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Leukofiltration enhances *Escherichia coli* control in equine blood bags[1]

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ABSTRACT.- Moura FMB, Silva KPC, Escodro PB, Bernardo JO, Pereira YVSP, Notomi MK. **Leukofiltration enhances *Escherichia coli* control in equine blood bags.** *Pesquisa Veterinária Brasileira 45:e07559, 2025.* Faculdade de Medicina Veterinária, Universidade Federal de Alagoas, Fazenda São Luiz, Viçosa, AL 57700-000, Brazil. E-mail: francyelly.moura@ceca.ufal.br

Leukoreduction by filtration (LRF) is widely used in human blood banks to reduce the transmission of infectious agents, including viruses, bacteria, and blood parasites. This study evaluated the efficiency of LRF in controlling *Escherichia coli* contamination in equine blood bags over 14 days of storage. This *in vitro* experimental study utilised blood samples inoculated with *E. coli.* Blood samples (450 mL per bag) were obtained from six hematologically stable horses. Fourteen equine blood bags were divided into control (unfiltered) and experimental (with a leukocyte filter) groups. Whole blood bags were refrigerated for two hours at 2-6 °C, after which each bag was inoculated with 1 mL of *E. coli*. The experimental group underwent bedside leukocyte filtration using pre-filters. Blood count, osmotic fragility, percentage of hemolysis, bacterial culture, and serum potassium, protein, albumin, and glucose levels were analysed. Tukey's and Pearson's chi-square tests were applied, with significance at p-value < 0.05 and 95% confidence interval. The filtered group showed an immediate 65.5% reduction in bacterial load, reaching 97.01% by day 14, compared to 72.99% in the control group. Although both groups showed a reduction in bacterial load during the 14 days of storage, the data indicate that filtration significantly contributed to a more rapid and pronounced decrease in *E. coli*. The main limitations were a small sample size (14 blood bags) and a relatively short storage period (14 days), limiting the study's generalizability and reducing its statistical power. Human leukocyte filtration efficiently reduces *E. coli* contamination in equine blood, without affecting red blood cell count or osmotic fragility. The findings suggest that leukoreduction may act as a complementary method to refrigeration in bacterial control of equine blood bags. Nevertheless, further studies with greater statistical power and extended storage durations are recommended.

INDEX TERMS: Blood bank, leukoreduction, bacterium, blood storage, leukocyte filtration, horse.

RESUMO.- [**Influência da leucofiltração no controle de *Escherichia coli* em bolsas de sangue equino.**] A leucorredução por filtração (LRF) é um procedimento comum em bancos de sangue humano e também tem sido usada para reduzir a transmissão de agentes infecciosos como vírus, bactérias e parasitas sanguíneos. Este estudo teve como objetivo avaliar a eficiência da leucorredução por filtração no controle quantitativo de *Escherichia coli* inoculadas em bolsas de sangue equino durante 14 dias de armazenamento. Amostras de sangue de aproximadamente 450 ml por bolsa de sangue foram obtidas de seis cavalos hematologicamente estáveis. Quatorze bolsas de sangue equino foram divididas em dois grupos: controle (sem filtração) e experimental (com filtro leucocitário). As bolsas de sangue total foram refrigeradas por duas horas entre 2-6 °C, após cada bolsa foi inoculada com 1 ml de *E. coli* diluída em caldo Brain Heart Infusion, contendo $1x10^8$ CFU/ml. No grupo experimental foram aplicados filtros leucocitários do tipo beira de leito com pré-filtro. Foram analisados hemograma, fragilidade osmótica, porcentagem de hemólise, cultura bacteriana, dosagens séricas de potássio, proteína, albumina e glicose. Para análise descritiva e comparação de médias entre grupos e entre tempos foram utilizados o teste de Tukey e teste qui-quadrado de Pearson para concentração bacteriana e eficiência de filtração, com significância fixada

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em valor de $p < 0,05$ em ambos os testes, sendo o intervalo de confiança de 95% (IC). Observou-se uma redução imediata de 65,5% da carga bacteriana no grupo filtrado, com redução acumulada de 97,01% ao final do período, comparado a 72,99% do grupo controle. O filtro leucocitário humano foi eficiente na leucorredução do sangue total equino inoculado com *E. coli*, sem redução quantitativa da série vermelha ou aumento da fragilidade osmótica. Embora ambos os grupos tenham apresentado redução na carga bacteriana ao longo dos 14 dias de armazenamento, os dados indicam que a filtração contribuiu significativamente para uma redução mais rápida e acentuada de *E. coli*. O estudo apresentou número limitado de amostras e período de armazenamento relativamente curto. Ainda assim, os achados sugerem que a leucorredução pode atuar de forma complementar à refrigeração no controle bacteriano em bolsas de sangue equino. Estudos com maior poder estatístico e duração estendida são recomendados para confirmar esses resultados e esclarecer os mecanismos envolvidos.

TERMOS DE INDEXAÇÃO: Banco de sangue, leucorredução, bactéria, cavalo.

