Research Article **Open Access**

Oral Application of Charcoal and Humic acids to Dairy Cows Influences *Clostridium botulinum* Blood Serum Antibody Level and Glyphosate Excretion in Urine

Henning Gerlach[1], Achim Gerlach[2], Wieland Schrödl[1], Bernd Schottdorf[3], Svent Haufe[4], Hauke Helm[5], Awad Shehata[1,6*] and Monika Krüger[1]

[1]*Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 29, D-04103 Leipzig, Germany*

[2]*Waldstraße 78, D-25712 Burg, Germany*

[3]*Carbon Terra GmbH Gutermannstrasse 25, D-86154 Augsburg, Germany*

[4]*WH Pharmawerk Weinböhla GmbH, Poststr. 58, D-01689 Weinböhla, Germany*

[5]*Südholzring 2, D-25693 Gudendorf, Germany*

[6]*Avian and Rabbit Diseases Department, Faculty of Veterinary Medicine, Sadat City University, Egypt*

[*]**Corresponding author:** Dr. Shehata A, Institute of Bacteriology and Mycology, Veterinary Faculty, University of Leipzig, An den, Tierkliniken 29, D-04103 Leipzig, Germany, Tel: +49 341 973 8181; E-mail: shehata@vetmed.uni-leipzig.de

Abstract

The present study was initiated to investigate the influence of oral application of charcoal, sauerkraut juice and humic acids on chronic botulism in dairy cows. A total of 380 Schleswig Holstein cows suffering from chronic botulism were fed daily with 400 g/animal charcoal for 4 weeks (1-4 weeks of study), 200 g/animal charcoal (5-10 weeks of study), 120 g/animal humic acid (11-14s week of study), 200g charcoal and 500 ml Sauerkraut juice/animal (13-16 weeks of study), 200 g charcoal and 100 mL Aquahumin/animal (15-18s week of study), 100 g charcoal and 50 mL Aquahumin (19-22 weeks of study) followed by 4 weeks without any supplementation. Bacteriological and immunological parameters investigated included *C. botulinum* and botulinum neurotoxins (BoNT) in faeces, *C. botulinum* ABE and CD antibodies, positive acute phase proteins (APPs) haptoglobin and LPS-binding protein (LBP) using serum ELISA, negative APP paraoxanase by its enzymatic activity and glyphosate in urine by ELISA. Neither BoNT nor *C. botulinum* was detected in feacal samples. From week six until four weeks before the end of the study, there was a significant reduction in antibody levels. All supplementation, except low doses of charcoal (200g / animal) alone, led to a significant reduction of *C. botulinum* ABE and CD antibody levels. There also 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. Haptoglobin, paraoxanase and LBP were significantly increased by the 24th week of the study. The positive APPs and *C. botulinum* antibodies were significant negative correlations. In conclusion, a charcoal-sauerkraut juice combination and humic acids could be used to control chronic botulism and glyphosate damage in cattle.

Keywords Humic acids; Peripartual cases; *C. botulinum*

Introduction

In recent years, an increased frequency of a new form of bovine botulism has been observed. This form of botulism differs from regular food-born botulism by its slow and chronic development with various unspecific symptoms. This protracted form may develop when small, sub-lethal amounts of BoNT are taken up and/or absorbed over several days or are generated in the hind gut [1,2]. Clinical symptoms of chronic botulism are most often peripartual cases with indigestion (constipation alternating with diarrhea), non-infectious acute laminitis, ataxia and stiff stilted gait, impossibility to get up (paralysis), apathy, engorged veins, positive venous pulse, edema in legs, udder, and dew-lap, retracted abdomen, forced respiration and unexpected death. The prevalence of C. botulinum in cattle can be determined by detection of botulinum neurotoxins (BoNTs) and/or *C. botulinum* vegetative bacteria or spores in the gastrointestinal tract or organs (liver, kidney, lungs and muscles [1,3,4]. A second way to verify chronic botulism is with specific antibodies for BoNTs [3,5,6] detected

natural specific antibodies in wild canine species, horses and dairy cows.

C. botulinum is an ubiquitous Gram-positive, spore forming, obligatory anaerobic bacterium that inhabits soil, dust and organic matter such as feces of animals and man, slaughterhouse wastes, residues of biogas plants, and bio-compost. It generates eight highly toxic neurotoxin isoforms (BoNT A-H) that are the most toxic substances known [7-12]. All isoforms, together with the related tetanus neurotoxin (TeNT) secreted by *C. tetani*, are $Zn2+$-endoproteases. The immunologically distinct neurotoxins (A-H) of *C. botulinum* are homologous proteins consisting of a heavy and light chain linked by an essential disulfide bridge. The light chain blocks the release of acetylcholine at the neuromuscular junction. Human cases are mostly caused by types A, B, or E, while animal diseases are mostly caused by types C and D [1,13,14]. Several C. botulinum strains produce two neurotoxins [11]. Physiological differences are used to divide *C. botulinum* strains into 4 physiological groups; group I, consisting of *C. botulinum* A and proteolytic strains of *C. botulinum* B and F; group II, consisting of *C. botulinum* E and nonproteolytic strains of *C. botulinum* B and F; group III, consisting of *C. botulinum*

C and D; and group IV, consisting of *C. argentinense* (BoNT G). Neurotoxigenic strains of other Clostridium species such as *C. butyricum*, (BoNT E, group V) and C. baratii (BoNT F, group VI) have also been identified [14]. BoNTs are produced as a 150 kDa single polypeptide chain. The protein is post-translationally proteolyzed to form a dichain in which the heavy chain (HC, 100 kDa) and light chain (LC, 50 kDa) are linked through a disulfide bond. HC is composed of two 50 kDa domains, with the N-terminal half involved in translocation and the C-terminal half involved in binding with nerve cells. BoNTs bind specifically to neuronal cells, enter the cytoplasm, and then cleave core proteins involved in the vesicular fusion machinery (SNARE proteins) by its metalloprotease activity to block the release of neurotransmitters. When BoNTs are produced by the bacteria, the BoNTs are found in complexes associated with protective proteins (progenitor toxins). These are the nontoxic, nonhemagglutinin (NTNH, 130 kDa) and several nontoxic hemagglutinins (NTHAs). BoNTs cannot penetrate intact skin, but the toxin is absorbed from mucosal surfaces or wounds. In foodborne and intestinal botulism, botulinum toxins are produced from *C. botulinum* and other BoNT producing Clostridia, which colonize the lumen of the intestine where the toxins are absorbed from the digestive tract. BoNT binds through a double-receptor system consisting of a protein receptor and acidic lipid-gangliosides with its heavy chain domain [15,16].

