SHARACELY DE SOUZA FARIAS

Donkey milk: animal health and welfare

São Paulo

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CORRECTED VERSION

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CERTIFICADO

Certificamos que a proposta intitulada "Leite de asinino: bem-estar e saúde animal.", protocolada sob o CEUA nº 8696141117 (ID 007216), sob a responsabilidade de **Adroaldo José Zanella** *e equipe; Sharacely de Souza Farias* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 27/11/2019.

We certify that the proposal "Jenny's milk: animal welfare, animal health. ", utilizing 14 Equines (14 females), 109 Swines (males and females), protocol number CEUA 8696141117 (ID 007216), under the responsibility of **Adroaldo José Zanella** *and team; Sharacely de Souza Farias* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 11/27/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 11/2017 a 11/2020 Área: Epidemiologia Experimental Aplicada As Zoonoses

Origem:	Animais de proprietários						
Espécie:	Equídeos	sexo:	Fêmeas	idade:	2 a 4 anos	N:	14
Linhagem:	Raça Pêga			Peso:	250 a 400 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Suínos	sexo:	Fêmeas	idade:	2 a 4 anos	N:	9
Linhagem:	landrace x large white			Peso:	250 a 400 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Suínos	sexo:	Machos e Fêmeas	idade:	1 a 35 dias	N:	100
Linhagem:	landrace x large white			Peso:	2 a 20 kg		

Local do experimento: Os asininos serão mantidos no Criatório Ximbó.. Os suínos serão mantidos na Universidade de São Paulo, Campus Fernando Costa, Pirassununga.

São Paulo, 20 de janeiro de 2020

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Date: _____/_____/_____

Committee Members

Prof. ____

Institution:___________________________ Decision:________________________

Prof. ____

Institution:___________________________ Decision:________________________

Prof. ____

Institution:___________________________ Decision:________________________

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ABSTRACT

FARIAS, S.S. **Asinine milk: animal health and welfare**. 2022. 118 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2022.

In Brazil, donkeys have lost their economic value and since 2016, have not been accounted for by the Brazilian Institute of Geography and Statistics (IBGE). As a consequence, their abandonment has been intensified. Most of these animals are found in the northeastern region of Brazil (80%) and their expressive numbers have caught the attention of Chinese markets, which have attempted to establish the slaughter and commercialisation of donkey skin as a means to reintroduce them into the brazilian economy. Therefore, this study aims to contextualise the social, economical and cultural situation of donkeys in Brazil, assess the welfare conditions of donkeys destined for slaughter, and explore possibilities of reintroducing donkeys in the brazilian economy through the sustainable and ethical production of asinine milk. A case study was conducted to assess the welfare of abandoned donkeys (Farias, 2021a), an experiment to assess the welfare of jennies during milking (Farias, 2021b), other about physical and chemical parameters of asinine milk of the Pêga breed and a study on the effects of supplementation with asinine milk on behaviour, productive parameters and immunity of piglets before and after weaning. The studies concluded that it is observable that asinine milk samples collected in this study did not suffer composition changes throughout lactation, which is in agreement with other studies with donkey and mare milk and supplementation with asinine milk did modulate the expression of genes related to inflammation, resulted in a decrease in salivary cortisol post-weaning in the afternoon, and did not alter the behavioural expression of stress in the tests conducted. Therefore, further studies on the supplementation with asinine milk are warranted. All data were analysed in the SAS software and for all presented tests, average pairs were compared to 5% probability. The objective of this study is to explore possibilities of reintroduction of donkeys in the brazilian social and economic scenarios through the sustainable and ethical production and commercialisation of asinine milk.

Keywords: Donkeys, dairy product, behaviour assessments, physiological assessments, pigs.

RESUMO

FARIAS, S.S. **Leite de jumenta: saúde e bem-estar animal.** 2022. 118pg. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2022.

No Brasil, os jumentos perderam sua funcionalidade econômica e não são contabilizados pelo Instituto Brasileiro de Geografia e Estatística (IBGE) desde 2016. Como consequência, seu abandono tem se intensificado. A maioria desses animais é encontrada na região nordeste do Brasil (80%) e seu grande número tem chamado a atenção dos mercados chineses, que têm tentado estabelecer o abate e comercialização da pele de jumento como forma de reintroduzi-los na economia brasileira. Portanto, este estudo tem como objetivo contextualizar a situação social, econômica e cultural dos jumentos no Brasil, avaliar as condições de bem-estar dos jumentos destinados ao abate e explorar possibilidades de reintrodução de jumentos na economia brasileira por meio da produção sustentável e ética de leite asinino. Foi realizado um estudo de caso para avaliar o bem-estar de jumentas abandonadas (Farias, 2021a), um experimento para avaliar o bem-estar de jumentas durante a ordenha (Farias, 2021b), outro sobre parâmetros físico-químicos do leite asinino da raça Pêga e um estudo sobre os efeitos da suplementação com leite asinino no comportamento, parâmetros produtivos e imunidade de leitões em fase de maternidade. Os dois últimos estudos concluíram que é observável que as amostras de leite asinino coletadas neste estudo não sofreram alterações de composição ao longo da lactação, o que está de acordo com outros estudos com leite de jumenta e égua e a suplementação com leite asinino modulou a expressão de genes relacionados a inflamação, resultou em diminuição do cortisol salivar pós-desmame no período da tarde, e não alterou a expressão comportamental do estresse nos testes realizados. Portanto, mais estudos são necessários. Todos os dados foram analisados no software SAS e para todos os testes apresentados, os pares médios foram comparados a 5% de probabilidade. O objetivo deste estudo é explorar possibilidades de reintrodução de jumentas no cenário social e econômico brasileiro por meio da produção e comercialização sustentável e ética do leite asinino.

Palavras-chave: Jumenta, laticínios, avaliações comportamentais, avaliações fisiológicas, suínos.

SUMMARY

1.BACKGROUND AND INTRODUCTION

Due to the increasing use of machines in rural properties and motorized vehicles for transportation, donkeys have lost their place in the national social and economic scenarios. This results in rising numbers of wandering animals dwelling near roadways (PEROZZI GAMEIRO; CLANCY; ZANELLA, 2021; RAW et al., 2021) with neglected needs (BIFFA; WOLDEMESKEL, 2006) and low welfare levels.

Donkeys are mammals of the Perissodactyla order, Hippomorpha suborder, Equidae family, *Equus asinus species*, and asinus species (GRINDER; KRAUSMAN; HOFFMANN, 2006). In Brazil, the three main donkey breeds are Nordestino, Brasileiro and Pêga (CARNEIRO; CAVALCANTE LUCENA; DE OLIVEIRA BARROS, 2018; ALVES; COSTA, 2022).

As most donkeys in Brazil are found in the northeast region and a significant number of of these animals are wandering, investors have attempted to establish the slaughter of donkeys, in predatory ways, as a mean of producing Eijao, a product utilized in traditional Chinese medicine. This has resulted in serious social, economic and welfare-related issues (WATERS, 2019). A group of 800 donkeys destined for slaughter were neglected in a farm that served as a holding facility due to the sudden prohibition of donkey slaughter in the state of Bahia, in the town of Euclides da Cunha (FONSECA et al., 2021).

This situation brought the slaughter of donkeys into question in Brazil. As a result, it was concluded that the production chain for donkey meat, to be considered ethically acceptable and sustainable, must be better planned and recent work demonstrated that there are other strategies, such as milk production, to be explored (FARIAS et al., 2021; FONSECA et al., 2021) which could be more sustainable thanthe disorganized and often illegal trade of donkeys to produce skins in order to meet the demands for Eijao. A recent report addresses a concerted effort carried out by stakeholders and interested parties in order to protect donkeys that were kept in a farm as a result of skin trade (GAMEIRO; REZENDE; ZANELLA, 2021).

Donkeys have and long and historical association with humans, including biblical accounts, such as the transport of the sacred family, and for the development of many countries, for example, as reported by D'Arval in 1912, when asinine milk was successfully used for feeding orphaned children in France.

Although the empirical knowledge of the health benefits of asinine milk exist since the roman empire, the adequate handling procedures for milking in this species are still questionable. A potential solution for the reintroduction of donkeys as an important species in the Brazilian socioeconomic scenario is their employment for dairy farming. However, in Brazil, there are limited research to assess the suitability of asinine milk for humans.

Donkeys possess physiological and anatomical characteristics that result in milk production that differs from that of other species, especially in terms of milk supply (D'ALESSANDRO; MARIANO; MARTEMUCCI, 2015; VINCENZETTI et al., 2017b). In this species, there are two peaks in the kinetics of milk ejection: cisternal (25-15%) and alveolar (75-85%), resulting in a production which is essentially alveolar, meaning it is synthesized in the lactation cells that surround the mammary alveoli (ALESSANDRO; MARTEMUCCI, 2009; MARTINI et al., 2020).

During the galactopoietic period, the intensity and duration of milk synthesis and secretion are affected by thyroid and growth hormones. Its production is dependent on its removal from the mammary gland (SEYITI; KELIMU, 2021; LI et al., 2022), and due to its low storage capacity which does not exceed 2,5 L (SALIMEI, 2011; SANTOS, 2017b). Milk ejection occurs through the suckling stimulus, which triggers the release of oxytocin, inducing the contraction of myoepithelial cells. However, the milking routine is more manageable when foals are not physically present, in terms of both human and animal safety and for an optimal milk extraction (VINCENZETTI et al., 2021).

In order to achieve efficient milking, jennies must be milked after 2 to 3 hours of physical separation from their foals (D'ALESSANDRO; MARIANO; MARTEMUCCI, 2015). Long intervals between milking events may cause rise of intra-udder pressure, inducing early ceasing of glandular activity, due to the udder size and its low storage capacity. Due to this, donkeys may need to be milked multiple times a day (POLIDORI; VINCENZETTI, 2012; ASPRI; ECONOMOU; PAPADEMAS, 2017; BHARDWAJ et al., 2020).

When faced with aversive handling situations, animals tend to adapt through physiological and behavioural alterations, which culminate in the "emergency" state, provoking changes in endocrine and metabolic parameters (COHEN; JANICKI-DEVERTS; MILLER, 2007) . These alterations can cause the activation of the hypothalamic-pituitary-adrenal axis (HPA).

In the first chapter we present our case study addressing the welfare assessment in a group of 800 donkeys destined for slaughter which were neglected in a farm that served as a holding facility due to the sudden prohibition of donkey slaughter in the state of Bahia, in the town of Euclides da Cunha.

In the second chapter we present data on the responses of jennies and their foals to short term separation. The separation of jennies from their foals may be a stress factor for these animals, which might trigger a rise in production of vasopressin, prolactin, corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH), stimulating the adrenal glands for secretion of glucocorticoids, such as cortisol (BOHÁK et al., 2013a), and possibly altering the jennies' behaviour (JOHNSON et al., 1992) . If they experience fear or are under effect of stress factors, alveolar milk ejection could also be affected, due to its relation to the oxytocin concentration and myoepithelial contraction (BRUCKMAIER; WELLNITZ, 2008). Moreover, milk composition and yield may be altered by factors such as nutrition, jennies age, handling, and climatological characteristics.

In the third chapter we present some data regarding milk composition in Brazilian Pega donkeys. Among the reasons asinine milk is so precious is the composition of its lipid fraction, which is rich in polyunsaturated and essential fatty acids (PUFAs and EFAs), and poor in saturated fatty acids (SFAs) (MASSOURAS; TRIANTAPHYLLOPOULOS; THEODOSSIOU, 2017). Asinine milk also has a large percentage of whey proteins (35-50% of the nitrogen fraction), the most important being α-Lactalbumin, β-Lactoglobulin, lactoferrin and lysozyme (MARTINI et al., 2018), and low casein content, comparable to human milk (VINCENZETTI et al., 2017a). The most represented casein found in asinine milk is β-casein, followed by low levels of α-s1, α-s2 and kappa caseins (INGLINGSTAD et al., 2010; LIAO et al., 2017). The lactose percentage is similar between the asinine and human milk, at nearly 5,3% (A., 2004; D'ALESSANDRO; MARTEMUCCI, 2012; MEDHAMMAR et al., 2012).

Based on its physicochemical properties such as content of proteins, sugars and lipids, asinine milk appeared to be the most similar to human milk (MARTINI et al., 2015), and can be used for hypoallergenic infants and adults in risk situations (ENRICO BERTINO , LAURA CAVALLARIN , FRANCESCO CRESI , PAOLA

TONETTO , CHIARA PEILA , GIULIA ANSALDI , MELISSA RAIA , ALESSIA VARALDA , MARZIA GIRIBALDI , AMEDEO CONTI MORO , SARA ANTONIAZZI , GUIDO E. § ELENA SPADA , SILVANO MILA, 2019; BERTINO et al., 2022).

In Chapter 4 we attempted to understand the impact of asinine milk in mitigating the stress responses in pigs subjected to maternal separation. Due to the lack of research in Brazil, before testing the potential health benefits of brazilian asinine milk in human nutrition, it is preferable that this product be tested in an animal model first (MARIANO, 2003). An excellent model for humans in studies of nutrition and other related fields (MILLER e ULLREY, 1987) is the domestic pig (Sus scrofa domesticus) due to its anatomical and physiological similarities.

Many studies have been carried out in piglets during the maternity stage due to the potential physiological, emotional, cognitive and immunological challenges they face (BERNARDINO et al., 2016). Therefore, we considered as relevant the realisation of this project which aims to not only contextualise the actual situation of donkeys in Brazil but also assess the welfare conditions of donkeys destined for slaughter, as well as explore possible means to utilize asinine milk as a nutraceutical and, in this mannter, reintroduce donkeys in the brazilian social and economic scenarios.

2.LITERATURE REVIEW

2.1 Donkeys in Brazil

Donkeys are mammals in the Perissodactyla order, Hippomorpha suborder, Equidae family, Equus genus and asinus species (GRINDER; KRAUSMAN; HOFFMANN, 2006).

It is estimated that there are 50,453,888 donkeys worldwide, of which 395,910 are in Europe, 2,843,306 are in South America and 822,255 are in Brazil (“FAOSTAT”,) (Food and Agriculture Organization of the United Nations, 2018). The majority of donkeys in Brazil (86,7%) are found in the Northeast region (Instituto Brasileiro de Geografia e Estatística, 2017).

The most important breeds of donkeys in Brazil are Nordestino, Paulista and Pêga (MARIANTE et al., 2009). Nordestino donkeys originated from north-african animals, and are considered rustic and well-adapted to the brazilian semi-arid environment, (MORI et al., 2003; ALMEIDA; SILVA, 2010) (ALMEIDA, 2009; OLIVEIRA, 2004) This breed is reported to be undergoing extinction risk (MARIANTE et al., 2009).

Paulista donkeys originated from crosses between Italian and Portuguese donkeys in Brazil, and are characterized by their ability to withstand heavy work in fields and travel long distances (ALMEIDA; SILVA, 2010)

Pêga donkeys originated in Brazil in 1810, in the municipality of Entre Rios de Minas, Minas Gerais (MCMANUS et al., 2010), They are of medium stature, with a minimum height at withers of 125 cm for males and 120 cm for females and their main function is the production of mules through breeding with horses, to be used in work and leisure (GIRARDI, 2012a).

Though donkeys have had historical roles and still hold great economic potential in Brazil, their population has not been accounted for by the Brazilian Institute of Geography and Statistics since 2016 (Instituto Brasileiro de Geografia e Estatística, 2017).

Donkeys, when wandering on highways, deprived of regular food source, and water and are at serious risk of accidents (FLÁVIO et al., 2014; PERUZZI GAMEIRO; CLANCY; ZANELLA, 2021), so their level of well-being is poor. For Broom and Molento (2004; 2011) the definition of welfare must be done in a way that allows a ready relationship with other concepts, such as needs, freedoms, happiness, adaptation, control, ability to predict, feelings, suffering, pain, anxiety, fear, boredom, stress and health.

Stressful events without resolution control generate disruption in the homeostasis of the organism which has to leave its comfort zone to adapt to an adverse situation (COHEN; JANICKI-ADVERTS; MILLER, 2007; POHL et al., 2017). A stressful environment is one characterized by any stimuli that demand an adaptive response from the animal to the new condition.

To ensure animal welfare, it is necessary that these animals have access to healthy food and a resting area and do not suffer fear or injury (COSTA et al., 2021). The deprivation of these factors can lead the animals to become debilitated and even die (FARIAS et al., 2021).

The lack of credible information sources and administrative registers to support the population estimatives can be ascribed to the diminishing use of these animals by rural properties (IBGE, 2012). This trend was possibly caused by rural exodus, rise in agricultural mechanization and government-issued incentives for the purchase of agricultural inputs, the increase in donkey meat consumption (FARIAS et al., 2021) and the exploration of donkey skin to meet consumption chinese traditional medicine demands, represented by Eijao (WATERS, 2019).

The lack of utilisation of these animals generates an increase of wandering animals, often living close to federal and state roadways (OCHIENG; ALEMAYAHU; SMITH, 2003; MARIANTE et al., 2009), with neglected needs (BIFFA; WOLDEMESKEL, 2006), and low welfare. However, the slaughter of donkeys in Brazil for meat consumption and Eijao production are questionable.

Eijao is a traditional Chinese remedy used for over 3,000 years. It is believed that collagen is the main active component of Eijao (Colla corii asini - CCA), and its claimed therapeutic properties are related to blood nutrition, optimization of immune response, metabolic equilibrium improvements, slowing of aging and treatment of gynecological diseases (LI et al., 2017).

However, due to the 75,4% decrease of donkey populations in China between 1994 and 2018 (10,89 million in 1994 to 2,68 million in 2018), around 90% of Eijao products in China are fabricated in the Shandong province from imported donkey skin(“China corta tarifa sobre couro de burro - CGTN”,) from countries that do not possess established cutures or business models that explore this type of product.

The demand for exportation of donkey skin for Eijao production, especially in Brazil and African countries (FONSECA et al., 2021; GAMEIRO; REZENDE; ZANELLA, 2021) generated a price increase for this product, which went from 20 yuan in 2000 to about 3,000 yuan in late December 2017 (“China corta tarifa sobre couro de burro - CGTN”,).

This resulted in intense pressure over donkey populations worldwide and led to the theft and illegal commercialization of donkeys, resulting in worries regarding their welfare and the livelihood of people who depend on them (BENNETT; PFUDERER, 2020; The Donkey Sanctuary, 2019).

Therefore, donkeys had been kept in vulnerable conditions and oftentimes illegally gathered for slaughter and trade of skin and meat, lacking all sanitary control, transportation guidelines and welfare conditions (FONSECA et al., 2021).

This resulted in a 28% decline of population in exporting countries between

2007 and 2017 (LESTÉ-LASSERRE, 2019). The unchecked exploration of these animals may be an important factor in the extinction of the species (QUEIROZ; GAMEIRO; ZANELLA, 2021), which has suffered a drastic population decline worldwide (“2018 | FAO | Organização das Nações Unidas para Agricultura e Alimentação”,).

According to FAO, the population of donkeys is already threatened with extinction, after decreasing from 1,31 million donkeys in 1994 to 822,000 donkeys in 2018, a 37,2% decrease Organização das Nações Unidas para Agricultura e Alimentação (2020).

In order to revert this situation, research is needed to structure the meat production chain and explore other possibilities such as asinine milk production due to the its nutraceutical potential, as seen in other countries where donkeys remain relevant in the economic and social scenarios, such as Italy and France (SALIMEI, 2011; VENEZIANO et al., 2011).

2.2 Physical and chemical properties of asinine milk

Before research can focus on commercial milk production, it is important to investigate the quality of milk produced by national breeds.

Milk is defined as an emulsion of fat in water, stabilized by a colloidal dispersion of proteins in a solution of salts, vitamins, peptides, lactose, oligosaccharides, caseins and other proteins, which include enzymes, antibodies, hormones, pigments (carotenes, xanthophylls, riboflavin), cells (epithelial cells, leukocytes, bacterias and yeast), CO2, O2 and oxygen, of neutral pH that varies between 6.6 and 6.9 (VINCENZETTI et al., 2008).

The composition of milk varies in function of many factors, including species, breed, lactation stage and milking management (CORRALES-HERNÁNDEZ et al., 2018). Regarding the milk of different mammals, it is known that many properties are similar between donkey and human milk, and different from cow milk (VINCENZETTI et al., 2008; ALESSANDRO; MARTEMUCCI, 2009; SANTOS, 2017b).

The average protein concentration (18 g/L) is similar to that of human milk (21 g/L) (21 g/L) (ALTOMONTE et al., 2019),), which may impact health as the high ingestion of proteins in babies fed with formula may be related to infant adiposity (LIND et al., 2018; CAVALCANTI et al., 2021).

Non-protein nitrogen (NPN) in asinine milk represents 10 to 16% of total nitrogen or 5 g/L. The concentration of essential amminoacids is 36 to 38% of protein, and free amminoacids are more readily available for absorption in the gastrointestinal tract. The bioactive enzymes spermine, spermidine and putrescine are present at 5-32 µg/L, 5-51 µg/L and 2-347 µg/L, respectively (SALIMEI, 2011).

The protein fraction of asinine milk serum is composed primarily of β-lactoglobulin (29% of serum), α-lactalbumin (22% of serum), immunoglobulin (11% of serum), serum albumine (6% of serum), lactoferrin (4% of serum) and lysozyme (21% of serum) (CHIOFALO; SALIMEI, 2014).

The β-lactoglobulin of asinine milk was identified in two mollecular forms, I and II, with 2 (A and B) and 4 (A, B, C and D) variants, respectively (SALIMEI 2012). It represents approximately 40% of serum proteins, less concentrated but also more digestible when compared to the same protein in cow milk (JONSSEN; DIJSRERVELD, 1983; VINCENZETTI et al., 2017b)

The α-lactalbumin concentration is higher in human milk (approximately 0.34 g/100 mL) and cow milk (approximately 0.21 g/ 100 mL) (MIRANDA et al., 2004; NADA, 2009)

The main source of Lactoferrin (LF) is maternal milk, and its concentration differs among mammal species, being the highest in human and asinine milk.

Bovine Lf (bLf) is present in high concentrations in colostrum (2-5 mg/mL) and low concentrations in mature milk (0.1-0.3 mg/mL) (Inoue et al., 1993; Pan et al., 2007), and has shown health benefits when tested in humans . During lactation, higher levels of Lf were reported in colostrum (0.88 mg/mL) (GARDNER; OZANNE; SINCLAIR, 2009) In human milk, Lf is the second most abundant protein (1 g/L) (0,25 mg/mL (GUBIC; AUTHORITY, 2015). In human milk, LF is the second most abundant protein (1 g/L) (JENSSEN; HANCOCK, 2009; HENNART; BRASSEUR, 2018).

The protein fraction of asinine milk is similar to that of human milk, with low levels of casein (CN) and immunoglobulins, which contribute to its use in the nutrition of children and adolescents(ZEGARSKA; JAWORSKI; PASZCZYK, 2001).

The casein of asinine milk is highly digestible, and the non-digestible fraction is also similar to that of horse and human milk, at 7%, 4% and 5%, respectively. This proportion is doubled in cow and goat milk after gastric digestion (INGLINGSTAD et al., 2010; TIDONA et al., 2011).

The lipid fraction of the milk is generally dominated by triacylglycerols (TAGs), with lower quantities of other classes such as diacylglycerols, monoacylglycerols, phospholipids (PLs), cholesterol, hydrocarbons and fatty acids (FAs) (ZEGARSKA 2003). There may also be limited quantities of glucolipids, fat-soluble vitamins, ether lipids, lactones, aldehydes and ketones

The total lipid content of asinine milk has been reported from 0.2 to 0.7% (CONTARINI 2017), 0.42% to 0.35% (MARTINI 2015) and 0.42% to 0.72% (MARTEMUCCI 2012), reaching values up to 1.4% (SALIMEI 2004) and 1.8% (GUO 2007). The wide range of lipid concentrations can be ascribed to its many influencing factors, such as lactation stage (MARTEMUCCI 2012), breed, milking management, location and feeding (CONTARINI 2017).

The lipid content of asinine milk is considerably lower than that of human milk, in which it represents 3.5 to 4% of the general composition (ASPRI 2017), and cow milk, which contains 3.5 to 3.9% of fat (ALTOMONTE 2019, VALLE 2018, SALIMEI 2012, TAFARO 2007, MARTINI 2018, CONTARINI 2017, CARMINATI 2017, GANTNER 2015, SMIDDY 2012, MARTINI 2014, MARTINI 2015, GASTALDI 2010, GUO 2007, CLAEY 2014). However, the compositions of the lipid fractions of donkey and human milk are comparable, especially regarding the poliunsaturated fatty acid (PUFA) content (MARTEMUCCI 2012, ASPRI 2017, AMATI 2010, CHIOFALO 2011).

The composition of the lipid content of asinine milk is 80 to 85% triacylglycerols, 5 to 10% phospholipids and 9.5% free fatty acids (FFAs) (CARMINATI 2017, GANTNER 2015).

Human and ruminant milk contains higher proportions of TAGs, of up to 98% (CLAEYS 2014), but smaller proportions of PLs, between 0.5 and 1.5%, and FFAs, between 0.7 and 1.5% (CLAEYS et al., 2014; CARMINATI; TIDONA, 2017a).

The milk fat globules in asinine milk average 2 microns of diameter for up to 70% of globules (MARTINI et al., 2018; ALTOMONTE et al., 2019), which means they are relatively small when compared to other species, including humans, which produce milk fat globules that average 4 microns diameter (VERONESI et al., 2014; MARTINI et al., 2015, 2018). In cows, milk fat globules average 3.5 to 5.5 µm (MARTINI 2003), and their size varies between 2.79 and 4.95 µm in sheep and 2.2 to 2.8 µm in goats (MIRANDA et al., 2004). In cows, milk fat globules average 3.5 to 5.5 µm (MARTINI 2003), and their size varies between 2.79 and 4.95 µm in sheep and 2.2 to 2.8 µm in goats (MARTINI 2013).

The smaller globules may be a consequence of the lower fat content, as higher areas of membrane from mammary epithelial cells became available to envelop milk fat, resulting in smaller units (MARTINI 2013, MARTINI 2014).

