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Efficacy of florfenicol and flunixin followed with vitamin E and/or C on selected oxidative and inflammatory mechanisms in young cattle under transport and adaptation stress

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Summary

Bovine respiratory disease (BRD) in cattle, due to the participation of numerous viral and bacterial etiological agents, predisposition factors, and environmental stresses, is a serious health and economic problem in herds of beef cattle. Given the broad scope of the problem of respiratory syndrome and the limited immunoprophylaxis, the aim of this study was to estimate the additive effects of flunixin with florfenicol followed with vitamin E or/and C in reducing the development of oxidative stress and inflammation in the first weeks in the feedlot.

The study was conducted on young Simmental cattle (n = 50) weighing approx. 160 kg. The animals were divided into 5 groups (n = 10 in each group). Group I was the control. The cattle received florfenicol and flunixin in combination with vitamins E or C (Group II and III), florfenicol and flunixin followed with vitamin E and C (Group IV), or florfenicol and flunixin without vitamins (group V). Blood for sera was collected on the day the drugs were administered and on days 3, 7, 14, 21 and 28 of the experiment. The level of oxidative stress was analysed on the basis of the concentration of nitrogen ions (NO) in the sera and of lipid peroxidation end products reacting with thiobarbituric acid (TBARS). Concentrations of haptoglobin (Hp) and serum amyloid A (SAA) in the sera were determined using EIA enzyme immunoassays.

The treatment used in the study, involving the application of florfenicol and flunixin with vitamin E or C, substantially reduced the level of oxidative stress, expressed as a reduced concentration of TBARS and NO ions, particularly in groups II and III. It was also confirmed that the treatment inhibited the concentration of acute phase proteins (Hp and SAA).

Keywords: antioxidants, cattle, feedlot, florfenicol, flunixin, respiratory diseases, stress

Bovine respiratory disease complex (BRDC) in cattle is caused by numerous viral (BRSV, PI-3, BHV-1, BVD) and bacterial (*M. haemolytica*, *Pasteurella* spp., *Klebsiella pneumoniae*, *Histophilus somni*) and *Mycoplasma bovis* etiological agents. The main predisposing factors are genetic predisposition and environmental stress (transport, adaptation, stress, poor nutrition, social stress, or microclimate), and currently approved feedlot management technology leads to serious health and economic problems in beef cattle herds (2, 11). Morbidity and mortality rates depend on numerous factors involved in the development of the syndrome. The incidence of viral infections is high but with a low mortality rate, while higher mortality is observed in the case of bacterial infection. Mixed viral-bacterial infections have the highest incidence rates and are associated with high mortality (7). Cattle, due to the anatomy and physiology of the lungs – associated with significantly reduced ventilation, low partial pressure of oxygen, and reduced clearance – are at increased risk of developing respiratory diseases (24).

Reduced elasticity of the alveoli and limited efficiency of gas exchange is conducive to accumulation of exudates, which impedes their evacuation.

Mechanisms contributing to activation of the local defence system induce inflammation by enhancing the release of pro-inflammatory cytokines, predominantly IL-8 and TNFα. IL-8 is a major chemoattractant for neutrophils and activates the cells to increased migration into the inflammatory focus (1).

Increased incidence of symptoms of respiratory disease in young cattle has been observed during the first 45 days in the feedlot, with a peak in incidence at 2-3 weeks. The increased sensitivity of young cattle at this time is also influenced by their weight on entering the feedlot. Young cattle weighing < 180 kg exhibit increased susceptibility to disease, which is linked to decreased resistance to environmental stressors associated with transport and adaptation. The lowest incidence is observed in young cattle whose weight on entering the feedlot is over 300 kg (3, 22). The increase in morbidity in young cattle with respiratory disease symptoms is also linked to low content of energy, protein, as well as macro- and micronutrients in their feed during exposure to the stress of transport and adaptation (7, 17).

An important factor in reducing respiratory disease morbidity in young cattle is effective immunoprophylaxis. In the United States it is a key element of prevention. Some European countries, such as Belgium, the Netherlands, Austria and Germany have introduced obligatory programs to control viral infections with BRSV, BHV-1 and BVD (9). Poland is currently one of the countries in which there are no obligatory immunoprophylaxis measures to reduce the transmission of pathogens. Many livestock farms have not implemented a prevention programme, while on others, vaccinations are performed only occasionally and are limited to the reproduction sector.

