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Appraisal of a new patented method for control of chicken coccidiosis

Rizwana Sultan[a], Asim Aslam[a], Muhammad Yasin Tipu[a], Habib ur Rehman[b], Ahsan Anjum[a], Werner Krull[c], Taha Kumosani[d], Houssam Shaib[e] and Elie K. Barbour[c,d]

[a]Department of Pathology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Lahore, Pakistan; [b]Department of Physiology, Faculty of Bio-Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan; [c]Department of Research & Development, Opticon Hygiene Consulting GmbH, St. Gallen, Switzerland; [d]Department of Biochemistry, King Abdulaziz University, Jeddah, Saudi Arabia; [e]Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon

ABSTRACT

The aim of this research is to appraise a newly patented method for control of chicken coccidiosis in comparison to a classical approach. The new method implements an invented Disinfectant (WSD) for decontamination of rearing surfaces, and a developed drinking water-herbal coccidiostat (EOBWE) supplement. The experimental design has 8 treatments (TRTs), with four pens/TRT, and 25 broiler chicks/pen. The floors of TRT1 to TRT7 are contaminated with 4.0×10^5 sporulated oocysts/m^2 of each of *E. acervulina, E. maxima, and E. tenella*, while TRT8-floor is left uncontaminated. The floors of TRTs 1 and 2 are disinfected with chlorine, while that of TRTs 3–6 are disinfected by WSD. Floors of TRTs 7 and 8 are deprived of disinfection. Birds in TRTs 1, 6, 7, and 8 are deprived of coccidiostat, while birds in TRTs 2 and 3 are fed salinomycin in their feed; birds of TRT 4 are administered EOBWE in drinking water, while birds of TRT 5 are administered simultameously both, the salinomycin and EOBWE. Birds in TRT 4 (WSD/EOBWE treated) and TRT 5 (WSD/salinomycin + EOBWE) had improved protection (lowest oocyst output and intestinal lesions) and enhanced production (lowest mortality, lowest feed conversion ratio, and highest live body weight) compared to the other five challenged treatments, associated with significant improvements compared to positive controls (TRT 7) ($P < 0.05$).

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Introduction

Coccidiosis is one of the most economic protozoan disease, affecting the poultry industry worldwide, with estimated losses, in the United Kingdom alone, of around £38 588 795, of which 98.1% of the losses involved the broilers sector (Williams 1999). Nowadays, the most prevalent method for control of poultry coccidiosis is by application of classical disinfection on surfaces of the farms, followed by supplementation of the offered feed with different coccidiostats (Tewari and Maharana 2011; Fatoba and Adeleke 2018). This adopted coccidiosis control programme by the poultry sector, since the year 1948, the time when the FDA had its first approval of coccidiostats (Sulphaquinoxaline and Nitrofurazone) (Conway and Mckenzie 2007; Peek and Landman 2011), revealed later in time a frequent emergence of resistance to the commercial disinfectants (Williams 1997; McDonnell and Russell 1999; Guimaraes et al. 2007; Souza 2012), and to the cococcidiostats offered in the feed (Jeffers 1974a, 1974b, 1989; Chapman 1978, 1982, 1984, 1997; Ryley 1980; Hamet 1986; Litjens 1986; McDougald et al. 1986, 1987; McDougald and Seibert 1998; Ruff and Danforth 1996; Zeng and Hu 1996; Zhou et al. 2000; Peek and Landman 2003; Peek and Landman 2004; Kadykalo et al. 2018). The resistance to disinfectants and/or coccidiostats resulted in severe losses due to high mortality (Beraa et al. 2010; Györke et al. 2016), unacceptable conversion of feed to live body weight, and lower body weight at the market age (Shirley and Lillehoj 2012; Chapman 2014; Gerhold 2016); moreover, the supplementation of coccidiostats in the feed resulted in sporadic detection of its residues in poultry products (Elliot and O'Keeffe 2003; Roudaut et al. 2013; De Lima et al. 2017); this situation created a concern in consumers, who are requiring safer food (Safefood 2005), which in turn led to a new strategy by some poultry industries, targeting the avoidance of using drugs in their operations (European Commision 2012; Rummo 2017).

There are a number of reports related to disinfection strategies, for reduction of environmental contamination by coccidial organisms that are of veterinary and human health importance (King and Monis 2007; Wainwright et al. 2007; Dumetre et al. 2008). In spite of the importance of the oocysts to the epidemiology of poultry coccidiosis, there is limited information about the use of chemical disinfectants for reduction of viability or infectivity of eimerian oocysts in the environment, especially on contaminated surfaces (Daugschies et al. 2013). Previous reports confirmed the presence of coccidian organisms in the environment of the raised poultry, including dust on surfaces, soil, litter, and in invertebrates (Reyna et al. 1983). Unfortunately, most documented studies implemented *in vitro* assays for studying the oocysts susceptibility to disinfectants, by contacting the oocysts with the disinfectant in liquid medium, while ignoring the importance of disinfection on

CONTACT Houssam Shaib hs45@aub.edu.lb

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Table 1. Invented WSD and EOBWE and their method of application, components, and mode of action.