The phospholipid content of asinine milk, when compared to human milk, has been described as relatively low (ALTOMONTE 2019, ZOU 2013), however asinine milk is rich in phospholipids by unit of fat (CONTARINI 2017). Some authors have found higher concentrations of phospholipids in asinine milk than human milk (CARMINATI 2017, GANTNER 2015) and ruminant milk (CONTARINI 2017).

Asinine milk is hypoallergenic primarily because of its low casein content (Vincenzetti et al. 2007), which is close to the casein content of human milk. The low casein is also related to the concentration of β-lactoglobulin, which also relates to its hypoallergenicity (BUSINCO et al., 2000; CARROCCIO et al., 2000).

The principal caseins of cow milk are αs1- and β-CN. The composition of caseins in asinine milk (Vincenzetti et al. 2008; Criscione et al. 2009) has shown the presence of αS-1 and β-caseins in different phosphorilated and glycosylated forms, while the presence of κ-casein and αS2-casein is present in very low concentrations, differently from cow milk (Bertino et al. 2010; Creamer 2002).

The tolerance mechanism may be related to the specific levels of the main allergenic components of milk. Asinine milk, which is similar in composition to human milk, is an adequate alternative to children with allergies to cow milk protein (HY, 2007; Altemonte, 2019).

The proteins of cow milk, namely β-lactoglobulin and β-casein, are considered the main food allergens which affect infants whose enzymatic system is still not fully developed as well as adults (Villoslada et al. 2005). Asinine milk is similar to human milk (11, 14, 16) and more readily available; it has been successfully utilised in two clinical studies, with 9 (17) and 21 (18) children with APLV, and was found to promote adequate nutrition and good palatability.

The lactose content is one of the major similarities between asinine and human milk (SALIMEI, 2011; MUNK et al., 2017; MARTINI et al., 2018), in which it is present at concentrations of 6 to 7% (GUO et al., 2007; VINCENZETTI et al., 2008). Lactose is a disaccharide formed by glucose and galactose, and is chemically defined as O-β-D-galactopyranosyl-(1-4)-β-D-glucose (ADAM 2004). It can only be synthetized in the mammary gland, and it is generated from the transfer of an uridine phosphorylase (UDP)-bound galactose to a glucose molecule, with catalyzation by a galactosyl transferase (ADAM et al., 2012).

Information on the presence of milk-saccharides in asinine milk (CLAEYS et al., 2014; ALTOMONTE et al., 2019), although they have been reported, are presented as a similar composition and smaller amount when compared to human milk(WANG et al., 2014; CARMINATI; TIDONA, 2017a). ligosaccharides were identified in asinine milk (3-sialyllactose-SL), 6-sialyllactose-milk (Ossides6-SL) and disial-lacto-tetraose (DS-Sial-lacto-tetraose), in kectose from 12 to 45 mg/L, 12 to 52 mg/L and up to 11 mg/L, respectively(BUSINCO et al., 2000; MONTI et al., 2012).

2.3 Nutraceutical properties of asinine milk

Asinine milk is used for therapeutical purposes, such as a complementary treatment option for tuberculosis, gastric ulcers and metabolic diseases (LI; LIU; GUO, 2018). It is also used by the Maasai people in Kenya, where it is offered immediately after milking to children in order to control coughing, pneumonia or prevent diseases such as colds (FERNANDO; STARKEY, 2004).

It is known that many components of asinine milk may have benefits to the immune system and cognitive functions, namely proteins, fatty acids, lysozyme and lactoferrin (GICHURE et al., 2020).

Moreover, asinine milk is rich in bioactive molecules that are optimal in the prevention of intestinal infecctions in infants (ALTIERI et al., 2016) (AMATI et al., 2010), and the elderly (AMATI et al., 2010), such as lactoferrin, lysozyme and the concentration of PUFAs (COSENTINO et al., 2015; JIANG et al., 2018).

Lactoferrin acts in the first line of defense in the host (CONNEELY, 2001). Generally, this protein presents many biological functions independently of the species in question, such as antimicrobial activity (JENSSEN; HANCOCK, 2009) and immunological functions (BROCK, 2002; WARD; URIBE-LUNA; CONNEELY, 2002). The antiviral activity in humans and other animals is ascribed to its ability to inhibit the replication of a wide range of viruses through the prevention of infecction of host cells, binding directly to viral particles or to receptors and co-receptors in host cells ((PAN et al., 2007; HU et al., 2019).

Lysozyme in mammals is most abundant in respiratory secretions (GANZ et al., 2003). The lysozyme content in asinine milk is exceptionally high, representing 21% of the milk serum protein level, with concentrations between 1 to 4 mg/mL (PRGOMET et al., 2007). It is poorly digested by human gastrointestinal tract enzymes, allowing it to act selectively upon the intestinal microbiota and favouring a healthy composition (RANGEL et al., 2015). The antimicrobial effect of lysozyme begins when it catalyses the hydrolisis of glycosyl bindings of mucopolysaccharides in bacterial cell walls (CHIAVARI et al., 2005).

The concentration and types of fatty acids found in asinine milk also contribute to its nutraceutical quality. The relatively small size of its fat globules influences its ingestion and digestive kinetics (MARTINI et al., 2015; ALTOMONTE et al., 2019). The higher proportion of milk fat globule membrane (MFGM) also translates to higher concentrations of its components, namely phospholipids, cholesterol and polyunsaturared fatty acids (CARMINATI; TIDONA, 2017; CONTARINI et al., 2017). Phospholipids may exert health benefits (CONTARINI et al., 2017) by their anti-inflammatory activity and by reducing the risk of cardiovascular diseases (DONATO et al., 2011) . Phospholipids derived from milk may affect many cell functions, including growth and development, molecular transport systems and absorption processes, as well as neurological functions such as memory, stress responses, development of neurological disorders and myelinization in the Central Nervous System (SPITSBERG, 2005).

An ω-3 PUFA, eicosapentaenoic acid (EPA), may also exert immunomodulatory effects, often acting in tandem with docosahexaenoic acid (DHA). EPA stimulates the production of prostaglandin D3 by endothelial cells, which counteracts the effects of prostaglandin D2, an AA-derived metabolite causing neutrophil adhesion and transmigration (GUTIÉRREZ et al., 2012).

The significant lactose content favours the absorption of calcium and phosphorus, which may contribute to bone development and health, which makes asinine milk interesting for growth and also prevention of osteoporosis (MONTI et al., 2007; ASPRI et al., 2018). It also acts as a prebiotic, representing a substrate that favours a beneficial microbiota composition, by promoting species such as *Lactobacillus spp, Bifidobacteria spp, Bacteoides spp Clostridium spp* (A., 2004) SZILAGYI 2004) and inhibiting Bacterioides and Clostridium (ITO 1993), with various effects on general host defense, prevention of gastrointestinal tract diseases and inflammatory processes (AMATI et al., 2010; MARTINI et al., 2015).

3. OBJECTIVES

3.1 General objectives

To explore possibilities of reintroduction of donkeys in the Brazilian social and economic scenarios, through the sustainable and ethical production of asinine milk.

3.2. Specific objectives

a To formulate a diagnosis of the living conditions of abandoned donkeys kept in a restricted area through welfare assessment;

b To investigate whether a separation period of 2 hours in a manual milking system is stressful for Pêga jennies and foals;

c To characterize the milk of Pêga donkeys in relation to some physical and chemical characteristics, as well as lipid and protein profiles;

d To evaluate production, behavioural and immunological parameters of piglets supplemented with asinine milk before and after weaning.

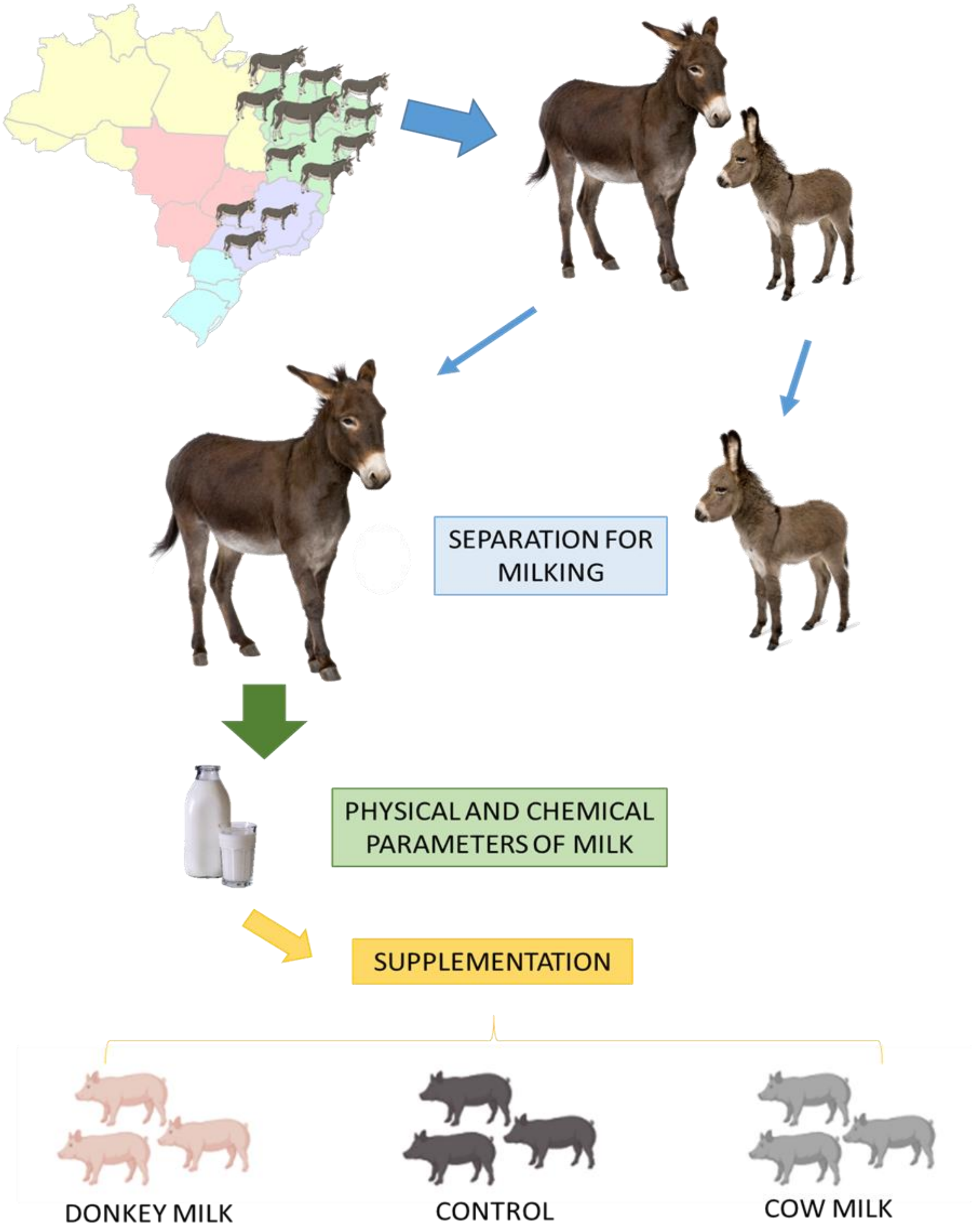
4. HYPOTHESIS

- It is possible to evaluate the welfare of donkeys housed in open areas, using the published AWIN welfare assessment protocol for donkeys;
- The milking management with two hours of separation between jennies and their foals promotes lower welfare for both jennies and foals;
- The physical and chemical profile of asinine milk of Brazilian breeds varies according to lactation days;
- Asinine milk improves production parameters as well as immunological and behavioural parameters of piglets during weaning.

5. GENERAL METHODOLOGY

The study was divided into three steps, as shown in the illustration (Figure 1).

Figure 1: Illustration of steps and distribution of chapters of this study.



5.1 Chapter 1 - Case study on the evaluation of welfare levels of donkeys destined for slaughter

In the first step, the welfare and living conditions of a group of donkeys which would be destined for slaughter were assessed. These animals were neglected and housed in a temporary holding farm for legal reasons. The AWIN protocol for the welfare assessment of donkeys was used, and the results were compiled and published in an article presented in Chapter 1.

5.2 Chapters 2- Welfare and productive performance of Pêga jennies

In this step, Pêga jennies from the Southwest region were milked, and behaviour and salivary cortisol concentrations were measured from both jennies and foals. The objective was to determine whether milking compromises the welfare of jennies and their foals, and whether the stress generated by the separation from the foals leads to changes in milk volume and composition. These resuls are published in an article presented in Chapter 2.

5.3 Chapters - 3 Assessment of physical and chemical qualities of asinine milk throughout lactation

For one year, milk was collected from a group of 14 jennies. Samples were collected before and after two-hour separation from the foals for milking, and were taken to a laboratory for physical and chemical analyses such as protein, fat, casein and total solids concentrations.

A regression analysis was carried out to verify how these components behaved throughout lactation. The results are presented in the article in Chapter 3.

5.3 Use of asinine milk to feed piglets in maternity phase

5.4 Chapter 4 – Can asinine milk mitigate the harmful effects of weaning stress in piglets?

The objective of this step is to evaluate the body weight gain, feed intake, molecular assessment of gene expression of NR3C1, IL-1B and HSD11, salivary cortisol levels and behaviour of piglets in maternity phase supplemented with Pêga asinine milk. The final article with the compiled results is presented in Chapter 4.

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CASE REPORT

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Case report: the use of the AWIN welfare assessment protocol to monitor a group of abandoned donkeys

Estudo de caso: o uso do protocolo AWIN de avaliação de bem-estar para monitorar um grupo de jumentos abandonados

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ABSTRACT

The objective of this study was to reach a diagnosis of the living conditions of abandoned donkeys kept in a restricted farm area through the assessment of their welfare level utilizing the AWIN protocol as a methodological tool. These animals were supposed to be sent to slaughter, but after the activity was temporarily banned, they were abandoned by traders. The protocol of welfare assessment was associated with general environmental and sanitary conditions. Information regarding the mortality rates was also gathered. According to the welfare assessment results, the living conditions of these animals were acceptable in some areas, despite the insufficient shade and shelter, a 3-month food restriction period, and a mortality rate of over 70%. These results demonstrate that welfare assessment protocols must be adapted to crises and databases for welfare indicators in diverse conditions must be created.

Keywords: Asinine. Assessment protocol. Behavioral assessments. Mistreatment. Northeast region.

RESUMO

O objetivo deste estudo foi formular um diagnóstico das condições de vida de jumentos abandonados mantidos em uma área restrita de uma propriedade através da avaliação de seu nível de bem-estar, utilizando o protocolo AWIN como ferramenta metodológica. Estes animais seriam destinados ao abate, porém, depois da suspensão temporária da atividade, foram abandonados pelos proprietários. O protocolo de avaliação de bem-estar foi associado às condições ambientais e sanitárias gerais. Informações sobre os índices de mortalidade também foram coletadas. De acordo com os resultados da avaliação de bem-estar, as condições de vida destes animais estavam aceitáveis em algumas áreas, embora não houvesse sombreamento e abrigo suficientes, um período de restrição alimentar de 3 meses e um índice de mortalidade acima de 70%. Estes resultados demonstram que protocolos de avaliação de bem-estar devem ser adaptados a situações de crise, e bancos de dados para indicadores de bem-estar em condições diversas devem ser criados.

Palavras-chave: Asininos. Protocolo de avaliação. Mensurações comportamentais. Maus-tratos. Região Nordeste.

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The world donkey population is approximately 50 million animals and has been decreasing in some regions (Food and Agriculture Organization, 2018), possibly due to rural exodus, increased mechanization in agriculture, and the rise in consumption of donkey meat. Another recent contributing factor is the exploitation of donkey skin to cater to the Chinese demands for traditional medicine, represented by the Eijao (Waters, 2019). In Brazil, the total donkey population is greater than 800.000 animals (Food and Agriculture Organization, 2018), and 86.7% of them are found in the Northeast region (Instituto Brasileiro de Geografia e Estatística, 2017).

In Brazil, donkey slaughter was recently prohibited by legal determinations, resulting in the abandonment of donkeys that were housed extensively in a farm area of 6ha in the city of Canudos, Bahia. Following the slaughter facility closures, there was an increase in mistreatment and mortality rates of the animals kept on the farm, which worked like a collection point.

The animals assessed in this study were housed in this farm after being captured and transported over distances that reached more than 1500 km, possibly in unsanitary conditions and without nutritional support, leading to their arrival at the farm in a severely debilitated state, which was further aggravated during their housing. As a result, the mortality rate for these animals was extremely high. This situation culminated in the employment of a national task force to investigate the living conditions of the donkeys at the farm.

The objective of this study was to reach a diagnosis of the situation through the assessment of the welfare level of donkeys kept on a farm, which worked as a collection point to bring together animals from various sources to be shipped for slaughter.

This study was approved by the Committee for the Use and Care of Animals in Research (CEUA) of the Federal University of Alagoas (UFAL), under the register nº 8696141117. Data collection was carried out between February 14-17, 2019, from 9 am to 2 pm when temperatures reached up to 36°C. The data was collected by two trained professionals as described in the AWIN protocol for donkey welfare assessment, which utilizes animal-based indicators to assess the welfare levels of animals (Animal Welfare Indicators, 2015) (Figure 1). The protocol was adapted to suit the conditions in the study, by conducting it without restraining or moving the animals.

The mortality rate was reported by the government veterinary team assigned to this situation. There were 800 donkeys on the farm, and as recommended by the protocol guidelines, 58 were assessed. They were chosen at random, from the groups of animals that were closest to the assessors.

The protocol holds as principles and criteria the good feeding (body condition score and water availability), housing (signs of thermal stress), and health (integument alterations, swollen joints, prolapse, hair coat condition, fecal soiling, discharges, abnormal breathing, coughing, and signs of hot branding), as well as appropriate behavior (expression of social behavior, stereotypes, and human-animal interaction) of animals. These correspond to the first level of welfare assessment, according to the AWIN methodology. Additionally, the environmental conditions of the property were also observed, such as the destination of carcasses and the presence of shade.

All collected data were submitted to descriptive analysis in Statistical Analysis System Institute (2013). Data are presented as percentages, the mean and standard deviation of assessments.

The assessed animals were 60.3% female (0.60 ± 0.49), and 100% of males were not castrated (Figure 2). The body condition scores (Figure 3) were considered ideal in 43.1% of animals (0.43 ± 0.49) and moderate in 29.3% (0.29 ± 0.45). There were no water troughs to be assessed, and the animals drank from natural water sources in which the water was scored as dirty (Figure 4). Thermal stress signs (Figure 5) were not present in 94.83%, of animals (0.94 ± 0.22).

Figure 1 – Trained professionals conducting the welfare assessment on the group of abandoned donkeys utilizing the AWIN protocol as a methodological tool. Source: The authors.

Regarding integument alterations, 60.3% presented skin lesions (0.60 ± 0,49), 22.41% presented alopecia (0.22 ± 0.42) and 13.79% presented deep wounds (0.13 ± 0.34). Swollen joints were not found in 84.48% of animals (0.84 ± 0.36), and 87.93% showed no signs of prolapse (0.87 ± 0.32) (Figure 6).

The coat condition was considered healthy in 81% of animals (0.81 ± 0.39). Fecal soiling was not found in 87.93% (0.87 ± 0.32). Abnormal breathing was absent from 94.83% of animals (0.94 ± 0.22), and coughing was absent from 100% (Figure 7). Ocular discharge was absent in 84.48% of animals (0.84 ± 0.36), nasal discharge was absent in 94.83% (0.94 ± 0.22) and 89.66% showed no signs of vulvar or penis discharges (0.89 ± 0.30) (Figure 8). Signs of hot iron branding were not found in 87.93% (0.87 ± 0.32) (Figure 9).

Based on the behavioral observations (Figure 10), we noted that 89.6% of donkeys were sociable within their group (0.89 ± 0.30), 96.55% showed no signs of stereotypes (0.96 ± 0.18), 55.2% usually did not avoid human interaction (0.55 ± 0.50), 58.6% stood still in response to human movement (0.58 ± 0.49) and 55.2% showed no alterations in tail position, even when parallel to a person (0.55 ± 0.50).

These results can be partially explained by the sample of animals assessed: the ones who could be assessed by researchers were closest to humans and showed more social behaviors, while feral or “less sociable” donkeys hid and could not be reached. In this sense, it was not possible to assess a large number of animals, as they fled in small groups to distant areas of the farm. It is well-known that donkeys are gregarious animals, adapted to living in small groups (Rudman, 1998), and due to their prey condition, when frightened, they present natural “fight or flight” responses (Burden & Thiemann, 2015).

The mortality rate for the animals between their arrival at the farm and the time of assessment was over 70%. On the farm, there was little shade available, and the animals could only drink from two natural water sources, exposed to the sun. The water was dirty and there were many carcasses scattered in the surroundings, which could lead to contamination and intoxication of animals (Figure 11).

Based on these results, the welfare assessment protocol underestimated the extremely poor welfare conditions of the animals. The unacceptable mortality rates reported for the animals and the environmental conditions of the farm were not part of the AWIN protocol. It is important to note that protocols for welfare assessment indicate risks of welfare issues, and not a complete picture of the animal’s welfare at the time (Rousing et al., 2001).

The high resilience and adaptability of donkeys (Davis, 2019) combined with the natural social isolation behavior of sick and threatened animals (Hart & Hart, 2019), may have generated the assessment of only the strongest and best-adapted individuals, as well as those with better human-animal relationships, which remained close to the assessors.

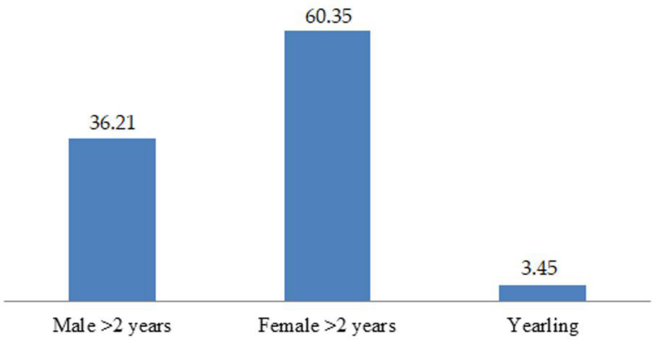

Figure 2 – Percentages of males older than 2 years, females older than 2 years, and yearlings (animals of both sexes aged between 1 and 2 years) in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

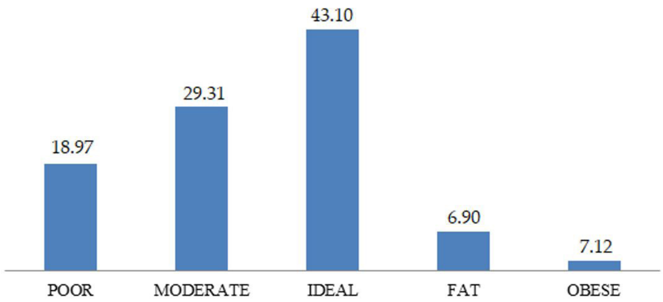

Figure 3 – Percentages of animals in each category of the Body Condition Score scale in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

Figure 4 – One of the natural water sources from where the abandoned donkeys drank at the farm, as part of the water availability assessment utilizing the AWIN protocol as a methodological tool.

