

Oral Application of Charcoal and Humic Acids Influence Selected Gastrointestinal Microbiota, Enzymes, Electrolytes, and Substrates in the Blood of Dairy Cows Challenged with Glyphosate in GMO Feeds

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Abstract

The present study was initiated to investigate the influence of oral application of charcoal, sauerkraut juice and humic acids on specific gastrointestinal microbiota and selected enzymes, electrolytes, and substrates in the blood of dairy cows fed GMO feeds containing glyphosate. A total of 380 Schleswig Holstein cows suffering from symptoms of chronic botulism were fed sequentially with 400 g/animal charcoal daily for 4 weeks (weeks 1-4 of the study), 200 g/animal charcoal (weeks 5-10 of the study), 200 g charcoal and 500 ml Sauerkraut juice/animal (weeks 11-14 of the study), 120 g/animal humic acids (weeks 15-18 of the study) 200 g charcoal and 100 mL Aquahumin/animal (weeks 19-20 of the study), or 100 g charcoal and 50 mL Aquahumin (weeks 21-22 of the study) followed by 4 weeks without any supplementation. There was a significant reduction of glyphosate in urine following supplementation with a combination of 200g charcoal plus either 500 mL sauerkraut juice or humic acid. From all of the parameters investigated in blood and urine, distinctive effects were only seen as a lack of manganese and cobalt and a significant reduction of creatinine excretion by urine. All other measured parameters such as creatine kinase (CK), alkaline phosphatase (AP), glutamate dehydrogenase (GLDH), glutamate oxaloacetate transaminase (GOT), and cholesterol, urea, and blood creatinine were not significantly changed. A significant increase of fecal Gram-negative bacteria and enterococci were only seen at week 8 (200 g charcoal/d) and week 20 (200 g charcoal+100 mL Aquahumin). In conclusion, a charcoal-sauerkraut juice combination and humic acids reduced glyphosate excretion by urine and led to the improved health of animals.

Keywords: Glyphosate; Charcoal; Sauerkraut juice; Humic acids; Fecal bacteria; Enzymes; Electrolytes; Substrates in blood

Introduction

Health and performance of high yielding dairy cows depend on a lot of interacting factors of the environment, feed and management [1]. Investigated 240 Danish dairy cows on eight farms (15 fresh calving, 15 high yielding cows at each farm) to evaluate relationships between the regular intake of glyphosate (N-(phosphonomethyl) glycine) in feed, as measured by excretion in urine, and changes in serum biochemistry, especially enzymes indicative of cytotoxicity such as AP, CK, GLDH, GOT; parameters demonstrating nephrotoxicity (urea, creatinine); a lipid pathway parameter (cholesterol); as well as the trace elements copper (Cu), cobalt (Co), manganese (Mn), selenium (Se), and zinc (Zn). They found increased blood serum parameters indicative of cytotoxicity such as GLDH, GOT, and CK and the lipid profile marker cholesterol in cows at all farms; and high urea levels in half of the farm animals. These results suggest a previously unanticipated impact of glyphosate based herbicides on food animal production.

Glyphosate is a highly effective, broad-spectrum herbicide that inhibits 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway that is necessary to synthesize aromatic amino acids and other aromatic components in higher plants, algae, bacteria and fungi [2]. The herbicidal action is generated by chelating manganese required in the reduction of the flavin mononucleotide (FMN) co-factor of EPSPS [3]. Glyphosate is the most extensively used herbicide worldwide. The intensive use of glyphosate, especially in GMO forage and grain crops, has led to its wide-spread contamination of different ecosystems where it influences plants, microorganisms, animals and many components of the food chain. Moreover, glyphosate and its primary metabolite aminomethylphosphonate (AMPA) have

been also detected in immature seed [4], harvested seeds [5], and ground water [6] accessible to farm animals.