The multi-etiological nature of the syndrome and the production technology of purchasing calves from different backgrounds with different immune status (often after antibiotic therapy), creates difficulties in selecting effective immunoprophylaxis. The use of live modified vaccines (LMV) is limited in young cattle exposed to stress conditions associated with transportation and adaptation. Immunization with an attenuated virus in combination with bacterial components may induce immune suppression, as demonstrated in cattle immunized with the PI-3 virus and bacterial antigens (1).

A beneficial procedure in reducing morbidity, widely distributed in Canada and the United States, is the use of broad-spectrum antibiotics (e.g. florfenicol, thiamphenicol, enrofloxacin, cephalosoporin, or oxytetracycline) in combination with non-steroidal anti-inflammatory drugs (NSAIDs) in groups of infected animals. The use of NSAIDs with antibiotics eliminates free oxygen radicals, inhibits phosphorylation, blocks the release of kinins and proteoglycan degradation, and also inhibits the migration and adhesion of neutrophils. In inflammatory processes associated with the respiratory tract, NSAIDs reduce the release of toxic peroxides causing proliferation of fibroblasts and collagen deposition. Furthermore, they inhibit the increased production of nitric oxide synthase in lung macrophages, thereby weakening the destructive effect of these cells on the bronchial mucosa. The antitoxic activity of NSAIDs involves inhibiting the production of malondialdehyde (MDA), and their antipyrogenic effect involves inhibiting the activity of endogenous pyrogens (IL-1, TNF) and prostaglandin PGE2 (12, 15).

Preventive measures undertaken in Poland to control respiratory disease are often limited to single breeding environments. The opening of the markets of the European Union, particularly the Balkan countries (Romania and Bulgaria), as well as non-EU Eastern Europe (Ukraine), enables the purchase and transport of calves from distant parts of Europe, which significantly impairs the control of pathogen transmission within herds, as well as effective immunoprophylaxis. As a result, the therapeutic efficacy of antibiotics is negligible and losses resulting from the cost of treatment failures and deaths surpass production results. When treatment is ineffective, further antibiotic therapy must be introduced, which additionally increases the cost of treatment and entails the risk of spreading drug-resistant microorganisms in animal populations. The average cost of treatment per animal is about 15.6 US dollars, and this amount increases to up to 92.3 USD due to losses resulting from reduced weight gain and reduced feed efficiency (23).

Given the broad scope of the problem of respiratory syndrome in feedlot cattle and the limited opportunities for effective immunoprophylaxis, the aim of this study was to estimate the additive effects of florfenicol and flunixin in combination with vitamin E or/and C in reducing the development of oxidative stress and inflammation in young cattle infected during the initial weeks in the feedlot.

Material and methods

Experimental design. The study was conducted on a group of young Simmental cattle (total n = 50) weighing approx. 160 kg. Before entering the feedlot the animals underwent clinical examination, including detection of secretions from the nose or cough, as well as measurement of rectal temperature, pulse and respiration. The young cattle were examined for the presence of serum antibodies against bovine syncytial virus (BRSV) by ELISA (Diagnostic Cypres, Belgium) according to the manufacturer's recommendations. In the feedlot the animals received a TMR feed mixture (highly productive soybean, canola, crushed grain, hay, straw, spent grain, and corn silage) in the amount of 3.5-4 kg of CJ 2 mix kg per animal and hay *ad libitum*.

The young cattle were divided into 5 groups of 10 animals each. Group I was the control. The animals received florfenicol and flunixin in combination with vitamin E or C (Groups II and III); florfenicol and flunixin followed with both vitamin E and C (Group IV); or florfenicol and flunixin without the addition of vitamins (group V). The

animals were tested clinically, including measurement of rectal temperature, repeated at 3, 7, 14, 21 and 28 days of fattening. Daily feed consumption and average daily weight gain were also monitored in the experiment.

Sera from the animals were collected on the day of administration of the preparations and on days 3, 7, 14, 21 and 28 of the experiment (26).

The experiment was approved by the Local Ethics Committee, no. 39/2009, 09.07.2009.

Procedure for obtaining sera. Serum was obtained from blood collected from the cattle into vacuum tubes without an anticoagulant. After one hour of incubation at 37°C followed by 4 h at 4°C the serum was centrifuged for 15 min/4°C at 3,000 rpm and stored until analysis at –20°C.