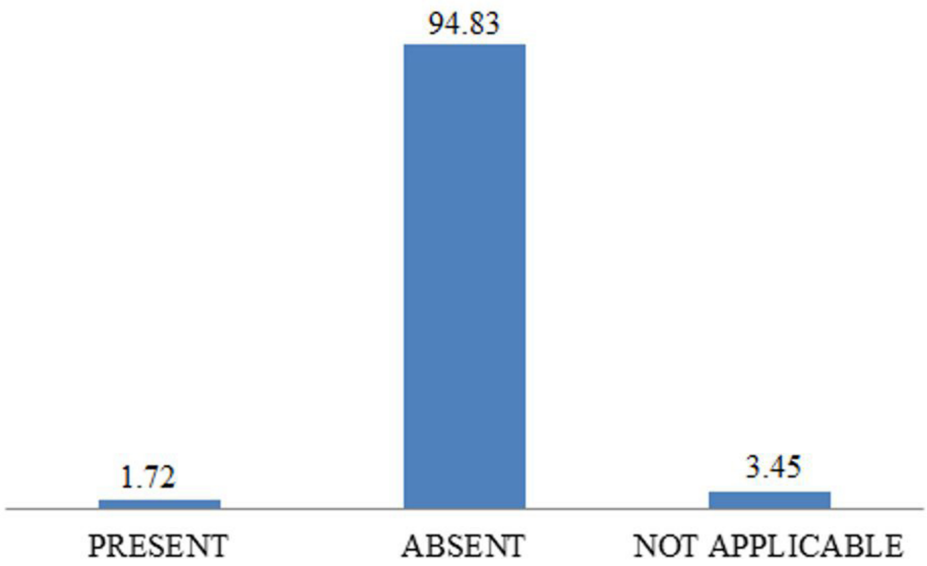

Figure 5 – Percentages of animals showing signs of thermal stress in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

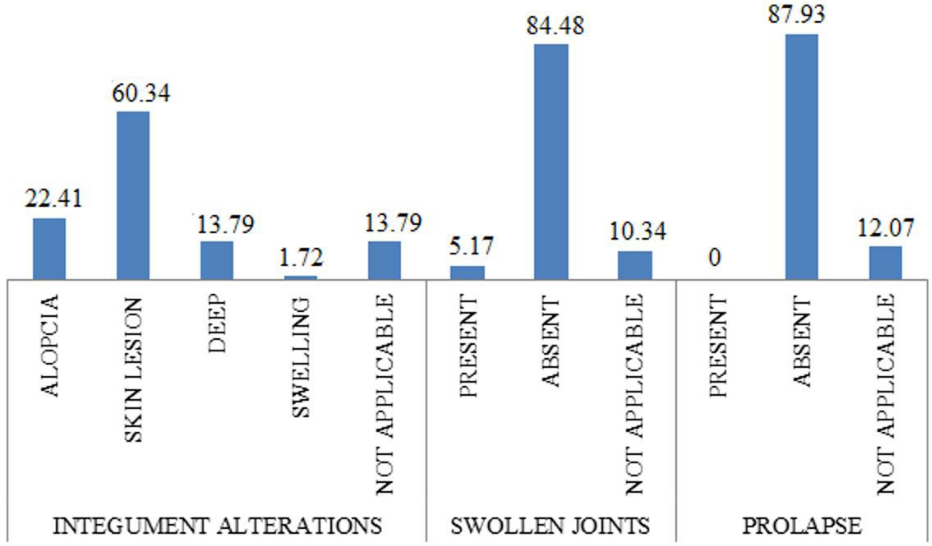

Figure 6 – Percentages of animals showing signs of injuries in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

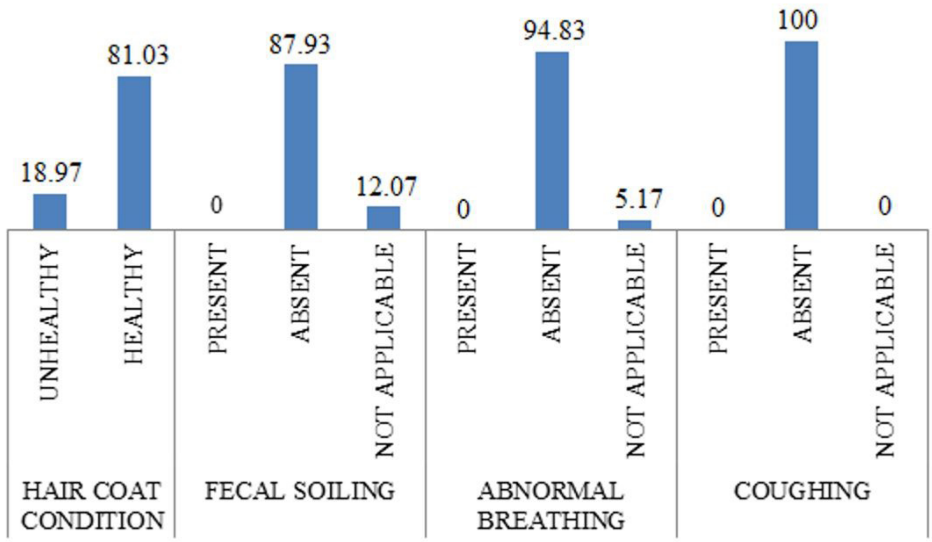

Figure 7 – Percentages of animals in each category of hair coat condition and showing signs of fecal soiling, abnormal breathing, and coughing in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

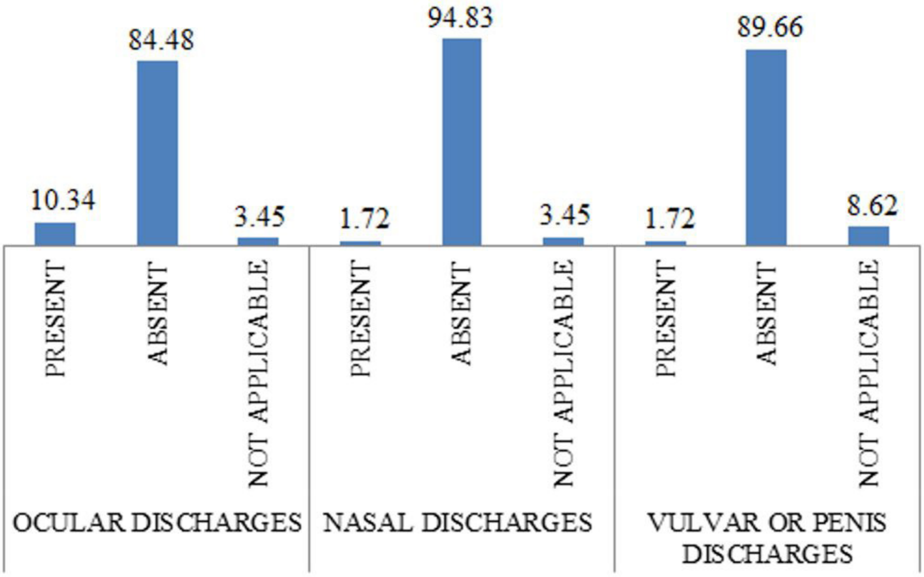

Figure 8 – Percentages of animals showing signs of ocular, nasal, and vulvar or penis discharges in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

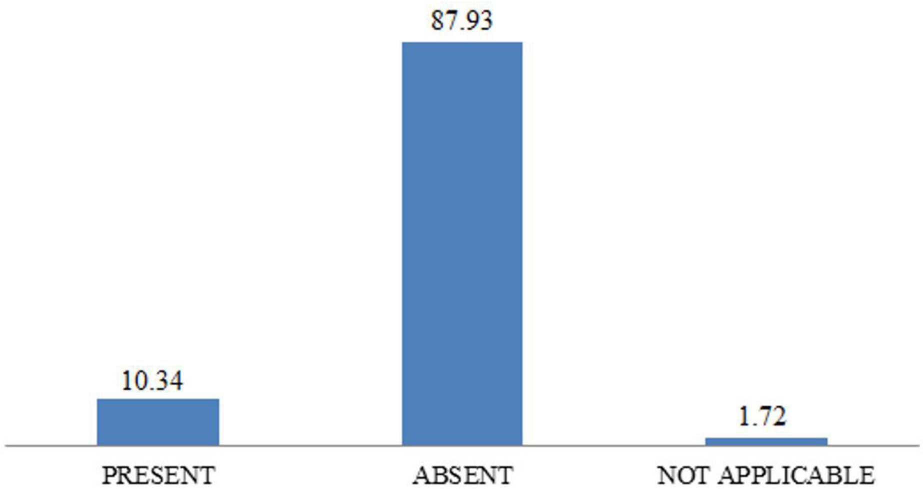

Figure 9 – Percentages of animals showing signs of hot iron branding in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

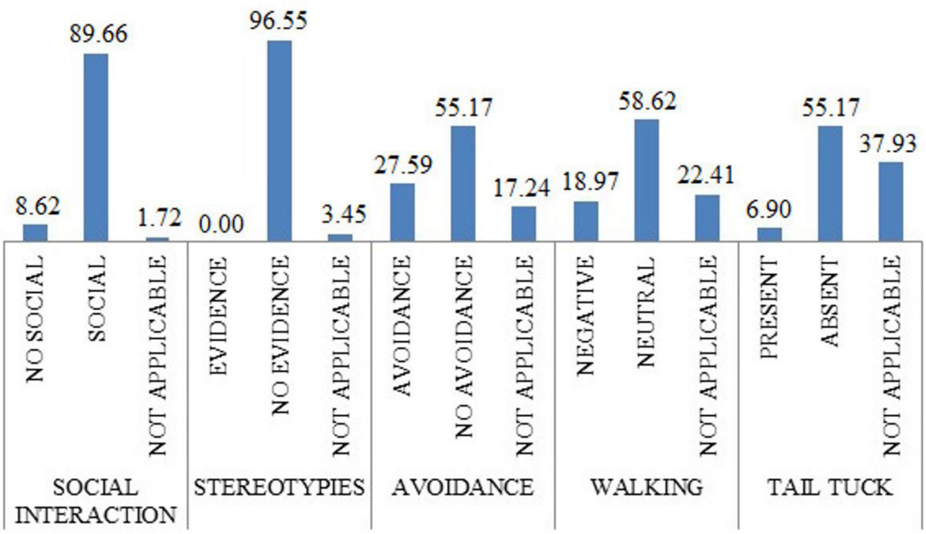

Figure 10 – Percentages of animals performing social interaction, stereotypes, and reactions to human-animal relationship tests (avoidance, walking, and tail tuck) in the group of abandoned donkeys at the farm utilizing the AWIN protocol as a methodological tool.

Figure 11 – Presence of carcasses among the abandoned donkeys in the farm in Canudos, BA, as part of the additional environmental conditions assessment conducted in the study. Source: The authors.

The high mortality rate could be explained by possible improper practices in their capture, transport, and the precarious living conditions on the farm. Little is known about the effects of transportation in donkeys (Fazio et al., 2013), but it is well reported as a stressful event for horses, which may lead to serious health disorders and death (Padalino et al., 2016).

The nutritional and environmental conditions on the farm may also have augmented mortality rates. It is known that donkeys are hindgut fermenters and require 1.3-1.8% of their body weight ingested as dry matter daily (Liu et al., 2020). Although they are highly tolerant of thirst, their daily water intake must be proportional to the ambient temperature and humidity, as well as their reproductive stage (Burden & Thiemann, 2015). Shade and shelter also were not sufficiently provided on the farm. Although the skin of donkeys is suited to direct sunlight and extreme heat (Knottenbelt, 2019), excessive light exposure may cause phototoxicity in equids (Williams & Barrif, 2011).

The lack of sanitary control, with no clinical evaluation, quarantine for sick animals, and removal of feces or carcasses from the pasture may also lead to various infections and parasite infestations to spread in the studied farm (Knottenbelt, 2019). Very few important requirements to maintain the health and welfare of the animals were met during the three months that they lived in the property before the welfare assessment. This may have generated a refusal for food and water, placing their health at further risk with the development of hyperlipemia, which may have contributed to their high mortality (Burden & Thiemann, 2015).

This data suggests the importance of conducting welfare assessments in crises, together with clinical veterinarian examinations. Examinations to assure the sanitary and nutritional conditions of animals must be collected before, during, and after their capture, transportation, and housing in the collection farms that function almost as warehouses. Some welfare protocols already include other indirect measurements, such as assessments of resources and questionnaires for farm owners (Pritchard et al., 2005), which could help to build a more complete picture of the situation.

Based on our results, we observed that the limited number of donkeys assessed in this period were in acceptable welfare conditions in all parameters but water quality, based exclusively on the AWIN protocol. However, due to various welfare challenges and their extremely poor overall condition, many animals did die right before the study was conducted. This demonstrates the need for adaptation of welfare protocols to crises, and refinement of welfare assessment techniques. It is also crucial to generate a database for donkey welfare worldwide so that a solid repertoire can be created regarding adequate welfare for diverse situations.

Conflict of Interest

The authors whose names are listed in this publication have no conflict of interest or financial or non-financial interest in the content discussed in this manuscript.

Ethics Statement

The current study was conducted after the review and approval carried under the protocol 21/2019 issued by the Universidade Federal de Alagoas.

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Article

Two Hours of Separation Prior to Milking: Is This Strategy Stressful for Jennies and Their Foals?

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Simple Summary: The economic importance of donkeys has decreased in Brazil, which has led to their mass abandonment. Asinine milk production is a potential solution to the reintroduction of donkeys into the Brazilian social and economic scenario. The milk has nutraceutical properties that make it valuable for human consumption, and thus a donkey dairy industry is likely to help stop their abandonment. That said, in any such industry, the welfare of jennies maintained for milk production must be guaranteed. Few studies have been published measuring the impact of milking management on the welfare of jennies and foals, and the potential behavioural and physiological challenges it may cause. It is also unknown whether these animals adapt to the milking routine. The goal of this study was to assess the impact of separating Pêga jennies from their foals for 2 h on indicators of welfare. Animal welfare was analysed through behavioural and hormonal assessments, their potential adaptive responses and effects on milk yield. Few significant alterations were found in behaviour, salivary cortisol concentrations, or milk yield as a result of the 2-h separation, which could indicate that the welfare of the animals was not compromised; however, the adaptation of jennies and foals to separation stress remains to be fully verified. The 2-h separation period, based on the reported data, is possibly not a stressful experience for the assessed group of Pêga jennies and foals. The reported protocol, which included frequent positive interactions with the animals, may be useful to assure acceptable animal welfare levels for donkeys in small-scale dairy production settings.

Abstract: The goal of this study was to assess whether or not a separation period of 2 h is stressful for jennies and foals, as measured by changes in behaviour, salivary cortisol, and milk production. This study was reviewed and approved by the Committee for the Use and Care of Animals in Research (CEUA) of the School of Veterinary Medicine and Animal Science of the University of São Paulo. Fourteen multiparous Pêga jennies (245 kg average body weight) and their foals were assessed from day 45 to 135 of lactation. Dams and foals were separated for 2 h prior to milking. Behavioural assessments and saliva samples were collected before and after separation, every 15 days, resulting in 14 samples per individual animal. Behavioural states (affiliative and inactivity) and events (agonistic, abnormal, eliminative and vocalisations) of the jennies were observed during 6 min in both periods. Moreover, milk yield was measured. Few significant behavioural and salivary cortisol changes were observed, and milk yield was not affected by cortisol levels in response to the separation. The 2-h separation period, on the basis of the collected variables, did not appear to be stressful for the assessed group of Pêga jennies or foals; however, their ability to adapt to milking routine stress remains to be investigated.

Keywords: animal welfare; behaviour; donkey; cortisol; milking management

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1. Introduction

Donkeys have been losing their relevance in Brazilian social and economic scenarios, having been less used in recent decades in their traditional role as animals of draft and burden. This trend can be ascribed, essentially, to the diffusion of mechanisation in agriculture [1], and the subsequent decrease in the number of donkeys used on rural properties. As a result, they have been omitted from official data and statistics collected by the Brazilian government [2], and their abandonment has been intensified, leading to increasing numbers of wandering animals with compromised welfare [3].

There are three registered Brazilians donkey breeds: Nordestino, Paulista, and Pêga [4]. Out of these breeds, the Pêga has been most developed for its genetic potential. It was developed in Brazil in 1810 [5], and is composed of medium sized donkeys, primarily bred to supply the market with mules [6]. Pêga donkeys are most commonly found in the Southeast region, and although they have a clear economic importance, productive donkey farms in Brazil are scarce and these animals are still generally left out of the social and economic scenario in the country.

In other countries, such as France and Italy, donkeys are still valued because of their milk which is used for human nutrition [7,8]. Asinine milk possesses similar chemical and organoleptic qualities to human milk [7,9], representing one of the best nourishment options, besides maternal milk, for human babies [10] that cannot be breast-fed and for consumers suffering from cow milk protein allergies [11,12]. The milk of Brazilian Pêga donkeys may have similar nutritional potential to that of Italian and French breeds, and thus their use for sustainable, high welfare, donkey dairy production is a possible means of reintroducing donkeys as an important species in the Brazilian socioeconomic scenario. The use of animals for milk production must guarantee their welfare. There is a limited number of studies regarding stressors that trigger physiological and behavioural changes, generated by the milking management in donkeys [13].

The milk storage capacity in this species is low (less than 2.5 L) [14], thus milk production is dependent on its removal from the mammary gland, generally by milking or suckling. In the latter, milk ejection is triggered by a foal's sucking, which triggers the release of oxytocin that in turn induces the contraction of myoepithelial cells [15]. Milking of jennies by humans, in terms of both human and animal safety and for optimal milk extraction, is more manageable when foals are not physically present [16].

In order to achieve efficient milking, jennies must be milked after 2 to 3 h of physical separation from their foals [17]. Long intervals between milking events may cause a rise in intra-udder pressure, inducing early cessation of glandular activity [18], due to the udder size and its low storage capacity. Therefore, donkeys may need to be milked multiple times a day [19].

The social structure of donkeys is composed of a territorial-based system [20–22], with complex hierarchies within groups [23]. The only permanent bond among donkey social structures is between jennies and their foals [24].

In precocious animals such as donkeys, the neonatal period is characterized by intense interactions between mothers and newborns, which are important for bonding and allow for the development of autonomy in the offspring, including motor, sensorial and cognitive processes [24]. In natural conditions, jennies begin approaching their foals less frequently after the first day post-partum, grazing at further distances while the foals rest and allowing them to interact with other animals [25]. During the first five days of life, donkey foals suckle every 3 to 10 min, and every 20 to 30 min by the 10th day [26]. In mule foals, the suckling frequency between 4 and 17 weeks varies between two and three bouts per hour [27].

Behavioural and social impairments have been reported in ungulates separated from their mothers for 2.5 h after birth [28,29]. The separation between jennies and foals may be stressful [30,31], and these animals could respond via behavioural and physiological changes [32]. When the restoration of homeostasis in response to a stressor is difficult, such as when animals cannot move to a more favourable environment, they may express behavioural signals such as vocalisations [33], stereotypic behaviour [34], increased inactivity [35–37] and altered social interactions [38]. Behavioural responses facilitate physiological adaptations, which may manifest via the activation of the autonomous nervous system and neuroendocrine system [39]. The stimulation of central circuits involving the amygdala, hypothalamus and periaqueductal gray (PAC) result in an increased frequency of eliminative behaviour [40], and increased releases of corticotropin-releasing factor (CRF), from the hypothalamus, and adrenocorticotropic hormone (ACTH), from the pituitary gland culminate in an increase in the secretion of glucocorticoids, such as cortisol [41], from the adrenal glands. Alveolar milk ejection could also be altered by fear or stress, due to the influence of these endocrine factors on oxytocin concentration and myoepithelial contraction [42,43].

To our knowledge, there are no scientific studies concerning the adaptive responses of jennies and foals to repeated separation and milking procedures, and so it is crucial to determine their behavioural and physiological changes when exposed to this routine. Therefore, further investigations assessing the welfare effects, if any, of milking procedures on dairy jennies and their foals are required.

This study aimed to investigate whether a separation period of 2 h in a manual milking system is stressful for Pêga jennies and foals, i.e., whether it generated changes in behaviour, or caused changes in salivary cortisol concentration and milk production, and thus, if the use of donkeys for sustainable, high welfare, donkey dairy production is a model for their economic reintroduction in Brazil. For this purpose, a manual milking protocol was proposed and implemented.

2. Animals, Materials and Methods

This study was reviewed and approved by the Committee for the Use and Care of Animals in Research (CEUA) of the School of Veterinary Medicine and Animal Science of the University of São Paulo, under the protocol number CEUA 8696141117 (ID 007216).

2.1. Animals, Housing and Management

The study was conducted in Criatório Ximbó, a donkey farm in the city of Laranjal Paulista in the state of São Paulo, Brazil. The city of Laranjal Paulista is located at an altitude of 536 m, at the coordinates 23°02′59″ latitude South and 47°50′12″ longitude West. The local climate is humid subtropical (Köeppen–Geiger classification), the yearly average temperature ranges from 13 ± 4.9 °C to 31 ± 4.7 °C, and the yearly average pluviosity is around 1177 mm.

On the farm, donkeys are kept in a semi-intensive system, and receive nutrition composed of native foliage and *Brachiaria decumbens*, as well as alfalfa hay supplementation.

The donkeys on the farm are kept in pastures during the day and are moved to stalls of 24 m^2 stalls (6 m × 4 m) overnight, in stable groups of 3 (Stall 1, 8 m^2 allowance per jenny) to 4 jennies (Stall 2, 6 m^2 allowance per jenny) and their foals in each stall. In the morning, they are released by simply opening the stall door. All animals assessed in this study were habituated to this daily routine.

The farm had no dairy production activity, and the 60 Pêga jennies on the farm were used for reproduction. Fourteen multiparous Pêga jennies (245 kg average body weight) were studied from day 45 to 135 of lactation. Of the fourteen jennies assessed, seven foaled in February 2018 and were assessed until June, and seven foaled in May and were assessed until September of the same year. No milk was collected during the first month of each foal's life as it was used exclusively for their nutrition in the month of June, around day 135 of lactation for the first group and day 45 of lactation for the second group, all fourteen jennies and their foals were assessed at once, resulting in some animals being separated for up to 3 h.

For data collection, the established groups of jennies were maintained and minimal changes to the already established farm routine were made.

2.2. Experimental Design

On each data collection day, while still inside of the pens, together with their group, the behaviour of jennies in Stall 1 was assessed and saliva was sampled from both jennies and foals. The animals were then separated for 2 h. The same procedures were repeated in Stall 2. In order to separate them, two people stood at the stall door and allowed the jennies to pass through while impeding the foals from following. The jennies were stimulated to leave the stall using visual and sound cues, such as raising hands and clapping, and the foals were stopped by standing in their path. During the separation period, the jennies were loose on the farm, while the foals were kept inside of the group stalls with no visual contact with their dams. Information regarding the stall of each animal, as well as the exact time of release from the stalls, was recorded on each data collection day in order to keep the groups and separation times constant throughout the study.

After 2 h of separation, the jennies were led, one by one, to the milking parlour. This was the first instance they were restrained, utilising a halter and loose lead rope to keep them from leaving the parlour. At this time, their respective foals were brought to the milking parlour from the stalls, in less than one minute, marking the end of the separation period. The jennies' behaviour was then assessed, and saliva samples were taken from both jennies and foals. The foals were not restrained, but were stopped from suckling by placing a hand between their mouth and the dam's teat.

It was observed that, from the third collection day onwards, for both the February and May groups, the jennies tended to wait at the milking parlour by the end of the separation time, and did not need to be brought back from elsewhere on the farm.

During milking, an additional safety measure was taken by firmly tying a lead rope to their hind limb and securing it to a fence. The milk yield was noted.

Separation of jennies and foals took place at 10:00 am. Saliva samplings were conducted, before separation, between 8:50 am and 9:50 am, and after separation, between 12:00 pm and 2:00 pm.

Behavioural assessments and saliva samplings were performed from day 45 to 135 of lactation, totalling 14 assessments per animal. All data were collected every 15 days, to assess the possible adaptation of these animals to the stress generated by the milking management routine.

2.3. Behaviour Assessments

For the behavioural assessments, jennies were identified with ribbons of different colours attached to their necks. The protocol used for behaviour assessment was focal sampling with continuous recording, performed directly by two trained assessors utilising a check sheet.

The occurrence of behavioural states (long-duration behaviours such as prolonged activities, measured in time intervals between the beginning and end of each episode) and events (instantaneous or short-duration behaviours) were observed in the jennies in the pre- and post-separation periods and were later evaluated.

The observed behavioural states were affiliative behaviour and inactivity, and the events were agonistic, abnormal and eliminative behaviours, as well as vocalisations. These behavioural categories were chosen as they could be affected by the presence of a stressor [33–38], and various aspects of behaviour were assessed in order to paint a complete picture of any alterations the jennies exhibited between the pre- and post-separation assessments. It was expected that, if these animals were stressed by the separation, the duration of these behavioural states would be altered with potential increases in inactivity [35–37] and decreases in affiliative behaviour [38] post-separation, and the frequency of occurrence of these behavioural events would increase post-separation [33,34]. For these assessments, recording sheets based on an experimental ethogram, developed in this study, were used (Table 1). All observations yielded focal observation data from each animal, with a 6-min duration for each jenny in each assessment [44].

Table 1. Experimental ethogram utilised to assess jennies' behaviour.

Affiliative	
Mutual grooming	Behaviour in which two donkeys use their teeth to simultaneously nibble any of each other's body parts.
Licking	Licking any part of the body of another donkey.
Body sniffing	Sniffing the neck, withers, flank or tail of another donkey which may or may not reciprocate.
Approaching	Moving to within 1 m of another donkey that does not immediately move away and staying there for at least 10 s without initiating physical contact with it.
Touching	Touching another donkey at the neck or head, which may or may not reciprocate.
Agonistic	
Kicking	Rapid lifting of one or both hind limbs off the ground, directed towards another donkey or the observer, in an attempt to hit them, with the ears laid back.
Pushing	Pressing head, neck, chest or shoulder against another donkey, making them move away.
Chasing	Rapid movement toward another donkey and pursuit for a distance of over three body lengths, with the ears laid back, head raised and mouth closed.
Biting	Extension of head and neck towards another donkey, with the ears laid back, head raised and mouth open, closing teeth on its body.
Fighting	Pursuing another donkey for a distance of over three body lengths, with ears laid back, head raised and mouth open, attempting to close teeth on its body.
Abnormal	
Biting the stalls or structures	Grasping of structures with incisive teeth, which may be followed by simultaneous arching of the neck and sucking of air (cribbing).
False licking	Behaviour in which the animal slowly places its tongue on the borders of the stall or trough while keeping it still and stiff, so the action does not represent true licking.
Pawing	Vigorous and persistent stomping of limbs on the ground.
Eliminative	
Urinating	Elimination of urine.
Defecating	Elimination of faeces.
Vocalisations	
Vocalisations	Expression of vocal communication, such as whinnies, snores, snorts, groans or screams.
Inactivity	
Inactivity	Absence of movement or other actions.

Behaviours considered abnormal were biting the stalls or structures, false licking and pawing. Eliminative actions were urinating and defecating [45].

The recorded vocalisations included various types of vocal communication sounds, such as whinnies, snores, snorts, groans and screams [33].

Social interactions were divided between affiliative and agonistic according to the performed action and response of the receiving animal. In the absence of signs of aggression [46], interactions were considered affiliative, and behaviours linked to aggression were considered agonistic. The observed affiliative interactions were grooming, licking, sniffing, approaching and touching [46,47]. Agonistic interactions were kicking, pushing, chasing, biting and fighting [36]. All social interactions were performed between jennies and foals or other jennies.

After the behavioural assessment of the jennies, saliva was sampled from all animals in the pre- and post-separation periods.

2.4. Saliva Sampling

Saliva samples were collected from each animal using an individual sampler developed for this study, which did not require the animals to be restrained. For the jennies, the collector was made of ground *rapadura* (sugarcane candy) wrapped in gauzes and a cotton string. The inclusion of *rapadura* was necessary to stimulate saliva production in the jennies. For the foals, only gauzes and cotton strings were used.

The samplers were presented to all animals by holding them stretched, and both jennies and foals voluntarily approached the assessors to chew on the samplers. Sometimes, the samplers were secured to the animals' necks with the cotton strings, while the procedure was carried out on the rest of the animals.

Jennies and foals chewed the collectors for 2 min, after which the strings were cut and discarded, and the gauzes were placed in 15 mL Falcon tubes with their respective identifications. The tubes were stored in sealed styrofoam boxes lined with reusable gel ice packs.

2.5. Milking

Milking procedures began with the cleaning of the jennies' udders and teats with soap and water and drying with paper towels. They were milked manually.

The milking stopped once the udders were fully emptied, after which they were cleaned and dried again. After these procedures, each teat was submerged in a post-dipping solution (Dermasoft 2.5%, composed of Povidone-iodine (2.5 g) and purified water (100 mL)) for at least 15 s.