Reducing the activity of glyphosate in the gastrointestinal tract with binders or neutralizers could be a solution to remove this contamination. Mazzei and Piccolo [7] found that glyphosate may spontaneously and significantly bind to soluble humic matter by non-covalent interactions at slightly acidic pH. These results were confirmed by *in vitro* neutralizing assays with glyphosate sensitive enterococci Shehata et al., with different humic acid products [8]. Gerlach et al. investigated the influence of oral applications of humic acids (WH67) and charcoal (another absorbent substrate) as well as a combination of charcoal and sauerkraut juice for treatment intervals of four weeks to Holstein Friesian dairy cows on one farm (380 cows) in Germany with clinical signs of chronic botulism [9]. They reported that nearly all supplementations, except low doses of charcoal (200 g/animal and day) alone, led to a significant reduction of *C. botulinum* ABE and CD antibody levels. There was a significant reduction in

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glyphosate excretion by urine after four weeks supplementation with a combination of 200 g charcoal plus 500 mL sauerkraut juice/d or 120 g humic acid/d [10]. The present paper reports the results of a sequential study with different treatments, each lasting four weeks, on a 380 cow dairy farm over 26 weeks. The above mentioned treatment parameters (selected enzymes, electrolytes, and substrates in blood serum or whole blood as well microbiological parameters of feces) were investigated after each change of supplementation (each four week period) or four weeks after finishing all supplementations. To get information about the influence of the treatments on the fecal microbiota, specific microbiological parameters of feces were investigated [11].

Material and Methods

Animals and supplementations

A Schleswig Holstein dairy cow farm of about 380 cows with average milk production of 9000L per year showed clinical symptoms of chronic botulism (stiff stilted gait, paresis, apathy, engorged veins on tarsus, positive venous pulse, mucous saliva, reduced tail tonus, small wounds in the udder region) in 10-15% of the cows. 60% of the cows suffered from Dermatitis digitalis (mortellaro). The entire animal population was involved in the various treatments (400 g or 200 g/d charcoal (≤ 8 mm diameter); a combination of 200g charcoal plus 500 mL/d sauerkraut juice; 120g/d powdered humic acid (WH67); or a combination of 100 mL liquid humic acid (Aquahumin) plus 200g charcoal/d for two weeks followed with a combination of 50 mL Aquahumin plus 100g charcoal/d for two weeks). After each completed treatment interval, blood specimens, urine and feces from 10 similar cows of the 1st, 2nd, and 3rd lactation and the dry cows group were collected for analytical investigation. The last polyvalent Clostridial vaccination (Covexin, Intervet) was on 01.11.2012. The treatment regime with charcoal, sauerkraut juice, humic acids and their combinations was changed every 4 weeks (supplementation periods) is shown in Table 1. All of the supplements were thoroughly mixed with the feed and given as a total mixed ratio (TMR). The TMR was composed of grass and maize silage (glyphosate concentration was not tested), concentrated mixed feed (1.93 mg/kg glyphosate), ground grains (0.51 mg/kg glyphosate), wheat straw (0.03 mg/kg glyphosate) and alfalfa hay (0.02 mg/kg glyphosate). After 31.03.2013, 10 kg draff/cow (0.01 mg/kg glyphosate) was fed. At each sampling point, each of the 40 treated cows was evaluated for clinical symptoms of chronic botulism (Table 2).

Collection of samples

Blood, feces and urine were analyzed 7 times at 4 week intervals with one exception (200 g charcoal after 6 weeks). Two blood specimens were taken from the *Vena coccygena mediana*. The coagulated blood sample was centrifuged at 3000 x g for 15 min and the serum samples were stored at -20°C while the whole blood sample was also stored at -20°C. Feces were taken from *Ampulla recti* and spontaneous urinations were sampled and stored at -20°C. All specimens were quickly cooled and sent to the laboratory.

Glyphosate testing of urine

Urine samples were diluted 1:20 with distilled water (aqua distilled, Braun, Germany) and tested for glyphosate by ELISA (Abraxis, USA) according to the manufacturer's instructions. Test validation was done with Gas Chromatography-Mass Spectroscopy (GC-MS) by Medizinisches Labor Bremen (Germany). The correlation coefficient between the two tests was 0.96 (Data not shown).

Analysis of blood serum and whole blood for selected enzymes, electrolytes, and substrates

Blood serum parameters indicative of cytotoxicity (alkaline phosphatase, AP; glutamate dehydrogenase, GLDH; glutamate oxaloacetate transaminase, GOT; and creatin kinase, and nephrotoxicity (urea and creatine in blood and urine), and cholesterol were determined. Trace elements (Mn, Co, Se, Cu, Zn) in whole blood were measured. All analyses were done by the accredited diagnostic laboratory, Labor Schottdorf MVZ GmbH Augsburg.