Evaluation of the concentration of nitrogen ions (NO). Production of NO ions was determined as nitrite ions using Griess reagent, since nitric oxide produced would be rapidly converted to nitrite ions (6) (Sigma-Aldrich, Germany). Absorbance was read with a spectrophotometer (Bio-Rad, SmartSpec™ PLUS, Germany) at 538 nm. A standard curve was performed using Griess reagent in accordance with the manufacturer's instructions (POCH-TEST S.A., UK). Based on the results we prepared a calibration curve showing the dependence of the absorbance of the ion concentration of NO in µg/ml.

The diagnostic assays were performed according to the manufacturer's instructions. A 0.25 ml volume of each serum was placed in a flask and 29.75 ml of distilled water was added. Then 5 ml of a working solution of Griess reagent was added and the contents of the flasks were mixed. The samples were incubated at room temp. for 30 min in the dark, after which distilled water was added to 50 ml and the samples were read using a spectrophotometer (BIO-RAD™ PLUS SmartSpec, Germany) at a wavelength of 538 nm. The resulting absorbance and mass of NO_2^- ions, read from the standard curve, were used to calculate nitrite x in the samples according to the following formula:

$$x = m/V$$

m – mass of NO ions contained in the volume V, read from the calibration curve in mg/ml

V – volume of the sample taken for photometric analysis, ml.

The result is reported as the ion content of NO in µg/ml.

Determination of thiobarbituric acid (TBARS). The TBARS concentration was determined according to Ledwożyw et al. (14). A 2.5 ml volume of 20% trichloroacetic acid in 0.6 M HCl was added to 0.5 ml of plasma or a granulocyte suspension with a density of 2×10^6 cells/ml and incubated at room temp. for 10 min. Then 1.5 ml of 0.67% thiobarbituric acid (TBA) in 1 M NaOH was added and the sample was incubated in a water bath at 100°C for 20 min. After cooling and the addition of 4 ml of n-butanol, the suspension was vortexed for 3 minutes and centrifuged at 1,500 × g/4°C for 10 min. The absorbance of the layer of the n-butanol zone was measured at a wavelength of 532 nm (SmartSpec Plus, BioRad, USA). The results were calculated from a standard curve prepared with different dilutions of malondialdehyde (MDA, Sigma, D) and expressed in mmol/g of protein. The total protein concentration in the test samples was measured using Bradford Reagent (Sigma) and read using an ELISA reader at a wavelength of 595 nm (Bio Rad, USA).

ELISA for estimation of haptoglobin (Hp) and serum amyloid A (SAA). Concentrations of Hp and SAA in the sera were determined using an enzyme immunoassay (EIA Tridelta, Ireland). The procedures for the tests were performed according to the manufacturer's instructions. For determination of haptoglobin concentration the sera were diluted 1 : 2, and to determine SAA concentration they were diluted 1 : 5. The absorbance value obtained was read with a microplate reader (BioRad, Germany) using the appropriate filters for the wavelength 630 nm for Hp, and 450 and 630 nm for SAA. The results are presented in g/L for Hp and mg/L for SAA.

Statistical analysis. Statistical analysis of the results was carried out using Statistica 10.0 software. Two-way analysis of variance (ANOVA) was used to compare the differences between experimental groups. The post-hoc effect was determined by Tukey's test. Comparative analysis of the results obtained in all experimental groups on each day of the experiment was performed using ANOVA with repeatable parameters. Correlations between parameters were determined using the Pearson correlation coefficient. Differences were considered significant for $P < 0.05$.

Results and discussion

The study confirmed the presence of BRSV in the young cattle, showing positive titres in the ELISA and immunodiffusion TRURSV assays. The percentage of positive titres was 34% and 32%, respectively, confirming BRSV infection in this group. The substantial (> 30%) percentage of infected animals indicates the prevalence of etiological agents of respiratory syndrome, resulting in an increase in rectal temperature to above 39.5°C. It also suggests that management should be introduced to reduce transmission of bacterial pathogens by progressively eliminating them from breeding environments.

The morbidity rate was at 10% in group 3 and 20% in groups 4 and 5. In group 2 there were no cases of general symptoms of respiratory disease (Fig. 1A). The morbidity in all animals was 20%, while mortality was 10% and only affected young cattle from the control (Fig. 1B).

The results obtained in the present study confirm those of other studies in the United States and Canada (19), in which feedlot cattle, following administration of only non-steroidal anti-inflammatory drugs in combination with antibiotics, had significantly reduced incidence (by more than 64%) of respiratory symptoms, while daily weight gain increased by about 8% in herds of beef cattle. In addition, a reduction was observed in populations of bacteria of the family *Pasteurellaceae*.