Milk yield was noted for each jenny on every assessment day.

2.6. Salivary Cortisol Analysis

The 15 mL Falcon tubes containing the saliva from jennies and foals were stored at –20 °C until the salivary cortisol analysis, which occurred between June 2018 and May 2019. For storage, the samples were thawed in the fridge and extracted from the gauze via centrifugation. The gauzes containing the samples were centrifuged for 15 min at 1000× *g*, and the extracted fluids were placed in 1.5 mL microtubes. These were then frozen again until analysis. The analysis was performed by trained professionals following EIA protocols, developed and validated by previously reported publications [48,49].

Additionally, *rapadura* was added to a standard curve, and no effect was observed in the performance of the assay.

2.7. Data Analysis

The data for the affiliative and inactivity behaviours were studied through the Poisson distribution, according to the PROC GLIMMIX of SAS, utilising a randomised block design with repeating measurements for the duration of occurrence of the observation in question, over time. Blocks were defined by the days of lactation. The model includes the effect of observation time in two different periods (before and after separation).

Data from the events of the behavioural categories agonistic, abnormal, eliminative and vocalisation were studied through the Poisson distribution, according to the PROC GLIMMIX of SAS, utilising a randomised block design with repeating measurements over time. Blocks were defined by days of lactation. The model includes the effect of observation time in two different periods (before and after separation).

For the salivary cortisol data, the Shapiro–Wilk test was conducted to analyse the normality of the residues, and the fixed effects were analysed by PROC GLIMMIX. The studied model included the effects of observation in two different periods (pre- and post-separation).

The milk yield data were analysed in a randomised block design. The statistical model considered the day of lactation to be a fixed factor and the animal (block) effect to be a random factor, defined by the RANDOM command. Fisher's Least Significant Difference was used when the fixed factors were significant for both analyses. The PROC CORR procedure was used for determining the Pearson correlation between milk yield and salivary cortisol concentration for the jennies.

All analyses were done in the Statistical Analysis Software 9.4 (SAS) [50]; the adopted significance level was set at p <0.05.

3. Results

3.1. Behaviour

The frequency of occurrence of abnormal behaviour, vocalisation, eliminative behaviour and agonistic behaviour of jennies on day 45, 60, 75, 90, 105, 120 and 135 of lactation, before and after 2 h of separation from their foals for manual milking, are presented in Figures 1–4.

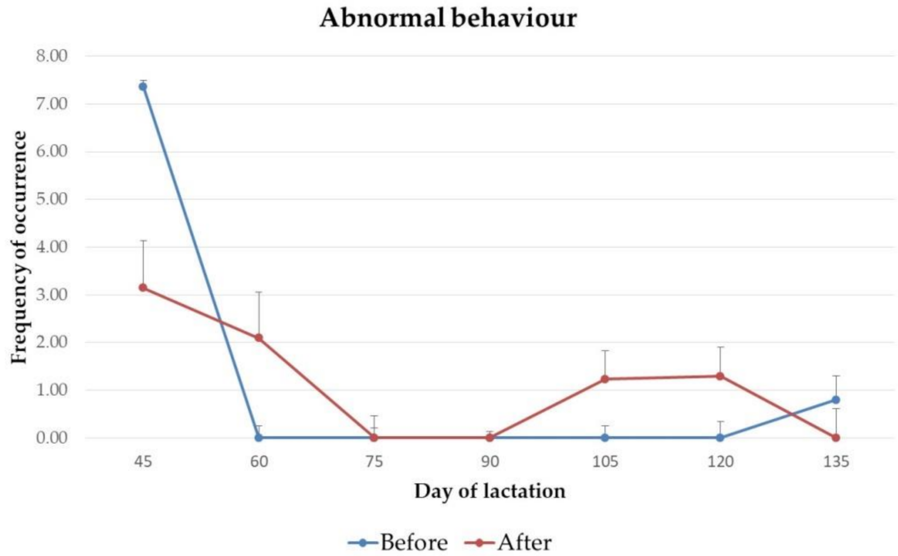

Figure 1. Mean frequency and standard deviation of occurrence of abnormal behaviour from jennies, before and after 2 h of separation from their foals for milking, on day 45 (p = 0.99), 60 (p = 0.99), 75 (p = 0.99), 90 (p = 1.00), 105 (p = 0.99), 120 (p = 0.99) and 135 (p = 1.00) of lactation.

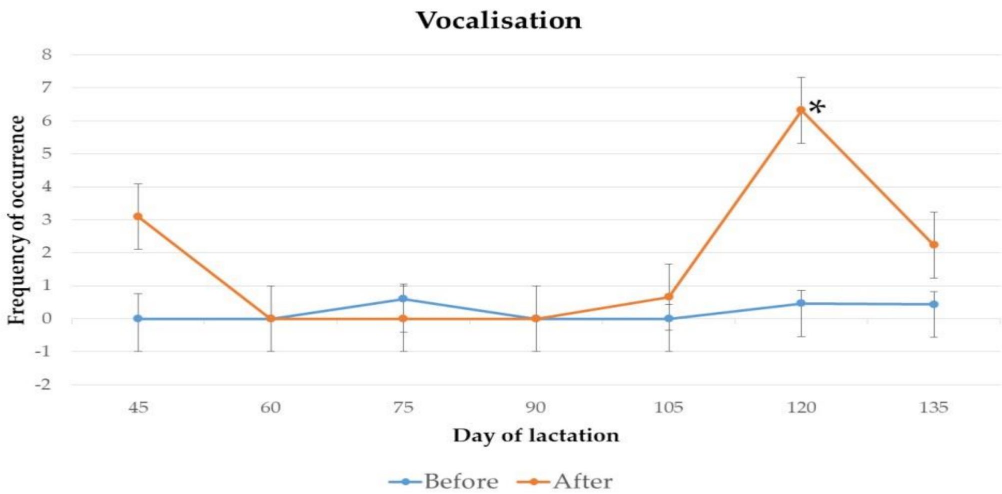

Figure 2. Mean frequency and standard deviation of occurrence of abnormal behaviour from jennies, before and after 2 h of separation from their foals for milking, on day 45 (p = 0.99), 60 (p = 0.99), 75 (p = 0.99), 90 (p = 1.00), 105 (p = 0.99), 120 (p = 0.03) and 135 (p = 0.09) of lactation. * indicates a statistically significant difference between frequencies.

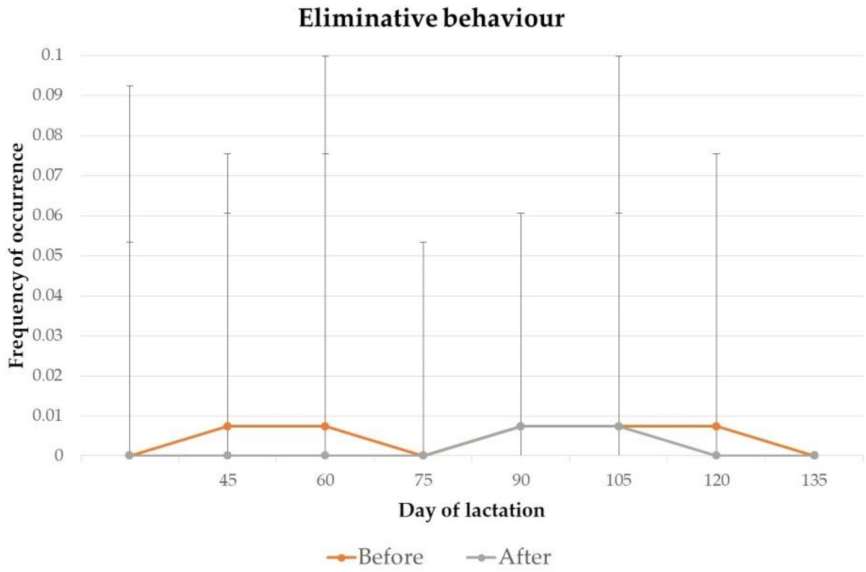

Figure 3. Mean frequency and standard deviation of occurrence of eliminative behaviour from jennies, before and after 2 h of separation from their foals for milking, on day 45 (p = 0.99), 60 (p = 0.99), 75 (p = 0.99), 90 (p = 0.99), 105 (p = 0.99), 120 (p = 0.99) and 135 (p = 0.99) of lactation.

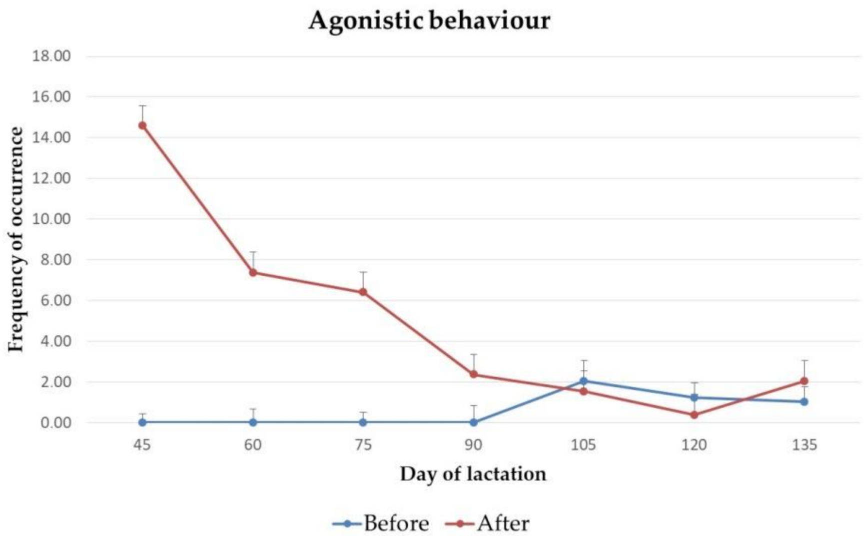

Figure 4. Mean frequency and standard deviation of occurrence of agonistic behaviour from jennies, before and after 2 h of separation from their foals for milking, on day 45 (p = 0.99), 60 (p = 0.99), 75 (p = 0.99), 90 (p = 0.99), 105 (p = 0.69), 120 (p = 0.40) and 135 (p = 0.40) of lactation.

Statistically significant differences were found for the frequency of vocalisations (p = 0.03) from jennies, on day 120 of lactation. No significant differences were found for the frequency of abnormal, eliminative or agonistic behaviours throughout lactation.

The duration of occurrence of affiliative behaviour and inactivity of jennies on day 45, 60, 75, 90, 105, 120 and 135 of lactation, before and after 2 h of separation from their foals for manual milking, are presented in Figures 5 and 6.

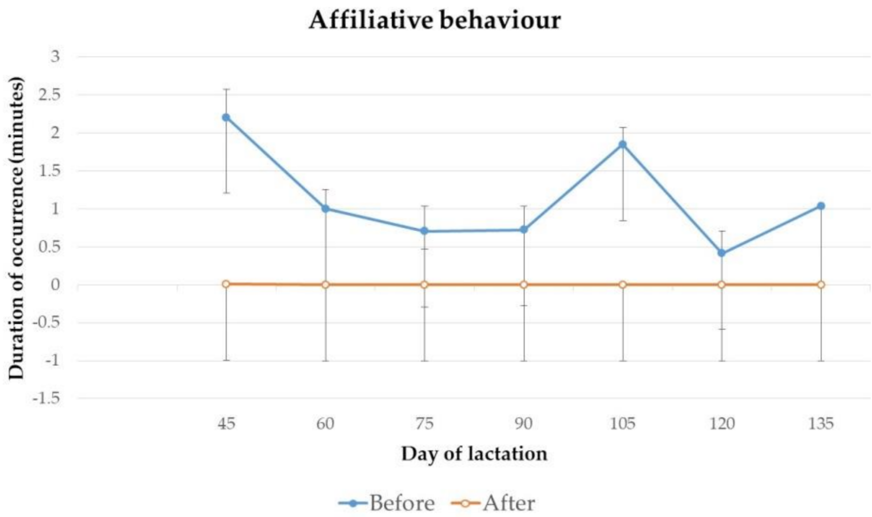

Figure 5. Mean duration and standard deviation of occurrence of affiliative behaviour from jennies, before and after 2 h of separation from their foals for milking, on day 45 (p = 0.99), 60 (p = 0.99), 75 (p = 0.99), 90 (p = 0.99), 105 (p = 0.99), 120 (p = 0.99) and 135 (p = 0.99) of lactation.

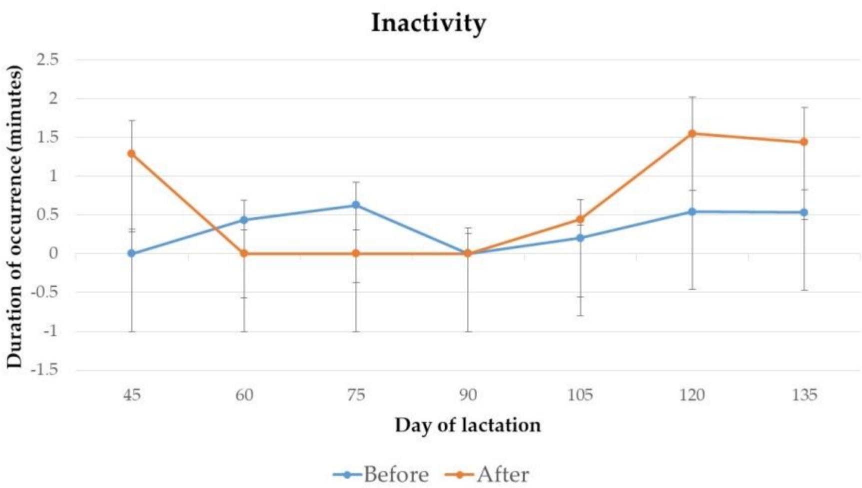

Figure 6. Mean duration and standard deviation of occurrence of inactivity from jennies, before and after 2 h of separation from their foals for milking, on day 45 (p = 0.99), 60 (p = 0.99), 75 (p = 0.99), 90 (p = 0.99), 105 (p = 0.44), 120 (p = 0.07) and 135 (p = 0.11) of lactation.

No significant differences were found for the duration of affiliative behaviour and inactivity from jennies throughout lactation.

3.2. Salivary Cortisol Concentration

The salivary cortisol concentration of jennies on day 45, 60, 75, 90, 105, 120 and 135 of lactation, before and after 2 h of separation from their foals for manual milking, are presented in Figure 7.

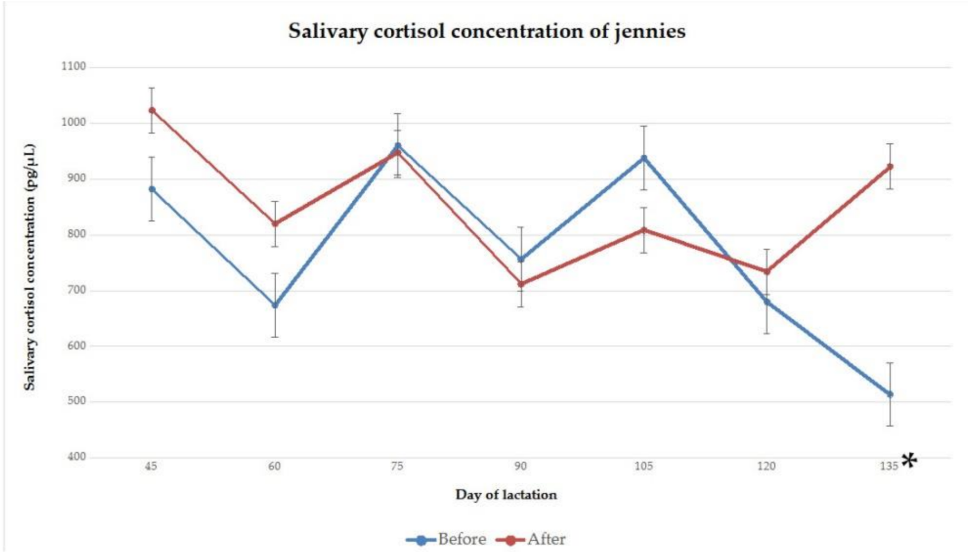

Figure 7. Mean and standard error of salivary cortisol concentration of jennies before and after 2 h of separation from their foals for milking, on day 45 ($p = 0.41$), 60 ($p = 0.40$), 75 ($p = 0.94$), 90 ($p = 0.79$), 105 ($p = 0.44$), 120 ($p = 0.77$) and 135 ($p = 0.02$) of lactation. * indicates a statistically significant difference.

A statistically significant difference between salivary cortisol concentrations of jennies before and after separation was found on day 135 of lactation ($p = 0.02$), but not on day 45, 60, 75, 90, 105 or 120 of lactation ($p > 0.05$).

The salivary cortisol concentrations of foals on day 45, 60, 75, 90, 105, 120 and 135 of lactation, before and after 2 h of separation from their dams for manual milking, are presented in Figure 8.

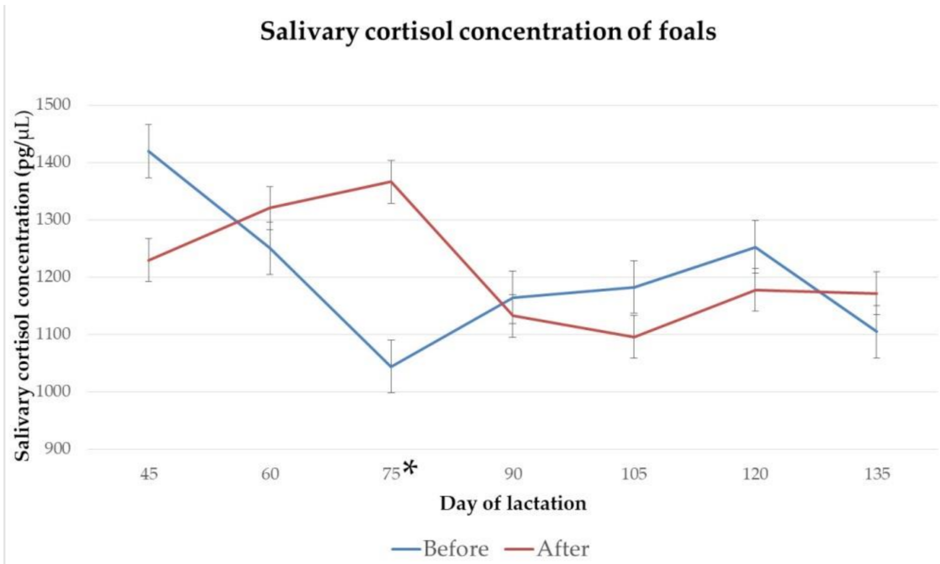

Figure 8. Mean and standard error of cortisol concentration of foals before and after 2 h of separation from their dams for milking, on day 45 ($p = 0.19$), 60 ($p = 0.63$), 75 ($p = 0.03$), 90 ($p = 0.82$), 105 ($p = 0.62$), 120 ($p = 0.61$) and 135 ($p = 0.64$) of lactation. * indicates a statistically significant difference.

A statistically significant difference between salivary cortisol concentrations of foals before and after separation was found on day 75 of lactation ($p = 0.03$), but not on day 45, 60, 90, 105, 120 and 135 or lactation ($p > 0.05$).

3.3. Milk Yield

The average milk yield of the Pêga jennies was 566.4 ± 205.2 mL/animal/milking.

There was no correlation found between the milk yield and salivary cortisol concentration pre- or post-separation (Table 2).

Table 2. Correlation between milk yield and salivary cortisol concentrations of jennies pre- and post-2 h of separation from their foals.

	Milk Yield (mL/day)	Cortisol before 2 h Separation (nmol/L)	Cortisol after 2 h Separation (nmol/pL)
Milk yield (mL/day)	1.00	−0.131	−0.044
Cortisol before 2 h separation (nmol/L)		1.00	0.432
Cortisol after 2 h separation (nmol/pL)			1.00

4. Discussion

4.1. Behaviour

Behavioural observation is considered the most reliable and immediate way to assess the perception and interaction of an animal with its environment [51]. However, the social behaviour of donkeys has not been sufficiently studied [35].

In this study, foals were separated from the jennies for milking management starting at 45 days of age. It is likely that the age-dependent reduction in the proximity between jennies and their foals partially explains the small behavioural responses reported in this study [25].

Additionally, jennies and foals were allowed to remain in physical proximity and maintained vocal communication, as the jennies were aware of the location of their foals during the separation period. Donkey foals begin drinking water and graze by themselves at four weeks of age [26], and mule foals have been observed at distances of 50 to 100 m from their dams starting from the 3rd week of life, and distances of over 100 m after the 11th week [27].

The increase in the frequency of vocalisations post-separation on day 120 of lactation, in comparison to the pre-separation period, may have been generated by various factors. Vocalisations are important to maintain the interactions between jennies and their foals, e.g., to signal the start of nursing bouts or direct the activities of the foal [25], and the increase might represent the fact that the animals were not in visual contact. It is known that equines utilise vocal communication to express many emotional states, ranging from curiosity, playfulness, and anticipation to distress signals, discomfort, frustration, and stress [23]. As this increase was only observed on one assessment day, it is not possible to determine if the increased frequency of vocalisations were a response to potential stress from the 2-h separation period, or an attempt to communicate in the absence of visual contact. The relevance of vocalisations as indicators of emotionality in animals must be analysed together with the other parameters.

The absence of significant differences in behavioural measures may indicate that both jennies and foals coped with the 2-h separation period with biologically acceptable responses that maintained good levels of animal welfare. When faced with routine changes, external stressors, or poor welfare conditions, animals tend to demonstrate behavioural signs such as a rise in inactivity [25–27], elevated frequency of urination and defecation [43,44], altered social interactions [28] and a rise in abnormal behaviours [26].

Animals also tend to perform greater amounts of abnormal and agonistic behaviour when responding to adverse situations, which may relate to stressors caused by housing problems and/or improper handling [26]. Changes in the environment and activities performed by the animals may generate alterations in the social environment [45,46]. Such changes were not observed in the present study, in which management alterations and separation between dams and foals for 2 h did not significantly impact social behaviour. The study population of purebred Pêga donkeys is unique as they encounter a wealth of human–animal interactions throughout all developmental stages, which may have mitigated their responses.

It is important to mention that the absence of alterations in social behaviour of the jennies in this study may be explained by the fact their social groups were not changed, minimising potential conflicts related to hierarchy, and might also indicate that the 2-h separation from their foals did not challenge social stability.

Affiliative behaviours among equids provide several social benefits [47]. Some known affiliative behaviours described for equines are mutual grooming, touching between the muzzle and body, playing, approaching, and following [36,49], though the occurrence of grooming and greeting are considered rare in wild jennies [48]. The occurrence of these actions is influenced by age, reproductive stage, hormones, social structures, and ecological conditions [47,49,50], and their quality and quantity may also be altered according to the quality of their habitat. Animals may display an increased frequency of affiliative behaviours to ease tensions or in situations of low perceived risk; contrarily, they may decrease their frequency to avoid imminent conflicts or in risky situations [47].

The results presented here may indicate an absence of stress in jennies when separated from their foals for 2 h, but further investigations are needed in regard to the normal social behaviours of donkeys and how they vary in response to adverse situations.

Even though no significant behavioural alterations were observed in the post-separation periods, further investigations are required in respect to the affiliative, agonistic, abnormal, and eliminative behaviours and inactivity in order to determine if the 2-h separation is a stressor for these animals. The study is unique in that it monitored the responses of purebred Pêga jennies and their foals. The animals were handled on a routine basis for other purposes, and this could have mitigated the response to the separation.

4.2. Salivary Cortisol Concentration

Cortisol was measured from saliva. This collection method is non-invasive [51–54] and reflects the biologically active portion of the total circulating concentration [52,53,55]. It is thus less likely to induce increases in cortisol concentration when compared to plasma cortisol sampling [54,56,57]. The aversive stimuli of drawing blood in dairy jennies may cause more intense stress than milking [31]. Significant differences between pre- and post-separation samples were only observed from jennies on day 135 of lactation, and from foals on day 75 of lactation. On all other assessment days, no significant differences between pre- and post-separation samples were observed. The significant rise in cortisol concentration in jennies after separation on day 135 of lactation, when compared to before separation, might have been the result of changes in management, which caused some animals to remain separated for longer than 2 h.

Few studies regarding the response and adaptation of jennies to milking have been performed [13,31], and no significant variance has been found in salivary cortisol concentrations before and after milking, even though donkeys can show great reactivity to milking procedures [13].

The average concentration of salivary cortisol from jennies, before milking was 790 pg/μL (217.93 nmol/L), taken between 9:00 am and 10:00 am, and 840 pg/μL (231.72 nmol/L), taken between 12:00 pm and 2 pm, before and after separation, respectively. The sampling period can alter salivary cortisol concentrations, which can reach values of 531.72 nmol/L when taken after milking [13]. In non-pregnant mares, basal salivary cortisol concentrations vary between 110.34 nmol/L and 331.03 nmol/L [58]. Significant differences have been reported between salivary cortisol concentrations of donkey stallions and equine mares or geldings [59,60], which have been ascribed to species variation [56].

The time of day in which samples are taken also affects results, due to circadian rhythms. Cortisol concentrations follow a clear diurnal pattern in horses, with the highest concentrations in the morning and the lowest in the late afternoon and evening [58,61–63]. This trend has also been observed in donkeys, with high plasma cortisol levels found in jennies milked at 8:00 am and lower values in groups milked at 4 pm [31].

As salivary cortisol levels were not measured throughout the day without routine changes, in the present study, and the circadian rhythm may influence basal cortisol levels, it is uncertain whether the absence of significant difference between cortisol levels before and after separation and milking is due to the absence of stress for the animals or lower basal levels at later times of the day [31].

It has been stated that inherent diurnal rhythms can be easily disturbed by minor challenges [59,61] and factors such as weather and ambient temperature, and interactions within groups may cause transient alterations [59]. Experimentally induced increases in salivary cortisol are often relatively small, and hardly exceed the range of physiological variations [54]. More research is needed regarding the variations in cortisol level in jennies according time of day and seasons of the year [13,31,56].