Microbiological investigations of feces

The 480 fecal specimens collected from the 40 selected cows every two weeks were investigated by conventional microbiological culture techniques. Fecal specimens (0.5 g in 4.5 mL PBS) were serially diluted in PBS for quantitative bacterial analysis. Dilutions were tested for total aerobic cell numbers (sheep blood agar, Oxoid, Germany), Gram negative cell numbers (Gassner agar, SIFIN, Berlin), enterococci (CATC agar, SIFIN, Berlin), *C. perfringens* (sheep blood agar with polymyxin B and neomycin), and yeasts (Sabouraud agar, SIFIN, Berlin).

Statistical analysis

The statistical program SPSS 15 (SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606) was used to analyze

Date	SP1*	SP2	SP3	SP4	SP5	SP6	SP7
	11.11.2012 -08.12.2012	9.12.2012 -20.01.2013	21.01.2013 - 17.02.2013	18.02.2013 -17.03.2013	18.03.2013 -31.03.2013	01.04.2013 -14.04.2013	15.04.2013 -14.05.2013
Supplements	400g CC ¹	200g CC	200g CC+ 500mL SJ ²	120g HA ³	200g CC+100mL AH ⁴	100g CC+50mL AH	Without supplements

*Supplementation period, 1CC=Charcoal (Carbo ligni, Schottdorf, Germany), 2SJ=Sauerkraut juice (Kronprinz Konserven, Meldorf, Germany), 3HA=humic acids WH67 (Pharmawerk Weinböhla, Germany), 4AH=Aquahumin (Pharmawerk Weinböhla, Germany)

Table 1: Overview of the various supplementation times

	06.01.2013	20.01.2013	17.02.2013	03.02.2013	03.03.2013	17.03.2013	31.03.2013	01.04.2013
Paresis	2/40	2/40	3/40	3/40	2/40	2/40	2/40	2/40
Cystitis	2/40	2/40	2/40	1/40	4/40	1/40	3/40	3/40
Diarrhea	5/40	0/40	3/40	7/40	0/40	5/40	0/40	2/40
Viscous saliva	0/40	0/40	0/40	0/40	1/40	2/40	1/40	4/40
Endometritis	0/40	1/40	0/40	0/40	0/40	0/40	0/40	0/40
Ataxia	0/40	0/40	0/40	0/40	0/40	0/40	0/40	1/40

Table 2: Clinical evaluation of cows at the various sampling points

data. The recorded parameters were tested for normal distribution by the Shapiro-Wilkinson Test. Normal distribution of the arithmetic mean and standard deviations were calculated and median and 1st and 3rd quartiles were calculated for abnormally distributed values. Significance tests were performed using Fisher's Exact Test and the Mann-Whitney U Test.

Results

Glyphosate results

There was a significant ($P < 0.001$) reduction of glyphosate output at week 14 (after 4 weeks of 200 g/d charcoal plus 500 mL sauerkraut juice/d) and at week 18 (after 4 weeks of 120 g/d humic acids WH67).

Microbiological investigation of faeces

Yeasts (about 10^{3-4} cfu) and *C. perfringens* (10^3 cfu) were not significantly different between any of the 12 investigation time points. The results of total aerobic bacteria, Gram-negative bacteria and enterococci are shown in Figures 1-3. The figures show a similar significant increase of the three bacteriological parameters at weeks 8 ($P < 0.001$) and 20 ($P < 0.001$). Aerobe bacterial counts also increased significantly ($P < 0.001$) at week 16 and week 22 ($P < 0.01$), and decreased significantly at week 18 ($P < 0.001$) and week 26 ($P < 0.01$).