INTRODUCTION

Leukoreduction by filtration (LRF) is routinely performed in human blood banks but remains infrequent in veterinary medicine. This procedure is characterised by the removal of leukocytes from blood components, resulting in an average leukoreduction of 97% (Andreu et al. 1988). In human medicine, pre-storage LRF can prevent non-hemolytic febrile autoimmune reactions to transfusion (Chien et al. 2024), as well as the formation of clots in blood bags (Wortham et al. 2003). Hébert et al. (2003) found that humans who received blood from pre-stored leukoreduced bags required fewer antibiotics and showed reduced mortality. Rajesh et al. (2015) showed that non-immune transfusion reactions were correlated with elevated cytokine concentrations during storage, and that the prior removal of leukocytes decreased the non-hemolytic febrile reaction to transfusion. LRF has previously been shown to be viable in equine blood, achieving a leukocyte reduction rate of 93.4% (Aguilar et al. 2008), without affecting erythrocyte concentrates or characteristics. Additionally, LRF has been employed to reduce the transmission of infectious agents, such as viruses, bacteria, and blood parasites (Dzik et al. 2000), as proven with human cytomegalovirus (Mainou et al. 2016), and is routinely applied in blood banks. Several studies have previously reported that leukoreduction of donated blood can achieve a reduction in the transmission of viruses, such as Epstein-Barr, human herpes 6–8, human T cell leukaemia/lymphoma virus (Buddeberg et al. 2008); bacteria such as *Chlamydia pneumoniae* (Ikejima et al. 2005) and *Escherichia coli* (Hinson et al. 2020, Braathen et al. 2021); and other infections such as leishmaniasis or trypanosomiasis (Buddeberg et al. 2008, Jimenez-Marco et al. 2017, Pereira et al. 2023).

The mechanisms underlying bacteria removal via LRF include phagocytosis by leukocytes trapped within the filter, adhesion to the leukocyte filtrate, direct adhesion to the filter, and complement-mediated cell death (Hinson et al. 2020). Although blood transfusions are lifesaving, they are associated with risks. Mortality, multiple organ failure, and generalised infections following transfusion can all lead to death (Blumberg & Heal 2022). Given the scarcity of studies in veterinary medicine regarding the efficacy of leukoreduction in controlling bacteria in contaminated blood bags, particularly in equines, this study aimed to test the hypothesis that filtration using human leukocyte filters would significantly reduce the *E. coli* load beyond what would be expected from refrigeration alone.

MATERIALS AND METHODS

Ethical approval. The animals used in this study were managed following the standard animal welfare practices. This study was approved by the Ethics Committee on Animal Use of "Universidade Federal de Alagoas" (UFAL).

Fourteen equine blood samples with a volume of 450 mL were stored in blood collection bags containing CPDA-1. Six hematologically stable adult horses were selected as donors, including one male and five females, with an average weight of 450 kg ± 50 kg. Two of these animals participated twice, respecting the interval between collections of 21 days. Health status was verified by physical examination, blood count, renal function (urea and creatinine), and liver function (AST/TGO and GGT).

Blood was collected from the selected animals into blood bags using routine techniques for the species (Jamieson et al. 2022). The volume of blood collected was approximately 450 mL (630 g) per bag. The units of whole blood were refrigerated for two hours between 2 °C and 6 °C, according to the methodology recommended by Brownlee et al. (2000). All animals were clinically monitored for 15 days after collection.

Inoculation of *Escherichia coli* colonies. Before initiating blood collections, bacterial colonies were cultured in laboratory test tubes containing 2 ml of brain heart infusion (BHI) broth, incubated for 18 hours at 37 °C, and refrigerated until inoculation. Prior to contamination of the blood bags, the inocula were grown on Levine Agar to ensure bacterial purity. Immediately after blood collection, 1 mL was inoculated into each bag containing 1 × 108 CFU/mL per inoculum following a technique similar to that described by Hinson et al. (2020). The strain of *E. coli* used was characterized as a multidrug-resistant, haemolytic strain from the American Type Culture Collection.

Leukoreduction of blood bags. After cooling the blood bags to a temperature between 1 °C and 6 °C for two hours, the bags were separated into two groups: experimental (n = 7) and controls (n = 7). In the experimental group, filters were applied to remove leukocytes from erythrocyte concentrates. The filters were Model BBS PF (Fresenius HemoCare, São Paulo, Brazil) for laboratory use, comprising a transfer bag and pre-filter, whereas the bags of the control group remained unfiltered. The filtration system comprised tubes with the proximal end connected to the blood bag, the leukocyte filter in the middle, and a transfer bag (satellite) located at the distal end. The blood bag was maintained at a height of 1.75 m, and gravity filtration was carried out, but each filter was saturated with blood before releasing the clip that allowed the blood to enter the satellite bag.

Storage and laboratory analyses. A sterile area was created using a Bunsen burner. Afterward, 5 mL blood samples were collected from each bag, with 4.0 mL stored in a tube without an anticoagulant for immediate analysis, and 1 mL sent for microbiological analysis. The tests were performed at four different times: (T0) two hours after blood collection and before leukofiltration; (T1) immediately after leukofiltration; (T7) seven days after collection; (T14) 14 days after collection. Hemogram analysis, osmotic fragility, and percentage of hemolysis were all performed. Blood counts were performed using an automated veterinary haematological counter, the Prokan Electric Impedance Method, with the measurement of globular volume by the microhematocrit technique, in a blood smear stained by rapid panoptic (methylene blue and eosin), and differential leukocyte count. Following

filtration, total leukocytes were quantitatively evaluated in a Neubauer chamber. To assess osmotic fragility, 25 µL of blood was added to 2.5 mL of NaCl solution at the following concentrations: 0.9%, 0.7%, 0.5%, 0.3%, and 0.1%, similar to the technique of Quadros & Brito Junior (2020). After 30 minutes of incubation at room temperature and centrifugation at 1616 RPM for 10 minutes, the absorbance of the supernatant was measured by spectrophotometry at 540 nm using a 0.9% NaCl solution as the blank. This technique was applied to evaluate minimum resistance (start of haemolysis), maximum resistance (100% haemolysis), and average corpuscular fragility (50% haemolysis).