The upper or small intestine is the most important site for absorption of BoNTs [17,18] but other mucous membranes also are able to absorb BoNTs [19]. BoNT complexes probably do not dissociate in the digestive tract. The whole toxin complex seems to be absorbed from the intestine into the lymphatics in the rat ligated duodenum loop assay. Molecular dissociation occurs immediately after BoNT complexes, designated progenitor toxins, are absorbed into the lymphatics [20]. Botulinum progenitor toxins are found in three forms with molecular masses of 900 kDa (LL toxin for type A), 500 kDa (L toxin for types A, B, C, D and G) and 300 kDa (M toxin for types A, B, C, D, E and F). The M toxin consists of BoNT and NTNHA with no hemagglutination activity, whereas the L toxin consists of BoNT, NTNHA and NTHA. The LL toxin is a dimer of L toxin [21]. NTNHA protects the BoNT from acidity and proteases in the digestive tract. NTHAs play an essential role in the effective absorption of the type C progenitor toxins to the small intestine. NTHA exists as subcomponents; HA-70, HA-33 and HA-17 [21]. The HAs of BoNT/A and B could disrupt the human epithelial intercellular junction through species-specific interaction with E-cadherin to presumably facilitate BoNT transport via the paracellular route [22-24]. NTHAs of BoNT A may bind to 9 different glycan sites of gut epithelial cells. Thus NTHA mediated absorption could be prevented by galactose and its derivates [25].

Treatment of BoNT intoxication is accomplished using specific polyclonal and monoclonal antibodies. Antitoxins given within 24 hour of the onset of disease can lower the death rate from botulism and shorten duration of the symptoms [16]. Antibodies only neutralize non-bounded BoNTs. Other possibilities for treatment could be peptide based inhibitors that mimic amino acid motives of the SNARE proteins to inhibit the catalytic part (endopeptidase) of the BoNT [15]. One obstacle for the endopeptidase inhibitors is how to deliver the molecules into the intoxicated nerve cells. Another way of treatment is mimicry of receptors. Binding of BoNTs to nerve cells in a two-step process is generally accepted. Binding to gangliosides is followed by high affinity binding to a protein receptor(s) [26]. Synaptotagmin has been proposed as the protein receptor for BoNT/A, /B, /E, and /G [15,27]. Aptamers, unique oligonucleotides with high affinity for their (proteins or small molecule) targets, are the newest treatment possibilities [16]. All of the above mentioned methods are mostly too expensive for food animals and cheaper methods are necessary. Neuvonen and Olkkola (1988) used charcoal to treat intoxications in humans. In the present study, *C. botulinum* types ABE and CD blood serum antibodies, feacal BoNTs and *C. botulinum* spores from 40 dairy cows in 4 different lactation states (10 each group) were investigated for 26 weeks [28]. The cows were supplemented with charcoal and humic acids in different doses to evaluate their BoNT binding capacity. Parallel haptoglobin [29] and LPS binding protein [30], as positive acute phase proteins (APPs), and paraoxanase [31,32] as negative APP were investigated. Urine was tested each 4 weeks for glyphosate to demonstrate the glyphosate neutralizing capacity of charcoal and humic acids, and to show a possible connection between glyphosate and chronic botulism.

Material and Methods

Animals and supplementations

A Schleswig Holstein dairy cow farm of about 380 cows with average milk production of 9000 L per year showed clinical symptoms of chronic botulism (flock 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 and 60% of the cows suffered from Dermatitis digitalis (mortellaro). The entire animal population was involved in the various charcoal (CC) (≤ 8 mm diameter) and powdery humic acid (WH67) or sauerkraut juice (SJ) and liquid humic acid (Aquahumin) supplement. Each treatment represented 10 identical cows of the 1st, 2nd, and 3rd lactation and the dry cows group for the full time of the investigation. Their last polyvalent clostridial vaccination (Covexin, Intervet) was on 01.11.2012. The treatment regime with CC, SJ and/or humic acids was changed every 4 weeks (supplementation periods) in Table 1 and given as part of the total mixed ratio (TMR).

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	400 g CC1	200 g CC	200 g CC+ 500 mL SJ2	120 g HA3	200 g CC+100mL AH4	100 g CC+50mL AH	Without supplements

*Supplementation period, 1CC=Charcoal (Carboligni, Schottdorf, Germany), 2SJ=Sauerkraut juice (KronprinzKonserven, Meldorf, Germany), 3HA=humic acids WH67 (PharmawerkWeinböhla, Germany), 4AH=Aquahumin (Pharmawerk Weinböhla, Germany).

Table 1: Overview at the various times of supplementation.

	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
Diahrea	5/40	0/40	3/40	7/40	0/40	5/40	0/40	2/40
Viscus 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 estimation of cows at the various sampling points.

The TMR was composed of grass and maize silage (glyphosate concentration 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 (Table 2).

Collection of samples

Blood, faeces and urine were analyzed 7 times at 4 week intervals with one exception (200 g CC over 6 weeks). Blood specimens were taken from the Vena coccygenamediana, coagulated blood centrifuged at 3000 x g for 15 min and the serum samples were stored at -20°C. Faeces were taken from Ampulla recti and spontaneous urination was 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 Medizinsches Labor Bremen (Germany). The correlation coefficient between the two tests was 0.96 (Data not shown).