The additive effect of the selected NSAIDs with antibiotics observed in the study has been confirmed in other studies (16), which showed that in the presence of non-steroidal drugs therapeutic concentrations of antibiotics in the lung tissue were obtained faster, significantly reducing the intensity of the disease process.

In the present study, the analysis of the percentage of morbidity with symptoms of respiratory disease in

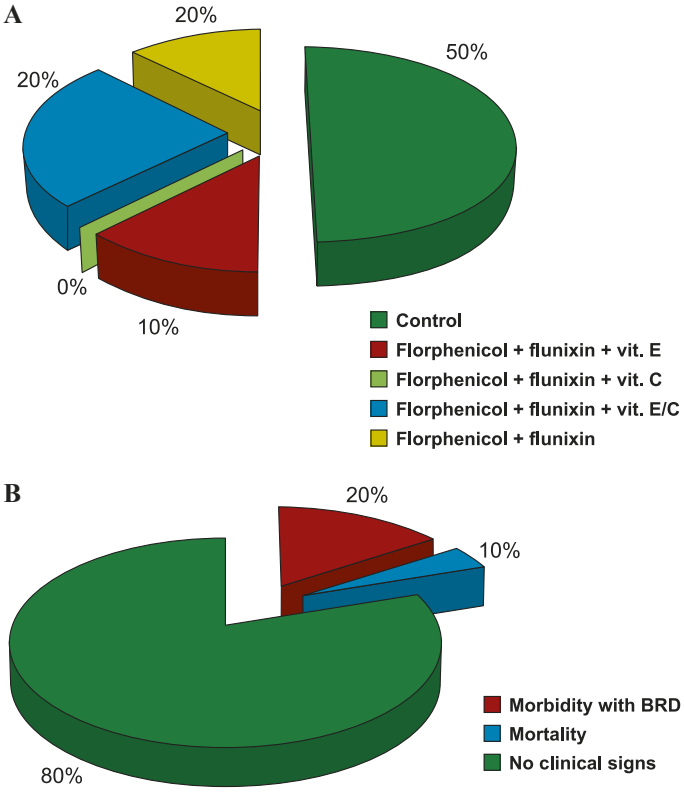

Fig. 1. Mean morbidity (A) and mortality (B) of young cattle in each experimental group

different experimental groups showed a reduction in morbidity of approx. 30% as compared to the control. It may be concluded that the treatment could reduce morbidity and intensity of the disease process in young cattle.

The results of our study are similar to those obtained by Chirase et al. (5), in which calves received only vitamins E and C, and the decrease in morbidity and mortality was significant in comparison to calves which did not receive antioxidants. According to Ekstrand-Hammarström et al. (8), after application of vitamin E, inflammation can be inhibited as a result of the inhibition of proinflammatory cytokine production, reduced production of acute phase proteins, and a reduced inflammatory response in the lung epithelial cells.

Despite slight differences in absolute values, there was no significant effect on average daily weight gain or daily feed consumption in the experimental groups as compared to the control. Average daily weight gain in the groups of young cattle ranged from 0.6 to 0.8 kg on all days of the experiment, while the average daily consumption in the first week in the feedlot ranged from 1.9 to 2.9 kg in all experimental groups. In the remaining days in the feedlot the animals consumed more than 3 kg, reaching a maximum amount of 4 kg on day 28 in the feedlot (Tab. 1).

The treatment used in the present study, involving the application of flunixin with florfenicol followed with vitamins E and/or C, significantly reduced the level of oxidative stress. This was expressed as a reduced concentration of end products reacting with thiobarbituric acid (TBARS) and inhibition of the production and release of nitrate ions (NO), which was particularly evident in the groups which received florfenicol and flunixin in combination with vitamins C or E (Tab. 2). A significant increase in TBARS concentration was observed on day 3 in groups 3 and 4.