Evaluation of bacterial growth. Bacterial growth was assessed using the 1 mL aliquot obtained from the bags of both groups, which were cultivated in 10% Sheep Blood Agar, MacConkey Agar, and Levine Agar and incubated for 48 hours at 37 °C.

Statistical analysis. BioEstat software (version 5.0) was used for all statistical analyses. The normality of the data distribution was evaluated using the Shapiro-Wilk test. Descriptive analysis and comparison of means between groups and timepoints were performed using Tukey's test and Pearson's chi-square test to determine the bacterial concentration and filtration efficiency. A 95% confidence interval was used for all estimates, and a p-value < 0.05 was considered significant in both tests.

RESULTS

Efficacy of leukoreduction

No significant differences were observed in the number of red blood cells (RBCs), hemoglobin concentration, globular volume, fibrinogen levels, serum levels of potassium, protein, albumin, and glucose between the groups ($p > 0.05$). However, significant differences in erythrocyte fragility and potassium levels were found after 14 days of storage ($p < 0.05$).

Whole blood bags collected from six horses inoculated with *Escherichia coli* initially presented (mean and 95% CI) with a value of 7.25×10^6 ($6.8\text{-}8.1 \times 10^6$) leukocytes. After filtration with bedside-type filters and a pre-filter, no white blood cells were identified in the automatic cell count or Neubauer's chamber (< 250.1 cells/mL). No quantitative reduction of the red series was observed, as there were no statistical differences in the comparison of the number of RBCs ($p > 0.05$), hemoglobin concentration ($p > 0.05$), globular volume ($p > 0.05$), or fibrinogen ($p > 0.05$), of the bags before and after filtration.

In the control group, the average number of erythrocytes in the blood was 6.86 (5.68-7.83) × 10^9, the average hemoglobin concentration was 12.3 (9.5-14.7) g/dL, the average globular volume was 29% (24-32), and the average fibrinogen concentration was 433.33 (325.24-541.41) g/dL. In the experimental group, the corresponding values are 6.84 (6.22-7.45) × 10^9, 11.8 (10.7–13.2) g/dL, 29% (26-32), and 300 (171–428) g/dL, respectively (Table 1).

In the experimental group, the average pre-filtration values were 1642 (range 1,364–1,968), and the post-filtration value was 2,099.40 (range 1,588–2,500), representing the concentration at which hemolysis began. The maximum resistance, corresponding to total haemolysis, was found at 0.3% NaCl. The pre-filtration average was 212 (range 117–553), while the post-filtration average was 1,158.19 (range 762–1,554). No significant differences were observed between samples with and without leukoreduction ($p > 0.05$) (Table 2).

Table 1. Mean values, standard deviation, and maximum and minimum (Max and Min) values of the number of erythrocytes (Erythro), hemoglobin concentration (Hb), mean corpuscular volume (MCV), leukocyte count (Leuco), and fibrinogen concentration (Fibri) of equine blood bags pre- and post-leukoreduction

		Erythro (x109)	Hb (g/dL)	MCV(%)	Leuko (x103)	Fibri (g/dL)
Pre-filtration	Average	6.86	12.3	29	7,250a	433.33
	SD	2.24	5.31	9	1,008	196.64
	Min	4.36	7.9	20	6,400	200.00
	Max	11.9	25.0	49	8,700	400.00
Post-filtration	Average	6.84	11.8	29	0b	300.00
	SD	0.74	1.47	3	0	268.33
	Min	5.81	9.80	26	0	100.00
	Max	7.89	14.00	35	0	400.00

SD = Standard deviation; a,b = Different letters indicate statistically significant differences between the groups ($p < 0.05$); Absence of letters indicates no statistically significant difference ($p > 0.05$).

Table 2. Average absorbance values of the osmotic fragility of red blood cells from equine blood bags with different concentrations of sodium chloride (0.9%, 0.7%, 0.5%, 0.3% and 0.1%) pre- and post-leukoreduction

		0.9%*	0.7%	0.5%	0.3%	0.1%**
Pre-filtration	Average	2,500	2,495	1,642	212	464
	SD	0.00	11.18	371.97	474.10	638.08
	Min	2,500	2,475	1,080	0.79	0,80
	Max	2,500	2,500	1,979	1,061	1,250
Post-filtration	Average	2,500	2,500	2,099.40	1,158.19	1,356.60
	SD	0.00	0.00	411.84	888.36	639.80
	Min	2,500	2,500	1,469	970	1,039
	Max	2,500	2,500	2,500	2,500	2,500

* Negative control, ** positive control; SD = standard deviation, Min = minimum, Max = maximum; Absence of letters indicates no statistically significant difference ($p > 0.05$).

LRF did not interfere with erythrocyte fragility immediately following the procedure. However, after 14 days, the RBCs in the leukoreduced bags were more susceptible to haemolysis, with an average of 2,053 (range 1,665–2,440), compared to the average of 2,124 in the unfiltered bags (range 1,709–2,538). This difference was statistically significant ($p < 0.05$) (Table 3).

Hemolysis was further evaluated based on the potassium concentration. No statistically significant differences were found ($p > 0.05$) in the potassium concentration before the procedure (average of 3.0 [2.8–3.1] mEq/dL) and after filtration (average of 6.4 [5.2–7.55] mEq/dL) (Table 4). In addition, there were no significant differences in other parameters such as total protein, albumin, or glucose (Table 5).