Analysis of free BoNT/A-E and C. botulinum spores in faeces

Preparation of faeces for detection of BoNT/A-E

Faecal samples were diluted 1:3 in PBS (Dulbecco, pH 7.4) containing 0.1% Triton X-100, 0.1% Tween 20 and 10 mM EDTA. The samples were thoroughly mixed and frozen at -20°C. After thawing, the diluted samples were centrifuged at 7000 x g for 15 min and the clarified supernatants were analyzed with BoNT-ELISA.

Indirect detection of C. botulinum spores

Rumen fluid and faecal samples were diluted 1:10 in RCM (0.5 g in 4.5 ml), vigorously mixed, and heated at 80°C for 10 min. Samples were incubated at 37°C for 7 d under anaerobic conditions and subsequently stored at -20°C until tested. After thawing, the sample was centrifuged at 7.000 x g for 15 min and the clear supernatant was analyzed for the type-specific soluble antigens of *C. botulinum* types A-E by ELISA.

BoNT-ELISA

BoNT/A-E were determined by an ELISA developed in our institute [2]. The standard volume was 100 µl per well and the standard incubation condition was 1 h at room temperature (1 h at RT) on a microtiter plate shaker (400 rpm). The coating buffer was 0.1 M NaHCO3 and the wash solution (WS) was 0.9% NaCl with 0.05% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). All washing steps were done in a Nunc-Immuno-Washer 12 (Nunc, Wiesbaden, Germany). After coating the ELISA wells with capture antibodies (3 mg/ml, BoNT-immunoaffinity purified-IgG from rabbits against BoNT/A-E, Institute of Bacteriology and Mycology, University of Leipzig, Germany) overnight at 4-6°C, they were incubated with 150 ml per well of 1% gelatin from cold water fish skin (Sigma-Aldrich, Taufkirchen, Germany) in 0.9% NaCl solution for 1 h at RT. The wells were washed twice with WS and loaded with the prepared faecal samples diluted 1:2 in 20 mMTris, pH 8.0, assay buffer [adjusted with 1 M HCl] containing 0.9% NaCl, 5 mM EDTA, 1% gelatin from cold water fish skin, 0.2% bovine serum albumin, 0.1 mg/ml rabbit IgG from normal serum and 0.2% Tween 20 (chemicals from Sigma-Aldrich or Fluka, Taufkirchen, Germany). After incubation, the wells were washed five times with WS and loaded with the detection antibodies conjugated with HRP and diluted in assay buffer. *C. botulinum* types A and B were detected with 2.5 mg/ml horse [Fab]2 from IgG against *C. botulinum* A and B (Novartis Vaccines and Diagnostics Co, Marburg, Germany). Types C and D were detected with 0.1 mg/ml of IgG from rabbits developed against BoNT/C and D (Institute of Bacteriology and Mycology, University of Leipzig). Type E was detected with 2.5 mg/ml IgG from horses against *C. botulinum* type E (WDT, Garbsen, Germany).

After incubation at RT, the plates were washed four times with WS and the HRP activity was determined by adding 100 µl/well of 3 mM H2O2 and 1 mM 3, 30, 5, 50-TMB. The substrate reaction was stopped

with 1 M H2SO4 (50 µl/well) and the optical density (OD) was measured with an ELISA-reader at 450 nm. The sensitivity, specificity, precision, limit of detection, and range of quantification were determined previously. Cross reactivity of antibodies with *C. tetani, C. perfringens, C. sporogenes, C. sordellii, C. novyi, C. butyricum, Bacillus cereus, Streptococcus agalactiae, Streptococcus zooepidemicus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Candida albica*ns and *Candida krusei* were all negative.

Evaluation of BoNT-ELISA

The relative units (RU) were calculated from the measured OD values as follow: (sample-OD minus twice the value of the control-OD [BoNT-negative sample of bovine faeces]) multiplied by 1000 and dilution factors per minute substrate incubation time.

Analysis of C. botulinum antibodies using ELISA

Solid phase antigen for ELISAs

C. *botulinum* types A (7272), B (7273), C (2300), D (2301), and E (2302) obtained from the National Collection of Type Cultures (NCTC) were used for preparation of ELISA antigens. Culture supernatant from *C. sporogenes* and *C. perfringens* (Isolated and identified by the Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University) served as a control antigen to study cross reactivity. All strains were cultured in reinforced Clostridial medium (RCM; Sifin, Berlin, Germany) and incubated anaerobically at 37°C for 7 days followed by freezing at 25°C. Supernatants were checked for BoNT-type with type specific ELISA [9]. After thawing and mixing, the culture suspension was centrifuged at 10,000 g for 15 min and the clear supernatant was separated. BoNT-proteins in the supernatants were detoxified with 20 mM formaldehyde (four additions weekly) and incubated at 37°C. Active formaldehyde groups were blocked by the addition of 100 mM lysine and 100 mM glycine in 100 mMTris/HCl (pH 8.0) solution and incubated at RT for 24 h. Complete detoxification was verified with the mouse test by Dr. F. Gessler (Miprolab, Göttingen, Germany), data not shown. The antigen preparation was washed with PBS (pH 7.4) and concentrated by ultrafiltration at a molecular weight cut-off of 50 kDa (viva- vivaspin 20, Sartorius Stedim Biotech, Göttingen, Germany). The protein concentration was measured with a spectral photometer (MBA 2000) and its integrated software (PerkineElmer, Norwalk, Connecticut, USA) and adjusted with PBS to 1 mg/ml.

Detection of IgG anti C. botulinum antibodies by ELISA

ELISA plates were coated with 100 ml/well of detoxified antigen from C. botulinum (1 mg/ml in 0.1 M NaHCO3) and incubated overnight at 4-6°C. Coated plates were washed twice with 0.9% NaCl containing 0.05% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany) followed by 135 µl of blocking solution (1% bovine case) mixed with 15 ml diluted serum sample (1:10 in 50 mMTris buffer, pH 8, containing 0.9% NaCl, 10 mM EDTA, 1% yeast extract, 1% BSA, 20% RCM and 1% Tween 20) and incubated for 1 h at RT on a microtiter plate shaker. After washing four times, IgG from rabbits against bovine IgG (Fc) conjugated with horse radish peroxidase (HRP) (Dianova, Hamburg, Germany) diluted 1:20,000 in assay buffer (50 mM Tris pH 7.4, 0.9% NaCl, 0.2% yeast extract, 0.1% BSA, 0.1% bovine Casein, 2% RCM and 0.1% Tween 20) was added to each well and incubated 1 h at RT.

