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THE EFFECT OF AN ANTIMICROBIAL MIXTURE ON *Cryptosporidium*

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Abstract

Cryptosporidium is an enteric protozoan parasite that causes gastrointestinal disorders in humans and in a wide range of animals, mainly in calves. As there is no available efficient treatment for cryptosporidiosis, in this study we evaluated the effect of Auranta 3001, a natural feed additive on animal growth, number of days with liquid diarrhoea and oocyst excretion, mean oocysts/gram faeces and on biochemical and physical parameters. The study showed that calves fed with Auranta 3001 as a feed additive administered prior to infection with C. parvum, significantly reduced ($P < 0.05$) the number of days with liquid diarrhoea, the number of days with oocyst excretion, the number of days of antibiotic administration and mean oocysts/gram faeces. Moreover, the prophylactic administration of Auranta 3001, significantly ($P < 0.05$) reduced the percentage of calves with fever and increased the body weight at day 56. However, significant differences were not seen between IgG, total protein intake and haematocrit percentage. This study showed the efficacy of Auranta 3001 in reducing cryptosporidiosis manifestations in calves.

Key words: *Cryptosporidium, calves, natural antimicrobial*

INTRODUCTION

The protozoan parasite *Cryptosporidium* sp. is the worldwide leading cause of diarrhoea in young calves of less than 6 weeks old, alongside with Rotavirus, Coronavirus and enterotoxigenic *Escherichia coli* K99 (Arsenopoulos et al., 2017; Cho and Yoon, 2014; Smith et al., 2014). During the first month of life, diarrhoea causes the syndrome of maldigestion and malabsorption which leads to delayed growth, increased morbidity and mortality, which can be up to 50% of weaning calves, having a direct and indirect negative economic impact in the farming industry (Cho and Yoon, 2014; Bartels et al., 2010; Gulliksen et al., 2009).

Once ingested, *C. parvum* oocysts release sporozoites that initially attach to the membrane of the epithelial cells, then it penetrates the small intestine wall where it resides for most of its life cycle. The ongoing infections can lead to increased intestinal damage and secretory diarrhoea (Yang et al., 2015; Cho and Yoon, 2014). The severity of the disease manifestation in humans and animals can vary depending on the *C. parvum* isolate as well as host predisposing factors (Enemark et al., 2003). In calves, cryptosporidiosis is mostly endemic, with more than 90% of dairy farms and 40% of beef farms being contaminated (Chako et al., 2010). The prevalence of *C. parvum* varies between countries, regions and farms. For example, in the UK, cryptosporidiosis in cattle dairy farms

range between 28% and 80% (Thomson et al., 2017; Smith et al., 2014). *C parvum* is reported to prevail among pre-weaned calves from 3.4% to 96.6% in different parts of the world (Thomson et al., 2017). In calves, the European Medicines Agency has approved only halofuginone lactate as an effective drug against *Cryptosporidium* spp., though it does not treat the disease and shows limited and temporary benefits in reducing infection or animal production (Connor et al., 2017; Chavez and White, 2018; Caccio and Chalmers, 2016).
In contrast to chemotherapeutics, the use of natural antimicrobials as feed supplements, active against *Cryptosporidium* spp. could represent a new and safe approach in pharmacotherapy of cryptosporidiosis (Valigurova et al., 2018). The aim of the present study was to investigate the efficacy of a combination of natural extracts for the prophylaxis and treatment of cryptosporidiosis in calves.

MATERIALS AND METHODS

Parasites

Cryptosporidium parvum oocysts were obtained from the American Type Culture Collection. Oocysts were stored in phosphate-buffered saline (PBS) at 4°C until use. Before use, they were incubated for 15 min in sodium hypochlorite (4%) at 4°C and washed (3x) with cold D-PBS (Dulbecco's phosphate buffered saline, Sigma, UK) followed by centrifugation for 10 min at 2500×*g*. Subsequently, the pellet was suspended in 1 ml D-PBS and the number of oocysts was determined using a haemocytometer.

Phytochemicals

The novel feed additive (Auranta 3001) was supplied by Auranta-Envirotech Innovative Products Ltd and contains lactic and citric acid, glycerine-based emulsifying agent, sodium hydroxide, sodium chloride, citrus extract (6%), grape seed extract (2%), oregano extract (1%).

Artificial infection

The feed additive, Auranta 3001, was provided to three calves, later included in the uninfected treatment group, for a period of 20 days starting from day 1 after their arrival at the farm. On day 0, nine calves were randomly assigned to three additional experimental groups and inoculated with $1x10^7$ *C. parvum* oocysts (three calves per group). In the prophylactic group, Auranta 3001 was administered prior to infection and then on a daily basis for a period of 20 days. The therapeutic group, was provided with Auranta 3001 starting at the time point of the first manifestation of diarrhoea and then daily for a period of 20 days. The calves of the infection control group (3^{rd} group), were left untreated. Preparation of inoculum and viable oocysts count was done as previously described (Anguish and Ghiorse, 1997; Jenkins et al., 1997). The feed additive was provided to the animals at a concentration of 25 ml/day for the first 5 days and 10 ml/day after day 5. Auranta 3001 was fed through a suckling bottle with milk replacer twice daily.