An increase in potassium concentration was observed during storage. In bags without filtration, the averages were 2.98 (2.80–3.16) mEq/dL, 5.38 (5.13–5.63) mEq/dL,

Table 3. Average absorbance values of the osmotic fragility of red blood cells from equine blood bags with and without leukoreduction under different concentrations of sodium chloride (0.9%, 0.7%, 0.5%, 0.3% and 0.15), at three different time points

		T0		T7		T14	
		UNFILT	FILT	UNFILT	FILT	UNFILT	FILT
NaCl 0.9%	Average	2,500	2,500	2,395	2,500	2,500	2,276
	SD	166	0	1,193	1,179	0	391
	Min	1,974	2,500	1,631	1,227	2,500	1,565
	Max	2,500	2,500	2,500	2,500	2,500	2,500
NaCl 0.7%	Average	2,500	2,500	869	1,177	2,053a	2,124b
	SD	179	0	938	1,132	448	418
	Min	1,929	2,500	0.811	0.835	1,449	1,444
	Max	2,500	2,500	2,500	2,500	2,500	2,500
NaCl 0.5%	Average	1,585	1,499	274	198	1,707	922
	SD	771	411	670	483	531	769
	Min	0.895	1,469	0.390	0.689	0.689	0.622
	Max	2,154	2,500	1,184	1,184	2,500	1,801
NaCl 0.3%	Average	218	548	223	174	1,032	867
	SD	581	601	545	426	387	874
	Min	0.587	0.562	0.425	0.515	0.775	0.515
	Max	1,061	1,338	1,338	1,140	1,924	2,221
NaCl 0.1%	Average	240	714	194	173	932	876
	SD	479	552	473	422	817	882
	Min	0.548	0.976	0.497	0.640	0.773	0.514
	Max	1,250	1,650	1,161	1,169	2,154	2,221

T0 = Day of collection, T7 = seven days after collection, T14 = 14 days after collection, UNFILT = unfiltered, FILT = filtered, SD = standard deviation, Min = minimum, Max = maximum, a,b = Different letters indicate significant differences ($p < 0.05$) between means; Absence of letters indicates no statistically significant difference ($p > 0.05$).

Table 4. Mean values, standard deviation (Devpad), maximum (Max) and minimum (Min) values of plasma potassium, total protein, albumin and glucose concentrations in equine blood bags with and without leukoreduction

		Potassium (mEq/dL)	Protein (g/dL)	Albumin (g/dL)	Glucose (mg/dL)
Pre-filtration	Average	3.0	5.38	2.7	485
	SD	0.78	0.53	1.04	166
	Min	2.7	4.6	1.0	105
	Max	5.6	6.36	5.3	565
Post-filtration	Average	6.4	5.44	3.0	443
	SD	1.62	1.59	2.04	142
	Min	3.0	4.31	1.5	271
	Max	8.4	12.67	8.7	688

SD = Standard deviation; Absence of letters indicates no statistically significant difference ($p > 0.05$).

and 7.18 (6.95–7.40) mEq/dL, at times T0, T7, and T14, respectively. In the experimental group (with filtration), the averages at the same intervals were 3.38 (2.23–4.53) mEq/dL, 5.12 (3.89–6.33) mEq/dL, and 7.58 (6.66–8.50) mEq/dL, respectively. This difference was statistically significant between times in both groups ($p < 0.05$).

Filtration and reduction in bacterial load

In this study, 14 whole blood bags containing 450 mL each of CPDA-1 originating from six different donors were inoculated with 1 × 108 CFU/mL of *E. coli*. Bacterial viability was confirmed by recovery of viable colonies in all bags immediately after inoculation, with a consistent average count of 6,951.76 CFU/mL (6,428.94–8,833.55), ensuring uniform initial contamination across samples.

Half of the bags (n = 7) were subjected to leukoreduction by filtration (LRF). Following filtration using bedside-type leukocyte filters with a pre-filter, the average bacterial count was reduced to 4,553.33 CFU/mL (2,885.58–4,859.41), representing a 65.5% decrease (range 780–10,000 CFU/mL). This reduction was attributed solely to the filtration process and associated leukocyte removal. Full data on filtration-related bacterial reduction are presented in Table 6.

After storage, a continuous decrease in the number of bacteria was observed in both groups. The control group presented an average colony count of 2,934.44 (2,244.10–3,624.78) CFU/mL of *E. coli* between T0 and T7 and 1,701.33 (968.45–2,411.98) CFU/mL between T7 and T14, representing reductions of 53.44% and 43.07%, respectively. The experimental group presented with an average colony count of 764.50 (493.35–1,110.86) CFU/mL at T7, and 111.28 (37.92–182.84) CFU/mL at T14, yielding a reduction of 79.02% between T0 and T7 and 79.26% between T7 and T14 (Fig. 1 and 2, Table 7). Considering the total decrease in bacterial quantity, non-LRF bags had a reduction of 72.99% (minimum = 1701; maximum = 6,951 CFU/mL) throughout the entire experiment, while LRF bags had a total reduction of 97.01% (minimum = 111.28; maximum = 4,553 CFU/mL) during the same period (Fig. 3 and 4). As such, there was a statistical difference in the comparison of the percentage reduction between time points (T0/T7/T14) and between groups (LRF and non-LRF); the *p*-value was < 0.001 in Pearson's chi-square test with 5% significance.

In the bags of the control group, throughout the entire study period, the levels of leukocytes oscillated between 4,700 and 7,900 × 10^3, and the difference between T0 and T14 was not statistically significant ($p > 0.05$).