Invented materials	Method of application	Components (%)	Mode of action (References)
WSD[a]	3% dilution in water; Application of 400 c.c./m^2 of surfaces; two hrs of contact time with microbes on surfaces.	Chloro-m-cresol polymeric biguanides (30%)	Microbial wall disruption; microbial multiplication arrest; chromosome condensation (Chindera et al. 2016; EU 2012)
		Organic peracetic acid (15%)	Protein denaturation (Tutumi et al. 1974)
		Inorganic phosphoric acid (15%)	Buffering effect (Kross and Kere Kemp 2000)
		Anionic dodecyl benzene sulphonic acid surfactant (10%)	Action on biofilm built up on surfaces (Bridier et al. 2011)
EOBWE[b]	0.0125% dilution in drinking water; Intermittent administration (3 consecutive days/week, *ad libitum*)	*Salvia libonitica* decoction (10% v/v)	Cavacrol effect on microbial outer structure and viability (Ultee et al. 1999)
		Eucalyptus essential oil (10% v/v)	1,8 cineol effect on microbial structure configuration and agglomeration (Li et al. 2014)
		Peppermint essential oil (10% v/v)	Perturbation of microbial structure; alteration of microbial attachment preventing infection (Trombetta et al. 2005)
		Saponin (45–55%)	Creation of Oil-in-Water emulsion, enhancing dispersion of EOBWE in drinking water (Luo et al. 2017)

[a]WSD: Wide Spectrum Disinfectant.
[b]EOBWE: Essential Oil Blend in Water Extract.

surfaces, and its evaluation by *in vivo* protocols. A useful *in vivo* protocol could include birds raised on surfaces contaminated with quantified sporulated eimerian oocysts (positive controls) versus raising others on similarly contaminated surfaces, after its treatment with a disinfectant that is under appraisal. It is worth noting that one study reported the development of bioassays to investigate the efficacy of disinfectants in reducing the viability of *Eimeria tenella* oocysts (Daugschies et al. 2002).

The paucity of research, targeting appraisal of disinfectants efficiency against oocysts on surfaces, worked as an impetus for concluding 13 trials during the last 7 years, compiled from nine countries around the globe, aiming at invention of a new method and its related substances, in an attempt to replace the adopted classical control programme of coccidiosis, while satisfying the new direction in poultry husbandry, by raising birds free of medication. The data of the 13 trials are documented recently (Barbour and Krull 2018; Barbour et al. 2018a, 2018b, 2018c).

In 2015, the World Health Organization (WHO) issued a 'Global Action Plan on Antimicrobial Resistance', developed through a collaboration between the World Animal Health Organization (OIE) and the Food and Agriculture Organization (FAO) of the United Nations; the WHO Global Action Plan called for a 'One Health' approach, including a trend in raising poultry free of drugs, aiming at control of drug resistance in poultry pathogens. The efforts of scientists in concluding the above mentioned 13 trials, resulted in an invention of new method for coccidiosis control that was in compliance with 'one Health' approach of WHO/OIE/FAO, allowing its patenting through the US Patent Office and internationally (Krull and Barbour 2017, 2018). The invention was based on the hypothesis of dual interception approach, by the implementation of an innovated Wide Spectrum Disinfectant (WSD), with high efficacy in inactivating oocysts of *Eimeria spp*. and wide spectrum of viruses and bacteria that are of economic importance in poultry health (Barbour et al. 2018b, 2018c), and another implementation of an innovated supplement in drinking water, constructed of natural Essential Oil Blend in Water Extract (EOBWE) of herbs, with anti-coccidial/antimicrobial and anti-inflammatory properties (Barbour et al. 2018b).

This research aimed at an *in vivo* appraisal of the efficiency of the novelty in the patented method that aimed at sustainable control of chicken coccidiosis, under environmental conditions of an open system in Lahore, Pakistan, and inclusion of a highly pathogenic challenge by Pakistanian strains of three *Eimeria spp*. (*E. acervulina, E. maxima, and E. tenella*), that had a history of significant economic losses in this country.

Materials and methods

Invented method and materials

The invented method, innovation of its materials, and mode of action of its active ingredients, are presented in Table 1. The developed method resulted from a compilation of 13 experiments, performed during the last 7 years in 9 countries, and documented in 2018 (Barbour and Krull 2018). Details related to Table 1 could be retrieved from recently published patents and manuscripts (Krull and Barbour 2017, 2018; Barbour and Krull 2018).

Experimental design for appraisal of the invented method

A Completely Randomized Design (CRD) was established for appraisal of the patented new method, targeted at control of coccidiosis, while avoiding the most prevalent protocol in poultry industry involving the supplementation of feed of broilers with cocidiostatic drugs. The CRD had eight different treatments, with four replicate pens/treatment, and stocking of 25 day-old meat type chicks/pen, totalling into 800 birds. The floor of 28 pens, allocated to the first seven treatments (TRT 1 – TRT 7) were contaminated with 4.0×10^5 sporulated oocysts/m^2 of each of three *Eimeria spp*. (*E. acervulina, E. maxima*, and *E. tenella*). A concise overview of the nature of the eight treatments is presented in Table 2; briefly, TRT 1 had the oocyst-contaminated floor disinfected classically by chlorine, and birds of this treatment were offered feed free of any coccidiostat (Chlorine/No Coccidiostat); TRT 2 had the oocyst contaminated floor disinfected with Chlorine and its birds were offered feed supplemented with salinomycin (Chlorine/Salinomycin); TRT 3 had the oocyst contaminated floor disinfected by the invented WSD and its birds were offered continuously the salinomycin in feed (WSD/Salinomycin); TRT 4 had the oocyst contaminated floor disinfected by WSD and

Table 2. Complete Randomized Design (CRD), composed of eight treatments with four replicate pens/treatment and 25 day-old birds/pen, aiming at comparing the new method to classical control programme of broiler coccidiosis.