HRP activity was determined by adding 100 ml/well of 3 mM H2O2 and 1 mM 3, 3´, 5,´5- tetramethylbenzidine (TMB) in 0.2 M citrate-buffer (pH 4.0). The substrate reaction was stopped with 1 M H2SO4 (50 µl/well) and the optical density (OD) was measured with an ELISA-reader at 450 nm. RCM without *C. botulinum* antigen served as a control antigen to determine the degree of non-specific solid phase binding of immunoglobulin on each sample (control OD). The control OD value was subtracted from each antigen specific OD value to calculate the Anti- *C. botulinum* IgG level relative to an internal laboratory standard (pooled blood samples from >3000 cows) that was defined as 100 percent.

Haptoglobin analysis

The Hp concentration in blood serum was determined by ELISA as described by Schroedl et al. [33]. Briefly, the coating antibody was IgG from rabbit anti-Hp (DAKO, Hamburg, Germany), which was diluted 1:3000. The standard was bovine plasma in which the Hp concentration was determined with a standardized colorimetric assay for bovine Hp (Tridelta Development Ltd., Greystones, Co. Wicklow, Ireland) and further checked against purified bovine Hp. The standard concentration ranged from 3 to 200 ng ml-1. The samples were diluted 1:1000 and 1:50000 in assay buffer (50 mMTris-HCl with pH 8.0, 0.15 M NaCl, 10 mM EDTA, 0.1% Tween 20 and 0.2% bovine casein, all from Sigma-Aldrich, Taufkirchen, Germany). The detection antibody was polyclonal IgG (rabbit) anti-Hp (DAKO, Hamburg, Germany) conjugated with horseradish peroxidase. The detection antibody was diluted 1:10000 in assay buffer. The detection limit, including the dilution factor of 1000, was 1 µg ml-1.

LBP analysis by ELISA

The LBP coating antibody was affinity purified monoclonal IgG2a (mouse) anti-LBP-human (mAb-Abi-202) at 1.2 µg/ml. The standard LBP-range in the ELISA was 0.3 to 20 ng/ml human LBP (LBP-standard serum). The samples were diluted 1:1,000 and higher. The assay buffer for dilution of the standard and plasma samples was 50 mM Tris HCl (pH 8.0), 0.15 M NaCl, 10 mM EDTA, and 0.1% Tween 20 (v/v). The detection antibody was affinity purified monoclonal IgG1 (mouse) anti-LBP-human conjugated with horseradish peroxidase (mAb-Abi-204) diluted 1:6,000 in assay buffer. The two mAbs and the standard serum were provided by Prof. Ch. Schuett, Institute of Immunology, and University of Greifswald, Germany.

Paraoxanase analysis

Paraoxonase/arylesterase activity was measured spectrophotometrically using paranitrophenyl acetate (PNPA) as a substrate. A stock solution was prepared using 1M Hepes buffer (pH 7.5), 400 mM p-nitrophenyl acetate in DSMO and 100 mM CaCl2. The working buffer contained 10 mM CaCl2, 10 mMHepes, and 2 mM p- nitrophenyl acetate in 50 mL distilled water. Blood serum specimens (25 µL) diluted 1: 10 in distilled water were applied to microtiter plates and 200 µL of the working buffer were added. After 3 s shaking, the optical density was measured at 405 nm wave length (t0) and remeasured 10 min later (t1). The paraoxanase activity in U/mL is calculated with the following equation:

Paraoxonase activity = (t1-to) x serum dilution x1000 = (t1-t0) x10x1000 = units/L

Statistical analysis

The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). A two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results

Effect of supplementation on glyphosate in urine

A significant reduction in glyphosate excretion (P<0.0001) was only seen at the 14th and 18th week of the study (Figure 1).
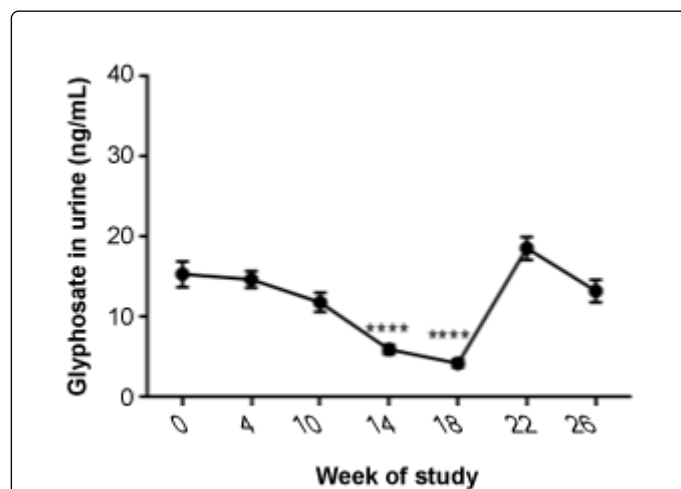
Figure 1: Dynamics of glyphosate excretion in urine with the application of 400 g charcoal daily(CC)the first four weeks (1-4 weeks) followed by 200 g CC daily for weeks 5-10, 200 g CC+500 ml Sauerkraut juice (SJ)daily weeks 11-14, 120 g humic acid (HA)daily weeks 15-18, 200 g CC+100 mL Aquahumin(AH) daily weeks 19-20, 100 g CC +50 mL AH weeks 21-22 and without supplementation weeks 23-26. A significant (P<0.0001) reduction of glyphosate in urine was detected only in weeks 12 to 19 (4 weeks daily of 200 g CC+ 500 mL SJ, 4 weeks daily of 120 g HA).

