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"Ozonated water for parasitic control in buffalo farms (OZO-PAR)"

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"Your beliefs become your thoughts,
Your thoughts become your words,
Your words become your actions,
Your actions become your habits,
Your habits become your values,
Your values become your destiny."

Mahatma Gandhi

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AD MAIORA SEMPER

List of abbreviations

DD	Boron-doped diamond
BPR	Biocidal Products Regulation
CDC	Center for Disease Control and Prevention
CI	Confidence interval
CNSA	Comitato nazionale sicurezza alimentare
COMBAR	COMBatting Anthelmintic Resistance in Ruminants
CON	Group Control
CPG	Cysts per gram of faeces
CREMOPAR	Regional Center for Monitoring of Parasitosis
CT	Concentration multiplied by contact time
Day 0	Day 0 (treatment)
Day 27	Day 27 (post-treatment)
DBL	Draper Biotech Limited
DBP	Disinfectant By-Products
DC	Direct current
DFA	Direct Fluorescent Antibody
DIF	Direct Immune Fluorescence
DPD	N,N-diethyl-p-phenylenediamine
EC	European Community
ECHA	European Chemical Agency
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
FCR	Feed conversion rate
FDA	Food and Drug Administration
FEC	Faecal egg count
FS	Flotation solution
GE	Group Eimeria
GG	Group Giardia
GI	Gastrointestinal
GRAS	Generally Recognized as Safe
HCl	Hypochlorous acid
IFA	Indirect Fluorescent Antibody
IMS	Immunomagnetic separation
JMP	Joint Monitoring Programme
NaCl	Sodium Chloride
NDB	National Data Bank
OD	Outsider diameter
OH	Hydroxyl radical
OPG	Oocysts per gram of faeces
ORP	Oxidation-Reduction Potential
OZO	Group Ozone

List of abbreviations

PCR	Polymerase Chain Reaction
PDO	Protected Designation of Origin
PEEK	Polyether ether ketone
Pes	Parasitic elements
PMMA	Polymethylmethacrylate
PTFE	Polytetrafluoethylene
PV	Predictive value
RFLP	Restriction fragment lenght analysis
SG	Specific gravity
TOL	Group Toltrazuril
UK	United Kingdom
UNICEF	The United Nations Children's Fund
UV	Ultraviolet light
WHO	World Health Organisation
ZnSO4	Zinch Sulfate... ...

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Abstract

Dairy water buffalo (*Bubalus bubalis*) farming plays an important role in the economy of several countries, including Italy, as buffalo milk is almost exclusively used for the production of mozzarella cheese. However, in intensive farming systems, infection of water buffaloes with intestinal protozoa, such as *Giardia*, *Cryptosporidium* and *Eimeria*, might threaten the profitability and sustainability of milk production.
These infections have constantly increased over the years through contamination of water, feed and environment by the infective stages of these parasites, e.g., *Giardia* cysts and *Cryptosporidium/Eimeria* oocysts. In particular, well water used for daily activities on the farm (e.g., watering the animals, cleaning the premises, etc.) could represent an important source of infection. Even conventional chlorination programs, at the dosages used in the official treatment plans, are not effective against intestinal protozoa. Sanitation of drinking water (using products such as ammonia, chlorine dioxide, hydrogen dioxide and ozone) for livestock animals has been suggested as a useful strategy to be directed at reducing and/or preventing the transmission of the infective (oo)cysts to the animals. Therefore, the adoption of appropriate control strategies against intestinal protozoa is a considerable challenge for water buffalo farms worldwide. Although metaphylactic approaches have been used successfully to control infections by intestinal protozoa in ruminant farms, reinfections are very common, thus requiring repeated treatments that, in turns, might increase the potential for developing drug resistance as well as contributing to the dispersion of antiparasitic drugs into the environment. In light of these concerns, the need to introduce eco-friendly and alternative strategies to control intestinal protozoa infections is a considerable challenge for preserving the health and welfare of water buffalo farms.

The general aim of this industrial PhD project was to introduce an innovative system for water ozone production to be used as a sanitation strategy to control intestinal protozoa (*Giardi*a and *Eimeria*) infections of water buffaloes in the Campania region of southern Italy. The specific aims presented in this thesis were: i) presenting the epidemiological scenario of intestinal protozoa in water buffaloes in the Campania region of southern Italy; ii) selecting a sensitive technique to detect *Giardia* cysts; iii) setting up an innovative ozone generator system; iv) conducting a series of *in vitro* tests to assess the effect of the ozone treatment on *Giardia* cysts and *Eimeria* oocysts.

Abstract

Chapter 1 provides a general overview of *Eimeria* spp. and *Giardia duodenalis* infections in livestock. In this chapter basic knowledge related to the taxonomy, life cycle, epidemiology and pathogenesis of *Eimeria* spp. and *G. duodenalis* are reported. Moreover, the different coprological, serological and molecular techniques used in veterinary medicine are described. In the face of this knowledge, control of both protozoa represents the principal challenge in water buffalo farms. Currently, conventional metaphylactic treatments are associated with increased resistance, thus, eco-friendly, alternative strategy to control intestinal protozoa are indispensable tools to reduce the risk of transmission of protozoal infections in water buffalo farms.

Chapter 2 provides an overview of the ozone gas, its general properties and current application in water sanitation treatment. This chapter details the chemical and physical properties of ozone, its strong oxidizing power and environmental factors (e.g., pH, temperature) limiting its working power. Moreover, in this chapter, the main industrial methods to generate ozone are described, by specifying advantages and disadvantages. For my thesis, here is described the use of ozone in water treatment as an alternative sanitizer.

Chapter 3 shows the results of an epidemiological investigation of *Eimeria* spp. in water buffaloes in Southern Italy. Compared with cattle, there is limited scientific knowledge about the health of water buffaloes so updated data on parasitic infections (as eimeriosis) is an interesting challenge in this species. Furthermore, the published studies on eimeriosis in large ruminants in Italy are few and focused mainly on treatment while the epidemiological data in Europe are scarce, not updated, and focused only on cattle. For this purpose, parasitological data on eimeriosis from a 10-year surveillance were analysed. The results shows that *Eimeria* spp., in the same way as *Giardia* spp., is a persistent and complex problem in water buffalo farms, and control strategies need to be implemented on farms.

Chapter 4 investigates a sensitive and cost-effective technique for the detection of *Giardia* cysts in faecal samples. While copromicroscopic techniques are well-established methods for the detection of *Eimeria* oocysts, some concerns still apply to the use of copromicroscopic methods for the detection of *Giardia* cysts. For this purpose, immunoassays and FLOTAC techniques were compared for diagnosing *Giardia* spp. infection. The results from the cost-effectiveness analysis, in combination with the sensitivity and specificity of the FLOTAC technique, suggest that the

FLOTAC technique can be used in the routine diagnosis of Giardia spp. infection in animals.

Chapter 5 describes activities carried out in the UK, at the University of Bristol and the Draper Biotech Limited (DBL), the latter being an industrial company specialized in air and water purification systems using ozone. Moreover, the training in the UK included visits to poultry and cattle farms designed by the industrial company as the experimental setting to study the effect of ozonated drinking water *in vivo*. Moreover, in the Aberdeen Angus farm (Salisbury, UK) I participate to instal the farm box pipefitting ozone generator. In this chapter the main part of the farm box is detailed. Furthermore, once in Italy, some preliminary *in vitro* tests using a well-water medium were performed to evaluate the effect of water ozonation (using an electrolytic cell within polycrystalline diamond electrode) on the viability of *Eimeria* oocysts and *Giardia* cysts collected from water buffaloes. Moreover, a preliminary *in vivo* test was performed in water buffalo calves to evaluate *Eimeria* oocyst output reduction and performance, like weight gain from the antiprotozoal treatment with ozonated drinking water. The results of the preliminary *in vitro* and *in vivo* studies on the effect of ozonated water on *Eimeria* and *Giardia* suggested that ozone could be a promising eco-friendly tool to control protozoa infections in water buffalo farms.

Chapter 6 reports the results of a proof-of-concept study aimed at evaluating the effect of ozone on the viability of *Eimeria* oocysts and *Giardia* cysts. For this purpose, *in vitro* tests were performed to determine the minimum concentrations of O_3 (mg/l) and the contact times (minutes) necessary to inactivate *Eimeria* oocysts and *G. duodenalis* cysts. The results of the present study showed different values of efficacy of ozonated water on the viability of *Eimeria* oocysts (33.0%) and *G. duodenalis* cysts (96.3%) isolated from water buffaloes. *Eimeria* spp. and *Giardia duodenalis* represent a persistent and complex problem in water buffaloes that impair their health, welfare, and production. Currently, conventional metaphylactic treatments are associated with increased resistance. The sanitation of drinking water for livestock animals could be a useful eco-friendly, alternative strategy to control the diffusion of both protozoa in intensive buffalo farms.

I General overview

Approximately 3% of the world buffalo population is hosted in the Mediterranean area, where the sub-species water buffalo (*Bubalus bubalis*) is farmed for dairy purposes and plays an important role in the economy of several countries. In Italy water buffalo farming is traditionally linked to the production of "Mozzarella di Bufala Campana" cheese, that was granted the Protected Designation of Origin (PDO) by the European Community (Regulation 1107/96 of 12 June 1996) in 1996 (Borghese and Moioli, 2011; Masucci et al., 2016; Minervino et al., 2020).
In intensive water buffalo farming systems, the infection of water buffaloes with intestinal protozoa, such as *Giardia*, *Cryptosporidium* and *Eimeria*, threatens the profitability and sustainability of milk production (Rinaldi et al., 2007; Cringoli et al., 2009; Bosco et al., 2017; de Aquino et al., 2020). These infections have been constantly increased in the years, therefore, even in intensive cattle and buffalo breeding which, for daily activities (e.g., watering the animals, cleaning the premises, etc.) use well water always potential candidates for contamination.
Some studies showed that well water is one of the main sources of *Cryptosporidium* and *Giardia* infections (Giangaspero et al., 2009; Budu-Amoako et al., 2011; Dreelin et al., 2014). The chlorination programs, at the dosages used in the official treatment plans, are not effective against intestinal protozoa (Caradonna et al., 2017). Sanitation of drinking water (using products as ammonia, chlorine dioxide, hydrogen dioxide and ozone) for livestock animals has been suggested as useful strategy to be directed at reducing and/or preventing transmission of the infective (oo)cysts to animals (de Aquino et al., 2020). Therefore, the adoption of appropriate control strategies against intestinal protozoa are a considerable challenge for water buffalo farms worldwide (Bosco et al., 2017; El Debaky et al., 2019). Although, metaphylactic approaches have been used successfully to control infections by intestinal protozoa in ruminant farms, reinfections are very common, thus requiring repeated treatments that, in turns, might increase the potential for developing drug resistance as well as contributing to the dispersion of antiparasitic drugs into the environment (Thompson, 2004; Daugschies et al., 2013; Bosco et al., 2015; Santin, 2020). In light of these concerns, the need to introduce eco-friendly and alternative strategies to control intestinal protozoa infections is a considerable challenge for the health and welfare of water buffalo farms.
In recent years, scientific and commercial interest in the use of ozone in various field of human and veterinary medicine has considerably increased

(Elvis and Ekta, 2011; Đuričić et al., 2015; Sciorsci et al., 2020). Leaving no secondary chemical residues, ozone has been formally approved by the U.S. Food and Drug Admistration (FDA) as an "*Antimicrobial Agent for the Treatment, Storage and Processing of Foods in Gas and Aqueous Phases"* (FDA, 2001). In Italy, the Ministry of Health recognized ozone as a "*Natural protection for the sterilization of environments contaminated by bacteria, viruses, spores*, etc." (protocol No. 24482 of 31/07/1996). The Ministry of Health with CNSA of 21/10/2010 also recognized the use of ozone in the treatment of air and water as a disinfectant and disinfestant agent. However, currently in Italy, from a regulatory point of view, ozone can be marketed and used exclusively as a sanitizer.

This industrial PhD project was aimed to introduce an innovative system for water ozone production to be used as sanitation strategy to control intestinal protozoa infections of water buffaloes in the Campania region of southern Italy.

II Water buffalo farming

The water buffalo (*Bubalus bubalis*) is a ruminant species important in the economy of several countries including Brazil, China, India, Vietnam and Italy (Bosco et al., 2017). According to a recent report of the Food and Agriculture Organization (FAO), global buffalo population, in 2019, amounted to about 204 million heads. The buffaloes are widely distributed in Asia (96.4%, mainly concentrated in India, China and Pakistan), in Africa with 2.9% (particularly in Egypt), in South America (0.7%) and with only 0.2% in Europe (Italy, Romania, Georgia, Bulgaria and Turkey) (FAOSTAT, 2019).

Water buffaloes are considered to have a dual purpose, primarily for dairy production, especially in Asia and Europe, but also for meat production (Borghese and Mazzi, 2005; Minervino et al., 2020).

In Italy, the Mediterranean buffalo produces high quality milk employed for production of "mozzarella", a fresh cheese with a Protected Designation of Origin (PDO) mark (European Commission. Available on line: https://ec.europa.eu/commission/presscorner/detail/en/IP_96_492).

The Mozzarella cheese manufacturing from milk of water buffalo is third-ranked in sales volume in. In the first quarter of 2020, Mozzarella cheese export from Southern Italy recorded a good performance (+4.4%). The sector grew by +19.4%, supported by the increase in exports especially to France and United Kingdom, first and third outlet markets, respectively (https://news.italianfood.net/2020/09/11/exports-how-mozzarella-di-bufala-pdo-is-getting-over-the-crisis/).

The southern provinces of Lazio (Latina and Frosinone), the Campania region (particularly the provinces of Caserta and Salerno), and other two provinces of southern Italy (Foggia and Isernia), represent the designed area of buffalo mozzarella cheese. In Italy there are 2,711 buffalo farms with a total of 402,796 animals. Lazio and Campania are the regions with the highest percentage of the total buffalo farms in Italy with 26.9% and 48.8%, respectively (National Data Bank—NDB on 31th December 2019).

The Italian buffalo herd increased to about 52% from 2005 to 2013 due to the increasing demand for Mozzarella cheese both on the national and international markets (Sabia et al., 2015).

The expansion of the dairy buffalo livestock over the years has also been accompanied by a substantial transformation of the farm managment. The modern intensive water buffalo breeding is likely to replace the cattle breeds and has almost completely replaced the traditional extensive/semi-extensive buffalo farming (Sabia et al., 2015; Bosco et al., 2017).