Trace elements in whole blood

Changes in the concentration of trace elements in whole blood are shown in figures 4 A-D. The Se was above the reference value of 70 – 100 $\mu\text{g/L}$ at all sampling times (mean concentrations of $157,61 \pm 40,52$; $157,48 \pm 19,73$; $153,68 \pm 14,79$; $159,00 \pm 16,89$ $\mu\text{g/L}$). Co increased significantly ($P < 0.05$) at week 26 but the concentration was well below the reference value of 21.2-107.2 $\mu\text{g/l}$ (Figure 4B). Cu was in the normal range (reference 1,020 -2,030 mg/l) at all sampling times although there was a significant ($P < 0.05$) increase at week 22 and 26 in relation to the starting point. Zn (Figure 4D) declined significantly ($P < 0.05$) from the starting point but all the mean levels were above the reference value of 0.7-1.3 mg/L. Mn was significantly reduced after supplementation with

different substrates (Figure 4C), and was well below the reference range of 0,36-1,8 $\mu\text{mol/L}$ at all of the sampling times.

Enzymes and substrates in blood serum

Enzyme and substrate analyses are shown in Table 3. Significant differences between the applied substrates were seen in GOT, urea, urine creatinine and glyphosate in urine. The other investigated parameters were within the reference range.

Discussion

This sequential study over 26 weeks at this dairy farm with a very high average *C. botulinum* type CD antibody level (about 600% of the normal dairy cow level) and a slightly higher average *C. botulinum* type ABE antibody level (about 150% of the normal dairy cow level), demonstrate the capacity of these animals to react to the *C. botulinum* bacterial cell components and neurotoxins with high antibody levels. The presentation of bacterial components and toxins by macrophages depends on the activity of amino peptidases, proteolytic enzymes that hydrolyze peptide bonds from the amino termini of polypeptide chains. Amino peptidases are ubiquitous enzymes that are widely distributed throughout the biological kingdoms and are found in many subcellular organelles, in cytoplasm, and as membrane components where they perform essential cellular functions. They may hydrolyze the first peptide bond in a polypeptide chain with the release of a single amino acid residue (amino peptidases in a strict sense) or they may remove dipeptides or tripeptides (dipeptidyl- and tripeptidylpeptidases) from polypeptide substrates. Most of the amino peptidases are metallo-enzymes but cysteine and serine peptidases are also included in this group. The strict metallo-aminopeptidases constitute the largest and the most homogenous class of these enzymes, and use one or two metal ions in their active sites to specifically release the N-terminal amino acid residues of polypeptides and proteins. Amino peptidases act in concert with other peptidases to complete diverse proteolytic pathways. They play a vital role in a range of biological processes and disease situations. Processes as distinct as angiogenesis, antigen presentation, neuropeptide and hormone processing, pregnancy

parameters	Week 0	week 4	week 10	week 14	week 18	week 22	week 26	Ref.
	Range (mean)	Range (mean)	Range (mean)	Range (mean)	Range (mean)	Range (mean)	Range (mean)	
CK (U/L)	69-2750(211)	56-450(131)	48-251(120)	66-675(146)	51-675(123)	57-1430(152)	11.3-196(106)	≤ 100
GLDH (U/L)	5.5-47(16.8)	3.1-46.6 (16.9)	3.3-46.8(16.8)	3-80(19.9)	3.1-186 (21.1)	3.5-96(13.9)	3.7-1756(63.2)	≤ 30
AP (U/L)	17-68(38.2)	17-77 (39.3)	17-76 (39.6)	15-87(38.5)	15-87 (39.9)	20-70 (38)	19-204(44.6)	≤ 300
GOT (U/L)	53-155(78.3)	44-116(69.5)*	43-113(70.8)**	53-173(85.5)**	53-178(84)**	51-148 (73)	46-159(81)	≤ 80
Chol. (mg/dL)	57-213(138.8)	47-177(116)	63-208(127.4)	30-220(131.1)	10-220(133.7)	3-218(73.9)	77-196(140)	> 75
Urea B (mg/dL)	14-46(22.6)*	12-30(22.2)*	6-39(25.7)	5-33(24.4)	5-38(26.5)	5-35(24.7)	7-106(29.5)**	20-30
Crea./U ^b (g/L)	0.2-2.2(1.2)*	0.2-2.9(1.1)	0.2-2.9(0.8)**	0.1-2.4(0.67)**	0-2.4(0.6)***	0-1.3(0.7)***	0-1.3(0.3)***	2.49-8.02
Crea./B ^a (mg/dL)	0.6-1.1(0.8)	0.5-1.3(0.8)	0.7-1.51(0.87)	0.2-1.2(0.79)	0.2-1.28(0.8)	0.6-1.2(0.8)	0.6-3.1(1.0)	1--2
Vit. B12 (ng/mL)	98-715(242.8)	114-2190(282)	106-601(225.1)	- ^c	-	-	-	
Glyphosate (ng/mL)	0.5-27.8(15,4)*	1.1-29.4(14,7)	0.5-28.4(11,9)	1.7-22(6,0)**	0.5-22(5,1)**	3.4-28.6(18,6)	1.3-36.4(12)	0
150-396								