Tab. 1. Average daily weight gain of young cattle and daily intake on particular days in the feedlot (average ± SD)

Group of cattle	Day of experiment									
	3		7		14		21		28	
	Body weight	Daily intake	Body weight	Daily intake	Body weight	Daily intake	Body weight	Daily Intake	Body weight	Daily intake
Group 1	159.8 ± 1.7	1.9 ± 0.3	162.0 ± 1.9	2.8 ± 0.3	166.6 ± 1.6	3.25 ± 0.29	170.8 ± 1.2	3.7 ± 0.35	177.1 ± 2.1	3.8 ± 0.3
Group 2	161.2 ± 1.9	1.9 ± 0.27	163.0 ± 2.1	2.7 ± 0.45	167.6 ± 1.9	3.2 ± 0.3	172.1 ± 1.8	3.75 ± 0.29	176.6 ± 1.8	3.9 ± 0.3
Group 3	161.5 ± 1.3	1.8 ± 0.3	163.6 ± 1.8	2.9 ± 0.35	167.8 ± 1.3	3.2 ± 0.32	172.3 ± 2.1	3.8 ± 0.3	176.5 ± 1.6	3.7 ± 0.27
Group 4	161.0 ± 2.1	1.7 ± 0.29	163.5 ± 2.2	2.8 ± 0.3	168.0 ± 1.5	3.1 ± 0.3	169.8 ± 1.3	3.65 ± 0.3	177 ± 1.9	3.9 ± 0.3
Group 5	159.2 ± 2.3	1.75 ± 0.28	161.7 ± 2.9	2.7 ± 0.3	167.4 ± 2.6	2.9 ± 0.27	171.9 ± 2.8	3.5 ± 0.34	176.5 ± 2.9	3.9 ± 0.25

Tab. 2. Mean concentrations of TBARS and NO_2 in sera obtained from young cattle on different days in the feedlot (mediana ± SE)

Group of cattle	Day of experiment									
	3		7		14		21		28	
	TBARS (µmol/l)	NO_2^- (µg/ml)	TBARS (µmol/l)	NO_2^- (µg/ml)	TBARS (µmol/l)	NO_2^- (µg/ml)	TBARS (µmol/l)	NO_2^- (µg/ml)	TBARS (µmol/l)	NO_2^- (µg/ml)
Group 1	3.9 ± 1.1	5.58 ± 1.7	3.9 ± 0.6	4.77 ± 2.01	3.90 ± 0.8[a]	8.10 ± 2.5	3.01 ± 0.8	15.53 ± 4.8[a]	3.00 ± 0.8	4.96 ± 1.6
Group 2	3.1 ± 0.5	5.03 ± 1.5	2.3 ± 0.6	3.90 ± 1.3	2.27 ± 0.7	5.19 ± 1.5	2.28 ± 0.5	5.03 ± 2.8	2.19 ± 0.7	3.3 ± 1.1
Group 3	3.8 ± 0.5	4.58 ± 2.8	3.12 ± 0.5	4.69 ± 2.3	3.08 ± 0.4	6.01 ± 2,0	2.58 ± 0.3	7.67 ± 2.5	2.75 ± 0.4	7.96 ± 2.3
Group 4	3.8 ± 0.7	10.8 ± 4.3	2.9 ± 0.7	7.32 ± 3.4	2.90 ± 0.4	7.65 ± 2.3	3.03 ± 0.3	7.45 ± 2.1	3.12 ± 0.4	5.44 ± 1.6
Group 5	3.4 ± 0.6	5.84 ± 2.9	3.02 ± 0.5	7.71 ± 4.3	2.70 ± 0.4	8.23 ± 2.1	2.3 ± 0.18	8.79 ± 3.7	2.40 ± 0.5	6.06 ± 2.5

Explanation: a – significant differences in comparison to control, $P \geq 0.05$

Tab. 3. Mean concentrations of haptoglobin and serum amyloid A in sera obtained from young cattle on different days in the feedlot (mediana ± SE)

Groups of cattle	Days of experiment									
	3		7		14		21		28	
	Hp g/l	SAA mg/l	Hp g/l	SAA mg/l	Hp g/l	SAA mg/l	Hp g/l	SAA mg/l	Hp g/l	SAA mg/l
Group 1	0.83 ± 0.3	227.2 ± 20.5	0.89 ± 0.2	218.6 ± 29.7	0.52 ± 0.26	243.8 ± 22.7	0.49 ± 0.28	244.8 ± 10	0.30 ± 0.21	190.3 ± 32
Group 2	0.43 ± 0.1[a]	201.8 ± 13.9	0.48 ± 0.1[a]	190.9 ± 15.8	0.40 ± 0.05	207,0 ± 13.7[a]	0.18 ± 0.19	206,0 ± 15.9[a]	0.17 ± 0.16	161.8 ± 21
Group 3	0.56 ± 0.2	209.2 ± 20.9	0.60 ± 0.1[a]	182.9 ± 17.9	0.48 ± 0.16	202.2 ± 21.0	0.23 ± 0.18	202.7 ± 21.6[a]	0.15 ± 0.1	165.4 ± 21.3
Group 4	0.8 ± 0.04[b]	200.7 ± 23.8	0.80 ± 0.3[b]	180.9 ± 23,0	0.60 ± 0.22	206.3 ± 24.2	0.23 ± 0.19	192.4 ± 22[a]	0.21 ± 0.1	167.0 ± 25.8
Group 5	0.69 ± 0.16	202.4 ± 33.6	0.76 ± 0.3	203.2 ± 22.3	0.58 ± 0.36	218.5 ± 24.7	0.17 ± 0.19	204.2 ± 24[a]	0.10 ± 0.16	163.9 ± 21.7