Introduction

Buffalo livestock management is usually performed in paddocks all year, with the same modern systems used for dairy cows and the animals are used to roll in puddles or lie on floor (Borghese and Moioli, 2016). Currently, the buffalo management is characterized by technologically advanced and automated systems (e.g., milking robots, automatic manure cleaning, the use of the pedometer for individual measurements of physiological/production parameters, etc.) for precision livestock farming. Thease characteristics have allowed buffalo farms to increase their milk production, as well as to a renewed interest in this livestock species.

III Parasitological scenario in water buffalo farms

The modern intensive water buffalo breeding together with constant supplies of concentrated and/or stored forages as well as the regular use of accurate protocols of anthelminthic control (targeted anthelmintic treatments only based on an accurate diagnosis) has strongly influenced the parasitological scenario of buffalo farms in central and southern Italy and have contributed to the decrease in helminth (nematoda, trematoda, cestoda) infections (Cringoli et al., 2009; Rinaldi et al., 2009; Bosco et al., 2017).
However, intensive breeding implies a high density of animals, thus leading to the spread of intestinal protozoa, e.g., *Eimeria*, *Giardia* and *Cryptosporidium* (Rinaldi et al., 2009). Noteworthy, *G. duodenalis* and *C. parvum* are also transmissible (directly or indirectly) to humans leading causes of morbidity worldwide (Thompson et al., 2008; Abeywardena et al., 2015; Bosco et al., 2017; Rivero et al., 2020). Parasitic infections of water buffaloes are considered common in tropical and subtropical countries where they cause huge economic losses as a consequence of deaths of infected animals, reduced rates of weight gains and the condemnation of infected organs after slaughter. Some parasites of buffaloes are also transmissible (directly or indirectly) to humans where they can cause significant clinical disease, such as schistosomiasis (in China and the Philippines), cystic echinococcosis, fasciolosis, cryptosporidiosis and giardiasis, everywhere.
Many studies have been conducted in recent years by the CREMOPAR (Regional Center for Monitoring of Parasitosis - Campania Region, Italy) reporting up-to-date information on parasitism caused by protozoa, in buffalo farms from central and southern Italy, where most Italian water buffaloes are bred (Bosco et al., 2017).
The overall findings of the above-mentioned studies are reported in the following table (Table 1) which summarizes the diverse parasitism of water buffaloes in central-southern Italy.

Table 1. The main parasitism of water buffaloes in central and southern Italy (Guarino et al., 2000; Capuano et al., 2006; Cacciò et al. 2007; Cringoli et al., 2007; Rinaldi et al., 2007; Cringoli et al., 2009; Rinaldi et al., 2009; Ciuca et al., 2020)

Parasites	**Farm Prevalence (%) (min and max)**
Protozoa	
Eimeria spp.	94.5-97.7
Giardia duodenalis	25.6-30.0
Cryptosporidium parvum	14.7-19.8
Neospora caninum (seroprevalence)	20.2-34.7*
Toxoplasma gondii (seroprevalence)	13.7-19.6*
Helminths	
Gastrointestinal strongyles	4.7-33.1
Strongyloides spp.	3.1-4.7
Fasciola hepatica	1.1-7.1
Dicrocoelium dendriticum	0.5-2.4
Paramphistomidae	2.3-7.1
Moniezia spp.	0.5-2.4
Echinococcus granulosus (larval stages)	8.0-12.4
Arthropoda	
Lice (*Haematopinus tuberculatus*)	2.8-11.0
Mange mites (*Psoroptes* spp.)	3.0-12.6

*Prevalence reported for animals

Infections caused by intestinal protozoa are a leading cause of neonatal diarrhoea, having a negative impact on the growth performance of buffalo calves.
In particular, *Eimeria* infections, better known as coccidioses, and *G. duodenalis* are widespread infections in buffaloes affecting calves all over the world constituting infections of economic concern to the beef and dairy industries due to reducing weigth gain and reproductive performance. Recent epidemiological surveys conducted in Lazio and Campania regions showed overall prevalence of *Eimeria* setup at 81.5% (Morgoglione et al., 2020); whilst *G. duodenalis* was present in buffalo farms with 30.0%, in Lazio region. Furthermore, isolates of *G. duodenalis* analysed using molecular techniques showed the presence of *G. duodenalis* assemblage A (zoonotic) and host-specific parasites (*G. duodenalis* assemblage E), suggesting that water buffaloes can contribute to environmental contamination with *Giardia* cysts potentially infectious to humans if their faeces are improperly disposed of.

IV Concluding remarks and needs for research

The control of intestinal protozoa in buffalo farming is certainly not easy to implement because of the considerable spread and resistance of oocysts/cysts of *Eimeria* and *Giardia* in the environment and in the water, even for months (Keeton et al., 2018; Adeyemo et al., 2019). As vaccination prevention measures are not yet available in ruminants (Thompson et al., 2008; Santin, 2020), a good integrated combination of rational treatments, hygiene and management are essential tools for controlling protozoan infections in buffaloes. Although, metaphylactic approaches have been used successfully to control *Eimeria* (e.g., toltrazuril and diclazuril) (Daugschies et al., 2007; Bosco et al., 2015) and *Giardia* (e.g., fenbendazole and albendazole) (Thompson et al., 2008; Santin, 2020) infections in ruminant farms, prophylactic measures are needed to reduce environmental contamination in order to limit the infection pressure (Daugschies et al., 2002).
Metaphylactic treatments with toltrazuril was very useful against *Eimeria* infections in water buffaloes and should also contribute to the reduction of environmental contamination with oocysts and limiting the infection pressure (Bosco et al., 2015; Keeton et al., 2018; Morgoglione et al., 2020); whilst for *G. duodenalis* anthelmintic drugs such as fenbendazole (O'Handley et al., 1997) and albendazole (Xiao et al., 1996) has proven their efficacy in cattle.

However, the efficacy of toltrazuril could be increasingly reduced by the development of *Eimeria* resistance in ruminants (Odden et al., 2018a, b) as has already been shown in poultry farms (Chapman, 1984; Noak et al., 2019; Snyder et al., 2021). Furthermore, the escalating spread of antimicrobial resistance (Sharma et al., 2018; WHO, 2020) and anthelmintic resistance (Vercruysse et al., 2018) emphasizes the need for a change toward more sustainable control approaches in order to prevent or reverse the development of resistance (Vande Velde et al., 2018).

Thus, prophylactic measures are needed to reduce environmental contamination in order to limit the infection pressure (Daugschies et al., 2002). Sanitation practices play an important role in the complex control of intestinal protozoa infection in water buffalo farms, reducing environmental contamination pressure and protect animals from infections.

Ozone is a powerful and reliable anti‑ microbial agent against bacteria fungi, protozoa, and viruses (Khalifa et al., 2001; Erickson and Ortega 2006; Elvis and Ekta, 2011; Marino et al., 2018; Sciorsci et al., 2020). The use of ozone is widely applied in drinking water and wastewater treatment in order to remove and inactivate waterborne bacteria such as *Salmonella* or *Escherichia coli* (Bialka and Demerci, 2007; Varga and Szigeti, 2016; Megahed et al., 2019) and protozoa, e.g., *Cryptosporidium* (Erickson and Ortega 2006; Pereira et al., 2008; Ran et al., 2010). Moreover, ozone is more efficient disinfectant than other chemical substances due to its high reactivity and strong oxidant power (Ding et al., 2019).

Therefore, ozone will not provide a long-lasting disinfecting residual in drinking water or in wastewater, rendering the treated water safe.

Ozone has successfully been used in dairy industries for the cleaning operation in milk processing and reducing the concentrations of pollutants in dairy wastewaters (Varga and Szigeti, 2016). However, in dairy farming ozone is still limited to few specific ozone therapy applications e.g., intrauterine, intravaginal and intramammary treatment to reduce antibiotic administration (Duričić et al., 2015; Sciorsci et al., 2020).

Considering the health implications and the economic potential of water buffaloes the development of appropriate control strategies are of extreme importance not only for animal welfare but also for public health, in support of the "ancient and new" One Health concept (Rabinowitz et al., 2013; Bosco et al., 2017; Innes et a., 2020).

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Capitolo 1

Infections by *Eimeria* and *Giardia* in water buffaloes

1.1 Introduction

Protozoa, from the Greek words "*protos*" (first) and "*zoon*" (animal) are ubiquitous unicellular organisms that belong to the Protista Kingdom (Taylor et al., 2007; Magill, 2013; Deplazes et al., 2016).
They are considered the most primitive organisms in the animal Kingdom and all vital functions are performed by a single cell.
They are eukaryotic organisms, and therefore all their genetic information is deposited in the chromosomes contained within a nuclear membrane. For this characteristic they differ from bacteria that have no nucleus and have only one free chromosome in the cytoplasm. They have a nucleus, a rough endoplasmic reticulum, mitochondria, lysosomes and a Golgi apparatus (Taylor et al., 2007). Furthermore, being organisms that conduct their own existence autonomously, they possess a series of subcellular structures and organelles with specific functions. The movement is guaranteed by the presence of a single flagellum or by several flagella. The flagellum is a contractile thread-like structure that originates from an organelle known as the basal body. The flagella are long and their movement is a complex whip-like undulation (Pampiglione and Canestri Trotti, 1999; Taylor et al., 2007). Some protozoa, such as the extracellular stages of *Eimeria* have no obvious means of locomotion (Taylor et al., 2007).
Protozoa occur as either free-living forms or harmless commensals, but their numbers include more than 17,000 parasitic species, aetiological agents of important infections in human and animals such as cocciodiosis (e.g., Cryptosporidiosis, Toxoplasmosis and Eimeriosis) and giardiosis (Hubalek, 2003; Deplazes et al., 2016). In some groups, reproduction is asexual, sexual or both (e.g., *Eimeria*) (Taylor et al., 2007; Magill, 2013).
Most of the protozoa that infect enteric tract have outline robust wall (e.g., cysts or oocysts) that enables their survival in the external environment allowing these parasites to infect other susceptible hosts through either horizontal-route and water or food-borne routes (Nichols, 2000, Rose et al., 2002; Pozio, 2003; Dawson, 2005; Baldursson et al., 2011; Cama and Mathison, 2015; Deplazes et al., 2016; Siwila et al., 2020;).
Protozoan-related morbidity and mortality in both human and animals worldwide are well documented (von Samson-Himmelstjerna et al., 2006; Coklin et al., 2007; Feng et al., 2009; Dixon et al., 2011; Fletcher, et al., 2012; Shaapan, 2016). Some species of protozoa, however, are significant causes of disease in domesticated ruminants, including water buffaloes, resulting in economic losses or because of their potential for zoonotic aspect

(Taylor, 2000; Cacciò et al., 2007; Feng and Xiao, 2011; Helmy et al., 2013; Robertson, et al., 2013; Villanueva et al., 2018; Volpato et al., 2017).
In buffaloes, the most common parasitic protozoa are the coccidia of the genus *Eimeria* and the flagellates of the genus *Giardia* (Rinaldi et al., 2007; Gupta et al., 2015; Bosco et al., 2017; Dubey, 2018; de Aquino et al., 2020; Morgoglione et al., 2020; Barburas et al., 2021).
Infections caused by these intestinal protozoa are a leading cause of neonatal diarrhoea, with negative impacts on health, welfare and production of animals, especially in young animals (Taylor, 2011; Cho and Yoon, 2014; Bosco et al., 2017; Minervino et al., 2020).

1.2 *Eimeria* spp.: Taxonomy, life cycle, epidemiology and pathogenesis.

Eimeria (Schneider, 1875) (Apicomplexa: Eimeriidae) are protozoan parasites belonging to the Coccidiasina (Coccidia), a group of obligate intracellular parasites of the intestinal epithelium of veterinary importance (and other sites) of most vertebrates worldwide (Shirley et al., 2005).
All members of the Coccidia replicate within the intestines of a definitive host progressing through sequential rounds of asexual (schizogony) and sexual (gametogony) reproduction, culminating in the production of oocysts that are shed into the environment with the faeces (Kemp et al., 2013).
Coccidians of the family Eimeriidae, such as species of *Eimeria* are monoxenes meaning that their development is restricted to a single host where they replicate rapidly to reach high numbers in the intestine causing acute enteritis of varying severity.
More than a dozen *Eimeria* species are common in cattle (*Bos taurus*, *Bos indicus*) and water buffaloes (Table 1.1).

Table 1.1 *Eimeria* species common in cattle (*Bos taurus*) and water buffalo

Eimeria species	**Host**	
	Cattle	**Water Buffalo**
E. subspherica	+	+
E. zuernii	+	+
E. ellipsoidalis	+	+
E. bovis	+	+
E. auburnensis	+	+
E. cylindrica	+	
E. canadensis	+	+
E. wyomingensis	+	+
E. brasiliensis	+	+
E. bukidnonensis	+	+
E. pellita	+	-
E. alabamensis	+	-
E. ankarensis	-	+
E. bareillyi	-	+
E. thianethi	-	+
E. azerbajdjhanaica	-	+
E. gokaky	-	+
E. ovoidalis	-	+

Of the numerous species of *Eimeria* in cattle, *Eimeria bovis*, *Eimeria zuernii*, *Eimeria auburnensis* and *Eimeria alabamensis* are considered highly pathogenic, and most outbreaks of bovine coccidiosis are associated with *E. bovis* and *E. zuernii* (Levine and Ivens, 1970; Levine, 1973; Bangoura et al., 2011; Floriao et al., 2013). On the other hand, *Eimeria bareillyi* (Gill et al., 1963; Sayin, 1968) has been documented to be the main pathogenic species in water buffaloes (Cringoli et al., 1998; Dubey et al., 2008, Dubey, 2018); this species is not transmissible to cattle (Figure 1.1).

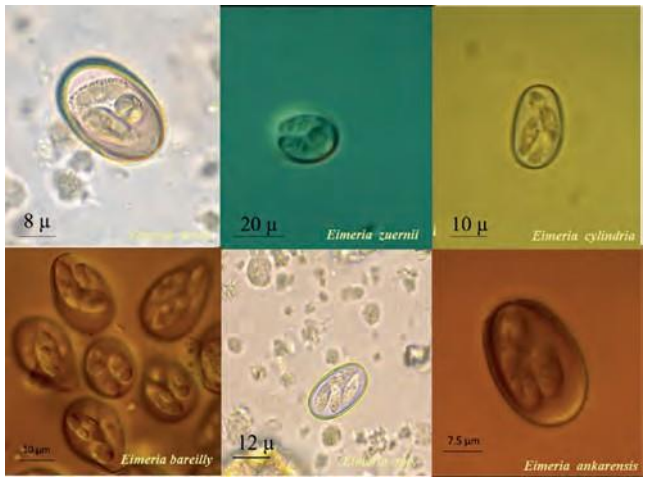

Figure 1.1 Oocysts of different *Eimeria* species observed under the microscope.