DISCUSSION

So far, little research has been conducted on the use of LRF in equine blood. However, similar to the study by Aguilar et

Table 5. Mean values, standard deviation (Devpad), maximum (Max) and minimum (Min) value of *Escherichia coli* colony-forming units in equine blood bags immediately following leukoreduction, and after seven and 14 days of storage

		T0	T1	T7		T14	
		UNFILT	FILT	UNFILT	FILT	UNFILT	FILT
Potassium (mEq/dL)	Average	2,98a	3,38A	5,38b	5,12B	7,18c	7,58C
	SD	0,78	1,10	0,24	1,62	0,22	0,88
	Min	2,7	5,0	5,0	3,0	7	6,5
	Max	3,2	5,6	5,6	6,3	7,5	8,7
Protein (g/dL)	Average	5,24	5,69	5,17	5,19	5,89	6,06
	SD	0,39	0,47	0,37	0,53	0,76	1,03
	Min	4,71	4,6	4,49	4,31	4,97	4,93
	Max	5,91	6,54	5,82	6,14	7	7,66
Albumin (g/dL)	Average	2,48	2,65	3,56	2,47	4,21	4,55
	SD	0,52	0,63	1,16	0,92	2,48	2,90
	Min	1,0	1,94	1,92	1,22	2,28	2,25
	Max	3,27	3,72	5,3	3,42	7,5	8,7
Glucose (mg/dL)	Average	492	507	347	463	471	527
	SD	38	54	104	66	182	135
	Min	105	422	271	135	271	351
	Max	538	565	541	550	688	

T0 = Day of collection, T7 = seven days after collection, T14 = 14 days after collection, UNFILT = unfiltered, FILT = filtered, SD = standard deviation, Min = minimum, Max = maximum; Uppercase (A-C) and lowercase (a-c) letters indicate statistically significant differences between means ($p < 0.05$); The absence of letters indicates no statistically significant difference ($p > 0.05$).

Table 6. Mean and standard deviation values of filtration interference of colony-forming units of *Escherichia coli* (*E. coli* CFU/mL), erythrocytes (Erythro), and leukocytes (Leuko) in equine blood bags immediately following leukoreduction

Product	Pre-filtration	Post-filtration	Performance
E. coli (CFU/mL)	6,951.76 ± 2,103.30a	4,553.33 ± 2,581.54b	65.50% reduction
Erythro (x10^9)	6.86 ± 2.24	6.84 ± 0.74	99.70% recovery
Leuko (x10^3)	7,250 ± 1,008	0 ± 0	100% elimination

a,b = Different letters indicate significant differences ($p < 0.05$) between means; Absence of letters indicates no statistically significant difference ($p > 0.05$).

al. (2008), who leukoreduced blood from healthy horses and obtained an erythrocyte concentrate with an average leukocyte reduction of 93.4%, the use of a human leukocyte filter in this study made it possible to obtain leukoreduced total equine blood. This was achieved without any quantitative decrease in the number of RBCs, hemoglobin, or globular volume. The result was considered a leukoreduced blood component when the count was less than 5 × 106 leukocytes after leukofiltration (Ferdowsi et al. 2021). These results show that the LRF system is efficient at obtaining leukoreduced erythrocyte units for transfusion in horses.

The absence of leukocytes may be influenced by temperature, as pre-cooling the bags before filtration has been shown to improve efficiency (Brownlee et al. 2000). Other factors include

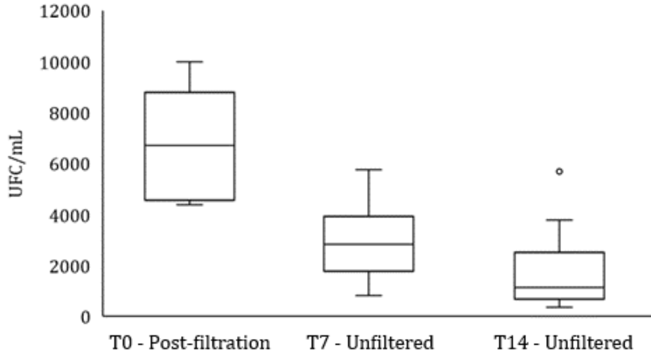

Fig. 1. Box plot showing the distribution of *Escherichia coli* concentrations (CFU/ml) in unfiltered bags on the day of collection (T0 = pre-filtration), seven days after collection (T7 = unfiltered), and 14 days after collection (T14 = unfiltered).

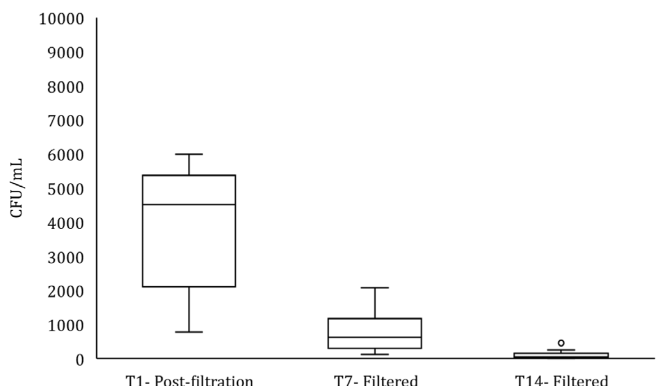

Fig. 2. Box-plot showing the distribution of *Escherichia coli* concentrations (CFU/mL) in filtered bags on the day of collection after filtration (T1 = post-filtration), seven days after collection (T7 = filtered), and 14 days after collection (T14 = filtered).