In horses, stressful events like separation from conspecifics acutely stimulates cortisol release [62,64], but they quickly habituate to these situations, and there are no lasting effects on diurnal rhythm. Furthermore, repeated stressful events also result in subsequent decreased cortisol levels [60].

The average concentration of salivary cortisol from foals before the separation from their dams was numerically higher than those of jennies, in agreement with studies performed in horses, which reported higher cortisol levels in suckling foals compared to their dams [59]. The elevated levels of cortisol in foals may be due to immaturity, following the same pattern found in gilts and humans [65–67], in which cortisol levels are initially high and gradually lower while forming a circadian rhythm.

The fact that the 2-h separation period, in all but two assessments, did not generate a significant difference between pre- and post-separation salivary cortisol concentrations in jennies or foals may indicate that this interval was not a stressful factor capable of altering the HPA axis, and may not compromise the welfare of the animals involved. Therefore, the ability of jennies and foals to adapt over time in response to stress remains unclear.

4.3. Milk Yield

The milk yield data differ from other studies, which, working with jennies of the Pêga breed in an extensive farming system in the drought season and without nutritional supplementation, reported an average milk yield in two daily milkings of 0.614 kg/day [68].

Ragusana donkeys receiving hay ad libitum and 3.5 kg feed/day have higher yields than those reported in this study, ranging from 0.56 to 0.59 kg/milking, from two and eight milkings, respectively [18]. This suggests that the difference in milk production of the different breeds could be linked to their diet [69].

The ejection of alveolar milk may be altered by stress, due to oxytocin concentration changes and myoepithelial contraction [42]. Jennies submitted to milking without previous training show lower milk yield when compared to jennies previously habituated to milking management, possibly due to reduced oxytocin supply via vasoconstriction or blocking of its receptors in the myoepithelial cells of the udder alveoli [13]. If we consider the density of Pêga asinine milk to be 1.03 g/mL [69], the milk yield was 0.6 kg/animal/milking, which represents approximately 0.25% of the jennies' average body weight. Since these animals may be milked two [10] to eight times a day [18], the total milk yield from these jennies may be up to 4.8 kg/animal/day.

Milk yield remained constant for all animals after the 2-h separation period on all days of lactation, and there was no correlation between the volume of milk produced and cortisol concentration pre- and post-separation, thus separation was not a stressor that impaired milk ejection. However, many other factors can alter milk ejection, and must be considered before concluding that there was no stressor present during the 2 h separation.

It is important to consider the fact that the animals had interactions with humans on a regular basis and some of the management practices involved short-term separation of jennies and foals.

5. Conclusions

The results presented in this study show that the behavioural categories assessed for the jennies were only mildly altered by the 2-h separation from their foals. The only behavioural variable that showed significant changes was vocalisation frequency, which may express social signalling in the absence of visual contact, and this was only observed on one assessment day.

The 2-h separation period also failed to generate significant changes in the majority of salivary cortisol concentration levels of jennies or foals, with the exception of one assessment in each animal category. Therefore, it does not appear to be a stressor capable of altering the HPA axis. Additionally, there was no apparent relationship between milk ejection and salivary cortisol concentrations.

We acknowledge several limitations to this study, such as the absence of a control group, the lack of true measures of basal salivary cortisol, and the low quantity of milk yield measurements to support more robust conclusions. Behavioural observations of foals will enhance our understanding of the impact of separation on their welfare. Further research is needed to determine whether the separation of these animals is indeed a stressor that could result in severe welfare problems. Additional studies are also required to determine the long-term consequences of the separation event, as well as the results of more frequent separation periods, on the lifelong trajectory of these animals.

Considering these results, it is important to emphasise that they are limited to one group of purebred Pêga jennies and foals in Brazil. It is possible that their responses were confounded by the fact that these animals were handled non-aversively on a regular basis. We hope that this study is useful to people interested in milking donkeys.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Publicly available datasets were analyzed in this study. This data can be found here: https://data.mendeley.com/datasets/7n9t5fn99b/2.

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8. Chapter 3

Physical and chemical parameters of asinine milk of the Pêga breed

ABSTRACT

Asinine milk has important nutritional and therapeutic properties that can benefit the diet of the elderly, convalescent or newborn. The nutritional composition of asinine milk stimulated the interest of this species for a new commercial activity. However, not much literature reporting the production aspects, physical and chemical characteristics, microbiology, or nutritional values of donkey's milk are available. Thus, this study was carried out to characterize the milk of Pêga donkeys raised in southeastern Brazil in relation to physical and chemical qualities presented throughout lactation. This study was conducted at Criatório Ximbó, in the city of Laranjal Paulista, state of São Paulo. Individual milk samples were collected from 14 multiparous lactating jennies. The jennies were adapted to milking procedures and milking was carried out every 15 days. Prior to milking, jennies and their foals were separated for 2 hours. During this period, the foals remained inside the stalls and the jennies were free to roam the farm. After the separation period of 2 hours, the jennies were taken individually to the milking parlour, during the sample collection days. The total volume of milk collected was measured and 5 mL were separated and destined for analysis at the Clínica do Leite in the Luiz de Queiroz College of Agriculture (ESALQ) in Piracicaba/SP. The analyses of composition included fat (GOR), total protein (PROT), casein (CAS), lactose (LACT), non-fat dry matter (ESD) and somatic cell count (CCS). The milk composition data of the donkeys was analyzed using the Proc Means procedure of SAS Software (version 9.3), for descriptive statistics. Furthermore, they were submitted to ANOVA using the PROC MIXED procedure of the SAS statistical package in order to verify the effects of age, lactation phases, lactation week and their interactions. The differences were assessed by the Tukey test ($P < 0.05$). The existence of the effect of lactation day in the concentrations of these nutrients was observed. The average milk yield of Pêga jennies was 632.01 ± 223.92 mL/animal/milking and the average body weight was 245 kg. There was an effect of lactation day in the concentrations of GOR, PB, LAC, ST, EAD and LogCSS 8. The concentration of CAS (1.23 ± 0.024%; P=0.182) and milk yield/day (623.3 ±

223.94 ml; P=0.682) were not influenced by lactation days. Based on the data above, it is observable that asinine milk samples collected in this study did suffer some composition changes throughout lactation, which agrees with other studies assessing asinine milk.

1. INTRODUCTION

The main objective of milk production is to meet the nutritional demands of neonates (GUHA et al., 2021). In regards to terrestrial mammals, cows contribute to 85% of the worldwide commercial milk production, while buffalo, does, ewes, camels and donkey jennies contribute with 11%, 2.3%, 1.4%, 0,2% and less than 0.1%, respectively (STEFANO GEROSA AND JAKOB SKOET, 2012). Although asinine milk production is smaller than that of other animals, it has been used as a remedy prescribed for infant nutrition since Ancient Rome, and its therapeutic qualities are widely known (CAROLI et al., 2017; CUNSOLO et al., 2017).

Studies show that asinine milk is an excellent source of lactose, proteins, minerals, vitamins and polyunsaturated fatty acids (CAROLI et al., 2017; JIANG et al., 2018; MARTINI et al., 2018). The concentration of lipids is smaller (3.5 to 4%) when compared to human milk, and the lactose concentration (5.8% to 7.4%) is similar (UNIACKE-LOWE; FOX, 2011; ALTOMONTE et al., 2019; SPADA et al., 2021). It also has a lower concentration of total solids, protein and minerals, when compared to cow milk (MEDHAMMAR et al., 2012).

Asinine milk also presents less total solids, protein and fat, but more lactose when compared to the milk of smaller ruminants (Park and Haenlein 2006).

Although studies reported that asinine milk is similar to human milk and its consumption is recommended, one of the main limiting factors for its availability is its production yield. The average production of milk by jennies is between 100 to 150 kg of milk per lactation period (LICITRA et al., 2019; GAI; WAHOME; BETT, 2022).

Production of asinine milk varies based on genetic, environmental and physiological factors, and both production and composition are influenced by management regimes and birth order (MARTINI et al., 2015; RANGEL et al., 2015).

Currently there is a worldwide initiative for research that aims to evaluate the properties of asinine milk and its use in human nutrition. Italy, China, Greece, France and the Balkans have become focal points for asinine milk characteristics and nutritional quality research (CAROLI et al., 2017; JIANG et al., 2018). These countries have focused efforts to increase asinine milk production, establishing farms and incentives to foster the establishment of donkey dairy production, which may be explored to meet nutritional demands and provide a livelihood for farmers, raising the economic importance of this species (BONELLI et al., 2019b; GUHA et al., 2021).

However, more research is needed especially with Brazilian breeds to better understand their milk properties and the best way to produce milk in a sustainable and etically acceptable way. It is important to mention that milk production from national donkey breeds is still not legislated, in Brazil, making the entrance of farmers into the dairy market more difficult (MESSIAS et al., 2022). There is a clear demand for research about the means of production, the diffusion of knowledge regarding the qualities of this milk and the legal aspects of its production.

2. OBJECTIVE

To characterize the milk of Pêga donkeys in relation to physical and chemical qualities throughout lactation.

3. MATERIALS AND METHODS

This research was conducted at Criatório Ximbó, in the city of Laranjal Paulista and state of São Paulo.

The city of Laranjal Paulista is located at the altitude of 536 m, coordinates 23º 02' 59" of latitute South and 47º 50' 12" of longitude West. The region presents subtropical humid climate (Köeppen-Geiger classification), with yearly average temperatures of 13 ± 4.9 °C to 31 ± 4.7 °C and pluviosity of 1,177 mm.

Data collection was carried out between April and November of 2018. The average temperatures and humidity levels during this period are presented in Figure 2 A and B. The meteorological data were obtained from the Center for Weather Forecasting and Climate Research (CPTEC – INPE).

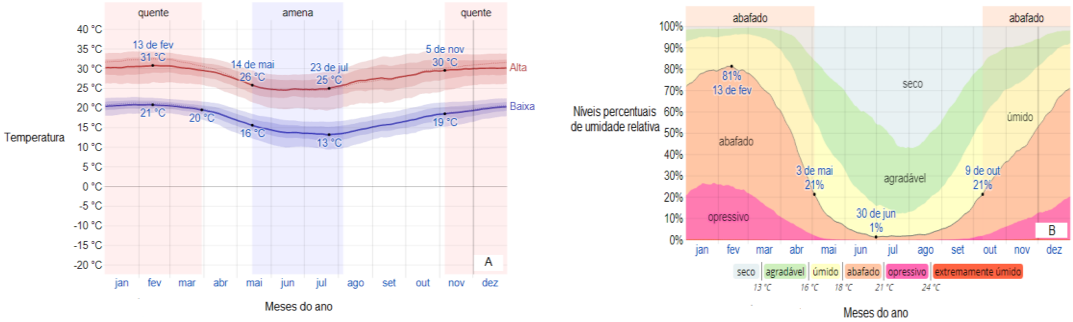

Figure 2: Meteorological data: temperature (A) and humidity (B) of Laranjal Paulista, SP, in the year 2018, obtained from the Center for Weather Forecasting and Climate Research (CPTEC-INPE).

At Criatório Ximbó, the production system for donkeys is semi-intensive, with free access to natural pasture associated with *Brachiaria decumbens*. Jennies are also supplemented with alfafa hay.

There was no dairy production activity at the farm prior to the study, and since one of the objectives was to assess the stress generated by milking management, as well as the composition and somatic cell count of milk throughout lactation, the jennies were adapted to milking procedures and milking was carried out every 15 days from 14 animals.

Of the 14 jennies, 7 gave birth in the summer and 7 in the spring. The births begun in the month of February and ended in the month of June 2018, and the lactation period lasts for 5 months.

Milking was not done for the first month post-partum as all the milk from this period was to be destined for the nutrition of the newborn foals. The experimental period lasted from March to October 2018 (Figure 3).

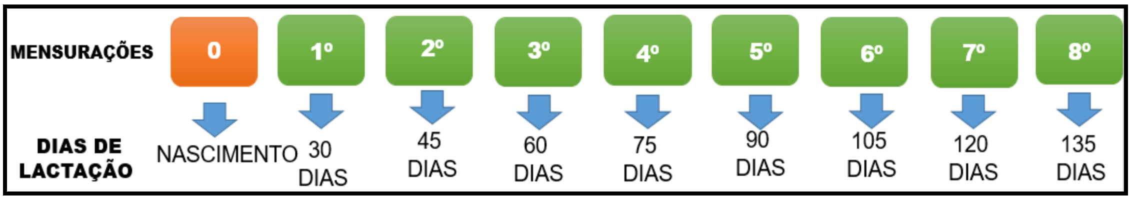

Figure 3: Measurements carried out throughout lactation of Pêga jennies.

In all data collection days, jennies were divided into groups of 3 or 4 individuals and respective foals and housed in two stalls of 90 m² (Figure 4).

Figure 4: Pêga jennies and foals divided into groups and housed into stalls.

Prior to milking, jennies and their foals were separated for 2 hours. During this period, the foals remained inside the stalls (Figure 5) and the jennies were free to roam the farm (Figure 6).

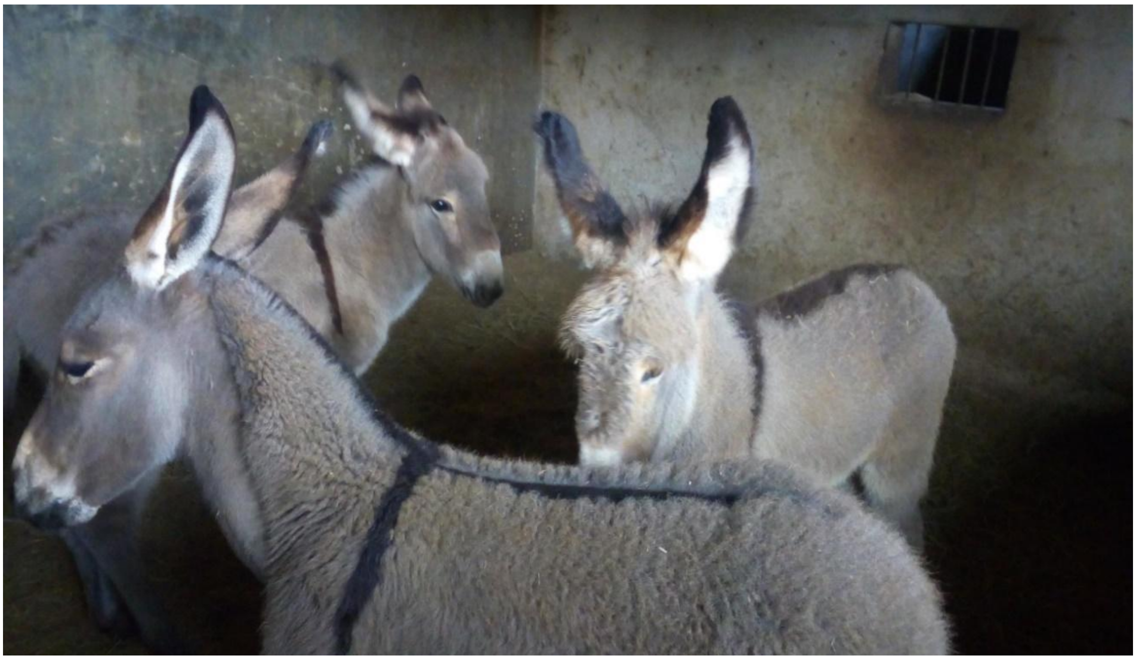

Figure 5: Pêga foals inside the stalls during the separation period.

Figure 6: Pêga jennies during the separation period.

After the separation period of 2 hours, the jennies were taken to the milking parlour individually.

In order to guarantee the safety of the individual milking the jennies and make the procedures easier, the jennies were restrained by being tied to the fence at their halter as well as one leg (Figure 7).

Figure 7: Restraining method for the Pêga jennies during milking.

At the start of milking, the foals were introduced into the milking parlour to maintain visual contact with their dams. Afterward, the udder and teats were cleaned with soap and water, and dried with paper towels.

Milking was carried out manually and ended when the udder was emptied. At the end, the udder and teats were again cleaned and dried, and dipped into an iodine solution (Dermasoft 2.5%, composed of povidone-iodine (2.5 g) and purified water (100 mL)), for 15 to 30 seconds.

The total volume of milk collected was measured and 5 mL were separated and destined for analysis at the Clínica do Leite in the Luiz de Queiroz College of Agriculture (ESALQ) in Piracicaba/SP, for analyses of composition of fat (GOR), total protein (PROT), casein (CAS), lactose (LACT), non-fat dry matter (ESD) and somatic cell count (CCS). Each collection tube was identified and stored in a polystyrene box with reusable gel ice packs for transportation to the laboratory.

3.1 STATISTICAL ANALYSES

To test the fixed effects of days in milk on the physicochemical characteristics of the milk, the data were analysed in a randomized complete block (animal) design using the PROC MIXED procedure of the Statistical Package SAS (Software version 9.3). Comparisons between means were made by the Fisher's Protected Least Significant Difference test. Linear and quadratic regressions were also performed to test the effect of days in milk using the PROC REG procedure. For all data, the significance was declared when P≤ 0.05.

4. RESULTS

The average milk yield of Pêga jennies was 632.01 ± 223.92 mL/animal/milking and the average body weight was 245 kg (Table 1).

Table 1: Average production values and physical and chemical composition of Pêga jennies milk.

Variable	**Mean ±SD**	**Minimum**	**Maximum**	**CV(%)**
Yield (mL/day)	632.01±223.92	200	1.100	35.94
Total Solids (%)	9.302±0.407	8.60	10.83	4.37
Protein (%)	1.627±0.240	1.06	2.58	14.78
Fat (%)	0.405±0.182	0.94	0.94	45.02
Lactose (%)	6.602±0.243	5.49	7.19	3.68
Casein (%)	1.237±0.232	0.67	1.800	18.81
ESD (%)*	8.831±0.270	8.03	9.540	3.66
Log CSS (10^3/ml)*	10.385±22.708	1.00	247.00	218.66

SD = standard deviation. *ESD = nonfat dry matter; CCS = somatic cell count.

There was an effect of lactation day in the concentrations of GOR, PB, LAC, ST, EAD and LogCSS (Figures 8, 9, 10, 11, 12 and 13). The concentration of CAS (1.23 ± 0.024%; P=0.182) and milk yield/day (623.3 ± 223.94 ml; P=0.682) were not influenced by lactation days (Figures 14 and 15).

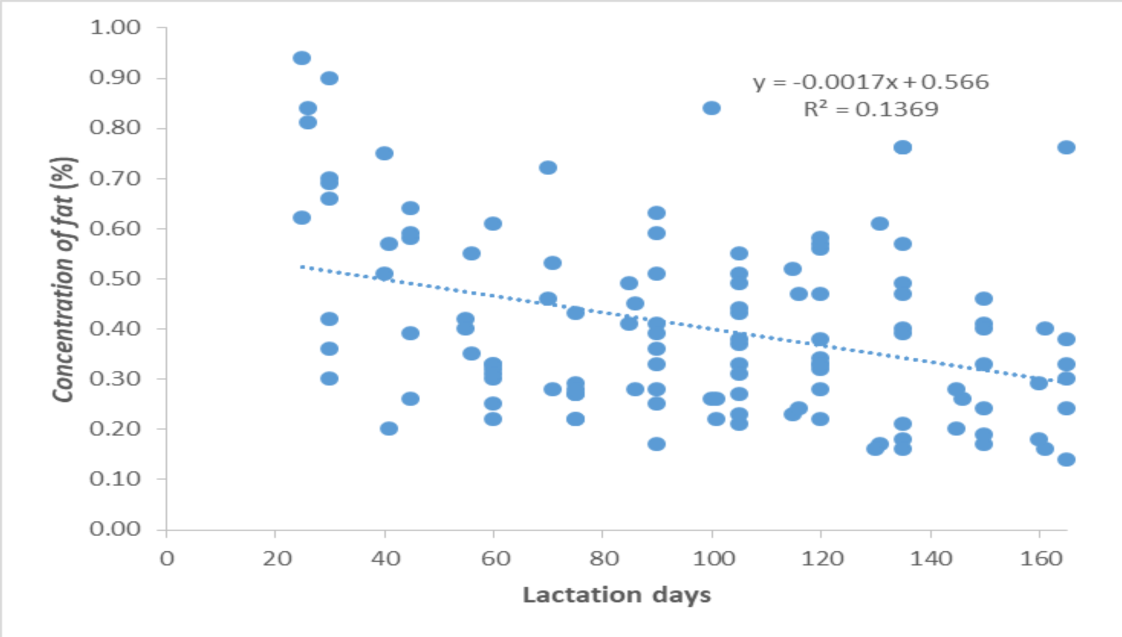

Figure 8: Concentration of fat in Asinine Pêga donkeys' milk throughout lactation.

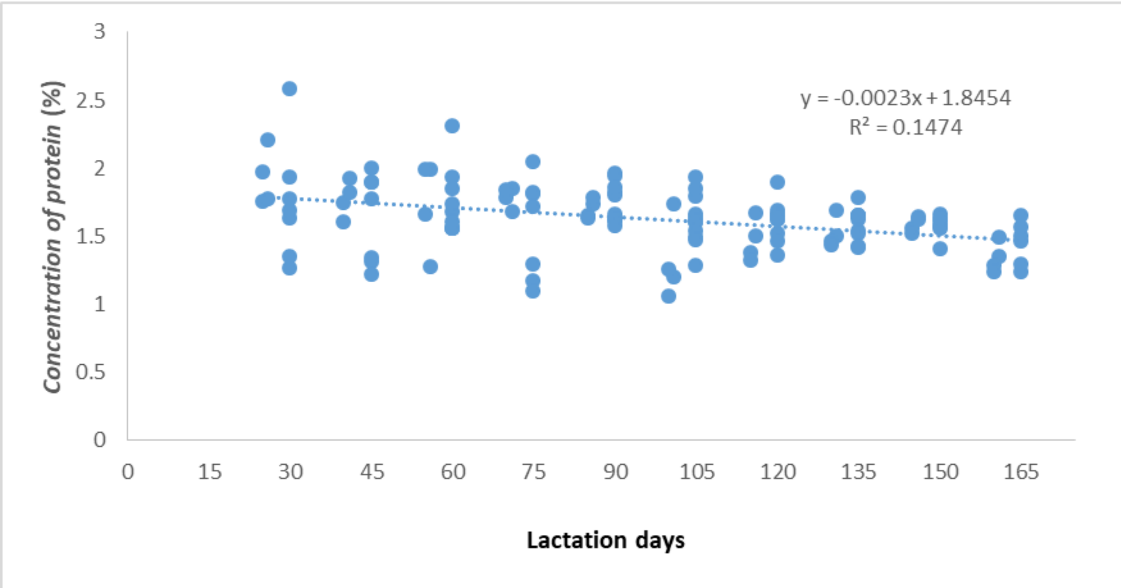

Figure 9: Concentration of protein Asinine Pêga donkeys' milk throughout lactation.

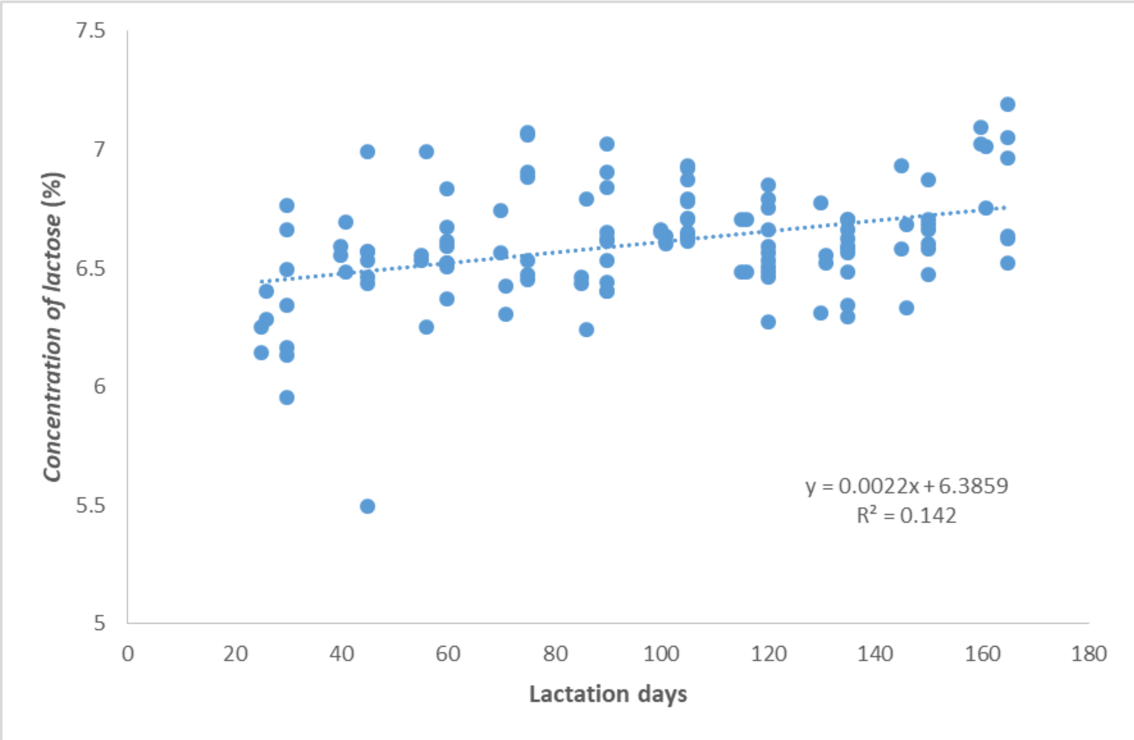

Figure 10 Concentration of lactose in Asinine Pêga donkeys' milk throughout lactation.