The combination of CC (200 g) and SJ (500 ml) as well as HA (120 g) reduced glyphosate in urine significantly.

Botulinum neurotoxin (BoNT) and C. botulinum in faeces

No BoNT or C. botulinum was detected in feacal samples.

Detection of C. botulinum IgG antibodies in blood serum

The dynamic effects of different supplementations on *C. botulinum* ABE and CD blood serum antibody levels over 24 weeks are shown in Figure 2.
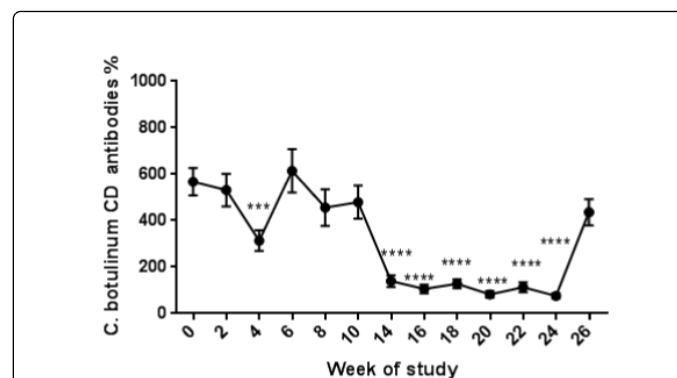
Daily supplementation with CC and/or humic acids initiated at week 6 significantly reduced antibody levels (P<0.01 at week 6, P<0.001 for weeks 8-24, and P<0.05 for week 26. The effect of different supplements on *C. botulinum* CD blood serum antibody levels over the 26 weeks is shown in Figure 3.

Supplementation with daily 400 g CC significantly decreased CD antibody (P<0.01) while a daily application of 200 g CC allowed the CD antibody level to increase. A highly significant (P<0.001) reduction in CD antibody was detected only after two weeks supplementation with 200 g CC plus 500 mL SJ. Antibody reduction was constant from week 4 to 24; however, four weeks after finishing supplementation (week 26), CD antibodies increased.
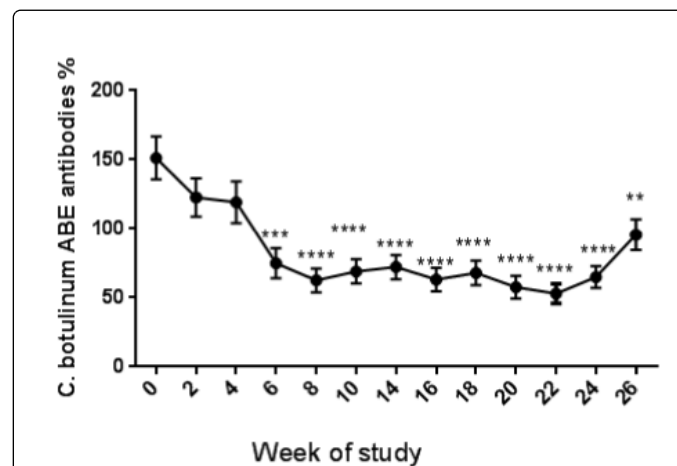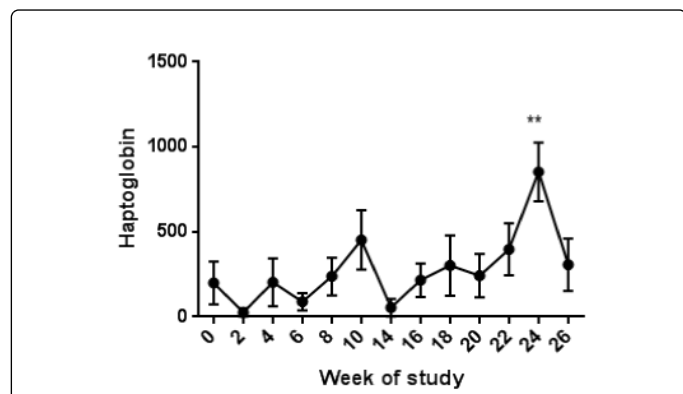

Figure 2: Dynamics of C. botulinum ABE antibodies in blood serum in relation to the daily application of 400 g charcoal (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC+500 ml sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CC +100 mL Aquahumin(AH) (weeks 19-20), 100 g CC +50 mL Aquahumin(AH) (weeks 21-22), and without supplementation (weeks 23-26). There was a significant reduction of antibody levels with a daily supplementation of charcoal or humic acids beginning from week 6 (P<0.01 for week 6, P<0.001 for weeks 8-24, and P<0.05 for week 26.

Figure 3: Effect of daily supplementation with 400 g CC (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC+500 ml sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CCl +100 mL Aquahumin(AH) (weeks 19-20), 100g CC +50 mL AH (weeks 21-22) and without supplementation (weeks 23-26) on the dynamic of C. botulinum CD antibodies in blood serum. There was a significant reduction in antibody levels from daily supplementation with charcoal and humicacids (P<0.01 and P<0.001) for weeks 14-24.

Detection of haptoglobin

Haptoglobin levels in blood serum were not significantly different with any of the supplements except for week 24 after they were taken off Aquahumin (Figure 4).

Figure 4: Haptoglobin in blood serum after the daily application of 440 g CC (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC+500 ml sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CCl+100 mL Aquahumin (AH) (weeks 19-20), 100 g CC +50 mL AH (weeks 21-22) and without supplementation (weeks 23-26). A significant (P<0.05) difference was only detected at week 24.