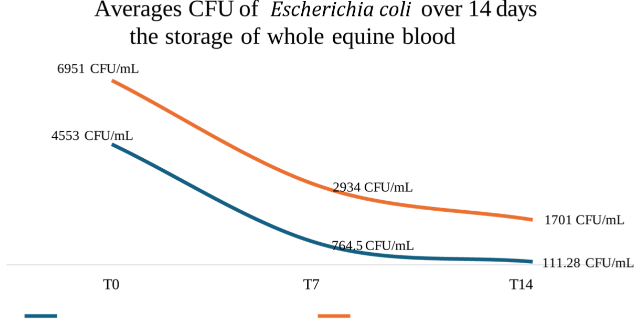

Fig. 3. Mean *Escherichia coli* concentration (CFU/mL) in the experimental and control groups, considering the evaluation time points.

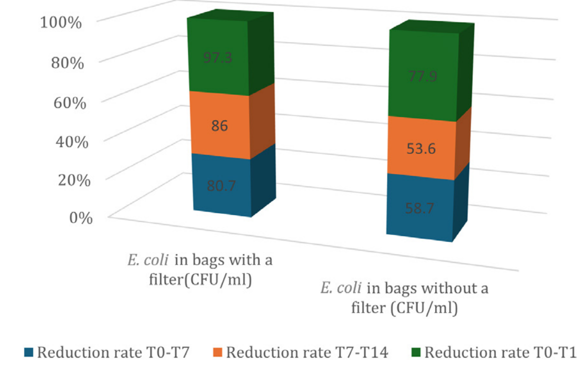

Fig. 4. *Escherichia coli* reduction rates (CFU/mL) in the experimental and control groups, considering the evaluation time points.

Table 7. Mean values, standard deviation (Devpad), maximum (Max) and minimum (Min) values of *Escherichia coli* colony-forming units in equine blood bags immediately following leukoreduction (T0), and after seven (T7) and 14 days (T14) of storage

		T0		T7		T14	
		CFU/mL	(%) re- duction	CFU/mL	(%) re- duction	CFU/mL	(%) re- duction
E. coli in bags with filter leuko (CFU/mL)	Average	4,553.33aA	(65.50)	764.50bA	(79.02)	111.28cA	(79.26)
	SD	2,562.6		620,10		141.25	
	Min	780		133		8	
	Max	10,000		2,080		460	
E. coli in bags with filter leuko (CFU/mL)	Average	6,951.76aB	(0)	2,934.44bB	(53.44)	1,701.33cB	(43.07)
	SD	2,044.1		1,349.09		1,405.5	
	Min	4,380		840		364	
	Max	10,000		5,760		3,800	

SD = Standard deviation; Different uppercase letters (A-C) in the same column and different lowercase letters (a-c) in the same row indicate statistically significant differences between means ($p < 0.05$); Absence of letters indicates no statistically significant difference ($p > 0.05$).

the type of fibre used in the filter, the speed of filtration, the number of platelets enhancing adhesion, and even the concentration of plasma proteins. During filtration, there is a gradual displacement of these proteins on the surface of the fibre, known as the Vroman effect; thus, the adhesion of leukocytes to the surfaces of the fibres will be largely influenced by pre-absorbed proteins from blood components, despite a lack of changes in the concentrations of total protein, albumin, or fibrinogen being observed between the control and experimental groups (Steneker et al. 1995).

Although the filtration process did not influence erythrocyte fragility, storage led to an increase in hemolysis over time in both groups, as has been observed in other studies, in which a gradual but significant reduction in the red series of bags stored for 42 days was identified (Barros et al. 2021). Erythrocyte stability may be related to the short storage period, which differs from the results of similar studies. Studies investigating the storage of leukoreduced human blood bags also verified an increase in osmotic fragility, elevation of MCV, and concentrations of hemoglobin and potassium, but with a reduction in glucose concentrations (Tayer et al. 2017).

According to Tayer et al. (2017), storage time influences biochemical parameters, hemolysis, and osmotic fragility. According to this study, the decrease in glucose, which was not observed in this study, as well as the increase in lactate concentration, may indicate erythrocyte glycolysis. Potassium dosage was analysed to relate to hyperkalemia due to hemolysis. An increase in potassium can trigger a change in the cation gradient and a consequent change in the sodium-potassium (Na+/K+) pump, resulting in irreversible changes in the membrane, such as changes in the size and shape of erythrocytes. However, the significant increase in potassium did not reflect an increase in MCV and RDW. Prior studies showed an increase in osmotic fragility and hematological changes lasting longer than 42 days, both with human and animal erythrocytes (Tayer et al. 2017, Barros et al. 2021), whereas this study lasted only 14 days.

Although hemolysis increased in both groups over the 14-day storage period, the filtration process itself did not lead to an immediate rise in osmotic fragility or erythrocyte lysis. Our results showed no significant differences in osmotic fragility immediately after filtration, suggesting that leukoreduction did not acutely compromise erythrocyte membrane integrity. However, by day 14, leukoreduced bags showed a slightly higher degree of hemolysis and potassium concentration, indicating that combining filtration and prolonged storage may influence erythrocyte stability over time. Therefore, we do not exclude the possibility that leukoreduction may have a delayed effect on membrane fragility when associated with extended storage, and we recommend further investigation into the long-term structural impact of filtration on equine RBCs.