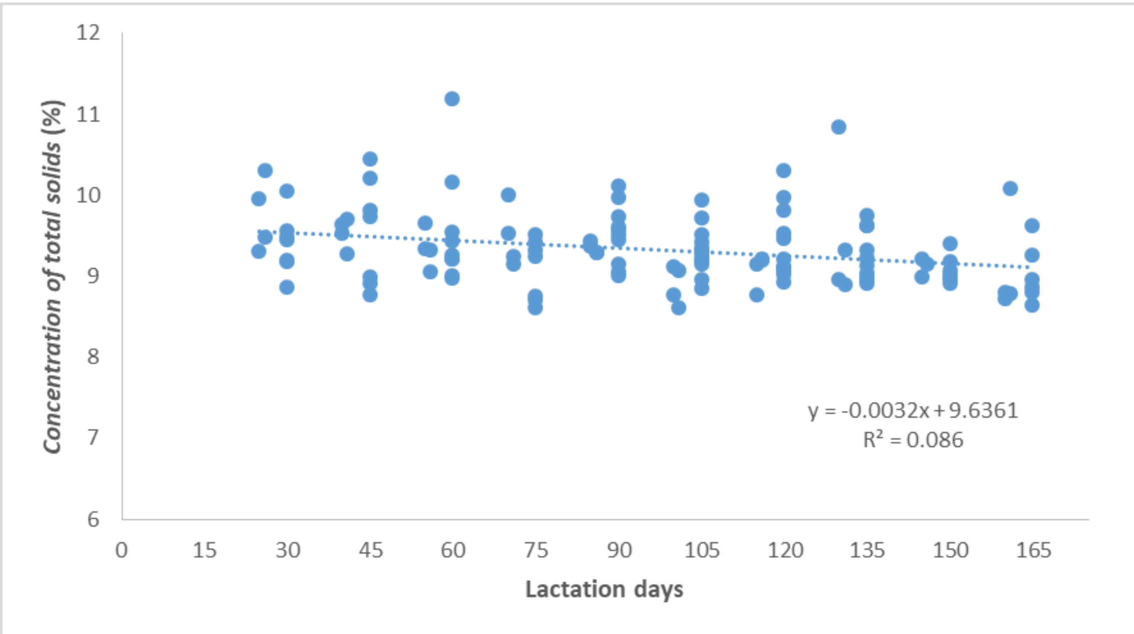

Figure 11 Asinine Pêga donkeys' milk solids non-fat content linear response (P=0.006) as a function of days in milk, $\hat{Y} = -0.0015x + 8.98$ (R2 = 0.10).

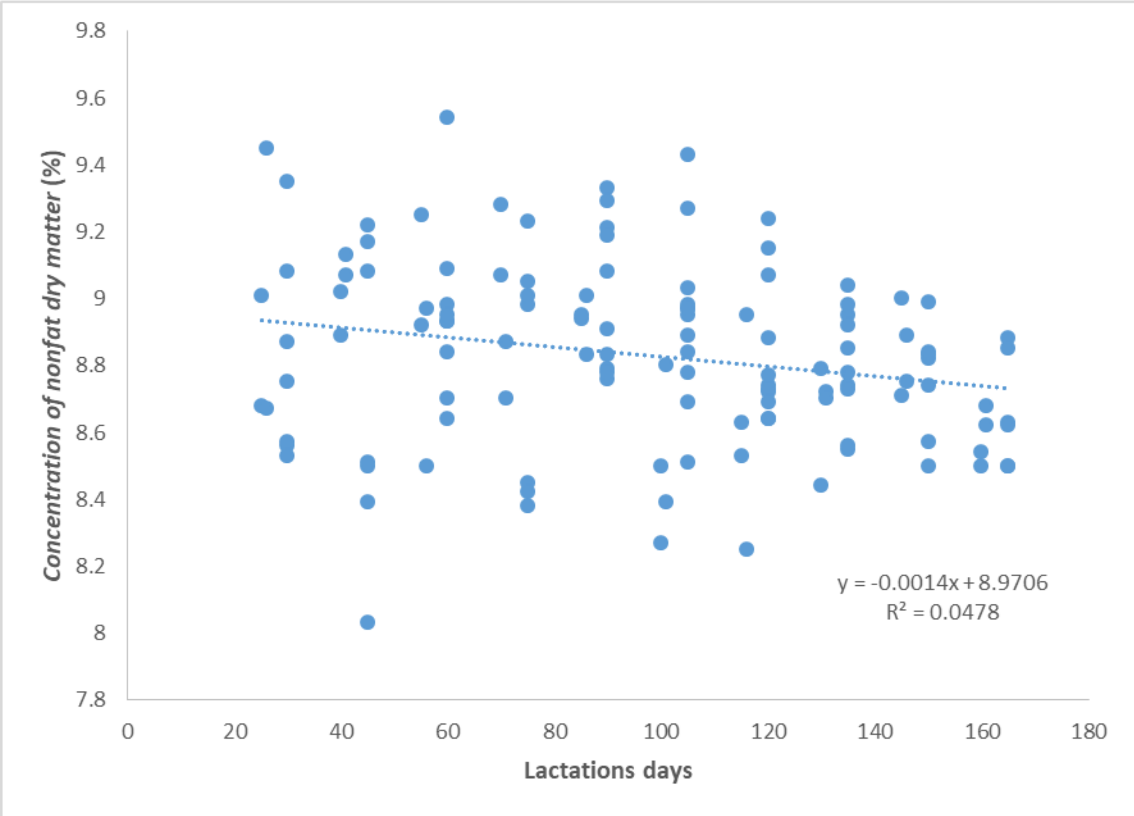

Figure 12 Concentration of nonfat dry matter in Asinine Pêga donkeys' milk throughout lactation.

5. DISCUSSION

Considering the density of Pêga milk is 1.03 g/mL (SANTOS, 2017), the milk yield was 0.632 kg/animal/milking, which represents approximately 0.25% of the jennies' average body weight. As these animals may be milked between 2 (ALESSANDRO; MARTEMUCCI, 2009; AMATI et al., 2010; MONTI et al., 2012) and 8 times a day (ALABISO et al., 2009), the total milk yield produced by the jennies of this study could reach upwards of 4.8 kg/animal/day.

Ragusana donkeys fed ad libitum hay and 3.5 kg of concentrate/day, authors have reported higher milk yield values than those of this study, with intervals of 0.59 to 0.56 kg/day of milk in 2 and 8 milkings, respectively (ALABISO et al., 2009).

The milk yield results in this study were higher than those found by Santos (2017), which worked with Pêga jennies maintained extensively, and reported an average milk yield with 2 daily milkings of 0.614 kg/day.

Differences in daily milk yield of the various donkey breeds may be related to their diet (CAROLI et al., 2017; SANTOS, 2017b), which also affects the daily production of milk fat (WIKING et al., 2006), as well as lactation age (YIN et al., 2013; LIU et al., 2020; LI et al., 2021).

The concentration of fat, protein, lactose, total solids, nonfat dry matter and somatic cell count did not remain constant throughout lactation, unlike casein and total milk yield.

These results are in agreement with other studies, which show a downward tendency in concentration of all milk components during lactation, with the exception of lactose (SANTOS; ZANINE, 2006). These were not the same results reported in a study done in Bahia, which also analysed Pêga asinine milk during the lactation period (SANTOS, 2017b).

Milk is the naturally optimal food for the initial phases of development, and its composition is compatible with the changing nutritional demands of newborns which explains the differences among the concentrations of its constituents along lactation (FONSECA et al., 2021).

The concentrations of protein (mean = 1.61), total solids (mean = 9.35), fat (mean = 0.52) and casein (mean = 1.23) are relatively low when compared to milk of other species, which is also reported by other studies (SANTOS; ZANINE, 2006).

The low values for total solids (ST) and low total protein content (PT) are characteristic of donkey and horse milks. A study done in Bahia with Pêga donkeys also found values of 6,23ºD (SANTOS, 2017).

When compared to human milk, it is known that asinine milk contains less fat, protein and inorganic salts, but similar concentrations of lactose (BHARDWAJ et al., 2020).

The mean value for lactose concentration in the samples was 6.6%. This result is in agreement with Santos (2017) and (CRAVEIRO DE SÁ ULIANA et al., 2016), which evaluated the milk of Pêga donkeys and reported values of 6.34% and 6.29%, respectively. This value is higher than that of cow milk, which contains approximately 5% lactose, and equal to that of mare and human milk (A., 2004; RANGEL et al., 2015; CARMINATI; TIDONA, 2017b), Lactose concentrations are lower immediately after birth and higher towards the end of lactation. In particular, lactose content is reported to increase from 4% at the start of lactation to 6% at 10 days, and being equal to values reported by other works at the 8th day of lactation (LICITRA et al., 2019).

Lactose stimulates the absorption of calcium, which is essential for bone mineralisation and development of the Central Nervous System in infants (BUSINCO et al., 2000). The high lactose content of asinine milk also suggests its use as a probiotic (CHIAVARI et al., 2005; PERNA et al., 2015), seving as a substract for the development of intestinal lactobacilli and therefore making it an ideal matrix for the preparation of probiotic beverages following incubation with *Lactobacillus rhamnosus* (COPPOLA et al., 2002).

The fat content found in this study was 0.405% in average. According to the Instrução Normativa 62 (Brasil, 2011), pasteurized skimmed milk must contain, among other physical and chemical requirements, a maximum fat concentration of 0.5% as a legal criteria. Therefore, the milk produced by jennies in the first group may be considered a naturally skimmed milk (BHARDWAJ et al., 2020).

When compared to cow milk, asinine milk has a lower fat content (SPITSBERG, 2005; KARRAR et al., 2022).

The average casein content in this study was 1.23%. This result is similar to that found by Santos (2017), at 1.20%. In comparison to the casein content of cow milk, this value is significantly lower, which means asinine milk may be an indication for people with allergies to cow milk protein. According to Carroccio et al. (1999), casein is the most important milk allergen for humans.

Studies of the composition of milks from various equid species (horses, donkeys and zebras) found that these species produce milk that is similar in total solids, fat, protein, non-protein nitrogen, lactose, calcium and phosphorus content (OFTEDAL; JENNESS, 1988; SALIMEI, 2011; UNIACKE-LOWE; FOX, 2011).

In a study about the composition of milk from mares of the Postier Breton breed, authors observed that total protein and mineral contents decreased, while lactose increased throughout lactation (DOREAU et al., 1992).

The protein, lactose and casein contents found in this study were similar to those reported in other donkey and human milk studies. The fat content also remained low, as in other donkey breeds (BALLARD; MORROW, 2013; CARMINATI; TIDONA, 2017b).

The lactose content of asinine milk is approximately 7% (Martini et al., 2014), which is similar to that of human milk and 4.6% higher than cow milk. The high lactose content is also responsible for its high palatability, as reported in a sensorial test conducted (BARNI et al., 2018).

The mean protein values found in this study were 16 g/L. This is in agreement with other studies that report a lower protein content in asinine milk than in cow milk, which varies between 13 and 28 g/L, but is still similar to the protein content of human milk (POLIDORI; VINCENZETTI, 2012; VINCENZETTI et al., 2017a; SPADA et al., 2021).

The low protein content in asinine milk also contributes to its lower renal load, and is similar to human milk and suitable for infants (SALIMEI & FANTUZ, 2012; RAGONA et al., 2016). Throughout lactation, there is a downward trend in protein content (ALTOMONTE et al., 2019).

The mean value for somatic cell count found in this study was 10.02 x 10^3 CS/mL, and is within the recommended standard values by MAPA in the Instrução Normativa nº 62, of December 2011 (cow milk standard), in which the highest acceptable CCS value is 400 x 10^3 CS/mL.

The somatic cell count results in this study reaffirm that it is suitable for human consumption, as it is within minimal quality requirements for raw milk, such as total bacterial count of 500,000 CFU/mL and maximum somatic cell count of 500,000 CS/mL (BOZO et al., 2013).

The somatic cell count values in this study are also in agreement with a study conducted by Santos (2017), in which the mean value was 31.32 x 10^3 CS/mL, also within the recommended standard values by MAPA.

The somatic cell count is a general indicator of mammary gland health, and there are not standard values for quality control of mare and asinine milk yet.

6. FINAL CONSIDERATIONS

It is observable that asinine milk samples collected in this study did suffer some composition changes throughout lactation, which is in agreement with other studies with donkey and mare milk.

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9. Chapter 4

Can asinine milk mitigate the harmful effects of weaning stress in piglets?

Abstract

Asinine milk has important nutritional and therapeutic properties that can benefit the diet of the elderly, convalescent or newborn. Early-life adversity, as weaning, may have programming effects on neuroendocrine and immune adaptation mechanisms in piglets and other animals. Studies focusing on changes generated by stress relied on molecular assessments and studies of emotionality. The present study aimed to evaluate whether supplementing piglets with asinine milk mitigate the outcomes associated with weaning. The study evaluated 48 piglets from 4 different litters, 12 per litter. After 20 days of birth, the animals were weighed and transported to the experimental facilities. At the experimental facility, the animals were allocated with their litter and dam in group pens to 28 days of age. The experimental design was organized by blocks, represented by4 sows, in 2 phases, before and after weaning, each block had 4 repetitions, piglets by treatments, and 3 treatments. Piglets were either supplemented with asinine milk (group A,), skimmed cow milk (group B,) or received no supplementation (control group). Piglets were either supplemented with asinine milk (group A, n = 16), skimmed cow milk (group B, n = 16) or received no supplementation (control group, n = 16). The supplementation took place 3 days before weaning and 3 days after weaning. The effects of supplementation with asinine milk and skimmed cow milk on the weaning stress response were assessed through salivary cortisol measurements, behavioural tests and mRNA quantification in plasma PBMCs from blood samples. RNA was isolated from HPA tissues and converted to cDNA by using Trizol reagent (Invitrogen) and High Capacity cDNA Transcription Kit (Applied Biosystems), respectively, according to the manufacturer's instructions The synthesized cDNA products were used as template for real time PCR (qPCR), and primers for the targeted genes *HSD11B1* (cortisona redutase), *NR3C1* (glucocorticoid receptor) and *IL1B* (inflammatory response inducer) . To test the effect of treatments (A, B and C) on weight and consumption of piglets, a randomized block design was used with repeated measurements in time by the PROC MIXED. To the molecular data of gene expression that were not normally distributed according to the Shapiro–Wilk test were transformed to natural logarithms.

Gene expression were analyzed using analysis of variance (ANOVA) with repeated-measures of time using the PROC mixed method, with animal considered as a random effect and group (asinine, bovine and control), day and their interaction considered fixed effects. To the cortisol, the weaning effect was determined by calculating a ratio between salivary cortisol concentrations of piglets before and after weaning, considering the collection period, morning or afternoon. The effect of the collection period was determined using a ratio between morning and afternoon salivary cortisol concentration of piglets, considering the weaning event, before or after. Principal component analysis (PCA) was performed to evaluate how the behavior measures explained the variance at each principal component (PC) on the Open field, New Object and Elevated Plus Maze Test, in each phase using the FactoMineR (Le et al., 2008) and factoextra (Kassambara and Mundt, 2020) software packages of the R Core Team software (2017). The final body weight of piglets and the milk intake for groups A and B were not affected by the supplementation (P = 0.589). The expression of the *IL-1B* gene was significantly down regulated in the group fed asinine milk (mean 8.11; p = 0.049) and up regulated in the group supplemented with skimmed cow milk (mean 15.55; p = 0.049), when compared to the control group (mean 14.50; p = 0.049). No significant difference (p > 0.05) was found between the groups supplemented with asinine milk, skimmed cow milk and the control group before and after weaning for the expression of *HSD11B2*. However, the expression of the *NR3C1* gene was significantly upregulated in the group supplemented with cow milk (mean 1.245; p = 0.04). Salivary cortisol levels from piglets before and after weaning when comparing groups according to time in relation to weaning, there was significant difference (p < 0.05) between groups in the afternoon period. For the behavioural tests, that the type of supplementation may not interfere with the behavioral variables studied in the tests. In conclusion, further studies are needed to investigate whether the inclusion of asinine milk represents an adequate strategy to mitigate the negative effects of stress.

1. INTRODUCTION

In pig farming, traditional weaning is carried out early and abruptly (LANGE et al., 2020), usually when piglets are between 21 and 28 days old (Council Directive 2008/120/EC)” . It represents a critical welfare-altering factor as it culminates in a variety of stressors such as the end of maternal care (WEARY; JASPER; HÖTZEL, 2008), change in food source (LANGE et al., 2020), mixing with unknown conspecifics (PITTS et al., 2000) , exposure to new environments on the way to the nursery (BØE, 1991; BARNETT et al., 1996) , as well as exposure to pathogens and procedures such as vaccination and medication administrations (BØE, 1991; WEARY; JASPER; HÖTZEL, 2008; BROWN et al., 2012).

These factors generate acute and chronic stress which can cause lasting changes in metabolism, altering the gut microbiota profile which results in post-weaning diarrhea (PWD) and release of pro-inflammatory cytokines which damage the epithelial lining of the digestive tract (HEO et al., 2013; VIGNERI, 2014) . These events can lead to temporary or permanent modulation of metabolism, the nervous system, and immunity in young individuals (YU; CHEN; CHANG, 2019). Alterations of behaviour, physiology, metabolism, and immune responses are consequences of this process (ESCRIBANO et al., 2019; CERÓN et al., 2022) .

Understanding the consequences of early life events in pigs is also crucial for translational biology purposes. Similarities in brain anatomy, neurodevelopment processes (HOLM; WEST, 1994; LIND et al., 2007) , and immune system anatomy, function and gene expression (DAWSON; REECE; URBAN, 2009; FREEMAN et al., 2012; DAWSON et al., 2013; MAIR et al., 2014) , make the porcine model one of the best species to investigate the effects of early life adversities in humans (MEURENS et al., 2012; POHL; MEDLAND; MOESER, 2015; POHL et al., 2017) .

Stress leads to the activation of a complex physiological and behavioural response system, known as the adaptive stress response (JOHNSON et al., 1992). This is achieved through the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adreno-medullar (SAM) axis (GODOY et al., 2018). The activation of the HPA axis leads to secretion of corticotropin-releasing hormone (CRH) and the endocrine cells of the anterior pituitary respond to CRH, synthetizing and secreting adrenocorticotropic hormone (ACTH) (SMITH; VALE, 2006; HERMAN et al., 2016) . ACTH reaches the adrenal cortex through the circulation, where the cells of the zona fasciculata secrete glucocorticoids (ANGELOUSI; MARGIORIS; TSATSANIS, 2020; ALLEN; SHARMA, 2021).

Among glucocorticoid hormones, the most important in the study of stress responses is cortisol (COOK et al., 2013) . Cortisol action is mediated by ligation to glucocorticoid receptors, such as *NR3C1* receptor (OAKLEY; CIDLOWSKI, 2013; NICOLAIDES; CHROUSOS, 2019; NICOLAIDES; CHARMANDARI; CHROUSOS, 2020). The homeostase of glucocorticoid receptors is important to ensure the proper functioning of the negative feedback which modulates the cessation of cortisol secretion by the adrenal cortex, once the response is no longer warranted (BINDER, 2009; ARNONE, 2021).

Exposure to chronic stress is considered especially harmful as it carries the most potential to result in long-term or permanent changes in physiology, behaviour, and emotion which influences the susceptibility and course of diseases (BRITIGAN; SERODY; COHEN, ; COHEN; JANICKI-DEVERTS; MILLER, 2007).

The impairment of the gut-brain axis is also proposed as a mechanism for the development of neurological diseases (MARRONE; COCCURELLO, 2019) , due to the observation that chronic treatment with antibiotics is associated with greater risk of psychiatric diseases (DICKERSON; SEVERANCE; YOLKEN, 2017) , the high comorbidity between psychiatric disorders and digestive tract diseases, and the efficacy of specific types of probiotics in the resolution of gastrointestinal symptoms related to stress, anxiety and depression (WHITEHEAD; PALSSON; JONES, 2002; LA FATA; WEBER; MOHAJERI, 2018; SKONIECZNA-ŻYDECKA et al., 2018).

The brain is an important target organ for stress hormones, with consequent modulations in the dynamics of the cell's molecular biology, which, in turn, lead to changes in gene expression and neuronal connectivity, and, consequently, changes in cognitive functioning, learning, memory processes, and activity of the neuroendocrine system itself (DE KLOET; JOËLS; HOLSBOER, 2005).

Changes generated by stress can be studied by molecular assessments and studies of emotionality. Molecular indicators are measurement tools that have been studied through simple and fast methods of gene regulation (AGRAWAL et al., 2003). Recently, several studies have been carried out investigating the abundance of mRNA from peripheral blood mononuclear cells (PBMC) (FERRAZ et al., 2021) through whole blood immune cells (YOSHINO et al., 2018) , to access gene expression in face to aversive stimuli linked to stressful events (CORBETT et al., 2021; DE GROOT et al., 2021).

For the study of emotions, there are a variety of methods proposed for measuring through behavior, many of which have been applied to pigs, such as the open field test, the novel object test and the elevated plus maze (MURPHY; NORDQUIST; VAN DER STAAY, 2014). The greatest interest for the study of emotion is the behaviors considered indicative of anxiety or fear (GOULD; DAO;

KOVACSICS, 2009). However, to better understand the causal mechanisms of stress-related disorders, it is necessary to explore more carefully the limits of species-specific defense mechanisms developed over the course of evolution (KOOLHAAS et al., 2010; KOOLHAAS; DE BOER; BUWALDA, 2016).

Studies in both animals and humans are necessary to better understand the interaction between gut microbiota, neural physiology and mental health, as well as the potential modulating effects of nutraceuticals. Asinine milk is considered a nutraceutical for its concentration of bioactive molecules, and is used in the treatment of people with immunodeficiencies and cardiac (CHIOFALO; SALIMEI, 2014; CAROLI et al., 2017) and psychological diseases (UNIACKE-LOWE; HUPPERTZ; FOX, 2010; VINCENZETTI et al., 2021) . It also has prebiotic potential, as it is rich in lactose and lysozyme. Lysozyme is resistant to human digestive enzymes and exerts selective action on gut microbiota (MONTI et al., 2012; TIDONA et al., 2014; CAROLI et al., 2017).

Asinine milk also carries high levels of Omega-3 fatty acids (GASTALDI et al., 2010), which are found in high concentrations in brain tissue and influence perceptive, intellectual, social and communicative functions, as well as growth and development processes (WYSOCZAŃSKI; SOKOŁA-WYSOCZAŃSKA; PĘKALA, 2016) . It also has antioxidant potential (MARTINI et al., 2018; SOUROULLAS; ASPRI; PAPADEMAS, 2018) , due to the presence of vitamin C similarly to human milk (CAROLI et al., 2017; VINCENZETTI et al., 2021) and the activation of synthesis pathways for antioxidant and detoxification enzymes (LIONETTI et al., 2012; TRINCHESE et al., 2015; MARTINI et al., 2018).

The ingestion of foods with adequate fatty acid profiles (LEI; VACY; BOON, 2016) , antioxidant capacity (DAWSON et al., 2013; ZHANG et al., 2019) and probiotic activity (SRIKANTHA; HASAN MOHAJERI, 2019) may have positive impact in the prevention and control of neurologic diseases and proper development of brain functions. Considering the fact that asinine milk possesses nutraceutical profile and weanling piglets are physiologically, emotionally and immunologically challenged, the goal of this study was to assess whether asinine milk supplementation has the potential to improve the immune and emotional system during stressful periods, as an early model for potential future interventions in human infants.

MATERIALS & METHODS

2.1 Location and ethical statement

The study was conducted in the Department of Preventive Veterinary Medicine and Animal Health (VPS) (-21,9484694, -47,4563268) of the School of Veterinary Medicine and Animal Science of the University of São Paulo (FMVZ/USP), in the city of Pirassununga and state of São Paulo, Brazil. Field data collection was carried out between May and June of 2021, and laboratory and behavioural test analyses were done between July and November of 2021. All procedures described in this study were approved by the Committee for the Use and Care of Animals in Research (CEUA) of the School of Veterinary Medicine and Animal Science of the University of São Paulo, protocol nº 8696141117 (ID 007216).

2.2 Animals and housing conditions

Four lactating sows and their litters were chosen for inclusion in the study. The minimum criteria for inclusion of animals in the experiment included a minimum number of 12 piglets per litter and a minimum age of 20 days. In total, 48 piglets were used in this study. All animals belonged to the TopGen Aphrodite® lineage (Large White x Landrace).

Sows and respective piglets were weighed and identified with non-toxic animal markers prior to transportation to the experimental facilities. Random numbers were assigned to the piglets at weighing, which would be used throughout the experiment to distribute them in supplementation groups. All animals were then transferred to the experiment stalls.

Sows were housed individually with only their respective piglets in stalls adapted with a heat lamp, rubber floor mats and hay (Figure 14 A and B) during the 10 days of experiment. For the first three days of housing in the experiment stalls, the animals were acclimatized and no procedures were conducted. After the adaptation period, the experimental procedures began with saliva samplings, behavioural tests and blood collection, followed by weaning and another round of saliva samplings, behavioural tests and blood collection.

Figure 14. Housing conditions provided for sows and their litters. The stalls measured 2.10 m x 3.50 m and were equipped with a feed trough and nipple drinkers at different heights for both the sow and piglets. The heat lamp was affixed to the inside of a metal crate with a metal floor and hay, and walls lined with black tarpaulin. The crate was attached to the stall walls to stop the sows from moving and turning them.

Piglets remained with their sows until 28 days of age, after which the sows were removed from the facilities and the piglets remained in the experiment stalls until 30 days of age.

When sows and piglets were housed together, the sows received 7 kg of lactation feed daily. No feed was offered to piglets while they were still nursing. Piglets only began receiving *ad libitum* nursery feed after weaning, containing at least 18% protein, 0.95% lysine and 3,300 kcal DE/kg, as per recommended by (ROSTAGNO et al., 2017) . Feed for both sows and piglets was distributed at 07:30 and 18:30 daily.

2.3 Experimental design, nutritional characteristics of the milk used, and management practices applied to the animals

The experimental design was organized by blocks (4 sows), 2 phases (before and after weaning), each block had 4 repetitions (piglets by treatments) and 3 treatments (supplementations), resulting in 16 piglets per experimental treatments.

Piglets were either supplemented with asinine milk (group A, n = 16), skimmed cow milk (group B, n = 16) or received no supplementation (control group, n = 16). Piglets in groups A and B were supplemented for 3 days before weaning and 3 days after weaning. Before the supplementation period began, all piglets were offered nursing bottles with sugary water in order to test their acceptability and get the animals used to the bottles which would be later used to offer the different types of milk. Piglets that approached or accepted the bottles had their identification numbers noted and were randomly distributed between groups A and B.