TRT[a]	Disinfectant	Coccidiostat	Challenge[b]
1	Chlorine[c]	–	+
2	Chlorine	Salinomycin[d]	+
3	WSD[e]	Salinomycin	+
4	WSD	EOBWE[f]	+
5	WSD	Salinomycin + EOBWE	+
6	WSD	–	+
7	–	–	+
8	–	–	–

[a]TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[b]The challenge was by contaminating the floor with 4.0×10^5 sporulated oocysts/m^2 of each of *E. acervulina, E. maxima, and E. tenella*.
[c]Sodium hypochlorite was diluted according to manufacturer instructions, applied in 400 ml/m^2, and for 2 h contact time against sporulated oocysts on the floor.
[d]Salinomycin inclusion rate in feed was at 60 ppm.
[e]WSD (Wide Spectrum Disinfectant) was used at 3% dilution, 400 ml/m^2, and for 2 h contact time against sporulated oocysts on the floor.
[f]EOBWE was used at dilution of 0.125%, and intermittent administration in drinking water at ages of 4–6, 10–12, 18–20, 24–26, 31–33, and 38–40.

its birds were offered intermittently the invented EOBWE in drinking water, for period of 3 consecutive days/week, complying with the new patented method of dual interception with WSD and EOBWE (WSD/EOBWE); TRT 5 had the oocyst contaminated floor disinfected by WSD and its birds were offered simultaneously the EOBWE in drinking water (intermittently, 3 days/week) and continuous administration of salinomycin in feed, in an attempt to study the synergism/antagonism between the two coccidiostats (WSD/EOBWE + salinomycin); TRT 6 had the oocyst-contaminated floor disinfected by WSD, and its birds were offered feed free of any coccidiostat (WSD/No Coccidiostat); TRT 7 had the oocyst contaminated floor deprived of disinfectant treatment and its challenged birds deprived of any coccidiostat (Positive Controls); TRT 8 had birds reared on the floor that is left without oocyst-contamination and its birds were not offered any coccidiostat (Negative Controls). It is worth noting that the bracketed denotation of the eight treatments, shown above, are used in the footnote of Tables 2–8.

Table 3. Comparison of the mean oocyte count per gram of intestine[1] in the 8 differently treated broilers at two different ages (d).

	Mean Oocyst count/g intestine $\times 10^4$ at age (d)	
TRT[2]	14	28
1	6.4^{c}	8.2^{b}
2	$4.2^{b,c}$	0.5^{b}
3	$4.4^{b,c}$	$0.4^{a,b}$
4	$3.8^{b,c}$	$0.1^{a,b}$
5	2.8^{b}	0.1^{a}
6	$6.0^{b,c}$	0.6^{b}
7	19.2^{d}	5.1^{c}
8	0.0^{a}	0.0^{a}

[1]Mean of each treatment at each age was averaged from 4 intestinal organs of each of 3 birds randomly sampled from each of the four pens/treatment.
[2]Denotations are bracketed after each treatment: TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[a–d]Means in a column followed by different alphabet superscripts are significantly different ($P < 0.05$).

Table 4. Mean gross intestinal lesion score[1] of broilers in the 8 treatments at six different ages.

	Mean[3] intestinal gross lesion score at six ages (d)					
TRT[2]	7	14	21	28	35	42
1	0.60^{a}	1.66^{b}	$1.60^{b,c}$	$2.40^{b,c}$	$1.66^{b,c}$	$1.00^{a,b}$
2	0.40^{a}	1.66^{b}	$1.40^{b,c}$	1.80^{b}	$1.33^{a,b,c}$	$0.67^{a,b}$
3	0.40^{a}	1.62^{b}	$1.35^{b,c}$	1.80^{b}	$1.33^{a,b,c}$	$0.67^{a,b}$
4	0.40^{a}	$1.33^{a,b}$	$1.20^{b,c}$	1.60^{b}	$1.00^{a,b}$	$0.43^{a,b}$
5	0.20^{a}	$1.28^{a,b}$	$0.80^{a,b}$	$1.33^{a,b}$	$0.93^{a,b}$	0.31^{a}
6	0.40^{a}	1.66^{b}	$1.60^{b,c}$	1.80^{b}	$1.33^{a,b,c}$	$0.67^{a,b}$
7	0.80^{a}	2.33^{c}	2.20^{c}	3.20^{c}	2.33^{b}	1.67^{b}
8	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

[1]Score '0' is given for absence of lesions; score '1' indicates presence of scattered petechiae; score '2' indicates presence of numerous petechiae; score '3' indicates presence of extensive hemorrhage; score '4' indicates presence of extensive hemorrhage, associated with dark colour of the epithelium (Johnson and Reid 1970).
[2]TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[3]The observed intestines are collected at each of the 6 times from three birds/pen, totalling into 12 intestines/treatment/time. The mean gross lesion score is accumulated from those present in each bird's ceca (*E. tenella*), middle ileum (*E. maxima*), and duodenum (*E. acervulina*).
[a–c]Means in a column followed by different alphabet superscripts are significantly different ($P < 0.05$).