LBP results

There was a significant increase in LBP in blood serum on week 20 (P<0.001) (Figure 5).
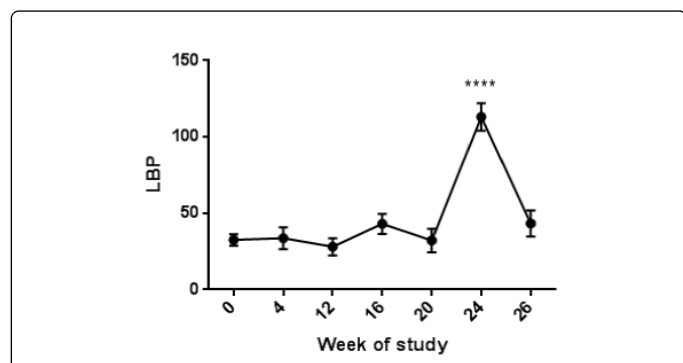
Figure 5: LBP in blood serum in relation to daily oral application of 400 g charcoal (CC) (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC +500 ml sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CCl+100 mL Aquahumin(AH) (weeks 19-20), 100 g CC +50 mL (AH) (weeks 21-22) and without supplementation (weeks 23-26). A significant (P<0.001) increase inLBP level was seen only at week 24.

Paraoxanase (PON) in blood serum

PON activity increased significantly only at 24-26 weeks (P<0.001) (Figure 6).
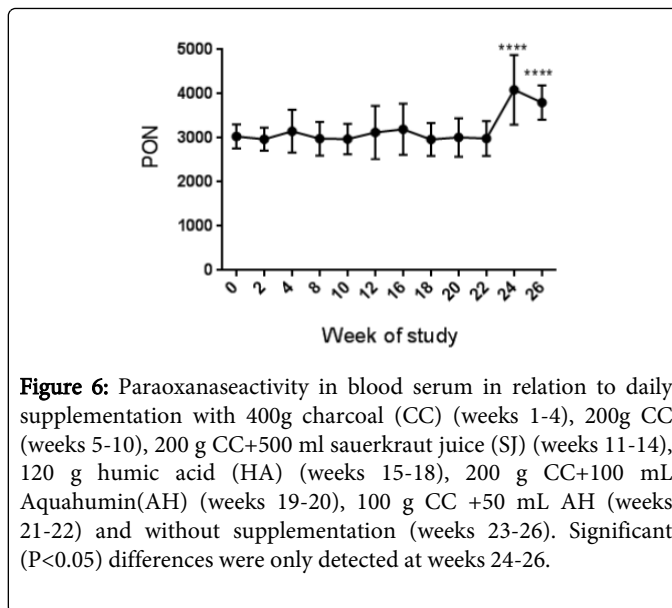
Figure 6: Paraoxanaseactivity in blood serum in relation to daily supplementation with 400g charcoal (CC) (weeks 1-4), 200g CC (weeks 5-10), 200 g CC+500 ml sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CC+100 mL Aquahumin(AH) (weeks 19-20), 100 g CC +50 mL AH (weeks 21-22) and without supplementation (weeks 23-26). Significant (P<0.05) differences were only detected at weeks 24-26.

Discussion

We investigated the effect of an oral application of CC and humic acids (HA) alone or in combination with SJ on blood serum *C. botulinum* ABE and CD antibody levels. Chronic botulism is characterized by the sub-lethal generation of *C. botulinum* progenitor toxins in the hind gut. The incorporation of the progenitor toxin and free BoNT from the gastrointestinal tract (GIT) into the body could happen via three different routes. Small concentrations of the progenitor toxin and BoNT bind with hemagglutinins (HA) or the HC part of the molecule can bind to receptors on the surface of epithelial cells and transcytosis can occur. Translocated HA disrupts the epithelial barrier. This is different with type A, B and C progenitor toxins. Type A and B HAs disrupt the epithelial cell line paracellular without causing cytotoxic effects in the epithelial cells of their susceptible hosts while type C HAs possibly evoke cytotoxic-barrier disrupting activity in the epithelial cells of susceptible animals. Damaged epithelial cells are not a barrier for progenitor toxins and BoNTs [20,23,24]. The damaged epithelial barrier permits the toxins to be distributed throughout the body by blood and lymph vessels. Based on this knowledge, it is very important to bind these toxins with CC. The very strong reduction of CD antibodies after the daily application of 400 g of CC shows this effect. These very high CD antibody levels without the application of a CD vaccine have not been reported previously. Such high antibody levels have only been observed in conjunction with vaccination [3]. Wang et al. showed good sorption of the hydrophobic herbicide terbuthylacin by CC [35]. Maybe the hydrophobic surfactant of the commercial herbicide Roundup also could be absorbed by CC [36]. Graber found that glyphosate can be absorbed by CC. Our results don`t support these results in animals [37]. Four weeks daily application of 400 g CC reduced the CD antibody level dramatically (Figure 3) but did not affect the excretion of glyphosate in urine (Figure 1). In our own investigation, we only found neutralization or absorption of a maximum of 300 μg glyphosate to 1 mg CC (data not shown). The daily application of 200 g CC in weeks 5-10 failed to reduce glyphosate excretion or *C. botulinum* type CD antibody levels. The mixed application of 200 g CC and 500 mL SJ significantly reduced the amount of glyphosate excreted and *C. botulinum* CD antibodies also significantly (P<0.001)