Explanations: a – significant differences in comparison to control ($P \geq 0.05$); b – significant differences in comparison to the remaining groups of cattle ($P \geq 0.05$)

The lowest statistically significant TBARS value ($p \leq 0.05$), as compared to the control, was observed in group 2 in all the experiments and in group 5 on days 14, 21 and 28 (Tab. 2). A significant increase ($p \leq 0.05$) in the concentration of nitrate ions as compared to the control was observed in group 4 on day 3 in the feedlot. The concentration of NO ions was lowest in the group receiving florfenicol and flunixin in combination with vitamin E on day 28 (Tab. 2).

As demonstrated in various studies, oxidative stress plays an important role in the development and course of respiratory syndrome in cattle, and reducing it can significantly decrease the incidence of respiratory symptoms in cattle (5, 18). Studies on humans infected with the syncytial virus (RSV) have also confirmed the therapeutic and prophylactic effect of antioxidant formulations in the prevention of respiratory syndrome (4). Another study (21) suggested the possibility of using assessment of redox status as a reliable diagnostic marker in evaluating the degree of sensitivity of animals to disease. Furthermore, a previous study by Urban-Chmiel et al. (25) also confirmed the inhibitory effect of vitamins E and C on both oxidative stress and the concentration of some acute phase proteins (Hp and SAA).

In the present study the analysis of the haptoglobin concentration in the sera showed the lowest concentration on days 3, 7 and 14 in the feedlot in the second group and on days 21 and 28 days in the third group of animals. The values obtained differed significantly ($p \leq 0.05$) from the values for the control and for group 4 on days 3 and 7 (Tab. 3). There were no significant differences in haptoglobin concentration between groups 4 and 5 or between these groups and the control, as the values obtained were very similar on all days. The concentration of SAA remained at a similar level on days 3, 7, 14 and 21 in the feedlot in all groups (Tab. 3). According to other authors (13, 20), these proteins could be indicators of an inflammatory process in the initial phase of respiratory syndrome in cattle. A significant correlation was also noted between haptoglobin levels and an increase in cases of calves with symptoms of respiratory syndrome. This is also confirmed by the correlation coefficients (r) obtained in the present study, in the range of 0.5 to 0.69, which show a strong correlation between experimental groups and the control (Tab. 4). This indicates a correlation between the influence of transport and adaptation stress in young cattle and the induction of acute-phase proteins during the first weeks in the feedlot.

Tab. 4. Mean values of the correlation coefficient ($r < 0.05$) between concentrations of haptoglobin and serum amyloid A in the sera obtained from young cattle on different days in the feedlot

Group of cattle	Correlation coefficient				
	Group 1	Group 2	Group 3	Group 4	Group 5
Group 2	0.69*	–	–	–	–
Group 3	0.49	0.18	–	–	–
Group 4	0.54*	–0.15	–0.02	–	–
Group 5	0.66*	0.02	0.56*	0.14	–

Explanation: *significant at $P < 0.05$

The inhibitory effect of florfenicol and flunixin followed with vitamin E or C on oxidative stress may play a significant role in reducing the causes of disease in young cattle during the first days in the feedlot. This is confirmed by the analysis of the correlation between the parameters observed in the experimental groups. High correlation coefficients ($r \geq 0.9$) indicate significant correlations in the case of both indicators of oxidative stress.

In conclusion, the use of an antibiotic and a non-steroidal anti-inflammatory drug in combination with antioxidants contributed to the protective effect of leukocytes involved in defence against *M. haemolytica* virulence factors. In addition, the treatments effectively limited the development of oxidative stress and inflammation in young cattle during the first week in the feedlot. The results form the basis for the potential adoption of the treatments described to support the prevention and control of respiratory disease and mortality in beef cattle following transport and adaptation to the feedlot.

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