When interpreting osmotic fragility results, it is essential to consider interspecies differences in RBC physiology. Equine RBCs are known to have a more stable membrane structure, lower metabolic rate, and distinct lipid and protein composition compared to human erythrocytes (Barros et al. 2021, Jamieson et al. 2022). These differences contribute to a generally lower baseline osmotic fragility in horses and may alter their response to storage lesions. Although we referenced human data (e.g., Tayer et al. 2017) for comparative purposes, we recognise that direct extrapolation must be approached with caution. Human studies offer a broader context and methodological parallels. However, the inherent species-specific characteristics of equine RBCs – such as greater membrane rigidity and lower permeability – must be accounted for when interpreting these comparisons. Future work should focus on expanding species-specific baseline data to allow for more precise physiological assessments.

Escherichia coli is among the bacteria that present a real risk of contamination in stored blood units, as confirmed by Miglio et al. (2016), who detected this bacterium in dog blood cultures after 35 days of storage. Further results revealed that the leukocyte filter was efficient in removing leukocytes and reducing the passage of a significant amount of *E. coli*.

Although the present study demonstrated a significant reduction in *E. coli* concentration after leukoreduction by filtration, it was not possible to determine the exact mechanism responsible for this bacterial reduction. Literature suggests various possibilities, including direct bacterial entrapment in the filter fibres, bacterial adhesion to retained leukocytes, or even phagocytosis by leukocytes before their removal (Steneker et al. 1995, Hinson et al. 2020). As our protocol did not include specific techniques to differentiate between these mechanisms, we cannot affirm which was predominant in our study. However, the immediate 65.5% reduction in bacterial count following filtration, prior to prolonged storage, suggests that the filter, possibly in combination with retained leukocytes, played a direct role. Future studies with microscopy or bacterial tracking techniques are warranted to elucidate this mechanism.

It is important to acknowledge that refrigerated storage plays a fundamental role in limiting bacterial growth, as previously demonstrated in studies such as Braathen et al. (2021) and Chen et al. (2008), which reported the self-limiting behaviour of *E. coli* in blood bags stored at 4 °C. In our study, both groups showed a gradual reduction in bacterial concentration over the 14-day storage period, confirming the expected effect of cold temperature. However, a significant and immediate decrease in *E. coli* load (65.5%) was observed in the leukoreduced group right after filtration, before the storage period could exert its influence. By day 14, the total bacterial reduction in this group reached 97.01%, compared to 72.99% in the control group, with a statistically significant difference between them ($p < 0.001$). Based on these findings, we affirm that the results support the hypothesis that leukoreduction by filtration contributes significantly to bacterial reduction beyond what would be expected from refrigeration alone. The filtration procedure appears to play an active and complementary role in bacterial control, likely through mechanisms involving direct bacterial entrapment or interaction with leukocytes retained by the filter.

These results were similar to those of a study on dog blood bags inoculated with *E. coli*, and the performance of RBC washing, followed by leukofiltration, with a reduction of 85.2% after washing and 99.9% following leukofiltration, thus verifying the capacity of the leukocyte filter to retain bacteria in contaminated bags (Hinson et al. 2020). However, the passage of *E. coli* was observed by Braathen et al. (2021) in studies using human blood, with the authors concluding that LRF performed two hours after collection did not protect all blood samples from the growth of *E. coli*. The difference

in results may be due to the direct binding of bacteria to different fibres (Tayer et al. 2017).

The discrepancy between our findings and those of Braathen et al. (2021), who reported persistent *E. coli* growth in leukoreduced human blood, may be explained by several factors. First, the filtration systems differ in design, pore size, and fibre composition, which can influence bacterial retention efficiency. The filter used in our study (Fresenius HemoCare model with a pre-filter) may have enhanced bacterial capture compared to the systems used in human studies. Second, methodological differences such as the timing of inoculation and filtration, initial bacterial concentration, and storage conditions may also have played a role. Third, interspecies variations – including erythrocyte and leukocyte morphology, immune activity, and biochemical environment – can significantly impact bacterial survival and interactions with filter fibres. Horses, for instance, have larger RBCs and different leukocyte behaviour, which may affect both the filtering process and the bacterial inactivation rate. Therefore, although inspired by human studies, our results reflect a unique combination of technical, biological, and procedural factors specific to the equine model used.

Although comparisons were made with human studies (McDonald & Blajchman 2008, Fung et al. 2014, Braathen et al. 2021), it is important to consider the physiological differences between human and equine blood. Equine RBCs are more stable in storage, exhibit different membrane lipid compositions, and have lower metabolic rates compared to human erythrocytes, which may influence osmotic fragility and hemolysis patterns (Barros et al. 2021, Jamieson et al. 2022). Furthermore, the leukocyte profile in horses differs significantly, particularly in terms of neutrophil predominance and cellular morphology, which may affect how leukocytes interact with bacterial pathogens and filtration fibres. These differences may also influence bacterial susceptibility and retention mechanisms within the filter. Despite these variations, human studies provide a valuable reference for understanding filtration dynamics, especially in the absence of extensive veterinary literature. Nonetheless, future studies specifically designed to characterise leukocyte behaviour and bacterial retention mechanisms in equine blood are necessary to confirm the applicability of human-based findings to veterinary transfusion practices.

Horse RBCs have an approximate diameter of 5.66 μm and can pass freely through the leukocyte filter. *E. coli*, with a smaller size of 1–3 μm × 0.4–0.7 μm, should not be retained by the filter's porosities. However, as observed by Dzik et al. (2000), who reported a reduction in the quantity of *Yersinia enterocolitica* (1–3 μm × 0.5–0.8 μm), with no comparative reduction of *Staphylococcus epidermidis* (0.5–1.5 μm), it is evident that, in addition to size, other factors influence the retention of microorganisms.