Sample analysis

Analysis of *C. parvum* oocysts in faecal samples was performed according to Bellosa et al. (Bellosa et al., 2011). On days 0, 28 and 56, blood was taken from the jugular vein, using collection tubes without anticoagulant. The whole blood was centrifuged at 3500 rpm × 10 min. Supernatants (serum samples) were stored at - 20°C until analysis. We measured plasma levels of total protein, albumin, urea, cholesterol, glucose and triglycerides using commercial kits using a BioPlus 2000® device. The levels of serum globulin was determined as total serum protein minus serum albumin. Rectal faecal samples were collected from the calves and faecal consistency was determined according to Grinberg et al. (Grinberg et al., 2002). Comparison between treated and control groups was done using Student's t-test, with a significance level of 0.05.

RESULTS AND DISCUSSIONS

Cryptosporidium sp. is a major cause of gastroenteritis in humans and animals. The protozoan parasite represents a highly problematic target for drug development (Checkley et al., 2015; Farthing, 2006; Tzipori et al., 1982). The only licensed drug for treatment of cryptosporidiosis in calves is

halofuginone lactate which is extremly toxic to multiple organs and systems at effective doses (Tzipori, 1998; Yvore and Naciri, 1989). Besides this, numerous substances like, paromomycin and nitazoxanide have been screened for potential anti-cryptosporidial activity in calves, but they showed no therapeutic effect (Fayer and Ellis, 1993; Grinberg et al., 2002; Theodos et al., 1998; Schnyder et al., 2009; Ollivett et al., 2009). Recently, the attention has been drawn towards plant extracts as an alternative remedy with anti-cryptosporidial activity. *Punica granatum*, *Allium sativum* extracts and curcumin have been shown to reduce faecal oocyst count, diarrhoea intensity and duration in neonatal calves and BALB/c mice (Weyl-Feinstein et al., 2014; Al-Mathal and Alsalem, 2012; Gaafar, 2012; Asadpour et al., 2018). *In vitro*, Auranta 3001 has been shown to reduce the invasiveness of *C. hominis* and *C. parvum* against HCT-8 and bovine primary cells and to reduce the virulence by downregulating *CpSUB1* gene expression (Ch Stratakos et al., 2017). The efforts to explore the *in vivo* efficacy of Auranta 3001 were undertaken. Auranta 3001 is a natural feed supplement consisting of a mixture of organic acids and plant extracts. Therefore, its anti-cryptosporidial potential was examined in the present study in calves by assessing the number of days with liquid diarrhoea, number of days with oocyst excretion, mean OPG, biochemical and physiological parameters.

The effect of the novel additive (Auranta 3001) on calves naturally infected with *Cryptosporidium parvum*

We have started with 61 calves in the control group and 58 calves in the treatment group enrolled in the study. By the end of the experiment (56 days), 4 calves from the control group died due to severe diarrhoea and exhaustion. However, no mortalities were over the entire 56 days study in the treatment group. By comparing the indices of animal health in the naturally infected calves, control and treated with the novel feed supplement, we can observe that overall the values of those treated were lower than those which were not given Auranta 3001. Specifically, the percentage of scouring calves and the number of days with scours during the study was significantly lower ($P < 0.05$) in the treated group when compared to the control group (Table 1).

Table 1. Comparison of physiological and biochemical parameters between the control and treatment group of naturally infected calves

	Control group	SD	Treatment group	SD
Scours (percentage of calves)	78.2	6.5	16.2*	0.3
Scours (duration in days)	7.2	0.5	4.8*	1.7
Faecal scores	2.44	0.08	1.28	0.2
Fever (percentage of calves)	45.8	0.2	14.3*	7.1
IgG (g/L)	8.1	0.5	8.5	0.6
Haematocrit (percentage)	31.0	1.0	32.8	1.0
Total protein (g/L)	58.3	0.8	59.6	0.05
Antibiotics (percentage of calves)	46.7	0.1	21.2	0.1

* indicates significance ($p < 0.05$); SD: standard deviation

The growth performance analysis of the calves indicated that initial body weights (BW) at day 0 were similar. On day 56 a significantly higher BW difference ($P < 0.05$) was observed in calves fed with Auranta 3001 (79.3 ± 0.5 kg) compared to the control group (72.7 ± 1.22 kg). The average daily weight gain in the period of acute infection (day 0-day 28) was twice as high in animals from the treatment group. In the convalescence period (day 29-day 56) the animals from both groups gained similar body weight. In the treatment group, the calves had a bigger affinity towards protein and glucose intake and hence increased levels of IGF-1 and urea. Plasma concentrations of TNF-α and growth hormone were unaffected by the dietary supplement ($P > 0.05$).