^a Crea./B-creatinine, blood, ^b Crea./U-Creatinine, urine, ^c - not tested. (N= 40 every two weeks)

*= $p < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3: Enzyme activity and substrate concentrations in blood serum and creatinine in urine of dairy cows supplemented with charcoal, charcoal with sauerkraut juice or humic acids

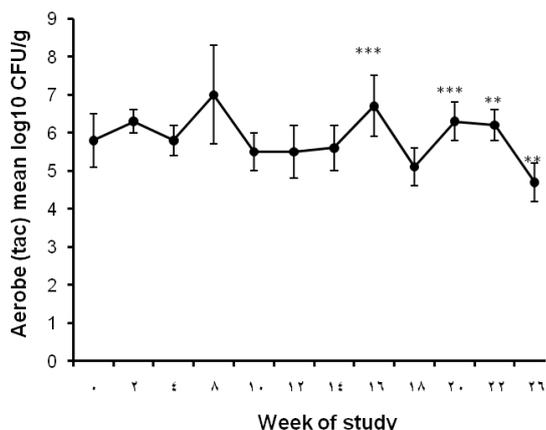


Figure 1: Total aerobe counts in feces of cows supplemented with 400g (week 0-4) or 200g (week 5-10) charcoal/d, 200g charcoal plus 500mL sauerkraut juice/d (week 11-14), 120g humic acid/d (week 15-18), 200g charcoal plus 100ml Aqua humin (week 19-20), 100g charcoal plus 50 mL Aqua humin (week 21-22), and without supplementation (week 23-26). *** P< 0.001, ** P<0.01, (N= 40)

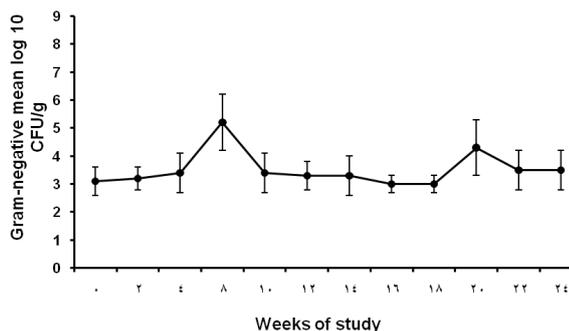


Figure 2: Gram-negative bacteria in feces of cows supplemented with 400g (week 0-4) or 200g (week 5-10) charcoal/d, 200g charcoal plus 500mL sauerkraut juice/d (week 11-14), 120 g humic acid /d (week 15-18), 200g charcoal plus 100ml Aqua humin (week 19-20), 100g charcoal plus 50 mL Aqua humin (week 21-22), and without supplementation (week 23-26). *** P< 0.001, (N= 40).

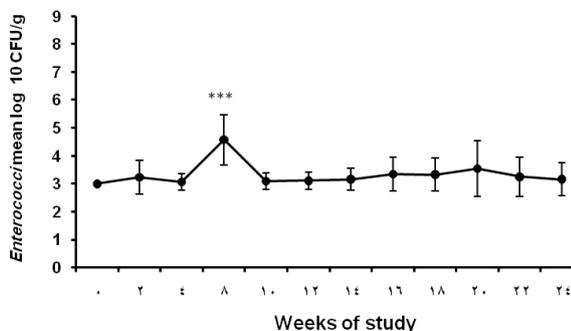


Figure 3: Enterococci in feces of cows supplemented with 400g (week 0-4) or 200g (week 5-10) of charcoal/d, 200g charcoal plus 500mL sauerkraut juice/d (week 11-14), 120g humic acid/d (week 15-18), 200g charcoal plus 100ml Aqua humin (week 19-20), 100g charcoal plus 50 mL Aqua humin (week 21-22) and without supplementation (week 23-26). *** P< 0.001, ** P< 0.01 (N= 40).