Milk supplementation was offered at 50 ml six times a day before weaning and at 100 ml three times a day after weaning, so that the total volume offered was a constant daily 300 ml for all piglets in groups A and B. The bottles were colour-coded to easily differentiate between asinine milk (pink bottles) and skimmed cow milk (blue bottles), and milk was offered to each piglet until they drank all of the bottle’s content or stopped showing interest in it. The volume of milk left in each bottle after each supplementation was noted in order to calculate the individual milk intake per piglet.

In the three supplementation days before weaning, the sows had to be removed from the stalls for the supplementation period as they did not respod well to the constant presence of the experiment team inside the stalls, or attempted to drink from the bottles themselves. The sows were kept together in a grass field adjacent to the stalls, with access to shade, mud, food and water, and could maintain limited contact with the piglets through the mesh stall doors.

The nutritional value of the asinine milk and skimmed cow milk are found in Table 2. The volume of milk offered to the piglets was determined according to (SKOK; BRUS; ŠKORJANC, 2008) , which reported that the average milk intake for piglets is between 43,1 ml/feed at two weeks of age and 43,9 ml/feed at four weeks of age. The volume of milk for each pre-weaning supplementation was determined at 50 ml in order to be close to a natural nursing session for these piglets.

Table 2. Means and standard errors of means of nutritional values found in samples of asinine milk and skimmed bovine milk used to supplement piglets before and after weaning.

	Skimmed bovine milk (%)	Donkey(%)
Proteins (% m/m)	2,9	2.94
Total fat (% m/m)	0,5	0.36
Lactose (% m/m)	4,3	4.41
Non-fat solids (% m/m)	8,4	8.00
Total Solids (% m/m)	*	8.36
Titratable acidity (ácido láctico/100 mL)	0,14 -0,18 g	0.17g
Mineral Fraction	*	0.65

The effects of supplementation with asinine milk and skimmed cow milk on the weaning stress response were assessed through salivary cortisol measurements, behavioural tests and mRNA quantification in plasma PBMCs from blood samples.

2.4 Experimental parameters

2.4.1 Salivary cortisol measurement

In order to assess salivary cortisol from piglets, saliva was sampled with the use of cotton rolls attached to dental floss. The cotton rolls were offered for the piglets to chew on, and once soaked with saliva, were removed from the piglets and placed in individually identified 15 ml falcon tubes. The tubes were kept in polystyrene boxes lined with reusable gel ice packs, and then stored at -20ºC. The samples were processed according to (SIEGFORD; RUCKER; ZANELLA, 2008).

Saliva samplings of all piglets were conducted one day before the start of supplementation and at the last day of supplementation, at 07:00 and 17:00. The assessment of salivary cortisol was realized through enzyme-linked immunosorbent assay (ELISA) as described by (MÖSTL; PALME, 2002).

2.4.2 Behavioural tests

2.4.2.1 Open Field and Novel Object test

In order to assess behavioural characteristics, the tests of Open Field and Novel Object were used as described by (PUPPE et al., 2007) and (TATEMOTO et al., 2020), aiming to assess the levels of fear and exploratory motivation in the piglets. All piglets were tested two days before supplementation began and again the day following the end of supplementation.

In each testing day, a randomized order for the piglets was generated and they were brought one at a time from their stall to the arena in 30 seconds. Piglets were tested individually by being placed inside the experimental arena (Figure 15 A) for 10 minutes. The first 5 minutes inside the arena were the Open Field test, and afterwards, an object was lowered from the ceiling through a pulley system for the 5 minutes of Novel Object test (Figure 15 B). The object used was a yellow bottle for the pre-weaning tests and a pink plushie for the post-weaning tests. All tests were recorded by a camera placed in the ceiling directly above the center of the arena, and the recordings were later analysed to assess the behaviour of each animal according to an etograma (Table 3).

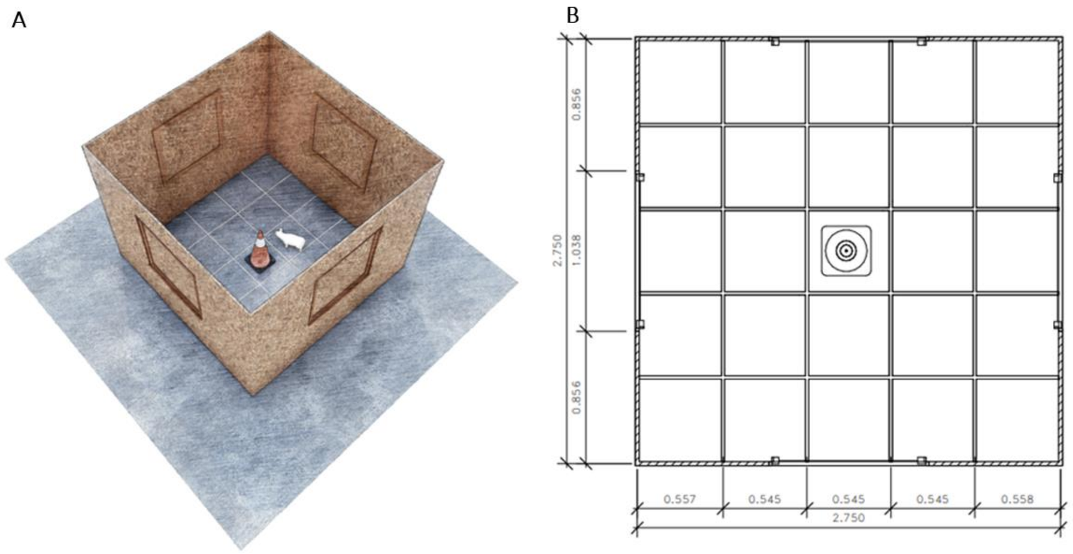

Figure 15. Open field and novel object test arena. The test arena measured 2,75 m x 2.75 m and had markings along the floor dividing the area into 25 similar quadrants.

Table 3. Definitions of behaviors assessed in the Open Field and Novel Object tests. Source: adapted from (ANDERSEN et al., 2000a, 2000b; MURPHY; NORDQUIST; VAN DER STAAY, 2014; TATEMOTO et al., 2020).

Behaviour	**Definition**
Jumping against wall	Piglet jumps against the walls of the arena.
Moving	Piglet walks in the arena.
Walking in center	Number of times the piglet crosses the center of the arena.
Time in center	Time spent by piglet in the central quadrants of the arena.
Time in edges	Time spent by piglet in the edge quadrants of the arena.
Time in the walls	Time spent by piglet to close to the walls of the arena.

Latency	Total time spent before piglet interacts with the object.
Interacting with object	Piglet interacts with the object.
Elimination	Piglet urinates or defecates.

2.4.2.2 Elevated Plus Maze Test

The Elevated Plus Maze Test (ANDERSEN et al., 2000a, 2000b) was placed in a room and surrounded by blue tarpaulin curtains so that piglets in the test could not see outside of it. The maze was raised 1 m above the ground and had 4 arms of equal length and width (1.2 m and 0.6 m), and 2 opposite arms had walls of equal height (0.45 m) (Figure 16). There were rubber mats on the floor along the maze arms to break the fall of any jumping piglets.

Figure 16: Elevated Plus Maze Test. The elevated measured 1.2 m long, 0.6 m wide and 0.45 m high.

Immediately after the Open Field and Novel Object tests, piglets were placed individually on the center of the maze and left for 5 minutes. The maze was sanitised between piglets if there was presence of urine or faces. A camera placed above the maze recorded all tests for subsequent behaviour analyses.

The behaviours analysed in the maze test were based on previously published works (RODGERS; JOHNSON, 1995; ANDERSEN et al., 2000), namely time spent in centre, time spent in open arms, time spent in walled arms, time standing still or exploring each arm, urinating, defecating, escape attempts and jumping.

2.4.3 mRNA expression assays

2.4.3.1 Blood sampling and peripheral blood mononuclear cells (PBMCs) isolation

Blood sampling from the piglets was carried out on the day before the start of supplementation and two days after the end of supplementation. The order of blood sampling was randomized on all days. The blood collection protocol was based on (MORENO, 1997) (Figure 7) and was done from the jugular vein. The isolation of PBMC was performed by Ficoll (Ficoll-Paque Plus, GE Healthcare) gradient protocol according to Pugliesi et al (2014) . Approximately 8 mL of blood was collected from each piglet into polypropylene tubes containing EDTA solution, which were immediately placed on ice and subsequently centrifuged at 2000 × g for 15 min at 4°C. Plasma was then stored at −20°C until analysis.

2.4.3.2 RNA isolation and Real-Time PCR

The *IL1B, HSD3B2*, and *NR3C1* genes were targets for our gene expression analysis. While, for the relative quantification of transcription, the arithmetic mean of the housekeeping genes *GAPDH* and *UBB* were included. RNA was isolated from PBMC cells according to a previously published protocol (89) , using Trizol reagent (Invitrogen). Reverse transcription was then performed using the High Capacity cDNA Transcription Kit (Applied Biosystems), according to the manufacturer's

instructions.

The synthesized cDNA products were used as the template for amplification transcription polymerase chain reaction real-time (RT-PCR) (Applied Biosystems, California, USA). The thermocycling profile consisted of 40 cycles of 15 s at 95 °C for denaturation and 12 s at 60 °C for annealing and extension, including a previous activation step of 95 °C for 10 min. The final stage included an analysis of the melting curve, verifying the presence of a single peak in the different PCRs. The primers for the targeted genes are illustrated in Table 3.

Table 4. Swine specific oligonucleotide forward (F) and reverse (R) primer sequence (5'-3'), amplicon length of the evaluated genes, and primer efficiency in the standard curve on qPCR.

Targetname	**GenBank ID**	**Primer (5'-3')**	**Amplicon**	**Efficiency (%)**
HSD11B1	NM_001004049.1	F: 5´ TGGTCATCCACACTGCCTCTAT 3´ R: 5' AGGGTCTGTTTGGGCTCATG 3'	90 bp	91,11
NR3C1	AF141371	F: 5' GATCATGACCGCACTCAACATG 3' R: 5' GCTCTCTGGCCTTGGAACAT 3'	68 bp	97,11
IL1B	XM_021085847.1	F: 5' TTTGAAGAAGAGCCCATCATCC3' R: 5' CCAGCCAGCACTAGAGATTTG 3'	119 bp	97,98
GAPDH	NM_001206359.1	F: 5´ TCCTGGGCTACACTGAGGAC 3´ R: 5´ ACCAGGAAATGAGCTTGACG 3´	123 bp	109,59
UBB	U72496.1	F: 5´ ACCAGCAGCGTCTGATTTTT 3´ R: 5´ CAAGTGCAGGGTGGACTCTT 3´	92 bp	100,03

The expression of each gene was quantified by determination of the threshold cycle value (CT) for the fluorescence of the SyBr green dye within the geometric region of the semi-log graph generated during PCR. In the exponential phase of the amplification curve, the quantity of cDNA is considered duplicated in each amplification cycle. The expression of the target genes was carried out using the comparative method (ΔΔ) CT as described by (PFAFFL, 2001) , being normalized in relation to the two reference genes mentioned above.

The expression of the *IL1B* gene was quantified via PBMCs mRNA assays in order to assess the effects of the weaning stress on the immune system, and the *NR3C1* and *HSD11B2* genes were similarly used to investigates the effects on the HPA stress response axis. *IL1B* was the chosen immunity-related gene as it codifies the *IL-1B* cytokine, which affects nearly all cells and organs and is an important mediator of several immunity-related disorders (Dinarello, 2009, 2010; Dinarello et al., 2012; Gabay et al., 2010; Sims e Smith, 2010; Mantovani et al., 2011). The *NR3C1* and *HSD11B2* genes, which codify the glucocorticoid receptor and the 11 B-hydroxysteroid dehydrogenase type 2 enzyme, are involved in HPA-axis-mediated stress responses and its dysfunctions (Polleto, 2006; (Wyrwoll et al., 2011; Bornstein, 2006).

2.5 Statistical analyses

2.5.1 Body weight and milk intake

To test the effect of treatments (A, B and C) on weight and consumption of piglets, a randomized block design was used with repeated measurements in time by the PROC MIXED of the Statistical Package SAS (Software version 9.3). Comparisons between means were made by the Fisher's Protected Least Significant Difference test. For all data, significance was declared when P≤ 0.05.

2.5.2 Molecular data

Data of gene expression that were not normally distributed according to the Shapiro–Wilk test were transformed to natural logarithms. Gene expression were analyzed using analysis of variance (ANOVA) with repeated-measures of time using the PROC mixed method in SAS version 9.2 (SAS Institute), with animal considered as a random effect and group (asinine, bovine and control), day and their interaction considered fixed effects. The ratio of gene expression between the gene expression after and before the treatments was compared by ANOVA using the PROC mixed method (SAS). Comparisons between groups within a day were made using the least significant difference test. Results are presented as arbitrary units.

2.5.3 Cortisol

The weaning effect was determined by calculating a ratio between salivary cortisol concentrations of piglets before and after weaning, considering the collection period, morning or afternoon. The effect of the collection period was determined using a ratio between morning and afternoon salivary cortisol concentration of piglets, considering the weaning event, before or after.

Grubbs test was performed to determine the presence of outliers considering the collection period and the weaning event. Data distribution was determined using the Shapiro-Wilk test. Subsequently, a nonparametric Kruskal Wallis test and Nemenyi post-test were performed to compare the ratios between the treatment groups. A significance level of 5% was considered. All analyzes were performed in the free software environment for statistical computing R, version 4.0.5 (RSudio Team, 2021).

2.5.4 Behavior tests

Principal component analysis (PCA) was performed to evaluate how the behavior measures explained the variance at each principal component (PC) on the Open field, New Object and Elevated Plus Maze Test, in each phase using the FactoMineR (Le et al., 2008) and factoextra (Kassambara and Mundt, 2020) software packages of the R Core Team software (2017).

To test the fixed effect of treatments (A, B and C) and phases (before and after weaning) on piglet behavior, a randomized block design was also used with repeated measurements in time with the PROC MIXED command of the Statistical Package SAS (Software version 9.3). Comparisons between means were made by the Fisher's Protected Least Significant Difference test. For all data, significance was declared when $P \leq 0.05$.

2. RESULTS

3.1 Body weight and milk intake

The final body weight of piglets and the milk intake for groups A and B (Table 5 and Table 6) were not affected by the supplementation (P = 0.589).

3.1.1Milk intake

Table 5. Average values for milk intake for piglets in group A and B piglets before (initial) and after (final) supplementation. SD: standard deviation from the mean; CV: coefficient of variation.

Group	Average ±SD	Minimum	Maximum	CV(%)
A	294.67 ± 11.69	258.30	300.00	3.96
B	243.68 ± 77.27	102.50	300.00	31.71
C	-	-	-	-

3.1.2 Body weight of the piglets

Table 6. Body weight of the piglets before (initial) and after (final) supplementation. Data are presented as mean of body weight ± standard error, minimum and maximum body weight, and the ratio of the standard deviation to the mean (CV%) for each one of the groups studied. SD: standard deviation from the mean; CV: coefficient of variation.

Group	Weight	N	Mean ±SD	Minimum	Maximum	CV(%)
A	Initial	16	7.22±1.83		10.3	25.39
	Final	16	8.81±2.66	5.8	14.0	30.27
B	Initial	16	7.54±1.89	5.1	10.8	25.10
	Final	16	9.12±2.64	4.0	14.0	28.96

C	Initial	16	6.79±1.68	4.1	9.9	24.83
	Final	16	8.63±2.56	5.0	13.5	29.67

3.2 mRNA expression assays

The expression of the IL-1B gene (Figure 17 A) was significantly down regulated in the group fed asinine milk (mean 8.11; p = 0.049) and up regulated in the group supplemented with skimmed cow milk (mean 15.55; p = 0.049), when compared to the control group (mean 14.50; p = 0.049).

When analyzing the genes related to the stress response by the HPA axis, there was significant up regulation in the group supplemented with cow milk (mean 1.245; p = 0.04) in the NR3C1 gene (Figure 17 B) when compared to the group that received asinine milk (mean 0.299; p = 0.366) and the control group (mean 0.315; p = 0.366) in the post-weaning period. Additionally, no significant difference (p > 0.05) was found between the groups supplemented with asinine milk, skimmed cow milk and the control group pre or post-weaning for the expression of HSD11B2 (Figure 17 C).

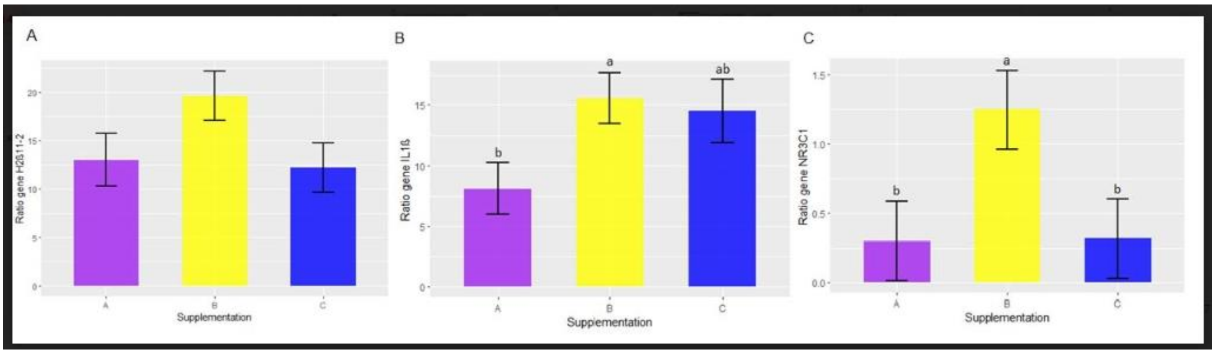

Figure 17. Descriptive analysis of the gene expression of the three evaluated genes, considering 2ΔΔCt values. Comparison between milk supplementation A (Asinine milk), B (Cow skimmed milk) and C (Control without supplementation) in the post-weaning period. Data represent averages ± standard error of the mean (SEM). When there were statistically significant differences (p < 0.05) between supplementation, the letter "a" or "b" were used.

3.3 Salivary cortisol

Regarding salivary cortisol, 20 data points were removed from the analysis as they were considered outliers in the Grubbs test. Salivary cortisol levels from piglets before and after weaning are shown in Figure 18. When comparing groups according to time in relation to weaning, there was significant difference (p < 0.05) between groups in the afternoon period (Kruskal Wallis test; p = 0.04), with the ratio of group A having higher values than that of group B and C (Nemenyi post-hoc test; p = 0.007).

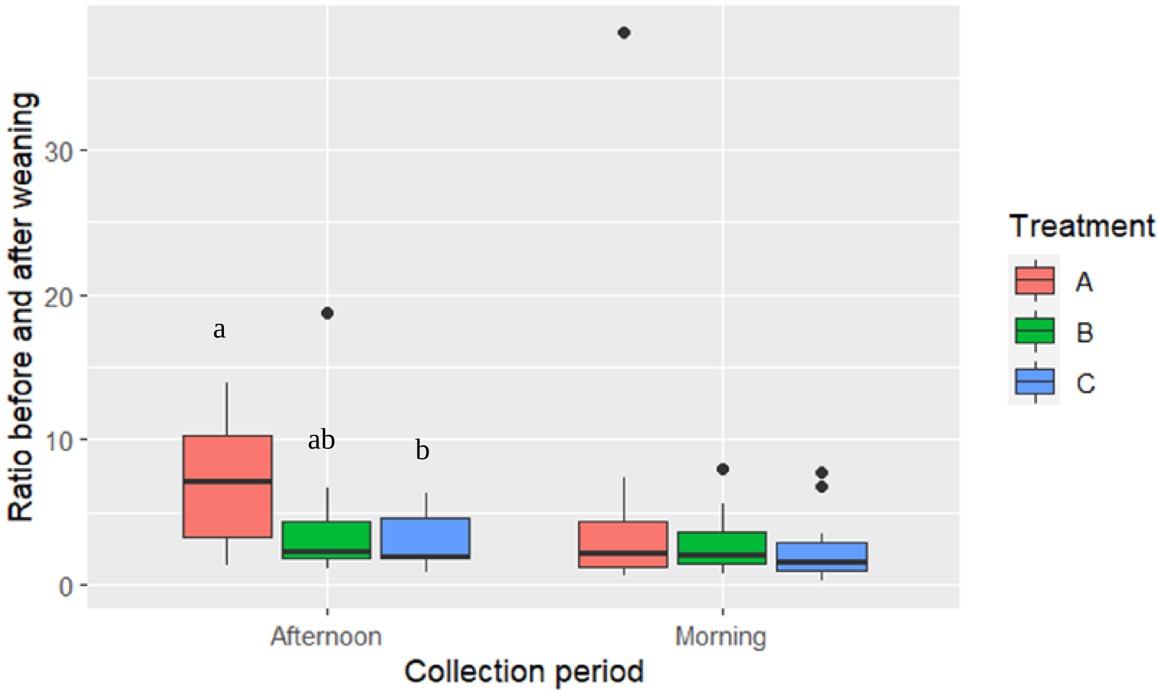

Figure 18. Distribution of the ratio calculated before and after weaning considering the period of collection and treatment of piglets. A: treatment with donkey's milk; B: treatment with cow's milk; C: control treatment with water.

3.4 Behavioural tests that the type of supplementation may not interfere with the behavioral variables studied in the

3.4.1Elevated Plus Maze

Both first PC of PCA referring to the behaviors measured, before and after weaning, explain 78.2% and 70.8% of the variation of the total data, respectively (Figure 19). Before the weaning phase, the PC1 explained 52.8% of the variation and the PC2 25.4%. After weaning, the values of 42.2% and 28.6% were found for PC1 And PC2, respectively.

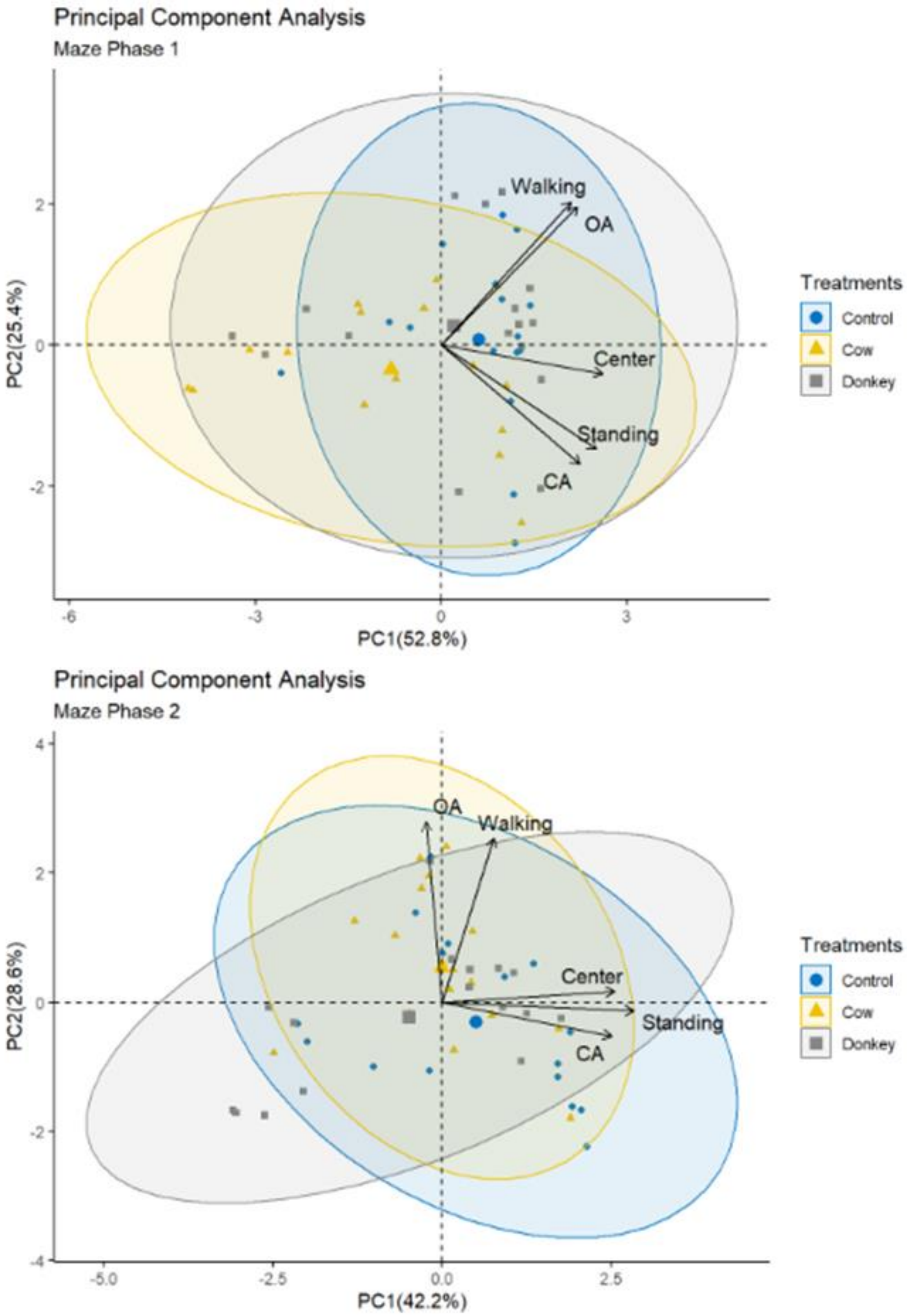

Figure 19. Principal component (PC) analysis of the piglet's behavior in the Maze test before and after weaning (n = 48) submitted to 3 supplementation treatments: Asinine milk, Cow skimmed milk and Control (without supplementation). Behaviors: OA (open arms), CA (closed arms), Center, Standing and Walking. Ellipses indicates the region with 95% confidence of the data for each treatment.

The walking behavior and the time spent in the open arm were the variable who explained more of the total variability of the data. The points match the PC1 and PC2 scores before and after weaning the piglets (Table 7). Most ellipses have intersections with each other, indicating that the type of supplementation may not interfere with the behavioral variables studied in the labyrinth test.