Quantitative appraisal of the eight treatments within the CRD

The quantitative appraisal of the eight treatments within the CRD relied on statistical analysis for comparison of means of six parameters including, Oocyst output, gross intestinal lesion score, histopathologic intestinal lesion score, mortality %, live body weight, and mean Feed Conversion Ratio (FCR). The parameters' means of each treatment were calculated from birds allocated to the 4 replicate pens/treatment.

Mean oocyst output

The oocyst output was determined in each pen at 14 and 28 days of age. The output was assessed by random sacrification of 3 birds/each pen at each of the two ages, and determining the oocyst count per gram of each intestinal organ. Briefly, three parts of each intestine (duodenum– affinity of *E. acervulina*, ileum – affinity of *E. maxima*, cecum – affinity of *E. tenella*) were rinsed in a saline solution to remove the fecal material, and a weight of 1–2 g of each rinsed organ was collected. Each weighed intestinal organ was homogenized and put in 56 ml volume of 35% NaCl solution to induce the floatation of the oocysts. The floated oocysts were put in two compartments of the McMaster chamber, allowing the debris to settle, and the oocysts to float within a period of 5 min. The oocysts were counted within the grid of the two chambers, under a compound microscope, and at a magnification of 10×. The obtained total counts were multiplied by 50 to obtain the count of the oocyst per gram of the intestinal organ. The calculated count was transformed to Log to the base 10 (Log_{10}) (Barbour et al. 2015).

Mean gross intestinal lesion score

The gross intestinal lesion score, ranging between 0 and 4, was recorded at 6 different ages (7, 14, 21, 28, 35, 42 day-old), by

Table 5. Mean intestinal[1] histopathologic lesion score[2] of broilers in the 8 treatments at six different ages.

TRT[3]	Mean histopathological score					
	7	14	21	28	35	42
1	2.20 ± 0.68^{e}	1.90 ± 0.74^{d}	1.60 ± 0.52^{d}	$2.20 \pm 0.63^{c,d}$	1.80 ± 0.42^{b}	$0.50 \pm 0.53^{a,b}$
2	$1.73 \pm 0.46^{c,d,e}$	$1.50 \pm 0.71^{c,d}$	$1.30 \pm 0.48^{c,d}$	$1.80 \pm 0.63^{b,c}$	$1.20 \pm 0.52^{a,b}$	0.30 ± 0.48^{a}
3	$1.47 \pm 0.74^{c,d}$	$1.30 \pm 0.48^{b,c,d}$	$0.90 \pm 0.74^{b,c,d}$	$1.70 \pm 0.67^{a,b,c}$	$1.10 \pm 0.53^{a,b}$	0.20 ± 0.42^{a}
4	$1.07 \pm 0.59^{b,c}$	$0.50 \pm 0.53^{a,b}$	$0.70 \pm 0.48^{a,b,c}$	$1.10 \pm 0.53^{a,b,c}$	$0.83 \pm 0.48^{a,b}$	0.10 ± 0.32^{a}
5	$0.67 \pm 0.49^{a,b}$	$0.50 \pm 0.53^{a,b}$	$0.30 \pm 0.48^{a,b}$	$0.90 \pm 0.42^{a,b}$	0.55 ± 0.32^{a}	0.10 ± 0.32^{a}
6	$1.90 \pm 0.70^{d,e}$	$1.70 \pm 0.48^{c,d}$	$1.30 \pm 0.67^{c,d}$	$1.90 \pm 0.57^{c,d}$	$1.32 \pm 0.52^{a,b}$	$0.40 \pm 0.52^{a,b}$
7	3.33 ± 0.82^{f}	3.10 ± 0.74^{e}	2.40 ± 0.52^{e}	3.40 ± 0.63^{d}	2.60 ± 0.52^{c}	1.70 ± 0.32^{b}
8	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[1]The microscopic observation of the intestines collected at each of the 6 times from three birds/pen, resulting in 12 intestines/treatment/time, including 3 intestinal organs/bird (ceca– *E. tenella*, middle ileum– *E. maxima*, and duodenum– *E. acervulina*), totalling into 12 intestines × 3 organs/intestine = 36 samples/Treatment.
[2]Score '0' is given for absence of histopathologic lesions; score '1' indicates a presence of mild inflammatory cell infiltration in the mucosa with intact epithelium and no submucosal or muscularis involvement; score '2' indicates a presence of extensive mucosal inflammatory cell infiltration and submucosal edema; score '3' indicates a presence of same histopathologic lesions as in score 2, in addition to extended infiltration towards the muscularis layer; score '4' indicates a presence of destructive mucosa associated with necrosis and hemorrhage (Adamu et al. 2013).
[3]TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[a–f]Means in a column followed by different alphabet superscripts are significantly different ($P < 0.05$).

randomly sacrificing 3 birds/each pen/per time, and recording the lesion score of each intestinal organ (duodenum, ileum, and cecum), based on the system that is previously documented (Johnson and Reid 1970); briefly, a score of '0' is given to absence of gross lesions; a score of '1' indicates the presence of small scattered petechiae; a score of '2' points at the presence of numerous petechiae; a score of '3' relates to occurrence of extensive hemorrhage, while the score of '4' indicates the presence of extensive hemorrhage associated with dark colour of the intestinal epithelium.