In the present study, a gradual reduction in the number of bacteria during refrigerated storage was observed in both groups, suggesting that leukocytes did not inhibit bacterial growth. These results were verified in a study using human blood bags that observed a lack of *E. coli* growth, combined with self-sterilisation, in both LRF and non-LRF groups during storage at 4 °C (Braathen et al. 2021). Indeed, *E. coli* is among the organisms reported to have the highest likelihood of self-sterilisation in stored human blood bags (Chen et al. 2008). Bacterial contamination of blood components does not always result in bacterial multiplication, as the organisms may not be able to survive the storage conditions, the presence of natural bactericidal agents in the blood component (for example, complement) may reduce their number, or they may even survive in the bag in low numbers, without the ability to multiply (McDonald & Blajchman 2008).

According to Fung et al. (2014), in routine veterinary medicine, bacterial concentrations may be undetectable immediately after collection. However, these pathogens can multiply during storage, particularly in blood components maintained at room temperature, such as platelet concentrates.

In human blood donation campaigns, collection may be performed in mobile blood banks, often by personnel with limited training and restrictions on hygiene procedures, thereby posing a higher risk of bacterial contamination in the donated blood. LRF use has been suggested as an alternative to reduce the risk of bacterial contamination (Braathen et al. 2021). This indication fits into the clinical procedures of veterinary medicine, in which blood banks are limited, and transfusions are performed by non-specialized professionals, often in environments unsuitable for procedures, or even in the field, as in the case of animal production.

Based on human hemovigilance data collected from multiple countries over several decades (Braathen et al. 2021), LRF has been identified as a safe procedure that does not increase the risk of bacterial infections in recipients. As such, the use of a leukocyte filter presents a simple technique, without the need for special equipment or environments, and allows for the reduction of transfusion reactions and transmission of infectious agents.

This study has several methodological limitations that must be acknowledged. The small sample size was determined based on the availability of animals and logistical and financial resources. The gender imbalance among the animals studied was due to convenience factors, with no intention to exclude or favour any particular sex. We suggest that future studies utilize power analysis as a statistical tool to determine an adequate sample size and gender variation for more precise results.

This study employed gravity-based leukoreduction, a method that, while practical and commonly used in veterinary settings, introduces inherent variability. Factors such as blood viscosity, storage temperature, and tubing height can influence the flow rate, which in turn affects the efficiency of both leukocyte and bacterial removal. Unlike pressure-driven systems, where flow is actively controlled, gravity filtration relies solely on gravitational force, making it more susceptible to variations in environmental and handling conditions. Although we standardised the filtration height (1.75 m) and ensured filter saturation before release, we acknowledge that unmeasured variability in flow rate could have influenced filtration performance. Future studies comparing gravity versus pressure-based systems may clarify whether active flow control enhances leukocyte depletion and bacterial retention in equine blood.

The use of the Neubauer chamber, although widely employed in laboratories, is susceptible to human error and has low sensitivity in quantifying residual leukocytes, which may affect the accuracy of the results. Its use was a practical choice in this study; however, future research should incorporate more

advanced technologies such as flow cytometry or hematology analysers with a low detection limit for residual leukocytes. Although the Prokan hematological analyser is used in veterinary settings, it is not validated for leukoreduction studies in equine blood and lacks the sensitivity required to detect residual leukocytes.

Osmotic fragility was assessed using five NaCl concentrations after 30 minutes at room temperature. Here, we emphasise that future studies should use a broader range of NaCl concentrations and incubation times to evaluate erythrocyte stability more comprehensively.

The study included a limited number of blood bags and a 14-day storage period. However, specific limitations related to longer timeframes were not investigated. Previous research, such as Barros et al. (2021), has assessed blood parameters and bacterial viability over 42 days, revealing more pronounced changes in erythrocyte integrity and hemolysis during extended storage. While our findings support the effectiveness of leukoreduction within a two-week timeframe, we recognise that longer storage periods could lead to different patterns of bacterial persistence or progressive cellular degradation. The 14 days were selected based on biosafety protocols and logistical constraints; however, future studies should investigate extended storage durations to determine the long-term efficacy of leukoreduction in equine blood.

Additionally, although the focus of the work was on *E. coli*, a bacterium commonly found in cases of blood bag contamination, using only this pathogen may not accurately simulate contamination in more complex hospital scenarios. Therefore, the effectiveness of filtration may vary for different infectious agents and storage periods exceeding 14 days. These limitations do not undermine the relevance of the results presented but rather highlight areas that can be improved in future investigations.

CONCLUSION

Filtration using a human leukocyte filter proved effective for leukoreduction of equine whole blood contaminated with *Escherichia coli*, without immediately compromising the red blood cell (RBC) series or osmotic fragility. Although both groups showed a reduction in bacterial load over the 14-day storage period, the data indicate that filtration significantly contributed to a faster and more pronounced decrease in *E. coli*, particularly immediately after filtration. However, we emphasise that the study has notable limitations, such as the small sample size and relatively short storage period. Even so, the findings suggest that leukoreduction may serve as a complementary measure to refrigeration for bacterial control in equine blood bags. Studies with greater statistical power and extended duration are recommended to confirm these results and clarify the mechanisms involved.

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Data availability statement.-The data supporting the conclusions of this study are openly available in personnel files and are available upon reasonable request.

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