and reproduction, protein turnover, memory, inflammation, tumor growth, cancer and metastasis, blood pressure and hypertension all involve one or more critical amino peptidases. A feature common to all metallo-aminopeptidase active sites is that the metal ion (in most cases zinc) is surrounded by a shell of hydrophilic groups that is embedded within a larger environment of hydrophobic groups [12].

After consideration of the immunological results (antibodies), it is not surprising that the immune system of the animals was able to process and present *C. botulinum* antigens to the immune system to express high CD and middle ABE antibody levels. The necessary Zn⁺⁺ ions were present above the reference level in the blood of the investigated animals throughout the time of this study. The other trace elements measured were either within the physiological range (Cu), above this range (Se), or very well below the reference ranges (Co and Mn). The estimated oversupply of Zn⁺⁺ (Figure 4D) seemed to especially influence the outcome of the immunological reaction.

Although Se levels on week 0, 14, 22 and 26 were above the reference level, the average GLDH levels were below the reference level of 30 U/L from week 0 to week 22. The average GLDH level reached 63.2 U/L only at week 26 and this was influenced by a very high maximum value of 1756 U/L (Table 3). CK was reduced at all sampling times compared to week 0, but the differences were not significant and the relatively high mean was caused by only one very high value of one cow (2750U/L). The average CK values of cows in this study were lower than the CK levels reported for Danish cows [1]. The increased CK, but low blood creatinine levels, low CK and the low blood creatinine levels indicate muscle cell damage is occurring although there were low, but no significant differences between the blood creatinine levels at any of the sampling times. Urine creatinine levels were different at some sampling times. Plasma creatinine originates from the degradation of creatine and creatine phosphate, which are present mainly in muscle. Table 3 shows significant differences between week 0 and weeks 10, 14, 18, 22 and 26. Urine creatinine levels decreased from the beginning to the end of this investigation even though blood creatinine levels remained unchanged. The reason for the reduced creatinine excretion in urine could be because of reduced muscle degradation by neutralization of glyphosate. Decreased CK (Table 3) after certain supplementations is an indication of this. Another reason could be from increased water intake and dilution of the urine ingredients. Unfortunately, the farmer didn't measure the water intake of the herd. Blood vitamin B12 levels were within the reference range at weeks 0, 4 and 10; however, it was not possible to determine vitamin B12 at the other sampling times.

Fecal bacteriological parameters were influenced by the different supplements. Aerobe total counts were significantly increased on weeks 8, 16, 20, and 22; but decreased on weeks 18 and 26; however, these cell counts were not influenced by Gram-negative bacteria at the same sampling times (Figure 2). The causes of these changes in aerobe bacterial counts are not known, but it could be from contamination of the charcoal and humic acids with spore forming aerobic bacteria. Gram negative and *Enterococcus* bacterial counts (Figures 2 and 3) were significantly higher at 8 and 20 weeks, but the population of *Enterococci* appears to be too low for an effective antagonism to *C. botulinum* over the full time period. In relation to the constant feeding of the cows with the glyphosate contaminated feed and the changing of different supplements, these results were surprising since Krüger et al. [10] reported antagonistic activity of *Enterococcus* at populations higher than 10³ cfu/g and the populations in this study appear relatively low. Under *in vitro* conditions more than 10⁵ cfu/g *Enterococcus* cells are necessary to antagonize *C. botulinum*, but under *in vivo* conditions