Mean histopathologic intestinal lesion score

The histopathologic intestinal lesion score, ranging between 0 and 4, was determined on 5 micrometer-sections of all intestinal samples that were subjected to gross lesion scoring, after staining the sections by H & E procedure (Feldman and Wolfe 2014). A Score of '0' is given to absence of microscopic lesions; a score of '1' indicates the presence of slight mucosal inflammatory cell infiltration; a score of '2' denotes the presence of extensive mucosal cell infiltration and submucosal edema; a score of '3' is given for the same observed lesions under score '2', when the lesions extend towards the intestinal muscularis layer, while a score of '4' is given to the presence of intestinal mucosal degeneration associated with necrosis and hemorrhage (Adamu et al. 2013).

Table 6. Mortality % up to 42 days of age.

TRT[1]	Mean mortality %
1	$13.00^{a,b}$
2	7.0^{b}
3	6.0^{b}
4	4.0^{b}
5	2.0^{b}
6	8.0^{ab}
7	19.0^{a}
8	2.0^{b}

[1]TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[a,b]Mean mortality % followed by different alphabet superscripts are significantly different ($P < 0.05$).

Mean mortality %

The frequency of the mean mortality %, up to market age of 42 day-old, is calculated from the averages of the weekly mortalities in the 4 replicate pens/treatment.

Means of live body weight and feed conversion

The mean weekly live body weight was obtained from averaging the weights of birds reared in the 4 replicate pens/treatment; in addition, the mean weekly Feed Conversion Ratio (FCR) was calculated by dividing the weekly consumption of feed/pen by the weekly live body weight gain/pen, and averaging the FCR obtained from the 4 replicate pens per treatment.

Statistical analysis

The comparison of means of each quantified parameter, except for frequency of mortality, was performed by Analysis of Variance (ANOVA), followed by application of the conservative Tukey's test, using Statistical Package of Social Sciences (IBM SPSS, version 23). The mean frequency of mortality % were compared among the eight treatments by the CHI-Square. Significant differences among the means of each quantified parameter, within the eight treatments, were reported at the significance level of $P < 0.05$.

Results and discussion

Invented method and materials

The innovated material mixtures of the WSD and EOBWE, their method of application, and mode of action of the components of each of the two mixtures, are presented in Table 1. The invention of the method and its innovated materials resulted from the data of compiled projects concluded during the last 7 years in 9 different countries (Barbour and Krull 2018; Barbour et al. 2018b, 2018c); briefly, the method of application of the WSD is by diluting it in water up to 3% (V/V), application of the diluted disinfectant at clean surfaces of the farm in 400 ml/m^2, respecting a contact time of 2 h, in an attempt to

Table 7. Mean[1] live body weight of broilers in the 8 treatments at six different ages.

TRT[2]	Mean body weight in grams at six ages (d)						
	1	7	14	21	28	35	42
1	42.9[a]	109.7[b]	299.4[b]	661.9[b]	1099.5[b]	1542.2[b]	2037.4[b]
2	42.9[a]	114.3[b,c]	310.1[b,c]	677.0[b,c]	1154.8[c,d]	1588.5[c,d]	2063.9[b,c]
3	43.1[a]	122.5[c,d,e]	320.1[d,e]	695.7[d,e]	1154.4[c,d]	1598.4[c,d]	2084.4[b,c]
4	42.1[a]	120.6[c,d]	332.4[d,e]	685.3[c,d]	1159.1[c,d]	1620.8[d]	2101.6[c]
5	42.2[a]	130.8[e,f]	346.9[e,f]	730.2[f]	1186.1[e]	1667.5[e]	2181.7[d]
6	43.4[a]	124.1[d,e]	325.8[d,e]	675.5[b,c]	1130.7[c]	1573.3[b,c]	2061.9[b,c]
7	42.9[a]	98.9[a]	273.6[a]	588.5[a]	924.4[a]	1399.7[a]	1770.8[a]
8	42.6[a]	133.6[f]	363.7[f]	710.7[e]	1214.8[f]	1744.3[f]	2336.1[e]

[1]The mean live body weight was calculated from the total weights of surviving birds in each of 4 pens/treatment, observed at each specific age.
[2]TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[a–f]Means in a column followed by different alphabet superscripts are significantly different ($P < 0.05$).

inactivate *Eimeria* spp. – oocysts that survived from previous reared flocks (Williams 1997; McDonnell and Russell 1999; Guimaraes et al. 2007; Beraa et al. 2010; Chapman et al. 2016; Györke et al. 2016). The 30% chloro-m-cresol polymeric biguanides of the WSD targets the disruption of microbial wall (Chindera et al. 2016; EU 2012); the 15% of the organic peracetic acid in the WSD denatures the poultry microbial proteins (Tutumi et al. 1974; Fathi et al. 2016; Nosrati et al. 2017); the 15% of the inorganic phosphoric acid in the WSD provides the buffering effect on different alkalinities in the water that is used for dilution of the WSD (Kross and Kere Kemp 2000; CDC 2016), while the 10% of the anionic dodecyl benzene sulphonic acid surfactant will help in detachment of developed biofilms on surfaces of the chicken rearing pens (Bridier et al. 2011; Díaz De Rienzo et al. 2015).