Effect of the Auranta 3001 on artificially infected calves

Altered faecal consistency and particularly diarrhoea was observed in all animals, though different in duration and severity (Table 2) between prophylactic, therapeutic and control groups. Prophylactic administration of Auranta 3001 has had more beneficial effect in reducing the number of days with liquid diarrhoea in calves hence it helped to significantly avoid the complications ($p < 0.05$). The effect of Auranta 3001 was further investigated on the number of days with oocyst excretion and number of oocysts shed [sum of the daily OPG (oocysts per gram of faeces) per calf] in all groups. The prophylactic administration of Auranta 3001 has had the most beneficial effect upon reducing the number of oocysts shed ($0.04x10^2$ OPG) and also the number of days (2 days) of oocyst excretion compared to the therapeutic (12.33 days) and the control groups (12 days). The number of oocysts shed and the daily mean OPG value was significantly lower ($P < 0.05$) in the prophylactic group compared to therapeutic and control groups. All calves tolerated Auranta 3001 treatment without compound related abnormalities.

Table 2. Effect of Auranta 3001 on the parameters of faecal consistency, number of days with observed oocyst excretion and mean OPG of calves artificially infected with *Cryptosporidium* oocysts

	No. of animals	No. of days where semi-solid faecal consistency	No. of days were diarrhoea was observed	No. of days with observed oocyst excretion	Oocysts/gram of faeces
Uninfected*	Animal 1	4	6	0	0
	Animal 2	4	6	0	0
	Animal 3	5	5	0	0
	Average	4.33	5.66	0	0
Prophylactic group**	Animal 4	11	2	3	$0.015x10^2$
	Animal 5	15	1	1	$0.012x10^2$
	Animal 6	9	1a	2	$0.1x10^2$
	Average	11.66	1.33	2	$0.04x10^{2b}$
Therapeutic group***	Animal 7	3	8	11	$4.2x10^6$
	Animal 8	4	9	14	$3.4x10^6$
	Animal 9	3	6	12	$5.1x10^6$
	Average	3.33	7.66	12.33	$4.23x10^6$
Control group****	Animal 10	1	12	13	$1.2x10^8$
	Animal 11	2	9	11	$2.4x10^8$
	Animal 12	1	11	12	$3.1x10^8$
	Average	1.33	10.66	12	$2.23x10^8$

[a]$p<0.05$: paired t test with control group
*Animals not infected but Auranta administered
**Auranta administered prior to inoculation (day 1) and then daily for 20 days
*** Calves received Auranta at the time point of the first manifestation of diarrhoea and then daily for a period of 20 days
****Infected but NO Auranta administered
- On day 0 animals were inoculated with $1x10^7$ *C. parvum* oocysts

The results of the study showed that prophylactic administration of Auranta 3001 significantly ($P < 0.05$) increased the number of days with semi-solid faecal consistency. In contrast with a study conducted by Schnyder et al. (Schnyder et al., 2009) on nine calves, the effect of nitazoxanide administered as a therapeutic and prophylactic treatment showed an increased number of days with liquid diarrhoea when compared with the infected but untreated control group.

The number of days with oocyst excretion and the mean OPG value were significantly lower ($P < 0.05$) in the prophylactic group when compared to the therapeutic and control groups. The number of days with oocyst excretion in the therapeutic group was similar to the untreated control group. Our findings showed that the prophylactic administration of the novel feed supplement could stop oocyst shedding without recurrence within 3-4 days after the infection.

Similar findings were described with halofuginone lactate administration as a prophylactic and therapeutic treatment, where the shedding of oocysts and the number of

scours (expression of diarrhoea) were significantly reduced at 4 and 7 days of age, but higher after 21 days of age, when compared to control (Trotz-Williams et al., 2011). The opinions on halofuginone lactate are controversial, as some researchers have found that a concentration of 0.1 mg/kg BW is increasing the number of scours per day (De Waele et al., 2010) and some have found that calves who are reared in good hygienic conditions, such as disinfected individual calf pens, halofuginone lactate was effective in delaying the onset of *Cryptosporidium* infection and diarrhoea, in reducing the number of calves that become infected and exhibit signs of enteritis, and decreasing the level of oocysts excretion (De Waele et al., 2010, Klein, 2008). In this study, the plasma urea concentration did not differ between the control and treatment groups, as the protein intake was similar in both of the tested groups.

CONCLUSIONS

In conclusion, our findings have shown that Auranta 3001 significantly reduced the number of days with diarrhoea, the number of *C. parvum* oocysts shed by calves and overall increased the general health of the calves. Therefore, the feed supplement could be considered as an effective alternative against cryptosporidiosis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experiments were approved by the Animal Research Committee according to the legislation in place (Law 471/2002 and government ordinance 437/2002) and under the supervision of National Sanitary Veterinary Agency. The ethics committee of Banat University of Agricultural Sciences and Veterinary Medicine „King Michael I of Romania“, from Timişoara approved this work.

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AUTHORS' CONTRIBUTIONS

FS performed the research and co-wrote the manuscript; AS, OG, LS, IP, EP, NP analyzed data, wrote the manuscript, NC and PW supervised the work and proof read the manuscript.

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