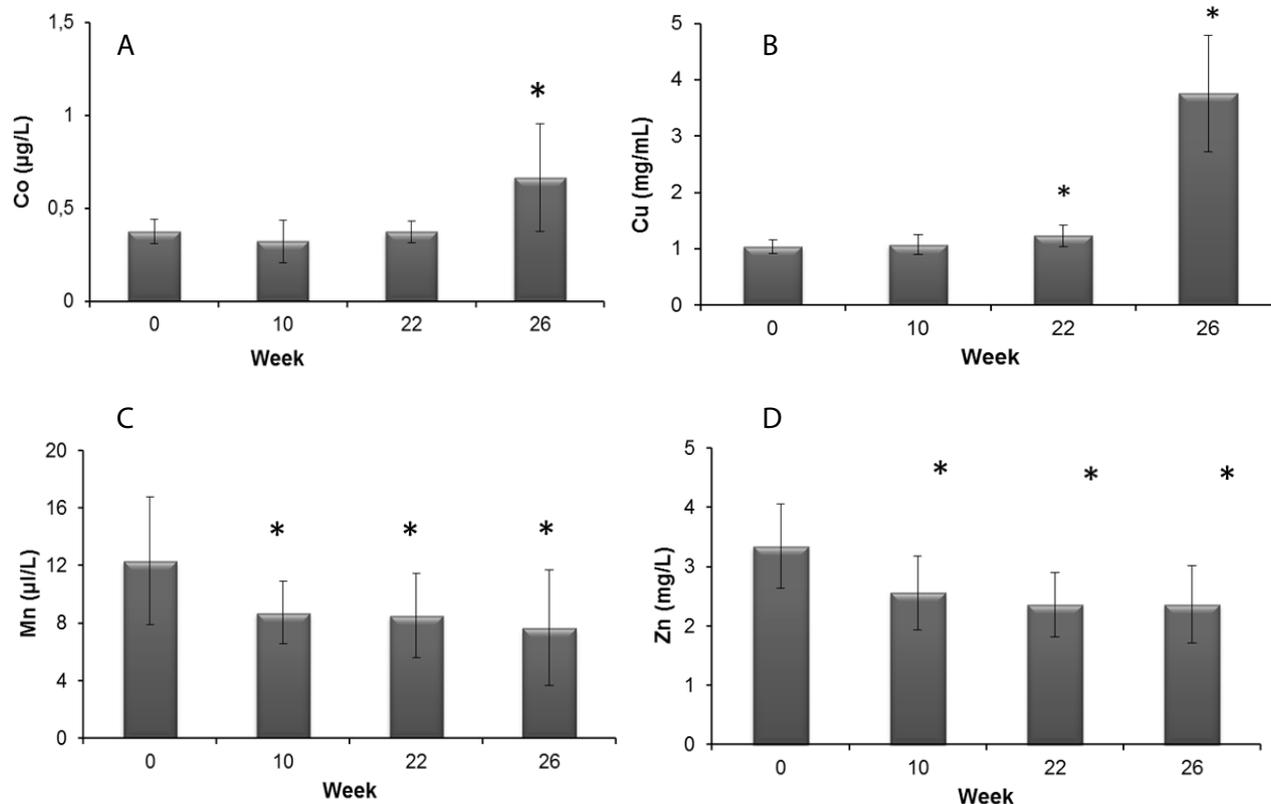


Figure 4: Trace element concentration (A. Co; B. Cu; C. Mn; D. Zn) in whole blood specimens of dairy cows supplementation with 400 or 200g/d charcoal and 200g charcoal plus 500mL sauerkraut juice (week 1-14), 120g/d humic acids and 100 or 50 mL/d Aquahumin plus 200 and 100g charcoal (week 15-22) and without substitution (week 23-26). A significant increase in Co occurred between 26 weeks and all the other sampling times ($P < 0.05$), Cu significantly increased at 22 and 26 weeks in relation to the starting point ($P < 0.05$), and Zn and Mn were significantly reduced from point 0 in relation to the other sampling periods ($P < 0.05$), (N= 40).

more antagonists than *Enterococci* are present since some strains of *C. perfringens*, *C. sporogenes* and *Bacillus cereus* also antagonize *C. botulinum* types A,B, F and E [12-14].

Conclusions

These results show that the application of charcoal, sauerkraut juice and humic acids in feed of cows can decrease the excretion of glyphosate by urine and influence enzymes and substrates of treated animals. This improved the animal's health and induced an appropriate immunological reaction to *C. botulinum* [9]. It must be noted that these animals were also deficient in manganese and cobalt so that additional mineral supplementation with these essential minerals should be evaluated.

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