Life cycle. Infection occurs by ingesting the sporulated oocysts (20-50 µm depending on the species) from the contaminated environment (Figure 1.2).

Figure 1.2 Schematic representation of *Eimeria* sporulated oocyst with four sporocysts, each with two sporozoites of banana-like appearance.

The life cycle is divided into three phases (Figure 1.3): merogony (asexual reproduction), gametogony (sexual reproduction) and sporogony with oocyst formation and sporulation.

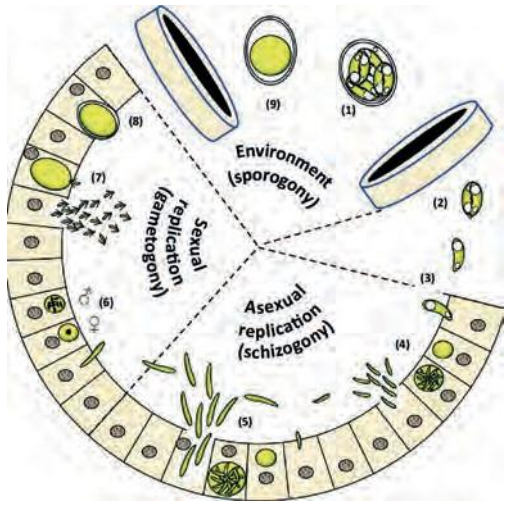

Figure 1.3 General schematic representation of *Eimeria* spp. life cycle (Blake and Tomley, 2014).

The first two phases take place in the host, the sprorulation phase takes place in the external environment.

Endogenus development occurs in the small intestine or in the small and large intestine (*E. bovis, E. zuernii*). After ingestion of sporulated oocysts, sporozoites are realesed into intestine and infect, in a species-dependent manner, cells of the jejunum or the ileum (Deplazes et al., 2016).

The subsequent development follows two different strategies according to the *Eimeria* species. *E. bovis*, *E. zuerni* and *E. auburnensis* form large schizonts (meronts I) within a period of approximately 2 weeks; other species, e.g., *E. alabamensis* create small meronts I within few days. These are followed by a second generation of smaller schizonts (Deplazes et al., 2016).

The 2nd merogony and the subsequent gamogony proceed in all species relatively quickly in enterocytes or in cell of the lamina propria or more distal sections of the intestina (Deplazes et al., 2016). During the gametogonic cycle, the zygote is formed from the fusion of micro and macrogametocytes, which is covered with a double wall and comes out with the feces in the form of non-sporulated oocysts. The sporulation of oocysts in the environment can be completed in 1-4 days, under suitable climatic conditions, longer times (e.g., weeks) are needed in colder climates.

Below a briefly description of the localization of some *Eimeria* species during the merogony and gametogony phases.
E. bovis – merogony in the second part of the small intestine; gametogonic phase occur in the cecum and colon. Shedding of oocysts appear in the faeces approximately within three weeks of infection.
E. zuernii – merogony in the second part of the small intestine and in the large intestine. Shedding of oocysts appear in the faeces approximately within three weeks of infection.
E. auburnensis – merogony phases at the second part of the small intestine. Shedding of oocysts appear in the faeces approximately within three weeks of infection.
E. ellipsoidalis – merogony phases at the second part of the small intestine. Shedding of oocysts appear in the faeces approximately within three weeks of infection.
E. alabamensis – The first stages of development occur in the nucleus of the epithelial cells of the intestinal *villi*. Gametogony occur in the third of the small intestine but it can also invade the mucous membrane of the cecum and colon. Shedding of oocysts appear in the faeces approximately within three weeks of infection.

Epidemiology

Eimeria bovis, *E. zuernii* and *E. auburnensis* are frequently found in large ruminants worldwide (Cringoli et al., 1998; Bangoura et al., 2011; Bahrami and Alborzi, 2013; Gupta et al., 2015; Teixeira Filho et al., 2016; Dubey, 2018; Morgoglione et al., 2020). The other species varies regionally. In most cases, mixed infection with several *Eimeria* species is observed.
Epidemiological investigation revealed that eimeriosis is common (up to 100%) in water buffaloes in Italy as in different parts of the world (Cringoli et al., 1998; de Noronha et al., 2009; Kan et al., 2013; Tomczuk et al., 2015; Gupta et al., 2016; Bosco et al., 2017; Dubey, 2018; Jahanzaib et al., 2017; El-Alfy et al., 2019; Bangoura et al., 2020; Morgoglione et al., 2020). Recent study reported an overall prevalence of *Eimeria* spp. of 81.5% in intensive water buffalo livestock in the Mediterranea area (Morgoglione et al., 2020), with a slight reduction compared to the previous decade (97.7%) reported by Bosco et al. (2017).
Generally, prevalence and intensity of oocyst excretion are higher in young animals than in adults. Water buffaloes can acquire *Eimeria* infection soon after birth (Barbosa et al., 1992; Guarino et al., 1997; Fusco et al., 1997;

Cringoli et al., 1998; de Noronha et al., 2009) and shed oocysts around an age of 2 weeks. The environment is the most important risk factor for coccidiosis in cattle and buffaloes (Gupta et al., 2016; Alcala-Canto et al., 2020). Husbandry system that allows intense contact between calves is considered a predisposing factor for infection (Kan et al., 2011; Mitchell et al., 2012; Sudhakara Reddy et al., 2015; Keeton, 2018). Coccidian oocysts are crucial for the survival of the parasites in the external environment and the transmission to suitable hosts (Kheysin, 1972). The oocyst wall of *Eimeria* is extremely robust. Hence, oocysts are resistant to disinfectants and chemicals, like sulfuric acid or potassium dichromate (Marquardt, 1966; Dubey et al., 1970; Kheysin, 1972), although they are sensitive to heat, cold, and desiccation (Kheysin 1972; Ryley 1973; Dubey 1998).
The resistance of *Eimeria* oocysts to the external environmental insults and to the most common disinfectants along with the high number of potential spreaders of oocysts, such as domestic animals or synanthropes (e.g., insects, rodents, ect), contribute to the difficulty experienced in attempting to exclude oocysts from livestock (Mai et al., 2009).

Pathogenesis
Eimeria bovis and *E. zuernii* are the most phatogenic coccidia species (Bangoura et al., 2011; Tomkuz et al., 2015). The large meronts I are not important in this respect but the release of meront II and gamonts is associated with distinct lesions in the large intestine. Depending on the intensity of infection, focal up to extended losses of epithelial cells and necroses of the mucosa, dilation of the blood and lymphf vessels, cellular infiltration and oedamas develop in the tissue. Due to the lesions, fluids, minerals, proteins, and blood are lost into the intestine (Deplazes et al., 2016).
Clinical coccidiosis in young buffaloes has been reported from India (Shastri et al., 1974; Shastri and Krishnmurthi, 1975; Shastri et al., 1976), Brazil (Bastianetto et al., 2008; Meireles et al., 2012) and The Netherlands (Dubey et al., 2008). The youngest affected calf was three weeks old (Bastianetto et al., 2008; Dubey et al., 2008) and the oldest was four months old (Shastri et al., 1976). *E. bovis*, *E. zuernii*, *E. auburnensis* and *E. bareillyi* are responsible of severe clinical disease due to intestinal lesions with effects on the digestive process and overall homeostasis. The infection becomes clinically manifest in cases of high parasitic load or when the animal's immune status is compromised by stressful factors such as transport, excessive density, extreme climatic events, inadequate nutrition or

intercurrent illnesses. The most frequent symptoms are weight loss, anorexia and diarrhea, often hemorrhagic. Anatomopathological lesions, most of the time, are reduced to a thickening of the intestine with hemorrhagic petechiae, but a small scrap of the mucosa is sufficient to highlight large quantities of gamonti and oocysts. Small whitish spots may be visible on the mucosal surface, which correspond to the presence of gamonti, the most pathogenic stage (Deplazes et al., 2016).

E. bovis is particularly pathogenic and causes haemorrhages, exfoliation of the mucous membrane of the cecum and colon that lead to severe enteritis and diarrhoea, up to dysentery with tenesmus in severe cases. The animal may present with fever, weakness and dehydration and if not treated with adequate therapy, the outcome can be fatal. The major pathological damage is caused by the gamontes that develop in the cecum and colon. At post-mortem examination, the mucosa appears congested, edematous, thickened and with widespread petechiae. Large amounts of blood may be present in the intestinal lumen. As the infection progresses, the mucosa is completely destroyed and the damage can extend to the submucosa. If the animal survives, the mucosa and submucosa are rebuilt.

E. zuernii is the most pathogenic species among bovine and buffalo coccidia, causing hemorrhagic diarrhea and is capable of leading to erosion and complete destruction of large areas of intestinal mucosa. It is one of the species most commonly associated with "winter coccidiosis", which affects calves after extreme climatic events that occur in the winter months.

Parasitosis is characterized by catarrhal enteritis involving both the small and large intestine. Acute infections in calves are characterized by hemorrhagic diarrhea.

E. zuernii can also cause chronic infection, with non-haemorrhagic diarrhea. The animals appear emaciated, dehydrated, weak and with shaggy fur.

E. alabamensis is also pathogenic. Diseased animals excret foamy, liquid faeces for some days accomplished by depression and weight loss. At anatomo-pathological examination, the infection manifests itself with catarrhal enteritis along the jejunum, ileum and cecum tracts with the presence of petechiae hemorrhages. Histologically, necrotic inflammation, destruction of epithelial cells and the presence of numerous schizonts in the nucleus of the epithelial cells of the villi are observed.

1.3 *Giardia duodenalis*: Taxonomy, life cycle, epidemiology and pathogenesis

Giardia duodenalis (Davaine, 1875) is a flagellated protozoan grouped in the Class Zoomastigophorea and Order Diplomonadida (Thompson, 2004; Taylor, 2006; Deplazes et al., 2016).
Giardia is worldwide distributed enteric parasite of >40 animal species (Ryan et al., 2018) including humans, rodents, birds, amphibians, dogs, cats and ruminants including cattle and water buffaloes (Thompson et al., 2004, Hunter et al., 2005; Feng et al., 2011; Bosco et al., 2018; Costa de Aquino et al., 2018). *Giardia* is also recognised as parasites of a diverse range of wildlife species including wild ruminants (Hunter et al., 2005).
Currently seven species of *Giardia* are considered valid: *G. duodenalis* (mammalia), *Giardia muris* and *Giardia microti* (rodents), *Giardia ardeae* and *Giardia psittaci* (birds), *Giardia agilis* (amphibians), *Giardia cricetidarum* (hamster) and *Giardia peramelis* (marsupials) (Lyu et al., 2018).
G. duodenalis a species complex consisting of several genetic subgroup (assemblages A-H, sub-assemblage and variants) with different host specificity (Table 1.2).

Table 1.2 *Giardia* species and genetic groupings (assemblages) within *Giardia duodenalis* recognised currently by the international scientific community.

Species/assemblages	Hosts
G. duodenalis	
Assemblage A (AI-AVIII)	Human, primates, ruminants, dogs, cats, livestock, rodents, wild animals
Assemblage B (B1-B4)	Human, primates, cattle, dogs, some species of wild animals
Assemblage C	Dogs and other canids
Assemblage D	Dogs and other canids, cats
Assemblage E	Cattle and other hoofed livestock
Assemblage F	Cats
Assemblage G	Rodents
Assemblage H	Marine vertebrate

G. agilis	Amphibians
G. muris	Rodents
G. ardeae	Birds
G. psittaci	Birds
G. microti	Rodents
G. cricetidarum	Hamster

Life cycle. The life cycle of *Giardia* is direct and includes only 2 stages: the cyst (infective stage) and the trophozoite (replicative stage) (Figure 1.4a-b).

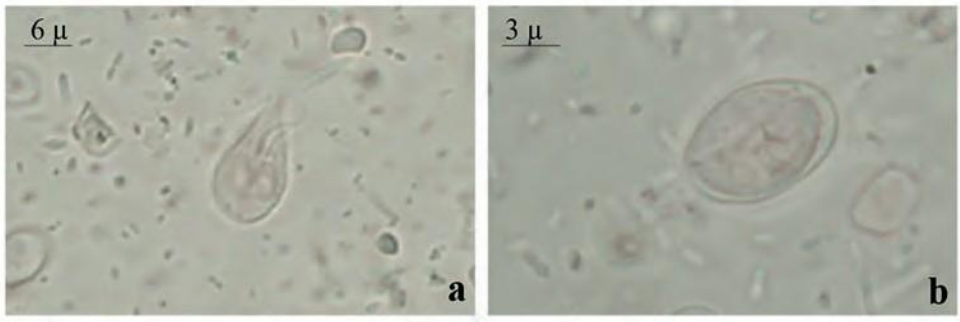

Figure 1.4. a *Giardia duodenalis*. Trophozoite; b *Giardia duodenalis.* Cyst

The trophozoite inhabits the small intestine. It has a bilateral and symmetric structure and resemble half a pear with a convex dorsal and flat ventral side, wich carries a large adhesion disk in its anterior part (Deplazes et al., 2016). Transmission is *via* horizontal route and occurs through direct contact with infected humans (anthroponotic transmission) or animals (zoonotic) and indirectly by contaminated water or food (water and foodborne transmission) (Rahman t al., 2002; Ryan et al.,2019; Dixon, 2020).
After ingestion, *Giardia* cysts excyst in the small intestine, releasing two motile, binucleated trophozoites, each with four pairs of flagellae and an adhesive disc for attachment to intestinal epithelial cells. Trophozoites reproduce asexually by longitudinal binary fission before encysting into environmentally resistant cysts in response to the presence of bile salts and

slightly alkaline pH (Feng et al., 2011; Dixon, 2020). Cysts is encysted when released into the faeces and troughs the intestine in faeces is spread in environment (Feng et al., 2011) (Figure 1.5).

