm interaction. The improved binding at 5 M and 7 M highlights the protective effects of optimal CPA concentrations in preserving the structural integrity of oviduct aggregates. However, the lack of significant difference between these two concentrations indicates a plateau in functional recovery, which mirrors the trends observed in viability. These findings highlight the intricate balance between maintaining structural integrity and preserving functional capabilities during cryopreservation (31).

Efficient vitrification and recovery of the aggregates may support the development of more refined *in vitro* fertilization protocols, improve sperm selection strategies, and serve as biological carriers for oviductal secretions that influence fertilization (32). The successful vitrification protocol of the aggregates offers different practical applications, including but not limited to their importance as a promising tool for studying sperm-oviduct interactions under near-physiological conditions, particularly in species like camels, where *in vivo* studies are limited.

5 Conclusion

In conclusion, this study demonstrates that aggregate size, cryoprotectant concentration, and cryodevice type significantly affect post-thaw viability, structural integrity, and sperm-binding ability of oviduct cell aggregates. Specifically, increasing aggregate size up to 150 µm improves viability, with no further benefit observed at 200 µm. Cryoprotectant concentration exhibits a clear dose-dependent effect: low levels (3 M) result in poor viability and structural damage, whereas higher concentrations (7 M) substantially enhance viability and preserve sperm-binding function. Among the cryodevices tested, the 0.25 mL straw outperforms both cryovials and 0.5 mL straws, achieving the highest post-thaw viability. Based on our findings, we recommend cryopreserving oviduct cell aggregates approximately 150 µm in diameter using a 7 M cryoprotectant concentration and 0.25 mL straw cryodevices to achieve optimal preservation outcomes. This optimized protocol offers a reliable model for the cryopreservation of oviduct aggregates, with promising applications in reproductive biology research and fertility preservation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Research Ethics and Integrity Committee under Approval Number REIC2024-FAC35. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ME-S: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. HA: Investigation, Methodology, Writing – original draft, Writing – review & editing. SS: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. KM: Conceptualization, Data curation, Formal analysis, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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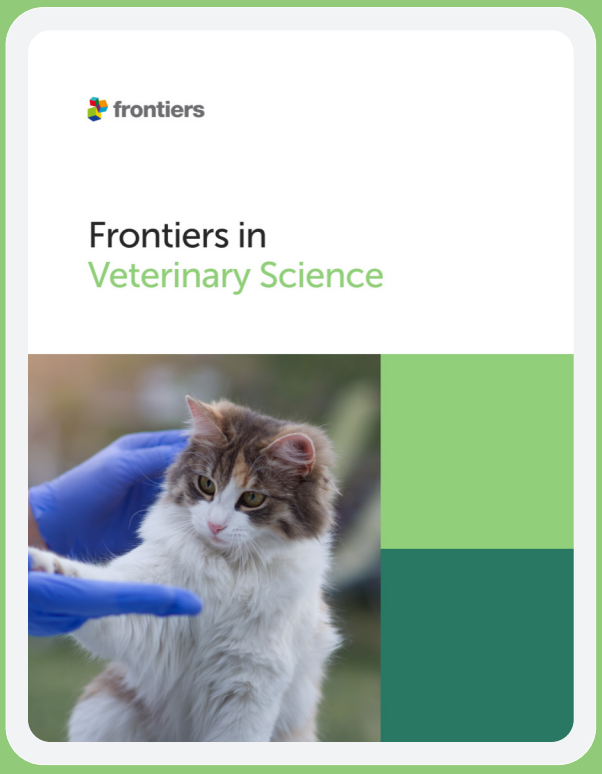

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