decreased (Figures 1 and 3). *C. botulinum* ABE antibodies were significantly reduced by all the treatments from week 4 on Figure 2. The application of HA (WH67) significantly (P<0.001) reduced glyphosate excretion and *C. botulinum* ABE and CD antibody levels. Krüger et al. demonstrated that glyphosate reduced the Enterococcus spp. bacteria that are antagonistic to *C. botulinum [34]*. Shehata et al. were able to neutralize the antibacterial activity of glyphosate with different humic acid preparations in vitro [38]. Results from the application of 200 g CC and 100 mL Aquahumin (liquid preparation) for 2 weeks compared with 100 g CC with 50 mL Aquahumin for two weeks showed that a definite amount of these substrates is necessary to absorb or neutralize glyphosate and/or *C. botulinum* toxins. Mazzei and Piccolo found that glyphosate may spontaneously and significantly bind to soluble humic matter by non-covalent interactions at slightly acidic pH [39]. Binding to matrices such as soluble fulvic and humic acids could be the reason. Glyphosate excretion was reduced with the soluble Aquahumin (Figure 1). It was not anticipated that the combination of 200 g CC and 500 mL SJ per day would be so very effective. Fermentation of cabbage to SJ is mostly done by Lactobacillus plantarum [40]. Lactobacilli produce exopolysaccharides (EPS), homopolysaccharides and heteropolysaccharides. These biopolymers are widely distributed in nature and can be the polymers of neutral (pentoses and hexoses) or anionic sugars (hexoses). They are released into the extracellular medium by Archebacteria and Eubacteria (both Gram positive and negative). Approximately 30 species of Lactobacilli are described as EPS producers. Among them, the best known are *L. casei, L. acidophilus, L. brevis, L. curvatus, L. delbrueckii, L. bulgaricus, L. helveticus, L. rhamnosus, L. plantarum*, and *L. johnsonii. L. plantarum* generates heteropolymers of glucose, galactose and rhamnose. Galactose and lactose inhibit the absorption of *C. botulinum* progenitor toxins to the sugar bearing receptors on epithelial cells of the GIT [41]. The sugar polymer concentrations in nutrient broth culture of Lactobacilli are in hundreds of mg per liter. EPS may also interact with proteins, mineral, ions and other compounds [42,43]. Zhang et al. (2013) identified antioxidant effects of *L. plantarum* that may involve scavenging reactive oxygen species (ROS), up-regulation of enzymatic and non-enzymatic antioxidant activities, and reduction of lipid peroxidation [44]. ROS and lipid peroxidation are induced by glyphosate [45,46]. The neutralization of glyphosate with humic acids from WH67 was reported by Shehata et al. [33]. The binding mechanism could be hydrogen bonding to phenolic groups of humic acid [47]. The positive acute phase proteins (haptoglobine, LBP) only significantly increased at week 24 and by week 26, both acute phase proteins (APP) were reduced but *C. botulinum* ABE and CD antibodies increased. Inflammation indicated by the significant increase of haptoglobin (P< 0.01) and LBP (P<0.001) may be induced by proliferation of *C. botulinum*. At week 26, when *C. botulinum* ABE and CD antibodies were high, the APPs were low. There is a negative correlation between LBP and *C. botulinum* ABE and CD antibodies (R2= -0.41 and -0.51, respectively). It is interesting that even though positive APPs increased, the negative APP paraoxanase also increased at week 24. This indicates that the anti-oxidative capacity of the cows increased, but the causes for this are unknown.

Conclusion

Daily oral application of 400 g CC) significantly reduced *C. botulinum* ABE and CD antibodies by absorption of *C. botulinum* toxins in the gastrointestinal tract but did not reduce glyphosate excretion in urine. This result was not repeatable with 200 g CC alone but 200 g CC plus 500 ml SJ reduced both glyphosate excretion and *C. botulinum* ABE and CD antibodies. The same excellent result was obtained highly significantly with 120 g humic acids. A certain amount of CC and/or humic acids are necessary to absorb and/or neutralize glyphosate and *C. botulinum* toxins.

References

1. Böhnel H, Schwagerick B, Gessler F (2001) Visceral botulism--a new form of bovine Clostridium botulinumtoxication. J Vet Med A PhysiolPatholClin Med 48: 373-383.

2. Krüger M, Große-Herrenthey A, Schrödl W, Gerlach A, Rodloff A (2012) Visceral botulism at dairy farms in Schleswig Holstein, Germany: prevalence of Clostridium botulinum in feces of cows, in animal feeds, in feces of the farmers, and in house dust. Anaerobe 18: 221-223.

3. Krüger M, Skau M, Shehata AA, Schrödl W (2013) Efficacy of Clostridium botulinum types C and D toxoid vaccination in Danish cows. Anaerobe 23: 97-101.

4. http://www.botulismus.org/index_htm_files/Boehnel_Rinder.pdf

5. Steinman A, Millet N, Frenkel C, King R, Shpigel NY (2007) Presence of antibotulinum neurotoxin antibodies in selected wild canids in Israel. J Wildl Dis 43: 548-550.

6. Steinman A, Kachtan I, Levi O, Shpigel NY (2007) Seroprevalence of antibotulinum neurotoxin type C antibodies in horses in Israel. Equine Vet J 39: 232-235.

7. Williamson JL, Rocke TE, Aiken JM (1999) In situ detection of the Clostridium botulinum type C1 toxin gene in wetland sediments with a nested PCR assay. Appl Environ Microbiol 65: 3240-3243.

8. Böhnel H, Lube K (2000) Clostridium botulinum and bio-compost. A contribution to the analysis of potential health hazards caused by bio-waste recycling. J Vet Med B Infect Dis Vet Public Health 47: 785-795.

9. Long SC, Tauscher T (2006) Watershed issues associated with Clostridium botulinum: a literature review. J Water Health 4: 277-288.

10. Bagge E, Persson M, Johansson KE (2010) Diversity of spore-forming bacteria in cattle manure, slaughterhouse waste and samples from biogas plants. J ApplMicrobiol 109: 1549-1565.

11. Barash JR, Arnon SS (2014) A novel strain of Clostridium botulinum that produces type B and type H botulinum toxins. J Infect Dis 209: 183-191.

12. Dover N, Barash JR, Hill KK, Xie G, Arnon SS (2014) Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis 209: 192-202.

13. Foran PG, Mohammed N, Lisk GO, Nagwaney S, Lawrence GW, et al. (2003) Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. J Biol Chem 278: 1363-1371.

14. Popoff MR (2014) Botulinum neurotoxins: more and more diverse and fascinating toxic proteins. J Infect Dis 209: 168-169.

15. Rummel A, Karnath T, Henke T, Bigalke H, Binz T (2004) Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. J Biol Chem 279: 30865-30870.

16. Cai S, Singh BR (2007) Strategies to design inhibitors of Clostridium botulinum neurotoxins. Infect Disord Drug Targets 7: 47-57.