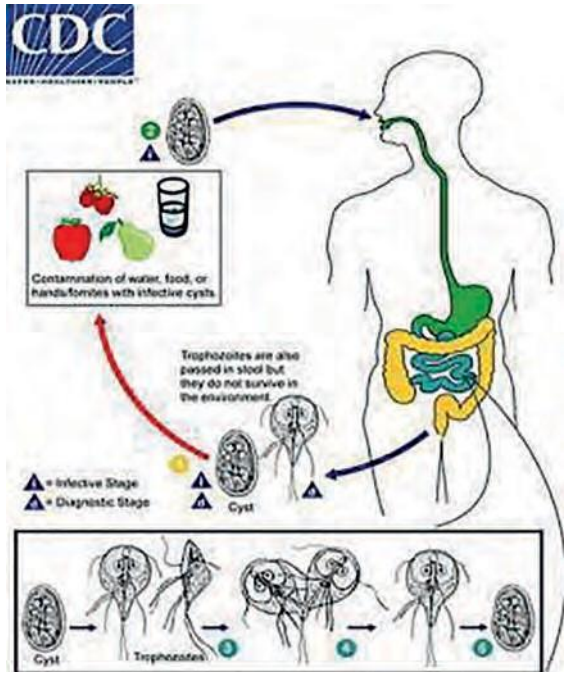

Figure 1.5 Life cycle of *G. duodenalis* (http://www.cdc.gov)

Epidemiology

Giardia cysts excreted in host faeces are responsible for the spreading of infection into the environment. Despite the number of excreted cysts is usually very large (e.g., 3 x 10^5 per g of faeces in calves) the infective dose is referred to very low numbers, e.g 10 cysts (Rendtorff, 1954). The excretion of cysts often lasts for several weeks or months. Cysts remain infective for up to approximately 3 months in a humid environment and 3 weeks in cool water, while trophozoites die off rapidly outside an infected host. Infection occurs by ingestion of *Giardia* cysts with contaminated water or food, or by faecal transmission, e.g., in animals by licking faeces or contaminated parts of the skin.

Worldwide, domestic animals, including ruminats, swine and carnivores are often infected by *G. duodenalis* (Feng and Xiao, 2011). In Europe

(Germany, UK, France and Italy) the averge prevalence of infections in calves is approximately 90% (Geurden et al., 2012).
In cattle, a prevalence of between 6.6% in New Zealand (Learmonth et al., 2003) and up to 57.8% in Canada and Australia (O'Handley et al., 2000) has been reported. A cumulative parasite prevalence of 73% to 100% in both dairy and beef calves has been observed in North America (Xiao and Herd, 1994; Ralston et al., 2003), and also a farm prevalence as high as 96% has been recently reported in Canada (Dixon et al., 2011).
Factors that contribute to the successful spread of giardiosis include large numbers of cysts released into the environment by infected hosts; cysts that are immediately infectious after excretion and that remain *viable* for extended times under the right conditions (cold temperatures and moisture). According to Kaplan (2019) infection rates vary greatly among studies and range from 1% to as much as 60% in cattle, sheep, and goats. However, in longitudinal studies the cumulative incidence increases to 100% in ruminants.
In Italy, Bosco et al. (2017) reported high prevalence of *G. duodenalis* in water buffalo farms (18.1% in animals and 30.0% on farms); moreover, the results showed the presence of assemblage A and E, suggesting that water buffaloes can contribute to environmental contamination with cysts potentially infectious to humans if their faeces are improperly disposed of (Rinaldi et al., 2007; Cacciò et al., 2005).

Pathogenesis
Giardiosis is an extremely common disease in ruminants, characterized by diarrhoea, weight loss, and malabsorption, but asymptomatic infections are also very common in animals (especially young) infected by this protozoon (Olson et al., 1995; Giangaspero et al., 2005; Castro-Hermida et al., 2007; Santin, 2020). The faeces do not contain blood and rarely mucus, because the parasite, and in particular the adhesive disc, does not damage the continuity of the intestinal wall but causes mechanical damage to the intestinal mucosa with flattening of the microvilli. Often the infection occurs in an asymptomatic form. Necropsy examination shows atrophy of the intestinal villi, hypertrophy of the crypts and an increase in intraepithelial lymphocytes. Trophozoites, adhering to the brush border of epithelial cells, may be microscopically visible between the villi. These alterations will induce an increase of the intestinal permeability which will cause malabsorption of electrolytes, nutrients and water, an alteration of the intestinal flora and impaired motility (Ankarklev et al., 2010). Diarrhea is

self-limiting in immunocompetent animals, while clinical manifestations and weight loss can be more severe when associated with co-infections with other pathogens (e.g., viruses, bacteria and other parasites) or immunosuppressive diseases (Scorza and Lappin, 2007). *Giardia* causes mechanical damage to the intestinal mucosa with flattening of the microvilli causing a decrease in the absorption of nutrients. In the long term this condition causes neonatal diarrhea with consequent slowing of growth, with low weight gains and with a delay in reaching the ideal weight for the first birth (about 80% of adult weight) (Castro-Herminda et al., 2005

Giardia infections in cattle are very important both from a clinical and production point of view, given the decline in animal performance (O'Handley et al., 2001; Olson et al., 2004). Young animals are more affected by this protozoon than older animals (Thompson, 2000; Olson et al., 2004; Bosco et al., 2018; De Aquino, 2020) and even represent the main cause of neonatal diarrhea (calves less than 30 days old).

Giardia is extremely common in ruminants which are frequently considered a major source of excretion *G. duodenalis* for humans (Thompson et al., 2007; Fen et al., 2011; De Aquino et al., 2020).

1.4 Diagnosis of *Eimeria* and *Giardia*

"An accurate diagnosis is the indispensable basis for veterinary interventions" (Deplazes et al., 2016).

In addition to the evaluation of symptoms, which are often not pathognomonic, laboratory diagnosis is needed to detect and/or count the cysts of *Giardia* and the oocysts of *Eimeria* spp. in infected animals.

Assays for detecting infection in faecal samples are important tools for diagnosing the disease (Pepe et al., 2019).

These parasites can be diagnosed by a wide variety of coprological, serological, and molecular techniques (de Aquino et al., 2020).

Copromicroscopic diagnosis of protozoa infections in water buffaloes can be either qualitative (thus providing only the presence/absence of oocysts/cysts) or quantitative, providing also the number of oocysts/cysts by faecal egg count (FEC). When quantification is pursued (FEC), protozoa elements are counted and usually expressed as the number of (oo)cysts per gram (OPG and CPG, respectively) of faeces. Qualitative and/or quantitative copromicroscopy in ruminants usually involves concentration of parasitic elements (e.g., oocysts and cysts) by flotation in order to separate protozoa elements from faecal material (Cringoli et al., 2010; Rinaldi et al.,

2014). Therefore, different procedures have been used in veterinary medicine.

1.4.1 Direct smear

Microscopical examination of wet mounts (fresh smear).
For *Giardia*, the traditional approaches, such as use of fecal smears have significant limitations due to the small size of the cysts. Moreover, shedding of cysts is intermittent, even in chronically infected individuals, thus requiring multi-day faecal examination (Pepe e al., 2019).

1.4.2 Flotation techniques (FT)

The parasitic elements (Pes) float in a flotation solution (FS) with a specificity gravity higher (SG) than water (sG 1.0) to give a suspension (Koutz, 1941; Ballweber et al., 2014).
Most of the FSs used in coprology are saturated and are made by adding a measured amount of salt or sugar (or a combination of them depending on the FS) to a specific amount of water to produce a solution with the desired specific gravity (SG). After preparing any FS, it is mandatory to check the SG with a hydrometer, recognizing that the SG of the saturated solution will vary depending on ambient temperature. It should be noted that the choice of FS is important but does not receive sufficient consideration by the scientific community, despite the substantial effect that the FS can have on the diagnostic performance of any flotation technique (Cringoli et al., 2004, 2017).
Flotation in tube. In literature, many diagnostic techniques, using different FSs were developed (Fulleborn, 1921; Stoll, 1923, 1930; Gordon and Whitlock, 1939; Whitlock, 1941; Eigenfeld and Schlesinger, 1944; Seghetti, 1950; Mayhew, 1962; Slocombe, 1973; Rossanigo and Gruner, 1991; Presland et al., 2005; Cringoli, 2004; Cringoli et al., 2017). The flotation in tube is the simplest flotation method. The faecal material is mixed with a FS into a tube. Then, a coverslip is placed on the surface of the tube and after 15 minutes the coverslip is removed to examine it under the microscope (MAFF, 1986). The main limit of this technique is that when the coverslip is removed from the top of the faecal suspension tube and then placed on a microscope slide, not all the floated PEs adhere to the underside of the coverslip. For these reasons flotation in centrifuge techniques were developed, e.g., Clayton- Lane, Wisconsin, Cornell-Wisconsin etc.
FLOTAC and Mini-FLOTAC techniques. The FLOTAC techniques allows a combination of flotation by centrifugation of a faecal sample suspension

and subsequent translation of the apical portion of the floating suspension. There are two versions of the FLOTAC apparatus: FLOTAC-100, which permits a maximum magnification of ×100, and FLOTAC-400, which permits a maximum magnification of ×400. FLOTAC-400 is a further development and improvement over FLOTAC-100, which is necessary for the detection of intestinal protozoa (Figure 1.6).

This method requires special equipment. To overcome these limitations, under the "FLOTAC strategy" of improving the quality of copromicroscopic diagnosis, a new simplified tool has been developed, i.e., the Mini-FLOTAC having an analytic sensitivity of 5 EPG (Cringoli et al., 2017). It is an easy-to-use and low-cost method, which does not require any expensive equipment (i.e., centrifugation requirement) or energy source, so to be comfortably used to perform FECs (Cringoli et al., 2017) allowing easy transfer and very simple application.

For the diagnosis of intestinal protozoa by using the FLOTAC and Mini-FLOTAC techniques the following flotation solutions have been recommended: saturated sodium chloride (NaCl) solution or Sheater (SG = 1.2) for *Eimeria* oocysts (Morgoglione et al., 2020) and saturated zinc sulfate (ZnSO4) solution (SG = 1.2 or 1.350) for detecting *Giardia* cysts (Pepe et al., 2019).

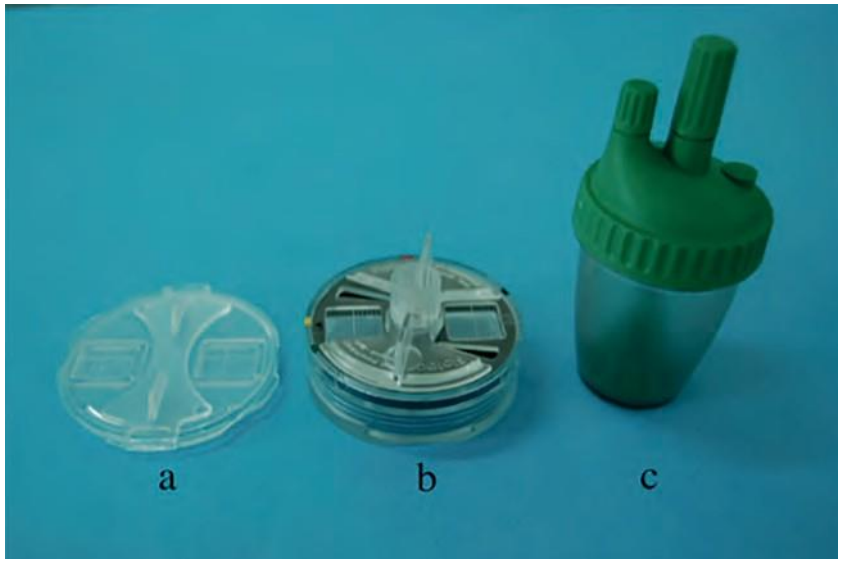

Figure 1.6 (a) Mini-FLOTAC (b) FLOTAC and (c) Fill-FLOTAC apparatus.

1.4.3 Stayning

Various staining procedures can be used to differentiate *G. duodenalis* cysts from coexisting protists and for excluding similarities from environmental or fecal debris.

Smear preparations stained by the trichome and iodine or iron hematoxylin methods can be utilized to assist in the detection of various stages of *G. duodenalis*. The most frequently used routine techniques for examining and to identify *Giardia* cysts are stained slides. The trichrome stains or with ferric hematoxylin or methylene blue safranin (Rajurkar et al., 2012) are useful for highlighting cysts

-*Trichrome stain*. Trophozoites appear as pear-shaped organisms, measuring 12 to 15 µm (range: 10 to 20 µm). Trophozoites contain two anteriorly placed nuclei and 8 flagella (rarely seen because they stain poorly). Cysts appear ovoid to ellipsoid in shape. Nuclei and intracytoplasmic fibrils are visible (CDC).

1.4.4 Immunological assays

Immunological methods offer several advantages over light microscopy in terms of sensitivity and specificity for the detection of *Giardia* cysts in diverse types of samples (Jex et al., 2008; Koehler et al., 2014).

Direct fluorescent-antibody (DFA/DIF), Immunofluorescence assays (IFA), combined with immunomagnetic separations (IMS) and fluorescent-monoclonal-antibody detection have all been used frequently for the detection of these parasitic protozoans (Jex et al., 2008; Koehler et al., 2014). While microscopy does not necessarily require expensive equipment or reagents and is, therefore, more accessible, it is labour intensive, subjective, and requires significant expertise. The use of fluorescent antibody staining (or immunofluorescence assay, IFA) has increased the sensitivity and specificity of microscopy but is still time- consuming and requires a skilled microscopist (Dixon, 2020). Furthermore, microscopical methods do not allow for the differentiation of species and assemblages that molecular methods can afford.

DFA tests offer the highest combination of sensitivity and specificity as traditional microscopic methods, requires less skill and increase laboratory efficiency by reducing labor, time, and costs. These immunoassays are considered the gold standard by many laboratories (Bench Aids- DPDx Laboratories). It is based on fluorescein-labelled antibodies directed against cell wall antigens of *Giardia* cysts and *Cryptosporidium* oocysts allow

visualization of the intact parasites, providing a definitive diagnosis in faecal samples (Johnston et al., 2003). Commercial DFA tests are available, such as MERIFLUOR® *Cryptosporidium*/*Giardia* test from Meridian Biosciences (Figure 1.7) as well as PARA-TECT™ *Cryptosporidium/Giardia* (Medical Chemical Corporation) and IVD® *Giardia/Cryptosporidium* test (IVD Research, Inc). The sensitivity and specificity of the most commonly used commercial DFA test, the MERIFLUOR DFA has been reported to be in range of 96–100% and 99.8–100%, for *Giardia* and *Cryptosporidium*, respectively; moreover, it is more sensitive than conventional staining techniques and it is easy to perform (Johnston et al., 2003)
Additionally, the high quality of the reagents results in minimal background fluorescence or nonspecific staining and enhances identification (Vohra et al., 2012).