Table 7. Contributions (in percentage) of the variables to the principal components (PC1 and PC2) measured elevated plus maze test.

Variable	Phase	PC1 (%)	PC2 (%)	Total
Open arms	1	17,7	29,2	46,9
Closed arms	1	18,3	21,7	40,0
Central region	1	25,0	1,30	26,3
Moving	1	16,0	31,3	47,3
Stopped	1	23,0	16,5	39,5
Open arms	2	0,269	53,5	53,8
Closed arms	2	29,3	1,89	31,2
Central region	2	30,2	0,195	30,4
Moving	2	2,74	44,3	47,0
Stopped	2	37,4	0,129	37,5

We analyzed the mean values of the behavioral responses of the piglets in the maze test. We found no differences between the mean values of times in the behavioral variables: walking, stay in center, stay in open arm, and stay in closed arm. Only the standing behavior differed (P=0.029) between the treatments studied (Figure 20). It demonstrates that the animals in the control group performed more movements than the other treatments.

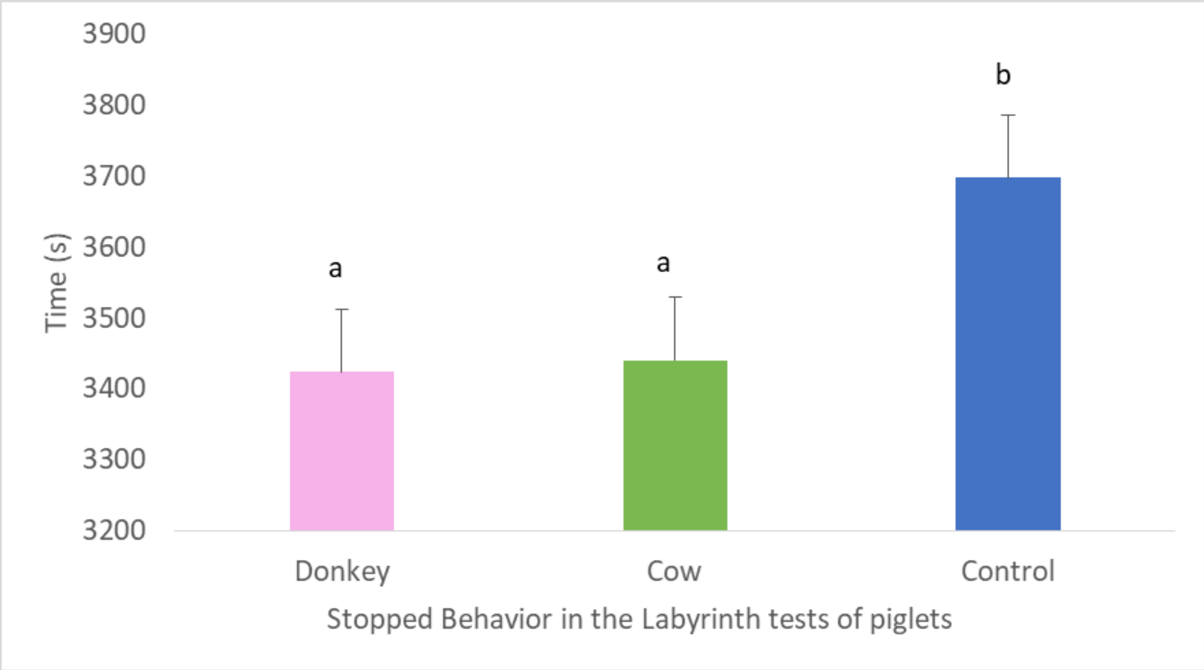

Figure 20. Stopped Behavior in the Labyrinth tests of piglets (n = 48) supplemented with donkey, cow and control milk (without supplementation) during the period from 25 to 31 days of life. The mean values of the columns followed by different lowercase letters indicate significant differences by Fisher's test (P<0.05) between treatments.

3.4.2 Open Field Test and Novel Object

Open Field Test: The first two PC from the PCA referring to the behaviors evaluated before and after weaning explain 92.3% and 95.6% of the total variation of the data, respectively. PC1 in the phase before weaning explained 50.5% of the variation when PC2 explained 41.8%. After weaning, values of 50% and 45.6% were observed for PC1 and PC2, respectively (Figure 21).

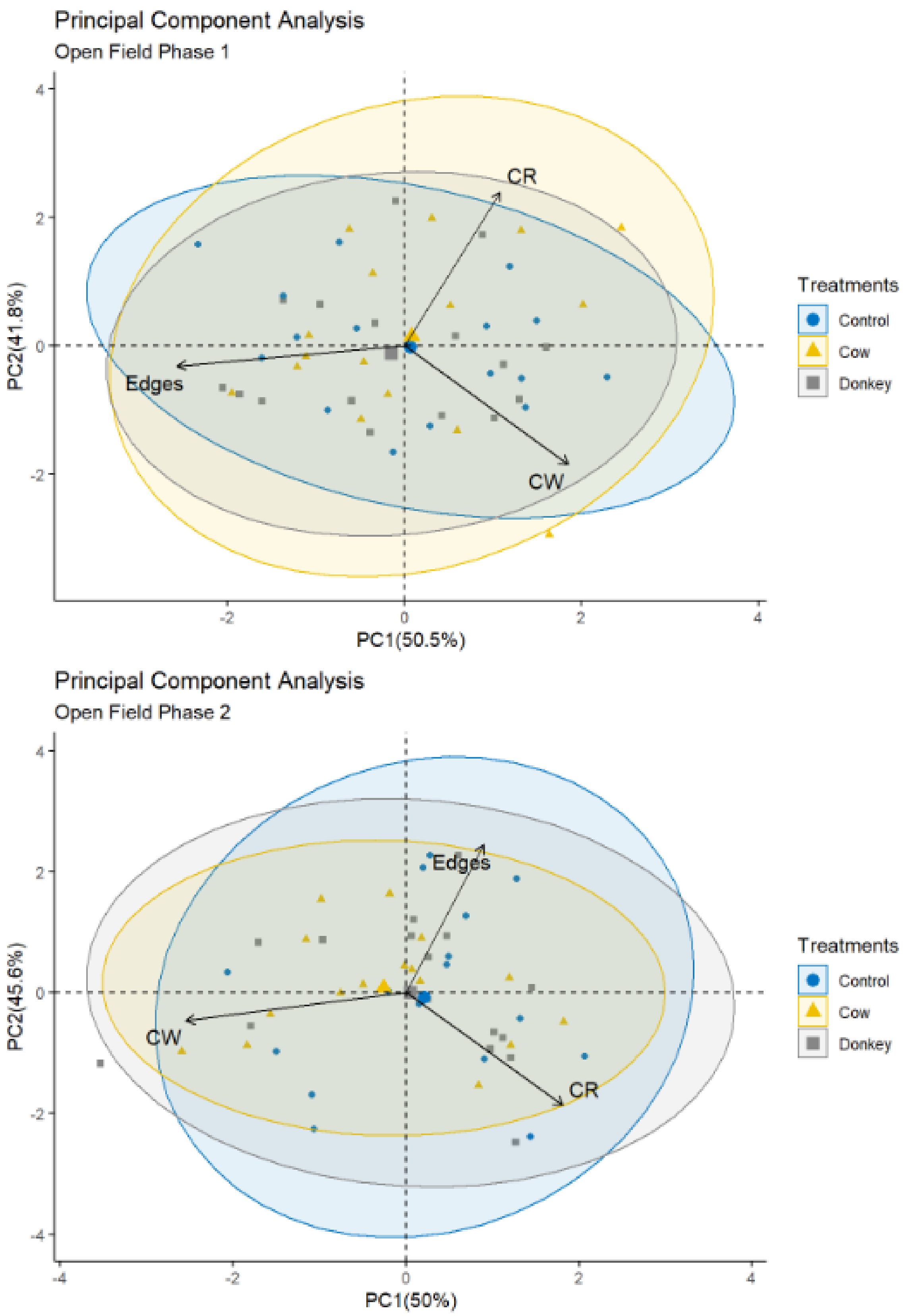

Figure 21. Principal component analysis (PCA) of the piglet's behavior in the Open field test before and after weaning (n = 48) submitted to 3 supplementation treatments: Asinine milk, Cow skimmed milk and Control (without supplementation). Behaviors: Edges (time in edges), CW (time close to the walls), CR (time in the central region of the Open field test arena).

The behavior permanence in the central region (Table 8) was the behavioral variable that possibly best explained the total variability of the data. Most of the ellipses have intersections with each other, indicating that the type of supplementation possibly did not interfere with the behavioral variables studied in the maze test.

Table 8. Contributions (in percentage) of the variables to the principal components (PC1 and PC2) measured during a Open field test.

Variable	Phase	PC1 (%)	PC2 (%)	Total
Time in edges	1	59,0	1,10	60,1
Time to close to the walls	1	30,5	36,9	67,4
Time in the Central region	1	10,5	62,0	72,4
Time in edges	2	7,60	61,8	69,4
Time to close to the walls	2	61,4	2,30	63,7
Time in the Central region	2	31,0	35,9	66,9

Further, the tests of comparison between the average pairs of the behaviors of climbing the wall, jumping against the wall, and excretion as a function of the treatments were analized. Only the behavior jumping against the wall differed (P=0.047) between the studied treatments (Figure 22). Demonstrating that the piglets supplemented with donkey's milk jumped more against the wall than the animals of the other treatments before the supplementation phase.

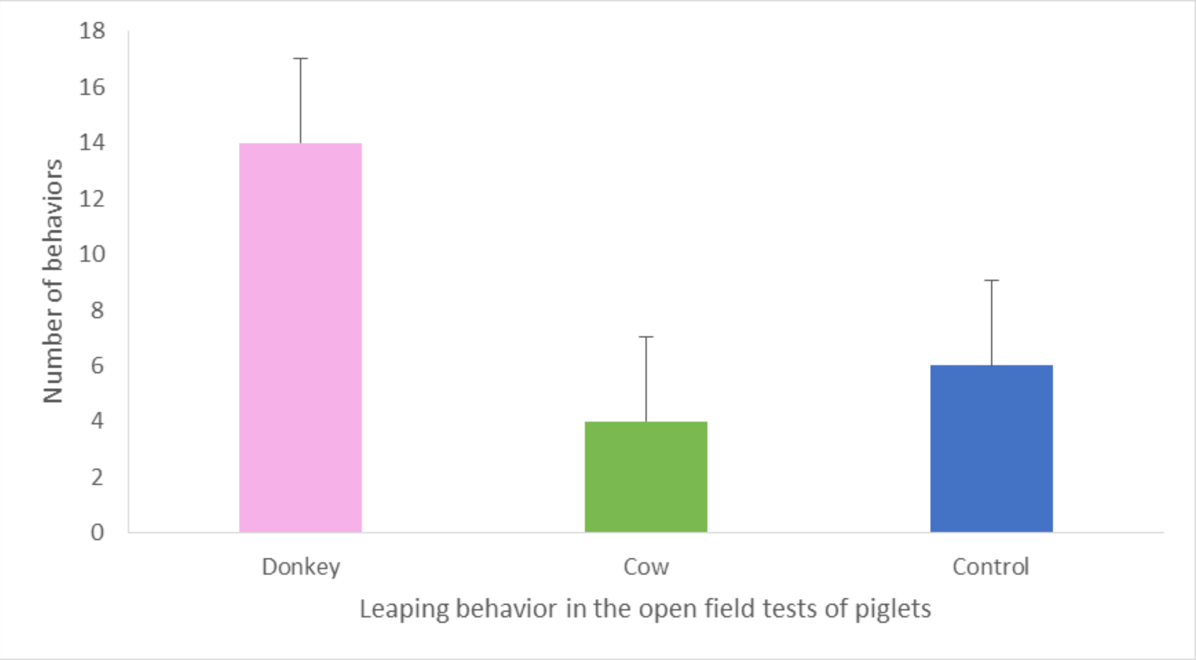

Figure 22. Leaping behavior in the open field tests of piglets (n = 48) supplemented with milk from: donkey, cow and control (without supplementation) in the phases before and after weaning. The mean values of the columns followed by different lowercase letters indicate significant differences by Fisher's test (P<0.05) between treatments.

Novel Object Test: In the evaluation of the average values of behavioral variables of piglets in the Novel Object Test, no differences were observed in latency (treatment: P = 0.124; phase: P = 0.126) or interaction time (treatment: P = 0.266; phase: P = 0.342) of piglets.

3. DISCUSSION

4.1 Weight and food intake

The absence of difference between the groups in these parameters is possibly explained by the low concentration of protein and fat in both asinine milk and skimmed cow milk. The average protein level of asinine milk is 1.5% to 1.8%, and the concentration of fat is 0.2% to 1.8%, similarly to human milk (UNIACKE-LOWE; HUPPERTZ; FOX, 2010; UNIACKE-LOWE; FOX, 2011; ALTOMONTE et al., 2019).

These results were expected, as the supplementation with both types of milk was not meant to enhance weight gain but to assess potential changes in stress response modulation at weaning. The acceptability of both types of milk during supplementation was considered adequate.

4.2 Immunity

The significant variation in IL-1B expression profiles between the experimental groups can probably be attributed to the fact that each supplemental milk used in the study possesses different nutritional properties (MARTINI, 2018; CUNSOLO, 2017), which favored varying intensities of immune reaction in response to the weaning challenges.

The down regulation of IL-1B expression in PBMCs of piglets fed asinine milk follows patterns observed in rats that received asinine milk for 4 weeks, which presented with lower serum concentrations of IL-1 and TNF-a (TRINCHESE, 2015; LIONETTI, 2012) and lower inflammatory state in muscle tissue (TRINCHESE, 2018) when compared to rats that received cow milk or no supplementation. These results differ from the findings of a study in which asinine milk was offered to elderly humans, which showed increased levels of plasma IL-1B, IL-8 and IL-6 (AMATI, 2010), however the latter results were beneficial in the specific context as the subjects were immunocompromised. Additionally, when asinine colostrum and milk were added to human PBMCs, they showed potential to modulate the expression of *IL-1B, TNF-a, IL-10*, and *IL-12* (TAFARO, 2007).

The fat profile of asinine milk, which contains high concentrations of PUFAs (MARTINI, 2018), might have influenced the down regulation of IL-1B expression observed in this study as these fatty acids are generally considered anti-inflammatory (reviewed by GUTIÉRREZ, 2019; HUSSON, 2016; YATES, 2014). Moreover, the lactoferrin content of asinine milk may also have contributed to this result, as the supplementation of lactoferrin in suckling piglets for 7 days has been previously associated with the decrease of IL-1B and TNF-a and increase of IL-10 concentrations in intestinal mucosa, favoring an anti-inflammatory profile (HU, 2019).

On the other hand, the up regulation in IL-1B expression in the group fed skimmed cow milk might be related to its lipid composition which contains higher concentrations of saturated fatty acids (SFAs), which may favors pro-inflammatory responses (reviewed by FRITSCHE, 2015), and poor concentrations of bioactive molecules such as lactoferrin (CUNSOLO, 2017), and does not contain lysozyme.

Besides cytokines, immunoglobulins could also have been used in this study as a parameter of immune response during weaning stress. The concentration of IgA in piglet salive may be modulated by stressful situations, reaching values of 500 mg/L to 800 mg/L from basal concentrations of 100 mg/L in the presence of stress (RIVERA-GOMIS et al., 2020; CERÓN et al., 2022). Future studies may explore the interaction between asinine milk supplementation and IgA response during a stress challenge such as the weaning period.

Further studies are needed to investigate whether the inclusion of asinine milk represents an adequate strategy to mitigate the negative effects of stressful events in early life. Future studies should consider improvements in the experimental design, such as supplementation times and concentrations, to determine the minimum periods and volumes needed that could bring benefits in relation to the stress response. Studies with varying amounts of asinine milk and using a larger sample size are needed to better answer the questions raised. Our study showed that piglets that received asinine milk presented lower post-weaning blood cortisol levels; however, further studies are needed to elucidate the molecular mechanisms involved in production, secretion and the receptors involved in this physiological axis, since our data were inconclusive.

4.3 Stress response - HPA axis

Regarding the gene expression profiles in response to weaning, no significant difference in expression of the 11 beta-Hydroxysteroid dehydrogenase type-II (11β-HSD2) enzyme, which converts cortisol to the inactive form cortisone (DOODY et al., 2017) was observed, but we did find a statistically significant upregulation in expression of the NR3C1 gene at post-weaning in the piglets fed skimmed cow milk, when compared to both other groups. In the assessment of salivary cortisol levels, we found that piglets fed with asinine milk had lower cortisol levels post-weaning in the afternoon samples, when compared to both other groups in the same time period.

The upregulation of NR3C1 expression, as seen after weaning in the piglets fed with skimmed cow milk, has been noted in animals which experienced early life stress (BOCKMÜHL et al., 2015) and in humans diagnosed with post-traumatic stress disorder (PTSD) (YEHUDA et al., 2014) . However, this result contradicts the findings of studies on weanling piglets (CORBETT et al., 2021) , which reported downregulation of NR3C1 expression in contrast to high levels of circulating cortisol.

Research about the dynamics of glucocorticoid receptor expression in response to stress is a relatively new field (MEANEY et al., 1996; MIFSUD; REUL, 2018) , and further studies are needed to elucidate the relevance and nature of glucocorticoids in relation to NR3C1 expression (YEHUDA et al., 2014) . Moreover, the present results refer to gene transcription, as they reflect mRNA levels, and may not reflect the effective protein expression of glucocorticoid receptors or their density in the Central Nervous System.

The results of mRNA expression of NR3C1 were not matched by the levels of salivary cortisol from the piglets, as there were no significant alterations in relation to salivary cortisol in piglets fed with skimmed cow milk. Instead, the group of piglets fed with asinine milk presented with lower cortisol levels in the afternoon post-weaning, in comparison to both other groups in the same time periods. We may suggest that these animals experienced less intense stress response in this period than the piglets fed with skimmed cow milk and the control group, as the exposure to stressors such as weaning causes significant activation of the HPA axis, expressed by the greater production of glucocorticoids (MELIN et al., 2004; LI et al., 2015; STERNDALE et al., 2022).

The decrease in salivary cortisol in the afternoon period could be assigned to the circadian rhythm of cortisol secretion, in which cortisol is physiologically produced at higher levels in the morning and lower levels in the afternoon (RUIS et al., 1997) . However, that is probably not the case in this study as the circadian rhythm of cortisol in piglets is only established at 16 to 20 weeks of age, and the afternoon decrease in cortisol levels is only present after 8 weeks (EVANS; CHRISTOPHERSON; AHERNE, 1988) , and all piglets in this study were no older than 5 weeks old.

The lower levels of salivary cortisol post-weaning in piglets fed with asinine milk could be resulting from various factors in the milk composition, such as the high concentration of lactose, polyunsaturated fatty acids (PUFAs) and lysozyme (BARLOWSKA et al., 2011; MASSOURAS et al., 2020) which are greater than those of cow milk (CHIANESE et al., 2010; CAROLI et al., 2017; MARTINI et al., 2018) and similar to human milk (CORCUERA et al., 2002; MONTI et al., 2007, 2012; GARAS et al., 2016). The specific composition of asinine milk could be relevant for this result as previous studies have elucidated valuable information. For example, researchers demonstrated that lysozyme in asinine milk represents 21% of the protein fraction (GUO et al., 2007; ASPRI; ECONOMOU; PAPADEMAS, 2017) This enzyme is poorly digested in the gastrointestinal tract, and acts primarily at gut level in microbiota modulation, favouring healthy configurations (CUMMINGS et al., 2004; CAPUANO, 2017; MARRA et al., 2021) . In addition, microbiota changes may influence the gut-brain axis (MARQUES et al., 2014; CONTE; SICHETTI; TRAINA, 2020) , potentially protecting the piglets fed with asinine milk from harmful effects caused by the weaning stress. Another factor that was previously explored involves the concentration of fat in asinine milk (TRINCHESE et al., 2015, 2018; VINCENZETTI et al., 2021) . Researchers demonstrated that it is lower than that of cow milk, however its composition is richer in Omega-3 PUFAs (MARTEMUCCI; D'ALESSANDRO, 2012; CHIOFALO; SALIMEI, 2014; ASPRI; ECONOMOU; PAPADEMAS, 2017) and its configuration of smaller fat globules favours digestive kinetics (CAROLI et al., 2017; ALTOMONTE et al., 2019) . The supplementation with Ômega-3 PUFAs may also have modulating effects on stress responses (MADISON et al., 2021) , and has been found to positively alter the stress response of piglets during weaning (LEE et al., 2005) .

Asinine milk is also rich in lactose, which is an important source of galactose. This carbohydrate is a key structural element in complex molecules that are crucial for early development (MARTINI et al., 2018), and may also positively alter the gut microbiota towards profiles that are compatible with favourable stress responses (CAROLI et al., 2017). It may also influence the acceptability by individuals (BARNI et al., 2018), especially children , and contributes to the fact that asinine milk is considered highly palatable (POLIDORI; VINCENZETTI, 2012; POLIDORI et al., 2016; BARNI et al., 2018; MARTINI et al., 2021) . The ingestion of palatable foods may have stress levels and mood altering effects (OLIVER; WARDLE; GIBSON, 2000; MACHT, 2008; TRYON; DECANT; LAUGERO, 2013; RANDO; SIMMONS, 2015) that may have contributed to the lower stress response in piglets fed with asinine milk.

Moreover, asinine milk oligosaccharides may have the capacity to modulate the proliferation, apoptosis and differentiation of intestinal cells (WANG 2019) and assist in brain development and cognition (MONTI et al., 2012; ALTOMONTE et al., 2019) , by being employed in the formation of myelin (++3) and providing sialic acid (++8 ++9) (UNIACKE-LOWE; FOX, 2011; HOBBS et al., 2021). Nevertheless, additional research utilizing longer supplementation periods, higher milk volumes and different types of milk are needed to assess whether they have modulating effects on systemic events that constitute stress responses. As in this study the supplementation time was only 6 days, there might not have been enough time to generate greater modulatory effects compatible with the broad range of health benefits potentially offered by asinine milk.

4.4 Behaviour

Although the salivary cortisol assessments indicate that piglets fed with asinine milk were less intensely stressed than those of other groups, the results from the behavioural tests do not reflect that finding. All groups performed similarly in the behavioural tests in regards to indicators of fear and anxiety, with the exception of a single variable: piglets fed with asinine milk jumped against the walls the most during the Open Field/Novel Object tests, in the post-weaning assessments, when compared to the other groups in the same period. It is not possible to tell from this single behavioural variable whether these animals were more or less stressed than the others.

It is known that in emotional tests the most basic and common result of interest is “movement”; however, this can be influenced by motor output, exploratory drive, freezing, or other behavior related to fear, illness, relative timing in the circadian cycle, among many other variables (GOULD; DAO; KOVACSICS, 2009).

The high concentration of Omega-3 PUFAs in asinine milk could potentially have generated greater behavioural effects in these piglets, as demonstrated by the supplementation of Ômega-3 and Ômega-6 PUFAs and the modulation of behaviour in piglets in other studies (Kai-Fong Ng, 2003). However, our results did not reflect these studies, possibly due to not enough time or volume supplemented.

Another important factor for consideration is that the behavioural tests in this study were conducted both before and after weaning, therefore the repetition could have influenced the reaction of all piglets in the second exposure to the arenas. Animals exposed to the Open Field tests a second time tend to be less active, less explorative and realise less vocalisations than in their first exposure to the arena (Donald, 2011).

Other studies using asinine milk to supplement piglets are unknown, and the use of behavioural measurements to assess the physiological significance of early changes in diet on the developing pig brain is not yet well established (Kai-Fong Ng, 2003). Further studies with varying supplementation times and volumes are needed to understand the possible modulation mechanisms of physiological and behavioural responses of piglets to stress. The inclusion of asinine milk for a longer period could be a suitable option, as other studies have found benefits of supplementing piglets with Omega-3 for upwards of 30 days (Fil, et al, 2019; Almeida, 2021; Costa, 2021).

4. CONCLUSION

Supplementation with asinine milk may have the potential to improve immunity parameters, as well as modulating the increase of salivary cortisol levels of piglets undergoing the stress of weaning, as a model for potential future interventions in human infants. Further studies should investigate the mechanisms behind the alterations found in cytokine gene expression and cortisol concentrations, as well as changes in other molecules which may be mediated by asinine milk supplementation.

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10 FINAL CONSIDERATIONS

There is a good possibility of reintroduce donkeys in the economic scenario of Brazil through the production of asinine milk, using ethically acceptable and sustainable production approaches. However, some issues are necessary to be solved before the implementation of the donkey dairy production chain. Since the slaughter of donkeys for skin production is still very problematic as it generates significant, and almost unsolvable, welfare and environmental issues milk production appear to be a better option. In this Ph.D. reseach some of the central issues related to the welfare of the donkeys, during milking, donkey milk composition and impact on the health, behaviour and welfare of pigs, which are relevant animal model for humans, were investigated. The initial issue, that the ideal separation time for milking and the disposition of milking that does not compromise the welfare of lactating donkeys and suckling foals was thought, explored, and tested. It was concluded that the separation time of 2h prior milking is ideal for the animals. It is also noted that the physicochemical parameters of Brazilian female donkeys of the Pêga breed maintain the same profile of parameters found in national and international studies.

Regarding the use of asinine milk as a nutraceutical feed source, some studies are still needed. However, it is believed that this is a promising scenario, as the inclusion of asinine milk used to supplement piglets in the farrowing phase demonstrates that we were able to modulate some negative effects generated by abrupt weaning.

Thus, it is evident that the resignification of donkeys in Brazil is very necessary. Given the welfare issues and the lack of sustainability associated with the skin trade, this approach will not meet the demands to resignify donkeys in the Northeast of Brazil. This work offered robust evidences that asinine milk production has tremendous potential to help in developing a sustainable, ethically acceptable new relationship with donkeys, which have a very important role for Brazilian cultural valuesHowever, more studies must be done in order to understand the implications of dairy donkey industry for animal welfare, sustainability and economic development.

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