The method of application of the EOBWE involves its dilution in drinking water at 0.0125% (v/v), and its intermittent *ad libitum* administration for three consecutive days/week, attempting to intercept at different points of the *Eimeria* spp. life cycle. The components of the EOBWE have the potential to modify the outer structure of the microbe, affecting its viability (Ultee et al. 1999; Li et al. 2014). The carvacrol molecule present in the decoction of *Salvia Libanotica* has the potential of inducing a change in the outer structure of the organism (Li et al. 2014; Pop et al. 2019); The 1, 8 cineol of the Eucalyptus essential oil potentiates a change in its structural configuration and agglomeration (Li et al. 2014; Jitviriyanon et al. 2016); the components of the peppermint essential oil potentiates its structural perturbation, altering its attachment to the intestine, resulting in significant reduction of its multiplication and magnitude of output (Trombetta et al. 2005; Barbour et al. 2015). while its saponin helps in creating an oil-in-water emulsion for enhancement of its homogeneous dispersion in drinking water (Luo et al. 2017; Fleck et al. 2019).

Table 8. Mean[1] feed conversion ratio (FCR) of broilers in the 8 treatments at six different ages.

TRT[2]	Mean FCR at six ages (d)					
	7	14	21	28	35	42
1	0.980[c]	1.170[b]	1.437[b]	1.611[b]	1.834[d]	1.955[d]
2	0.974[b,c]	1.151[a,b]	1.425[b]	1.584[b]	1.783[c,d]	1.898[c,d]
3	0.967[a,b,c]	1.141[a]	1.421[b]	1.599[b]	1.752[c]	1.879[b,c,d]
4	0.963[a,b,c]	1.142[a]	1.419[b]	1.574[b]	1.726[b,c]	1.816[a,b,c]
5	0.958[a,b]	1.141[a]	1.391[a,b]	1.504[a]	1.675[b]	1.807[a,b]
6	0.977[b,c]	1.157[a,b]	1.432[b]	1.609[b]	1.783[c,d]	1.902[c,d]
7	1.002[d]	1.204[c]	1.525[c]	1.716[c]	1.948[e]	2.052[e]
8	0.952[a]	1.137[a]	1.367[a]	1.459[a]	1.579[a]	1.764[a]

[1]The FCR in a pen = Consumed feed in a pen divided by Live broiler weight in a pen. The mean in each treatment is calculated by adding all FCR in 4 pens divided by 4.
[2]TRT 1 (Chlorine/No Coccidiostat), TRT 2 (Chlorine/Salinomycin), TRT 3 (WSD/Salinomycin), TRT 4 (WSD/EOBWE), TRT 5 (WSD/EOBWE + Salinomycin), TRT 6 (WSD/No Coccidiostat), TRT 7 (Positive Control), and TRT 8 (Negative Control).
[a–e]Means in a column followed by different alphabet superscripts are significantly different ($P < 0.05$).

Experimental design for appraisal of the invented method

The experimental design presented in Table 2, and detailed under Materials and Methods section, had challenged birds in its first seven treatments, while TRT 8 was assigned for the negative controls, left without challenge. It is worth noting that the protocol of contaminating the floor with sporulated oocysts of pathogenic *Eimeria spp*. resembles the usual mode of transmission of oocysts, surviving in the farm environment from previous flocks to newly introduced birds (Jenkins et al. 2019). This protocol is adopted by the German Veterinary Society (DVG 2007), and by other researchers in the same field of specialization (Danforth et al. 1977; Jeffers et al. 1988; Bafundo et al. 1989; Lee and Lee 2007).

Quantitative appraisal of the eight treatments within the CRD

The comparative quantitative appraisal among the eight different treatments, within the CRD strategy presented in Table 2, targeting the control of coccidial infection in poultry, follows standard protocol used in previous researches (Holdsworth et al. 2004; European Food Safety Authority 2011; Leung et al. 2019). The protocol includes in most published works a determination of the oocyst output in intestinal organs (Table 3), recording the gross and histopathologic intestinal lesion scores (Tables 4 and 5, respectively), and observation of the production parameters namely, mortality %, live body weight, and feed conversion ratio (Tables 6–8, respectively).