Figure 1.7 MERIFLUOR® *Cryptosporidium*/*Giardia* test

1.4.5 Sporulation

Sporulation techinique is used to identify *Eimeria* species.
Oocyst suspensions diluted 1:10 with a 2.5% aqueous potassium dichromate (K2Cr2O7) solution, mixed thoroughly, are placed in Petri dishes (or in wide-surfaced containers) and incubated in a stirring water bath at 26° C for a period from 3 to 10 days. Please note for good oxygenation use for example a 250 Erlenmeyer flask and do not fill with more than 80-90 ml of suspension of oocysts. The sporulation time varies according to the species (deNorah et al., 2009).

Sporulated oocysts were placed into glass bottles kept at 4 °C until they could be processed and identified (Duszynski and Wilber, 1997; Duszynski et al., 1999).

1.4.6 Molecular assays

The application of molecular techniques has resulted in expanded knowledge regarding the taxonomy and epidemiology of *Eimeria* spp. and *G. duodenalis* (Carvalho et al., 2011; Kumar et al., 2013; Xiao and Feng, 2017;). Molecular diagnostics are widely used to differentiate Eimeria spp. and *G. duodenalis* species or *Giardia* genotypes (Dixon et al., 2012; (Chapman et al., 2013).

Eimeria spp. Molecular assays for the diagnosis of *Eimeria* spp. infection are known to have greatly improved through more effective DNA extraction techniques and more sensitive PCR techniques (targeting the ITS-1 region of *Eimeria* spp.) for identifying protozoa (Reginato et al., 2020). In according to Zaho et al., 2001 and Tang et al., 2018 highlight that PCR results can be influenced by the DNA extraction methods used.
Therefore, the PCR is a more reliable, sensitive and less time-consuming approach for diagnosis of Eimer*ia* (Kawara et al., 2010; Malek and Kura, 2018;). Attention is needed to the restriction fragment length analysis (RFLP) rapid, inexpensive, accurate technique to detect infections of various protozoans of cattle (Pyziel et al., 2020).

G. duodenalis. PCR-based methods are rapid and objective compared to microscopy have produced excellent results both in terms of specificity and sensitivity (McGlade et al., 2003) and allow for the determination of the species / assembly through DNA sequencing, detection of multiple targets by multiplexing, and quantitation of results. Although, using a combination of PCR and restriction fragment length analysis (RFLP) sequencing are not need (Groth and Wetherall, 2000; Amar et al., 2002; Cacciò et al. al., 2002). Therefore, such methods as polymerase chain reaction (PCR), real-time PCR, or multiplex PCR together with DNA sequencing can identify species/assemblages with high sensitivity and specificity, and these techniques can be employed to identify sources of transmission as well as the zoonotic potential of these two parasites (de Aquino et al., 2020).
Filtration, flocculation, flow cytometry, immunomagnetic separation, immunofluorescence with monoclonal antibodies and PCR techniques are

the methods currently in use to detect the presence of *Giardia* in water (Slifko et al., 2000).

1.5 Treatment, prevention and control strategies

Eimeria- Control strategies are based generally on methaphilatic treatment and good management pratices to reduce environmental contamination. Normally all animals in a farm should be treated, as even those showing no symptoms are likely to be infected.
Calves should be treated immediately after first signs of coccidiosis with toltrazuril (15 mg/kg b.w., p.o.) or diclazuril (1 mg/kg b.w. p.o.) (withdrawal period for toltrazuril: 63 days; diclazuril: none). Best efficacy is seen in animals that are treated during prepatency.
Natural control methos, e.g., treatment with essential oils, has been used against coccidiosis in poultry (Bozkurt et al., 2016; Sidiropoulou et al., 2020) as well in lambs (Dudkoet al., 2017) and in cattle (Grandi et al., 2016).
Giardia-There is no recommended treatment for infection in calves. Several benzimidazole anthelmintics (e.g., albendazole, fenbedanzole) have been demonstrated effective by a significant decrease in cyst shedding and may prove to be of benefit.
Trials with vaccines against *Giardia* infection in cats, dogs and calves have so far not shown convincing protection (Anderson et al., 2004; Uehlinger et al., 2007).
Prevention of infections is superior to treatment because subclinical production losses and potential permanent damage unresponsive to treatment are cost-prohibitive for producers and have animal welfare implications (Siwila, 2017; Santin, 2020).
Moreover, the control of both intestinal protozoa in buffalo farms is certainly not easy to implement because of the considerable spread and resistance of their (oo)cysts in the environment and in the water for months (Keeton et al., 2017; Adeyemo et al., 2019); thus, prophylactic measures are need to reduce environmental contamination in order to limit the infection pressure (Daugschies et al., 2002).
However, recurrens and reinfections in contaminated environment are common. Therefore, protozoan infections can be reduced through avoidance of overcrowing and stress, and attention to hygiene. Proper hygiene regime (cool and dry, disinfection procedures), raising of food and and drinking

water sanitation troughs, for example, can help avoid contamination by reducing the levels of infections (Daugschies and Najdrowski, 2005).

1.6 Discussion

Eimeria spp. and *G. duodenalis* are common and widespread in dairy farms, including water buffaloes in different parts of world including Italy (Daugschies and Najdrowski, 2005; Rinaldi et al., 2007; Gupta et al., 2016; Bosco et al., 2017; Ojeda-Robertos et al., 2017; Tavassoli et al., 2018; Bangoura and Barsley, 2020; Morgoglione et al., 2020; de Aquino et al., 2020 Volpato et al., 2018; El Afy et al., 2019 Hailu et al., 2020).
These parasites are causative agent of diarreha, poor growth, weight loss, reduced productivity and even death in ruminants (Rinaldi et al., 2007; Bosco et al., 2017; Gillhuber et al., 2014; Dixon, 2020). In general, young buffaloes are more affected by these parasites than are older animals (Cringoli et al., 1998; Helmy et al., 2014; Dubey, 2018; de Aquino et al., 2019, 2020).
Both protozoa may impair health, welfare, and production of buffaloes resulting in important economic losses (Olson et al., 1995; Bosco et al., 2017; Cho and Yoo, 2014; Villanueva et al., 2018; Keeton et al., 2018; de Aquino et al., 2020, Santin et al., 2020).
As regard to *G. duodenalis*, the livestock cycle is thought to maintain Assemblage E (anthropo-zoonotic assemblage) within the livestock group (Thompson et al., 2004). Thus, *Giardia* infection represent a public health concern due to its zoonotic nature and water and foodborne transmission (Feng and Xiao, 2011; Rahman et al., 2020). Giardiosis is included in the World Health Organization (WHO) neglected diseases initiative owing to its burden and association with poverty (Levine et al., 1990; Thurman et al., 1998; Hoque et al., 2002; Leclerc et al., 2002; Leung et al., 2019).
In view of these considerations the protozoa infections control represents the principal challenge in water buffalo farms.
To date, as vaccine prophylaxis measures are not yet available in ruminants (Thompson, 2008; Santin, 2020), best combination of rational treatments, hygiene and sanitation are indispensable tools to reduce the risk of transmission of protozoal infections in water buffalo farms.
As described in Bosco et al. (2015) metaphylactic treatments with toltrazuril was very useful against *Eimeria* infections in water buffaloes and should also contribute to the reduction of environmental contamination with

oocysts and limiting the infection pressure prevention (Keeton et al., 2018; Morgoglione et al., 2020); whilst for *G. duodenalis* the use of anthelmintic drugs, such as Albendazole (Xiao et al., 1996) and Fenbendazole (O'Handley et al., 1997), and paromomycin (Geurden et al., 2006), have proven their efficacy in cattle (Thompson et al., 2008) and in water buffaloes (de Aquino et al., 2020). Moreover, the treatment of calves with febendazole was also helpful in improving the structure and function of microvilli of the intestinal mucosa after seven days from the start of treatment (O'Handley et al., 2001). However, chemotherapy treatment of *Giardia* is controversial in ruminants (Santin, 2020). As reported by O'Handley et al., (2000) no prolong effect has been detected using fenbendazole or paromomycin sulphate, where differences in mean body weight, average daily weight gain, or feed intake between the control and treated groups were not significant.
However, the efficacy of toltrazuril could be increasingly reduced by the development of *Eimeria* resistance in ruminants (Odden et al., 2018a, b) as has already been shown in poultry farms (Chapman, 1984; Noak et al., 2019; Snyder et al., 2021).
Treatment alone is not sufficient for controlling infections in ruminants because intestinal protozoa infections are continuous due to low dosed infections (Rendtorff, 1954; Daugschies and Najdrowski, 2005). Reinfections occurs rapidly and, contributing to the high level of environmental contamination by cysts/oocysts (O'Handley et al., 2000; Geurden et al., 2006).
Indeed, prophylactic recommended methods for controlling infections in ruminants should include good breeding practices, complete cleaning and disinfection of housing, removing and eliminating faecal content or wet garbage, cleaning feeders and drinking fountains (Santin, 2020; de Aquino et al., 2020), in combination with proper therapeutic treatment plan.
Thus, sanitation practices play an important role in the complex control of intestinal protozoa infection in water buffalo farms, reducing environmental contamination pressure and protect animals from infections.
In the case of waterborne transmission, a multiple barrier approach, including limiting access of people and animals to watersheds and reservoirs, and treatment using flocculation, filtration and disinfection, is necessary to minimize the risk (Efstratiou et al., 2017; Omarova et al., 2018; Dixon, 2021). Water treated with chlorine still poses a risk as the infectious stages of many parasites, including *Giardia,* are much more resistant to chlorine than are bacterial pathogens. However, many filtration methods routinely used in water treatment are effective in removing parasite, while

other technologies used in the water industry, such as ultraviolet light, ozone, and irradiation, can also be effective in inactivating parasites (Betancourt and Rose, 2004; Collivignarelli et al., 2018).

During last years, the scientific community, as well as the industries, focused their interest on finding alternative methods to control of protozoal infections. One of these is the use of ozone (O3), a strong agent oxidant that for its features has been readily used in many air and water treatment processes (Rice et al., 1981; Rakness, 2011; Donofrio et al., 2013; Martinelli et al., 2017). In fact, unlike other compounds, O3 doesn't leave any chemical residual. For these reasons, the system of sanitization with O3 has been recognized by the Food and Drug Administration as a safe agent and, in Italy, the Ministry of Health, with protocol n.24482 of 31/07/96, followed by the European Directive 2003/40/CE, recognizes the ozone (O_3) as "Natural presidium for the sterilization of environments contaminated by bacteria, viruses, spores, etc." Some studies demonstrated a high efficacy of O_3 in inactivating waterborne protozoan (e.g., *Giardia, Blastocystis, Cyclospora*, etc.) (Kalifa, 2001; Omarova et al., 2018).

Alternative therapeutic approaches based on ozone in ruminants (Đuričić et al 2015) could be useful to control GI infections as demonstrated in poultry against *Eimeria* (Liou et al., 2002).

Furthermore, the escalating spread of antimicrobial (Sharma et al., 2018; WHO, 2020) and anthelmintic resistance (Vercruysse et al., 2018; Rose et al., 2020) emphasizes the need for a change toward more sustainable control approaches in order to prevent or reverse the development of resistance (Velde et al., 2018). Thus, new sustainable low-cost and eco-friendly control methods against protozoa are urgently required in livestock farms worldwide.

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Capitolo 2

Water ozone treatment as an alternative sanitizing technology: an overview

2.1 Properties of ozone

Ozone (Schönbein, 1839), from the Greek word "*ozein"* (to smell), is an inorganic molecule (CAS n. 10028-15-6) with the chemical formula O_3 determined in 1865 by J.L. Soret (Rubin, 2001; Gottschalk et al., 2009) (Figure 2.1).

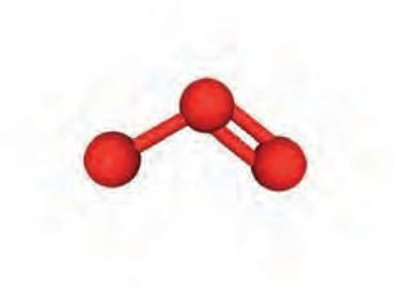

Figure 2.1. Three-dimensional representation of the ozone molecule computed by PubChem. (https://pubchem.ncbi.nlm.nih.gov).

Ozone appears as a colourless to bluish gas that condenses to a dark blue liquid, or blue-black crystals (Lide, 1993). Ozone is a gas at room temperature and pressure and it has a pungent odour less than 2 ppm (Budavari, 1989; Lewis, 1993; Takeuchi, 2005). It is an allotropic modification of oxygen that can exist in all three physical conditions, much less stable than the diatomic allotrope O_2, breaking down in the lower atmosphere to O_2 (dioxygen). The main chemical and physical properties of ozone are shown in table 2.1.

Table 2.1. Main chemical and physical properties of ozone.

Properties	**Value**
Molecular wheight, g/mole	47.998
Boiling point, °C	-111.9
Densitivity, kg/m^3	2.14
Melting Point, °C	-193
Solubility in water, mg/l, 20°C	570
Electrical potential, V	2.075
Energy, Kj/mol	142,3

Ozone is a very strong oxidizing agent, capable of reacting with a large number of organic and inorganic compounds (Cuerda-Correa et al., 2019) directly or through the formation of hydroxyl radicals (Gottshalk et al., 2009; Ngwenya, 2013; Tan, 2015; Singh 2015).

Like peroxide, ozone reactions are most effective in systems with acidic pH. The oxidation reactions proceed with extremely fast, pseudo first-order kinetics. Radicals (-OH) produced by the ozone decomposition in water are the second strongest oxidizers after fluorine (Cretin and Le 2015; Varga and Szigeti, 2016). Its high oxidation potential (E = +2.07 V) confers to ozone a broad‑ spectrum antimicrobial property. Furthermore, the absence of the formation of dangerous by products during the treatment of water with ozone have increased the importance of its application in water treatment during the past decades (Cuerda-Correa et al., 2019).