17. Bonventre PF (1979) Absorption of botulinal toxin from the gastrointestinal tract. Rev Infect Dis 1: 663-667.

18. Sakaguchi G (1982) Clostridium botulinum toxins. PharmacolTher 19: 165-194.

19. Park JB, Simpson LL (2003) Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. Infect Immun 71: 1147-1154.

20. Fujinaga Y (2010) Interaction of botulinum toxin with the epithelial barrier. J Biomed Biotechnol 2010: 974943.

21. Kouguchi H, Watanabe T, Sagane Y, Ohyama T (2001) Characterization and reconstitution of functional hemagglutinin of the Clostridium botulinum type C progenitor toxin. Eur J Biochem 268: 4019-4026.

22. Sugawara Y, Matsumura T, Takegahara Y, Jin Y, Tsukasaki Y, et al. (2010) Botulinumhemagglutinin disrupts the intercellular epithelial barrier by directly binding E-cadherin. J Cell Biol 189: 691-700.

23. Jin Y, Takegahara Y, Sugawara Y, Matsumura T, Fujinaga Y (2009) Disruption of the epithelial barrier by botulinumhaemagglutinin (HA) proteins - differences in cell tropism and the mechanism of action between HA proteins of types A or B, and HA proteins of type C. Microbiology 155: 35-45.

24. Matsumura T, Jin Y, Kabumoto Y, Takegahara Y, Oguma K, et al. (2008) The HA proteins of botulinum toxin disrupt intestinal epithelial intercellular junctions to increase toxin absorption. Cell Microbiol 10: 355-364.

25. Lee K, Gu S, Jin L, Le TT, Cheng LW, et al. (2013) Structure of a bimodularbotulinum neurotoxin complex provides insights into its oral toxicity. PLoSPathog 9: e1003690.

26. Simpson LL (2004) Identification of the major steps in botulinum toxin action. Annu Rev PharmacolToxicol 44: 167-193.

27. Nishiki T, Tokuyama Y, Kamata Y, Nemoto Y, Yoshida A, et al. (1996) Binding of botulinum type B neurotoxin to Chinese hamster ovary cells transfected with rat synaptotagmin II cDNA. NeurosciLett 208: 105-108.

28. Neuvonen PJ, Olkkola KT (1988) Oral activated charcoal in the treatment of intoxications. Role of single and repeated doses. Med Toxicol Adverse Drug Exp 3: 33-58.

29. Lai IH, Tsao JH, Lu YP, Lee JW, Zhao X, et al. (2009) Neutrophils as one of the major haptoglobin sources in mastitis affected milk. Vet Res 40: 17.

30. Bannerman DD, Paape MJ, Hare WR, Sohn EJ (2003) Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. J Dairy Sci 86: 3128-3137.

31. Bionaz M, Trevisi E, Calamari L, Librandi F, Ferrari A, et al. (2007) Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J Dairy Sci 90: 1740-1750.

32. Turk R, JuretiÄ‡ D, Geres D, Svetina A, Turk N, et al. (2008) Influence of oxidative stress and metabolic adaptation on PON1 activity and MDA level in transition dairy cows. AnimReprodSci 108: 98-106.

33. Schroedl W, Jaekel L, Krueger M (2003) C-reactive protein and antibacterial activity in blood plasma of colostrum-fed calves and the effect of lactulose. J Dairy Sci 86: 3313-3320.

34. Krüger M, Shehata AA, Schrödl W, Rodloff A (2013) Glyphosate suppresses the antagonistic effect of Enterococcus spp. on Clostridium botulinum. Anaerobe 20: 74-78.

35. Wang JY, Lin KD, Hou ZN, Richardson B, Gan J (2010): Sorption of the herbicide terbuthylazine in two New Zealand forest soils amended with biosolids and biochards. J Soils Sediments 10: 283-289.

36. Kim YH, Hong JR, Gil HW, Song HY, Hong SY (2013) Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. Toxicol In Vitro 27: 191-197.

37. Graber ES, Tsechansky L, Gerstl Z, Lew B (2012) High surface area biochar negatively impacts herbicide efficacy. Plant Soil 353: 95-106.

38. Shehata AA, Kühnert M, Haufe S, Krüger M (2014) Neutralization of the antimicrobial effect of glyphosate by humic acid in vitro. Chemosphere 104: 258-261.

39. Mazzei P, Piccolo A (2012) Quantitative evaluation of noncovalent interactions between glyphosate and dissolved humic substances by NMR spectroscopy. Environ SciTechnol 46: 5939-5946.

40. Yu Z, Zhang X, Li S, Li C, Li D, et al. (2013) Evaluation of probiotic properties of Lactobacillus plantarum strains isolated from Chinese sauerkraut. World J MicrobiolBiotechnol 29: 489-498.

41. Fujinaga Y, Inoue K, Nomura T, Sasaki J, Marvaud JC, et al. (2000) Identification and characterization of functional subunits of Clostridium botulinum type A progenitor toxin involved in binding to intestinal microvilli and erythrocytes. FEBS Lett 467: 179-183.

42. Yang Z, Li S, Zhang X, Zeng X, Li D, et al. (2010) Capsular and slime-polysaccharide production by Lactobacillus rhamnosus JAAS8 isolated from Chinese sauerkraut: potential application in fermented milk products. J BiosciBioeng 110: 53-57.

43. Badel S, Bernardi T, Michaud P (2011) New perspectives for Lactobacilli exopolysaccharides. BiotechnolAdv 29: 54-66.

44. Zhang L, Liu C, Li D, Zhao Y, Zhang X, et al. (2013) Antioxidant activity of an exopolysaccharide isolated from Lactobacillus plantarum C88. Int J Biol Macromol 54: 270-275.

45. El-Shenawy NS (2009) Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. Environ ToxicolPharmacol 28: 379-385.

46. Beuret CJ, Zirulnik F, Giménez MS (2005) Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. ReprodToxicol 19: 501-504.

47. Albers CN, Banta GT, Hansen PE, Jacobsen OS (2009) The influence of organic matter on sorption and fate of glyphosate in soil--comparing different soils and humic substances. Environ Pollut 157: 2865-2870.