Oocyst output

The oocyst output in challenged broilers (Table 3), subjected to dual intervention with the invented WSD and EOBWE materials

(TRT 4), had a significant reduction in oocyst output compared to positive controls (TRT 7) at 14 days of age (15.4×10^4 vs. 19.2×10^4, respectively) ($P < 0.05$) and at 28 days of age (0.1×10^4 vs. 5.1×10^4, respectively) ($P < 0.05$). This result indicates an efficacy in control of the oocyst output by the dual effect of WSD and EOBWE, and their new method of application. The interception by WSD alone in TRT 6 was more effective in reducing the oocyst output by the raised broilers at the age of 30 days (0.6×10^4), compared to that obtained by broilers raised on oocyst– contamintated floor that was classically disinfected with chlorine alone (TRT 1) (8.2×10^4) ($P > 0.05$). This is most likely due to the multivalent active ingredients present in the WSD (DVG 2007; Díaz De Rienzo et al. 2015; CDC 2016; Fathi et al. 2016; Nosrati et al. 2017; Barbour and Krull 2018), and the documented inefficiency of commonly used chlorine in inactivation of hard oocysts (Sundermann et al. 1987; Korich et al. 1990). The supplementation of salinomycin in the feed of broilers raised on chlorine– disinfected floor (TRT 2) did correct for the lower effectiveness of chlorine used alone in TRT 1, resulting in improvement of oocyst output reduction at 15 (4.2×10^4) and 30 (0.5×10^4) days of age; however, this reduction was still insignificantly higher than that obtained by broilers subjected to the new method implemented on broilers of TRT 4, and at the two respective ages (3.8×10^4 and 0.1×10^4) ($P > 0.05$).

Gross intestinal lesions

The differences seen in reduction of oocyst output in TRTs 1–6 at 15 and 30 days of age, compared to the highest output obtained by the positive control birds in TRT 7 (Table 3) did result in similar means of gross intestinal lesion score in all challenged birds of TRTs 1–6 at both the 14 and 28 days of age ($P > 0.05$) (Table 4); however, the six means of gross intestinal lesion score obtained by TRTs 1–6 were significantly lower than that obtained by the positive controls of TRT 7 at 14 and 28 days of age ($P < 0.05$). By the market age of 42 days, the healing process in the intestines of surviving birds resulted in insignificant differences among the mean gross intestinal lesion score of TRTs 1,2,3,4 and 6 compared to that of the positive controls (TRT 7) ($P > 0.05$). This observation is in agreement with previous documentations, showing that birds exposed to sporulated oocysts as early as 3 days of age had their significant intestinal injury at 10–17 days of age (Idris et al. 1997); it was reported that these injuries came as a result of ascending count in oocysts (Long and Rowell 1958; Chapman et al. 2016); however, the healing process in the surviving birds leads to a significant decline in intestinal inflammation by the market age of around 40 days, an observation coinciding with maximum decline in oocyst output (Long et al. 1975). It is worth noting that only birds in TRT 5 kept a lower significant gross lesion score at 42 days of age compared to the positive controls of TRT 7 ($P < 0.05$); this highest efficiency in protection of the intestines in birds of TRT 5 is most likely due to the synergism between the salinomycin offered in the feed and the EOBWE administered in drinking water. The salinomycin is known to inhibit the transformation of sporozoites to trophozoites, leading to their elimination at 30–72 h after ingestion of oocysts. The drug also affected schizonts during initial nuclear replication by either destroying or significantly delaying their maturation (Chappel 1979). A different mode of action is expected from the active ingredients of the EOBWE, including lysis of the oocysts by the EOBWE (Barbour and Krull 2018), which is most likely due to its main active ingredients including, the effect of the eucalyptol molecule (1, 8-cineole), known generally to modify the microbial structural configuration, and agglomeration of its inside structure (Li et al. 2014; Jitviriyanon et al. 2016); in addition, the menthol in peppermint is reported to cause perturbation of the microbial structure, resulting in alterations of their attachment component, and inability of the microbe to replicate (Trombetta et al. 2005; Barbour et al. 2015). Moreover, the superior activity of carvacrol on outer structure of organisms and their viability is documented previously (Ultee et al. 1999; Trombetta et al. 2005; Li et al. 2014; Pop et al. 2019). To our knowledge, this is the first observation, proving the synergism between salinomycin and EOBWE in protection against intestinal injuries by *Eimeria* organisms.

Histopathologic intestinal lesions

The higher sensitivity in detection of histopathologic intestinal lesions microscopically, compared to the unaided eye observation of gross lesions (Conway and Mckenzie 2007), helped in detection of significant differences among challenged birds at younger age of 7 days (Table 5), with the highest mean histopathologic score obtained by the positive control birds of Trt 7 ($P < 0.05$). The differences in histopathologic intestinal lesions of challenged birds were narrowed, effective the age of 35 days, and reached to insignificant differences among all challenged and treated birds (TRTs 1–6) by the age of 42 days, ($P > 0.05$). This insignificant difference in histopathologic intestinal lesion scores among the TRTs 1–6 is most likely due to the healing process in survivors, a finding that is in agreement with a previously published document (Long et al. 1975).

Production parameters

The quantified and adopted production parameters in this research were according to protocols used in evaluation of coccidiosis control programmes (Morehouse and Baron 1970; Holdsworth et al. 2004; European Food Safety Authority 2011; Leung et al. 2019), including the parameters of mortality, live body weight, and feed conversion ratio. It is of paramount importance in presenting the production parameters to attempt relating it to oocyst output (Idris et al. 1997) and associated intestinal lesions (Johnson and Reid 1970).