In water ozone is 49.0 mL/100 mL (at 0 °C), tenfold than oxygen, thus causing an immediate reaction with any biomolecule in biological fluids. Its density (2.14 kg/m^3) is higher than that of air, letting it concentrate close to the ground in indoor environments.

When ozone decomposes in water, the free radicals' hydrogen peroxy (H_2O_2) and hydroxyl (OH) that are formed, have great oxidizing capacity

and play an active role in the disinfection process. It is generally believed that bacteria are destroyed by ozone because of protoplasmic oxidation resulting in cell wall disintegration (cell lysis). The effectiveness of disinfection depends on the susceptibility of the target organisms, the contact time, and the concentration of the ozone (EPA, 1999).
The oxidation reactions proceed with extremely fast, pseudo first-order kinetics. Due to the high reactivity and instability, ozone should be produced on site (Askenaizer, 2003; Eriksson, 2005; Collivignarelli et al., 2018).
Ozone is used as a disinfectant for air and water; used for a wide range of applications such as bleaching waxes, textiles and oils, water treatment for taste and odor control; mold and bacteria inhibitor in cold storage; bleaching agent (EPA, 1998). For decades it has been widely used in a number of industrial processes, such as municipal and industrial drinking water and wastewater treatment such as a disinfectant as well as a chemical oxidant, to oxidize or destroy organic chemicals in the waste stream (EPA,1999; Okada and Naya, 2012; Ikehata et al., 2018). Furthermore, ozone has been used in chemical synthesis, food and beverage, agriculture, air pollution control, medical and dental applications (U.S. FDA 2001; Okada and Naya, 2012; Jegadeeshwar et al., 2017; Brodowska et al., 2018).
While ozone's oxidative power and multiple effects make it a good choice for many industrial processes, it remains underutilized in many areas due to its relatively high capital and operating costs (Gottshalk et al., 2009).

2.2 History of ozone

In 1785 the Dutch scientist van Marum described for the first time the "odor of electricity" in his experiment with electrical discharge oxygen from the largest static electricity machine of that period (Rubin, 2001). However, we wait up to 1839 when the German chemist Christian Friedrich Schönbein noting the smell at the anode of an electrolytic cell, formally classifies and names ozone (Gottschalk et al., 2009). Schönbein immediately recognizes and begins experimentation on the disinfecting qualities of ozone. In 1845, spurred intensive research on ozone's properties reported its ability to bleach colors and its danger to living beings through inhalation on a mouse, finding out its strong oxidizing properties, similar to chlorine.
Almost 20 years later, in 1865, Soret postulated the molecular formula of ozone: O_3.

The development of the first ozone generator by Werner von Siemens in Germany in 1857, and its ability to produce larger quantity of ozone, spurred widespread investigations of its multipurpose capabilities. Quickly, a wide range of applications – from killing microorganism (1873), food preservation agent (1909), improving ventilation of buildings (1912) to cleaning and sterilizing drinking water (1886) and waste water - were investigated (Gottschalk et al., 2009).
The technology spread quickly in Europe, particularly in the Netherlands (1893) where the world's first water treatment plant was installed; but also in France (1898) and in the rest of the world such as in the USA, where in 1896 the genius Nikola Tesla patented the first O_3 generator and later founded the "Tesla Ozone Company (Elvis and Ekta, 2011).
Moreover, ozone was applied in its first application in therapeutic use in 1880, in Michigan (US) where Dr. John H. Kellogg used ozone to treat the Diphtheria.
Since its discovery ozone has been used for more than a century for water treatment, although its use has increased enormously in number and fields (e.g., food processing, packaging cleansing and equipment sterilization) when U.S. Food and Drug Administration (FDA, 2001) recognized as Generally Recognized as Safe (GRAS) for bottled (Betancourt and Rose 2004; Gottshalk et al., 2009; Miller et al., 2013; Varga and Szigeti, 2016; Martinelli et al., 2017).
In Italy, the Ministry of Health, followed by the European Directive 2003/40/CE, recognized ozone as a "Natural protection for the sterilization of environments contaminated by bacteria, viruses, spores, etc." (protocol No. 24482 of 31/07/1996). The Ministry of Health with CNSA of 21/10/2010 also recognized the use of ozone for the treatment of air and water as a disinfectant and disinfestant agent.
Currently, ozone has being applied in several fields of human and veterinary medicine therapy (Elvis and Ekta, 2011; Sciorsci et al., 2020), in drinking water treatment (Rice et al., 1991), in food industry (Brodowska et al., 2018) but also in dairy industry (Varga and Szigeti, 2016).

2.3 Production of ozone

Ozone is well known for its protective role in the earth's ecological environment. In nature, ozone is formed from oxygen by the action of

electrical discharges and short-wave-length ultraviolet (UV) rays within the Earth's atmosphere (Takeuchi, 2005).

Most ozone (about 90%) resides in a stratosphere layer above the Earth's surface from 10 to 17 kilometers and extends up to about 50 kilometers. The ozone in this region is commonly known as the ozone layer which absorbs most of the Sun's ultraviolet (UV) radiation (Gottshalk et al., 2009). The remaining ozone is in the lower region of the atmosphere, which is commonly called the troposphere.

Generally, in industrial and commercial fields, ozone is generated by the exposure of air or pure oxygen, between two electrodes, where high-voltage (electrical discharge) or UV radiation has been applied in order to convert molecules of oxygen to molecules of ozone. Because of its relatively short half-life, ozone cannot be stored, thus it must be produced on-site and on-demand. Ozone (O_3) is created from oxygen (O_2) in nature as well as by ozone generators for commercial or industrial applications. However, ozone quickly reverts back to molecular Oxygen (O_2).

The half-life of ozone in water is a lot shorter than in air (Miller et al., 2013) (Table 2.2). In water, ozone quickly degrades to oxygen, although ozone is extremely soluble in water (at 27 °C, ozone solubility is 580 mg/L).

Several factors contribute to the solubility of ozone in water such as ozone concentration, pH, temperature, purity of water, fluid-dynamic conditions, presence of UV radiations, and concentration of organic and inorganic carbon molecules (Kadre et al., 2001; Gardoni et al., 2012; Miller et l., 2013).

Ozone solubility decreases at higher temperatures and is less stable. On the other hand, the reaction rate increases by a factor of 2 or 3 every 10° C.

Mainly, ozone dissolved in water cannot be applied at temperatures above 40° C, because at this temperature the half-life of ozone is very short (Sharma et al., 2013).

Gaseous ozone		Aqueous ozone	
Temperature (°C)	Half-life[a]	Temperature (°C)	Half-life (min)
−50	3 months	15	30
−35	18 days	20	20
−25	8 days	25	15
20	3 days	30	12
120	1.5 h	35	8

Table 2.2. Half-life of ozone influenced by temperature in gaseous and aqueous medium. (Miller and Brandão, 2013)

Ozone can be generated predominantly by three methods: electrical discharge (Corona discharge), ultraviolet (UV) radiation and electrolysis of water (Christensen et al., 2013; Wei et al., 2017). Each ozone generation occurs with the use of electricity. In addition to photochemical and electric discharge methods, ozone can be produced by thermal and chemonuclear methods (Horvarth et al., 1985).
Among these technologies Corona discharge and UV use air or pure oxygen as feed gas.
The corona discharge techonology accounted for the largest share of the ozone generator market in 2019 (https://www.marketsandmarkets.com/Market-Reports/ozone-generator-market-87276855.html?gclid=Cj0KCQjwyZmEBhCpARIsALIzmnKA0k2875Vc8sg6SOvqrCQ954Z_isDi0eOuRtTszBRZZW9p-kxgQmcaAkigEALw_wcB).

2.3.1 Electrical (Corona) Discharge Method

In 1857, von Siemens developed the first industrial ozone generator, which was based on corona discharges (Gottschalk, 2009).

Corona discharge method involves a flow of dry air (supplied by a compressor) or oxygen (O_2), supplied by an oxygen generator, passed between two high voltage electrodes separated by a dieletric material such as ceramic or glass, generally (Figure 2.2).

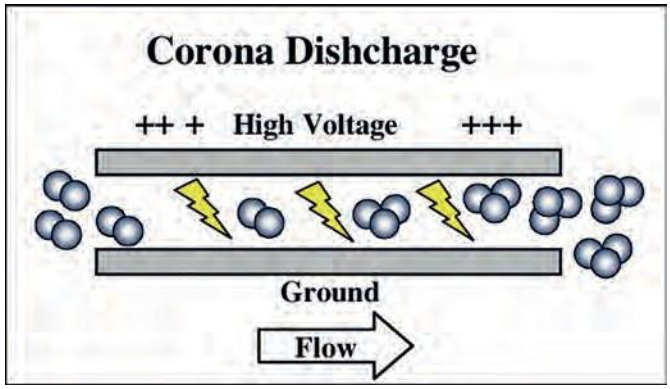

Figure 2.2. Schematic representation of Corona discharge method: Oxygen is forced between high voltage plates to simulate corona discharge. The oxygen is broken apart and recombines into ozone (Gonçalves, 2009).

An ozone production unit with corona-discharge consists of the following parts: oxygen source, dust filters, gas dryers, ozone generators, contacting units and torch destruction. In the ozone generator, the corona-discharge element is present, which provides a capacitive load. In here ozone is produced from oxygen as a direct result of electrical discharge. This corona-discharge ruptures the stable oxygen molecule and forms two oxygen radicals. These radicals can combine with oxygen molecules to form ozone (Figure 2.3). The excessive heat of the electrodes is often cooled by cooling water, or by air.

$$O_2 + e^- \longrightarrow O + O + e^-$$
$$O_2 + e^- \longrightarrow O_2^\circ + e^-$$
$$O + O_2 + M \longrightarrow O_3 + M$$
$$O_2^\circ + O_2 \longrightarrow O_3 + O$$

Figure 2.3 Scheme of Corona discharge reaction. The oxygen molecule is broken and recombines into ozone.

Ozone generation by corona-discharge is most common nowadays and has most advantages. Advantages of the corona-discharge method are greater

sustainability of the unit, higher ozone concentration. The generation of ozone is very energy-intensive, with some 90% of the power supplied to the generator being utilized to produce light, sound and primary heat.
In large-scale systems in industrial application, Corona apparatus has higher cost effectiveness. However, ozone generation is influenced by important factors such as oxygen concentration inlet gas, humidity and purity of inlet gas, cooling water temperature and electrical parameters.
In addiction, corona ozone generator also produces nitrogen oxides as a by-product. Use of an air dryer or oxygen concentrator can reduce or eliminate nitric acid (HNO_3) formation by removing water vapor and increase ozone production. At room temperature, nitric acid will form into a vapour that is hazardous if inhaled (Shindu et al., 1998)
It might be generated by the exposure of air or another gas mixture which contains oxygen to a source of energy such as a high-energy electrical field (corona discharge method), ultraviolet radiation (phytochemical method), or conversion of oxygen molecules (O_2) to ozone (O_3) (chemical method).

2.3.2 Ultraviolet (UV) Method

Ultraviolet or UV light is the light that has a higher frequency than the visible light (Figure 2.4). The visible light spectrum is the segment of the electromagnetic spectrum that the human eye can view. More simply, this range of wavelengths is called visible light. Typically, the human eye can detect wavelengths from 380 to 700 nanometers (https://science.nasa.gov/ems/09_visiblelight).
Ultraviolet (UV) light has shorter wavelengths than visible light occupaying the range between 100 – 380 nm. Moreover, UV light in the range from 160 – 240 nm is capable to generate ozone from oxygen. Ozone is created by the photolysis of the oxygen molecule (O2). This will disrupt the molecule and create valent oxygen atoms (O) that will then attach to any individual oxygen molecules (O2) to create ozone (O3).
Ozone can be produced commercially from an ozone generator using the UV light. A shortwave, low pressure UV lamp produces can be used for this purpose. These lamps will produce UV light with two peaks in the UV light band, one at 254 nm, and another at 185 nm. The 185 nm light is what is referred to as an “ozone producing” lamp, while the 254 nm light is referred to as a “germicidal” lamp.

Otherwise, the UV light (254 nm) will photolyze ozone back to oxygen. It is a simple-to-use dry process which is inexpensive to set up and operate. It can produce near-atomically clean surfaces, in air or in a vacuum system, at ambient temperatures (Kohli, 2019).
UV/Ozone generation, at large scale, presents a very high specific energy demand (~ 1 kW h g-1) due its low efficiency and, therefore, it is very expensive. Compared with the corona process UV/ozone production is not a very efficient to produce large amounts of ozone. However, the UV-light is very suitable for producing ozone in small amounts e.g., for laboratories proposes, odour elimination, etc. A great attractive of photochemical ozone production is reproducibility due to the easy control of the rate of ozone production by controlling lamp source power (Silva et al., 2003).

2.3.3 Electrolysis of water method

Electrolysis is electrochemical procedure in which water molecules (H_2O_2) are split into hydrogen and oxygen atoms applying a potential difference at electrodes, through an electrical external source.
It is well known that water is more favorable to be oxidized to oxygen than ozone, because the oxygen evolution occurs under a much lower anodic potential than that for ozone evolution as shown in the following reactions. In this process, oxidation takes place near anode generating hydrogen ions (H^+) and oxygen (O_2), and reduction takes place near cathode generating and hydroxyl ions (OH^-) and hydrogen (H_2).
The electrolysis of water is generally believed to produce ozone via a 6-electron process as shown by the following equations (Silva et al. 2003, 2006).

$$3H_2O \rightarrow O_3 + 6H^+ + 6_{e^-}\ E^\circ = +1.51V$$

The current efficiency for ozone production can be influenced by many parameters such as temperature and pH.
In this process, water is introduced to the anode side of the electrolysis cell, electrolytically decomposed, converted oxygen in water to ozone. Such apparatuses can enable ubiquitous and low-voltage operation as home electronics (Okada and Naya, 2012).
Therefore, no mass transfer from the gas to the liquid phase is involved and only very fine gas bubbles are formed so that the gas is immediately dissolved in the water. And since the absence of gaseous ozone means that another chemical is avoided and all its related safety precautions, the use of this method is in compliance with FDA regulations and the concept of Green

Chemical Processes (Silva et al., 2003); thus represent another advantage in the production of ozone for foodstuffs and pharmaceuticals (Gottshalk, 2009).
This method is very simple, efficient, and effective. Electrolytic ozone is especially useful for sanitizing water systems. Because the ozone can be created inside the water system, contamination is kept to a minimum and can be well managed and controlled.
The electrolytic generation of Ozone plays an important role in the history of Ozone, because the experiments conducted by Schobein on the synthetic generation of Ozone envisaged its formation starting from the electrolysis of sulfuric acid. This solution has the advantage to require fairly simple equipment so it can be considered for small-scale productions or for use in remote areas. Contrary to the corona discharge, no nitrogen oxides are generated as a by-product in the ozone generator and the ozone yield is significantly higher based on the amount of electricity used.
The potential advantages are considerable: low voltage current (DC) is used, no preparation gas is foreseen, few and simple devices, generation directly in the fluid (water) to be treated, produced in - situ. On the other hand, the main disadvantages are due to the potential corrosion and erosion of the electrodes and on the fact that the water to be treated must have low conductivity. In order to produce ozone, electrolysis system require a suitable electrode such as membrane (Okada and Naya, 2012) or new generation of boron-doped diamond (BDD) electrodes cell (Yao et al., 2011). Boron doped diamon (BDD) is high qualitative alternative to the standard. However, these two types of electrodes also suffer from high cost or short lifetime (Wang and Chen, 2013).
In the recent years, electrochemical ozone production by electrolysis of water has gained some attention in certain applications (Meas et al., 2011).

2.4 Measurement of ozone

Commonly used analytical methods designed to measure ozone levels in water can grouped into physical, physicochemical, and chemical methods.
The choice of the method to use might depend upon application, accuracy needed, budget and available resources.
Physical methods are based on measuring intensity of absorption in the UV, visible, or infrared region of the spectrum.

The UV absorbance method was developed mainly for measuring the ozone concentration in air, but it is also applicable to dissolve ozone in the water – because the UV radiation at 254 nm wavelength is absorbed only by ozone (Majewsk, 2012). The most common commercial ozone analyzers use measurement of optical absorption at 254 nm over an extended path length (Finlayson-Pitts et al., 2000). For accurate determination of gaseous ozone, the UV spectrophotometric method should be used. Nowadays, a wide variety of ozone sensors are commercially available to monitor levels in the working environment. They are usually UV analysers, equipped with a cell that measures concentrations from 0.1 to 100 ppm v/v, that trigger an alarm as soon as the ozone concentration rises above 0.1 ppm.
The physicochemical methods measure physical effects of ozone reaction with different reagents (e.g., KI).
The DPD (N, N-diethylp-phenylenediamine) is a common method used to measure chlorine. It is a US EPA-approved method for measuring chlorine in municipal water supplies. This method is a colorimetric test in which DPD reacts with oxidizers in water (Figure 2.4).
Chemical reaction produces pink color. Its absorption was determined at 510 nm with spectrophotometer. The range of direct determination was 0-1.00 mg/L. The detection limit was 0.01 mg/L (Song et al., 2000). The DPD method also measures other disinfectants that may be present, including chlorine, bromine and iodine.
This is an inexpensive way to measure ozone but is only useful if it is known that no other disinfectants are present.

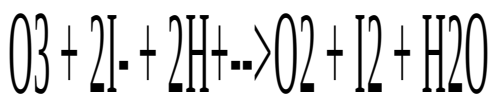

Figure 2.4 Equation reaction between the ozone and potassium iodide in buffered solution.

Chemical methods measure the quantity of the reaction products that are released when ozone reacts with an appropriate reagent (e.g., KI or HI) or the reduction in the molecular weight of a polymer.
Ozone can oxidize potassium indigo trisulfonate from blue to colourless. The concentration of aqueous ozone is determined by the decolorization of indigo trisulfonate (600 nm, pH below 4) whenever the ozone cannot be measured directly by its UV absorption (Bader and Hoignè, 1981). This

method is not expensive, relatively selective and is characterized by fast reaction, stability of the sample after adding reagents < 4-6 h. Furthrmore, secondary products do not interfere; on the other hand, the method needs calibration.
Another way to measure ozone concentrations is to utilize a probe or an electrode that measures oxidation-reduction potential, commonly referred to as ORP or Redox. ORP is not a measurement specifically for ozone but rather all oxidizing agents, including other disinfectants such as chlorine, chlorine dioxide and peroxide. It is commonly used to measure the disinfection of pools and spas. The disadvantage of this method is the interference of high turbidity in water. This can cause ORP readings to be below what is expected (potentially, even a reducing or negative value). Using ORP to measure ozone can only be done accurately if measuring clean water systems that have low turbidity levels.

2.5 The use of ozone in the water treatment

The physicochemical properties of ozone, i.e., its relatively high solubility in water and a high redox potential (which destroys the structure of microorganisms), have enabled its commercial application in the 1880s for deodorisation of industrial waste and disinfection of drinking water (Koppenol, 1982; Kubiak, 2003).
Therefore, thanks to its rapid decomposition into oxygen and the absence of residues, it shows important advantages for application in drinking water and waste water treatment as well as food industries (Rice, 1996; Rodríguez et al., 2008; Wei et al., 2016; Iakovides et al., 2019; Vitali and Valdenassi, 2011; Galdeano et al., 2018).
Ozone has been used continuously for the treatment of drinking water since 1906, when it was first installed in the city of Nice, France, for disinfection purposes. Although many water treatment plants throughout the world still utilize ozone primarily for disinfection, most modern plants rely on ozone to perform one or more oxidation functions (Rice et al., 1981).
The European Community's (EC) environmental regulations aim to sets standards for drinking water and to protect public health from the adverse effects of any contamination (Council Directive 98/83/EC) while for wastewater aim to reduce the pollution of surface water caused by municipal wastewater discharge (Council Directive 98/15/EC) (Collivignarelli et al.,

2018). The Italian legislation (Decree Italian Law 152/2006 and following modifications) provides that wastewater treatment plants with a high capacity of work, with the exclusion of treatment plants that apply natural technologies such as constructed wetlands or lagoons, must be equipped with a disinfection phase (Collivignarelli et al., 2018).

Conventional water treatment includes a series of processes (coagulation, flocculation, clarification through sedimentation, filtration and disinfection) that when applied to raw water sources contribute to the reduction of microorganisms of public health concern (Betancourt and Rose, 2004).

Water treatmen includes mainly two phases. A first phase (oxidation process) of primary ozonation, followed by flocculation and filtration phenomena aims to eliminate heavy metals or organic substances that can be completely destroyed by the oxidizing power of ozone. A second phase (disinfection process) of prolonged ozonation destroys any pathogenic microorganism such as bacteria, virus and protozoa (e.g., *Cryptosporidium* and *Giardia*) and is then followed by filtration on activated carbon which blocks the micropollutants resulting in drinking water (Rice et al., 1981; Collivignarelli et al., 2017; Ikeata and Li, 2018).

Disinfection generally represents the last barrier against pathogen microorganisms, its effectiveness is a crucial point for public health (Martines-Huitle and Brilla, 2008).

Moreover, the disinfection efficiency is affected by several interferences, such as ferrous and manganese ions, nitrites, sulphides and organic substances, that reduce the concentration of oxidizing disinfectants with the consequent reduction of microorganisms' inactivation (Collivignarelli et al., 2017). Thus, oxiding inorganic and organic compounds is necessary for the effictiveness of disinfection process.

Ozone oxides the iron, manganese, and sulfur in the water to form insoluble metal oxides or elemental sulfur. These insoluble particles are then removed by post-filtration. Organic particles and chemicals will be eliminated through either coagulation or chemical oxidation (Lee and von Gunten, 2016).

Despite the availability of many disinfection processes based on different action mechanisms, the conventional processes, which are consolidated technologies, represent the most used treatments. Nowadays, chlorine-based disinfectants are commonly used in Italy (mainly due to their efficiency, low cost and easy use), despite the fact that they may bring by-products to the disinfection process.

The natural disinfection processes could represent valuable solutions, due in particular to the absence of chemical reagents.

2.6 Ozone as an alternative sanitizer

Safe and readily available water is important for public health, whether it is used for drinking, domestic use, food production or recreational purposes.
The WHO/UNICEF Joint Monitoring Programme for Water Supply, Sanitation and Hygiene (JMP) has estimated that in 2017, 29% of the global population (2.2 billion people) lacked "safely managed drinking water"– meaning water at home, available, and safe (WHO | Water supply, sanitation and hygiene monitoring, 2019).
Indeed, disinfection of drinking water and the control of pathogens (e.g., bacteria, virus and protozoa) is of vital importance.
Producing high quality drinking water is a constant challenge since the quality requirements continue to rise as more and more chemical pollutants and microorganisms, such as the cysts and oocysts of parasites (*Giardia*, *Cryptosporidium*) are identified in source waters and concern over disinfection by - products increases.
In general, disinfection treatment has the purpose of breaking down all the bacterial or protozoa load still present in the effluent to reduce the likelihood of infection as much as possible. Water disinfection is accomplished with chemical disinfectants and chlorine (added to water as a gas or solid) is widley used and the specific disinfection is referred to as chlorination. Recent studies have shown that well water is one of the main sources of infection of *Giardia duodenalis* (Giangaspero et al., 2009) and its cysts are unlikely to be inactivated by routine chemical disinfectants or sanitizing water treatments (Caradonna et al., 2017).
However, chlorine use in food applications is associated with various problems, such as the production of several carcinogenic disinfection by-products (DBP), including trihalomethanes and haloacetic acids, derived from the reaction between chlorine and organic material. This concern has prompted some European countries to ban its use for washing organic produce. Indeed, disinfection process must inactivate all pathogens as quickly as possible without causing the formation of residues or by-products harmful to health. Therefore, the disinfection rate of ozone is much higher than that of chlorine based on the CT index as reported by the World Health Organization. Higher dosage value or a longer reaction time must be used

with chlorine. But a dosage of high chlorine rentail the risk of formation of chlorinated by-products. On the other hand, a low dosage of chlorine can promote resistance to chlorine in the bacterial load.

Advantages of ozone disinfection are: (i) it is a highly effective disinfectant for all groups of microorganisms, particularly viruses and bacteria; (ii) it produces very few disinfection by-products; and (iii) ozone generators can treat high volumes of water. The disadvantages of ozone are: (i) it can produce bromate as disinfection by-product if the water has bromide in it; (ii) there is no lasting residual effect; and (iii) reduced efficacy in cold water. (Betancourt and Rose 2004). The disinfectant action of Ozone is expressed through its strong oxidative capacity which in a short time is able to inactivate and destroy microorganisms by breaking the bacterial cell membrane (cell lysis). Chlorine, on the other hand, it spreads through the bacterium's cell wall and causes its death by attacking enzymes. These concerns have induced renewed interest in ozonation and in the ozone- based advanced oxidation processes.

Treatment plants in most European countries tend to use ozone as an oxidation treatment. Ozone has been, for decades, an essential part in the treatment of the largest water treatment plants in big cities such as Paris, Moscow, Helsinki and, in Italy, Turin, Bologna, Ferrara and Pesaro (Gottschalk, 2009; Collivignarelli et al., 2018; Remondino and Valdenassi, 2018).

Furthermore, Ozone treatment is particularly useful in industrial processes, such as municipal and industrial wastewater treatment, drinking water disinfection, food and beverage and in advanced water reclamation and potable reuse applications (Okada and Naya, 2012; Ikehata and Le, 2018).

In future perspectives, the role of natural disinfection treatment should be taken into account due to the control of emerging contaminants as well in dairy farming where the sanitation aspect is limited to milk line production (i.e., against intestinal protozoa which are not currently regulated in Italy). Being a natural substance, the potential environmental sustainability of the use of ozone in other areas (such as water disinfectant; pesticide action in agriculture; and antibiotic, anti-inflammatory and antiviral actions in animal husbandry and fish farming) are of great interest.

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Objiectives

Ozone (O_3) is a well-known antimicrobial agent with several potential applications in the dairy farms, including intensive water buffalo farms (Varga and Szigeti, 2016; Hassan et al., 2017; Megahed et al., 2018).
High reactivity, oxidizing power, capability to react with organic molecules containing double or triple bonds and spontaneous decomposition to a nontoxic product (O_2) make ozone an effective and safe sanitising molecule for microbiological control in water treatment and livestock industries applications. The inhibitory and lethal effects of ozone on pathogenic microorganisms have been observed since the 19th century, and the most cited explanation of these effects is based on the disruption of their external shell Korich et al., 1990; Erikson and Ortega, 2006; Zhang et al., 2011; Megahed et al., 2018).
Ozone has been used for decades in many countries worldwide, and has been recently recognised by the Food and Drug Administration (FDA) as a safe agent. As for Italy, the Ministry of Health, with protocol n.24482 of 31/07/96, followed by the European Directive 2003/40/CE, recognizes O3 as a "*Natural protection for the sterilization of environments contaminated by bacteria, viruses, spores, etc*.". Moreover, ozone has been used with success to inactivate contaminant microflora on food industries (e.g., fruit, meat, vegetables, etc.), poultry, aquaculture and in drinking and waste water sanitation industries.
Although the use of ozone in veterinary medicine can be traced back more 30 years, it is still rarely employed in dairy farms for the treatments of few diseases (e.g., mastitis, vaginitis, enteritis) to reduce the use of antibioticsa. Furthermore, despite ozone is effective against the majority of microorganisms, few studies have been conducted so far about its effectiveness against intestinal protozoa, e.g., *Eimeria* and *Giardia*.

The overall aim of the thesis was understanding the possible application of ozonated water in intensive water buffalo farms in order to limit and control the spread of intestinal protozoal infections.

The specific objectives were:

- To investigate the epidemiology of *Eimeria* spp. in water buffaloes in southern Italy, thus completing the knowledge about intestinal protozoa in this livestock species as epidemiological data on *Giardia duodenalis* and *Crytosporidium parvum* in water buffaloes had been already published (Rinaldi et al., 2007 Cacciò et al., 2007). Furthermore, the published studies on eimeriosis in large ruminants

in Italy are few and focused mainly on treatment (Veronesi et al., Veronesi et al., 2011; Bosco et al., 2015) while the epidemiological data in Europe are scarce, not updated, and focused only on cattle. For this purpose, parasitological data on eimeriosis from a 10-year of active and passive surveillance program were analysed (Chapter 3).