Mortality

The cumulative mortality % up to 42 days of age was the highest in the control positive birds of TRT 7 (19.0%), followed by birds of TRT 1 (13.0%), and of TRT 6 (8.0%), with insignificant difference among these three TRTs ($P > 0.05$) (Table 6). The mean gross intestinal lesion score presented in Table 4 showed highest values by birds of TRTs 1, 6, and 7 at the age of 21 days, with consistency in keeping the highest gross lesion score in birds of TRTs 1 and 7 through all the following ages (28, 35, and 42 days). However, the consistency in

keeping the highest mean histopathologic intestinal lesion score included birds of all three treatments (TRTs 1, 6, and 7) and through all studied ages of 7, 14, 21, 28, 35, and 42 days (Table 5). This observation is of paramount importance in future investigations, reemphasizing the stronger relationship between the mean histopathologic intestinal lesion score and mortality caused by *Eimeria* challenge in the followed protocol for appraisal of coccidiosis control programmes in broilers (Long and Joyner 1984; Holdsworth et al. 2004; Conway and Mckenzie 2007; European Food Safety Authority 2011; Leung et al. 2019). It is worth noting that the new method of dual interception by WSD and EOBWE was able to reduce the mortality to 4% in birds of TRT 4, a significantly lower frequency of mortality compared to positive control birds of TRT 7 ($P < 0.05$); moreover, the dual interception by WSD for disinfecting the oocyst-contaminated floor and synergism between salinomycin in feed and EOBWE in drinking water of birds in TRT 5 was able to reduce the mortality further to a lowest frequency of 2%, a frequency equivalent to that obtained by the negative controls ($P > 0.05$) (Table 6). The effectiveness of WSD against sporulated oocysts contaminating the floor (Barbour and Krull 2018), the salinomycin mechanism in inactivation of *Eimeria* microorganisms (Chappel 1979; Yang et al. 2019), and the additional inactivation of *Eimeria* oocysts and other wide spectrum microbes by the essential oil blend and decoction of the *Salvia libanotica* present in the EOBWE (Jitviriyanon et al. 2016; Barbour et al. 2018a; Pop et al. 2019) could have worked together, enabling the high survival in oocyst-challenged birds of TRT 5.

Live body weight

The consistent pattern of highest histopathologic lesion score in birds of TRTs 1, 6, and 7 (Table 5) did reflect on the slower growth of these birds, resulting in a consistent pattern of lowest live body weight at ages between 21 and 42 days (Table 7). This observation reinforces the importance of histopathologic intestinal lesion score in predicting the live body weight of challenged birds (Idris et al. 1997; Murakami et al. 2014; Nabian et al. 2018). It is worth noting that birds of TRTs 4 and 5, that had the lowest histopathologic intestinal lesion score among all challenged birds (Table 5), did result in the highest body weight among all challenged treatments at the market age (Table 7). Previous documented works described the negative impact of intestinal lesions, resulting from *Eimeria spp*. injuries, on digestion and absorption of nutrients in chicken (Adams et al. 1996; Tan et al. 2014; Adedokun and Olojede 2019).

Feed conversion ratio

The consistent pattern of highest histopathologic intestinal lesion score obtained by birds of TRTs 1, 6, and 7 (Table 5) did also reflect on obtaining the inefficient highest feed conversion ratios by birds of these treatments, and at all studied ages (Table 8). On the contrary, the lowest histopathologic intestinal lesion score observed in birds of TRTs 4 and 5 did result in the efficient lowest feed conversion ratios, effective the age of 21–42 days. These results are in agreement with previous documentation (Teirlynck et al. 2011), showing the negative impact of intestinal lesion on feed conversion ratio of chicken (Sharma et al. 2015; Pop et al. 2019).

Conclusion

In conclusion, the dual interception against *Eimeria spp*. challenge, implementing the invented method, in birds of TRT 4, did result in significant reduction of oocyst output, gross and histopathologic intestinal lesion scores, mortality, feed conversion, and in higher body weight compared to that obtained by the positive controls (TRT 7). The quantified microbiologic, pathologic, and production parameters were in favour of better protection and production in *Eimeria spp*.– challenged broilers of TRT 4 compared to birds of TRT 2 that were subjected to classical dual interception with chlorine disinfectant for decontamination of oocyst-contaminated floors and salinomycin supplementation in feed. In addition, the dual interception by chlorine and salinomycin (TRT 2) or by WSD and EOBWE (TRT 4) resulted in better protection and production than that obtained by a single interception by either chlorine (TRT 1) or by WSD (TRT 6) disinfectants. Moreover, The three interceptions with WSD for decontamination of surfaces and the administration of salinomycin in feed and EOBWE in drinking water of birds in TRT 5 had an additional improvement of the protection and production over that obtained by birds of TRT 4, providing an evidence of higher efficacy against broiler coccidiosis by the synergism between these two coccidiostats.

Disclosure statement

No potential conflict of interest was reported by the authors.

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