- To investigate a sensitive and cost-effective technique for the detection of *Giardia* cysts in faecal samples. While copromicroscopic techniques are well-established methods for the detection of *Eimeria* oocysts, some concerns still apply to the use of copromicroscopic methods for the detection of *Giardia* cysts. For this purpose, immunoassays and FLOTAC techniques were compared for diagnosing *Giardia* spp. infection (Chapter 4).
- To gain knowledge on innovative ozonated water generators with a polycrystalline diamond electrode and their application in water buffalo farms. For this purpose, as part of my industrial PhD programme, I spent seven months in the UK, the University of Bristol and the Draper Biotech limited (DBL), the latter being an industrial company specialized in air and water purification systems using ozone. Furthermore, some preliminary *in vitro* tests using a well-water medium was performed to evaluate the effect of water ozonation on the viability of *Eimeria* oocysts and *Giardia* cysts collected from water buffaloes. Moreover, a preliminary *in vivo* test was performed in water buffalo calves to evaluate *Eimeria* oocyst output reduction and performance, as weight gain from the antiprotozoal treatment with ozonated drinking water (Chapter 5).
- To evaluate the effect of ozone on viability of *Eimeria* spp oocysts and *Giardia duodenalis* cysts. For the purpose, *in vitro* tests were performed to determine the minimum concentrations of O_3 (mg/l) and the contact times (minutes) necessary to inactivate *Eimeria* oocysts and *G. duodenalis* cysts (Chapter 6).

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Chapter 3

A 10-Year Surveillance of *Eimeria* spp. in Cattle and Buffaloes in a Mediterranean Area.

*Based on the manuscript: **Morgoglione ME**, Bosco A, Maurelli MP, Alves LC, Saralli G, Bruni G, Cringoli G, Rinaldi L. 2020. A 10-Year Surveillance of *Eimeria* spp. in Cattle and Buffaloes in a Mediterranean Area. Front Vet Sci. 7, 410.

3.1 Abstract

Coccidiosis due to *Eimeria* spp. are widespread parasitic infections in cattle and water buffaloes and may impair health, welfare, and production of these livestock species. The aims of this study were (i) to investigate the prevalence and seasonal dynamics of eimeriosis and (ii) to characterize the *Eimeria* species in large ruminants in a Mediterranean area, in order to plan effective control strategies. Parasitological data were obtained from a 10-year surveillance program (2010–2019) on 3,631 farms (2,089 buffalo and 1,542 cattle farms) sampled in central and southern Italy. Pooled fecal samples were analyzed using the FLOTAC technique with an analytic sensitivity of 2 oocysts per gram of feces (OPG) utilizing a saturated sodium chloride flotation solution (specific gravity = 1.200). *Eimeria* species identification was performed by morphometric analysis after a one week incubation of oocysts in a 2.5% potassium dichromate solution. The results showed high prevalence of *Eimeria* (up to 100%) in both cattle and buffaloes in the 10 years of surveillance, even if a slight reduction was reported in the last three years. The overall prevalence of eimeriosis was 91.7% (95% confidence interval, 95% CI = 90.2–93.1) in cattle farms and 81.5% (95% CI = 79.8–83.1) in water buffalo farms. The mean OPG value was 66.8 (min = 2; max = 8,065) in cattle and 55.9 (min = 2; max = 15,415) in water buffaloes, but this difference was not statistically significant ($p > 0.05$). In total, nine species of *Eimeria* were found in cattle the most prevalent being *Eimeria bovis*, *E. ellipsoidalis*, *E. cylindrica*, and *E. zuernii*, whereas in water buffaloes eight species of *Eimeria* were found, the most prevalent being *E. ellipsoidalis*, *E. auburnensis*, *E. bovis*, and *E. zuernii*. Mixed infections were common in both ruminant species. The seasonal pattern showed a higher prevalence of eimeriosis in cattle in spring (86.9%) whereas in buffalo farms the prevalence was higher in winter (82.3%) and summer (82.4%). In conclusion, the 10-year surveillance program indicates that eimeriosis is common in cattle and water buffaloes and therefore continuous effective control strategies are needed.

3.2 Introduction

Coccidiosis due to *Eimeria* spp. are widespread parasitic infections in cattle and water buffaloes and may impair health, welfare, and production of these livestock species (Das et al., 2015; Dubey, 2018a; Keeton and Navarre, 2018). Animals become infected by the horizontal route, ingesting sporulated oocysts from contaminated feed, water, or pasture or by licking contaminated hair coat (Lassen et al., 2014; Das et al., 2015; Keeton and Navarre, 2018). Outbreaks in cattle and water buffaloes are associated with several factors, including the species of Eimeria, the age of the animals, immunological status of hosts, the dose of the oocysts ingested, and farm management and environmental factors (Makau et al., 2017; Lee et al., 2018; Alcala-Canto et al., 2019). More than 20 *Eimeria* species are described in cattle (Lopez-Osorio et al., 2020), and among them, 12 species can affect also water buffaloes (*Bubalus bubalis*) (Cringoli et al., 1998; Dubey, 2019) although coccidia are usually hostspecific parasites. *E. zuernii*, *E. bovis*, and *E. auburnensis* are the most pathogenic species in both hosts worldwide (Tomczuk et al., 2015; Cruvinel et al., 2018), while *E. bareillyi* is a pathogenic species specific only for water buffaloes (Dubey, 2018b). Usually adult animals are asymptomatic, although they can be a reservoir for younger ones (Reddy et al., 2015; Bangoura et al., 2020), whereas calves can show gastrointestinal (GI) signs, such as diarrhea, dysentery, dehydration, debilitation, and even death (Makau et al., 2017; Lopez-Osorio et al., 2020). Compared with cattle, there is limited scientific knowledge about the health of water buffaloes so updated data on parasitic infections (as eimeriosis) is an interesting challenge in this species where knowledge regarding the health consequences of the most common pathologies as well as their economic impact on the entire dairy food chain are still almost rare (Ciuca et al., 2020). Indeed, considering the health and welfare implications, as well as the economic losses due to *Eimeria* infections in ruminant livestock, the knowledge of their geographical distribution, prevalence, and intensity of infection is important to understand the dynamic of infection in relation to biotic (such as age) and abiotic (such as seasonality) factors (Lee et al., 2018) especially in areas where dairy cattle and water buffalo farms coexist and play a major role for the economy of the region (Ciuca et al., 2020). The published studies on eimeriosis in large ruminants in Italy are few and focused mainly on treatment (Veronesi et al., 2011, 2013; Bosco et al., 2015), while the epidemiological data in Europe are scarce, not updated, and focused only on cattle (Rinaldi et al., 2004; Bangoura et al., 2011;

Tomczuk et al., 2015; Bosco et al., 2017; Raue et al., 2017). For these reasons, the aims of this study were (i) to investigate the prevalence and seasonal dynamics of eimeriosis and (ii) to speciate the *Eimeria* in large ruminants in a Mediterranean area, in order to plan effective control strategies.

3.3 Materials and Methods

3.3.1 Study Area and Design

The study was conducted in three Italian regions: Lazio (latitude = 41°53′35″N; longitude = 12°28′58″E) in the Center, Campania (latitude = 40°49′34″N; longitude = 14°15′23″E) and Basilicata (latitude = 40°38′21″N; longitude = 15°48′19″E) in the South. The study area extends over 40,898 km^2 from the Apennines to the Tyrrhenian Sea where cattle and water buffaloes are bred. The entire area is characterized by high heterogeneity with hills and mountains inland and lowlands mainly near the coast. This area is characterized bymild and wet autumns/winters with an average monthly temperature of 9°C and hot and dry springs/summers with an average monthly temperature of 22°C (World maps of köppen-geiger climate classification).

Parasitological data were obtained by the Regional Centre for Monitoring of Parasitosis (CREMOPAR, Campania Region, Southern Italy) from a 10-year program (2010–2019) of active and passive surveillance on 3,631 farms (cattle and water buffalo farms) (Fig. 3.1). Data related to cattle farms in the Lazio region and water buffalo farms in the Basilicata region were fragmented, so they were not included in the study. Moreover, analysis of yearly prevalence and seasonal dynamics of cattle and buffalo coccidiosis was performed only in Campania region, because full data were available through all the years of this study, due to the continuous monitoring service offered by the Department of Agriculture of the Campania Region, through the activities of CREMOPAR.

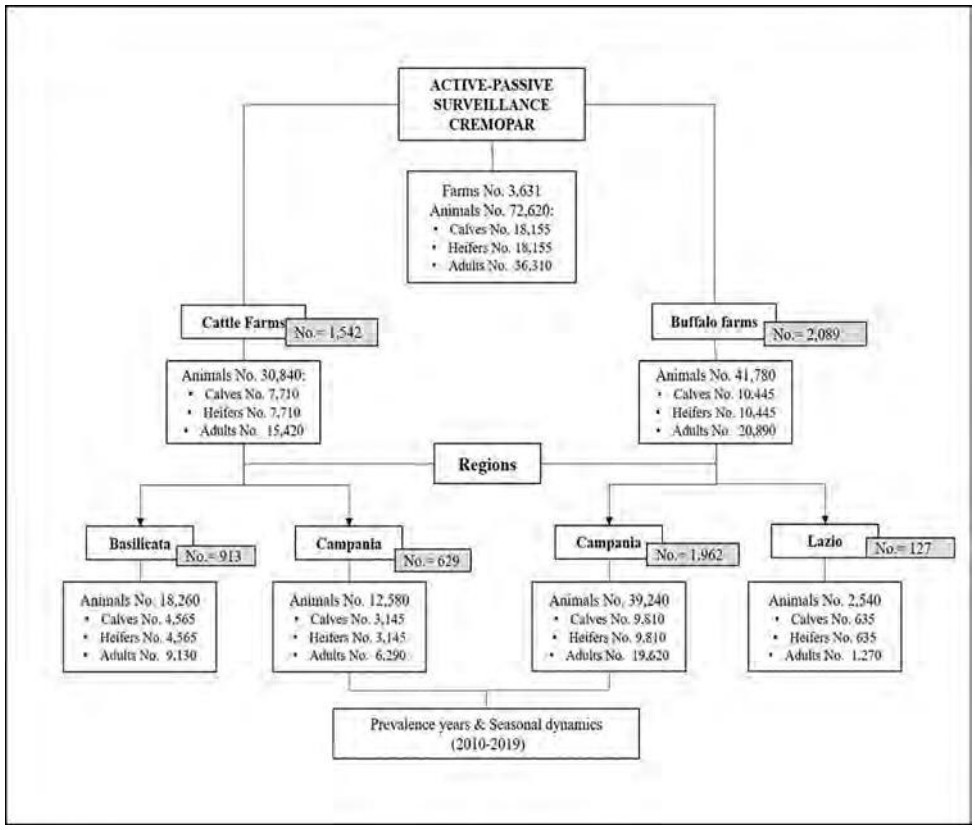

Figure 3.1 Study setup of the analyzed data from the 10-year surveillance program, with the total number of cattle and water buffalo farms, total number of animals and age categories in the Italian regions involved (Lazio, Campania and Basilicata).

3.3.2 Farm Management

3.3.2.1 Cattle Farms

Cattle (*Bos indicus* and *B. taurus*) are the most common world widespread species of large ruminant livestock. Cattle are raised in diverse production systems ranging from capital-intensive, specialized beef and dairy grass-based and feedlot systems (Gilbert et al., 2018). In the study area, cattle are raised for meat and/or milk production. The dairy farms are characterized by an intensive farming system, with suitable buildings and modern equipment to guarantee animal welfare, in order tomaximize the production (Guerci et al., 2013). On the other hand, the meat production is mainly characterized by an extensive farming system, with daily grazing and sheltering in part-time housing. This system allows the animals to graze on

poor soils with minimal vegetation. In the study area, the two productive realities coexist: dairy farms are spread in the plain and in the foothills area, while the beef cattle are on grazing and marginal land. The Italian cattle population amounts to more than 5 million onto 145,363 farms (Fig. 3.2A). The numbers of cattle farms in Campania and Basilicata represent 7.3% and 1.9% of the Italian farms, respectively (National Data Bank—NDB at 31th December 2019).

3.3.2.2 Buffalo Farms

Water buffalo (*B. bubalis*) farming is important for the economy of several countries, including Brazil, China, India, Vietnam, and Italy. Mozzarella cheese manufacturing from milk of water buffalo is third-ranked in sales volume in Italy (Pdo Buffalo Mozzarella Drives Italian Cheese Export: https://news.italianfood.net/2017/02/07/pdo---buffalo---mozzarella---drivesitalian---cheese---export/). The modern intensive water buffalo breeding is likely to replace the cattle breeds and has almost completely replaced the traditional free-range/semi-free-range buffalo farming (Cringoli et al., 2009; Bosco et al., 2017). Currently, the buffalo management is characterized by technologically advanced and automatic systems (e.g., milking robots, automatic manure cleaning, the use of the pedometer for individual measurements of physiological/production parameters, etc.). The southern provinces of Lazio (Latina and Frosinone), the Campania region, and other two southern provinces not included in the study area (Foggia and Isernia) represent the area of buffalo mozzarella cheese with the Protected Designation of Origin (PDO) mark (European Commission: https://ec.europa.eu/commission/presscorner/detail/en/IP_96_492). In Italy, there are 2,711 buffalo farms (Fig. 3.2B) with a total of 402,796 animals. Lazio and Campania are the regions with the highest percentage of the total buffalo farms in Italy with 26.9% and 48.8%, respectively (NDB at 31th December 2019).

3.3.3 Copromicroscopic Analysis

A total of 72,620 fecal samples were collected directly from the rectum of animals involved in the study. In each farm, individual fecal samples (at least 20 g) from 20 animals were collected according to three age groups: 5 calves (0–6 months), 5 heifers (7–12months) and 10 adults (>12months). The collected samples were stored by vacuum packaging (Rinaldi et al., 2011) and sent to the laboratories of CREMOPAR. In the laboratory for each

farm, 4 pools of feces (one for calves, one for heifers, and two for adults) were prepared, taking 5 g of each individual fecal sample (Rinaldi et al., 2019). Pooled samples were analyzed by the FLOTAC technique with an analytic sensitivity of 2 OPG, using a sodium chloride flotation solution (specific gravity = 1,200) (Cringoli et al., 2010).
In order to sporulate the oocysts and identify the *Eimeria* species, the fecal samples from each positive farm (OPG ≥50) were pooled into one sample (at least 10 g), diluted 1:10 with a 2.5% potassium dichromate solution and incubated in a container at 26–28°C for one week, oxygenating the samples several times a day (de Noronha et al., 2009). The *Eimeria* species were identified using the morphometric keys of Eckert et al. (1995) and de Noronha et al. (2009).

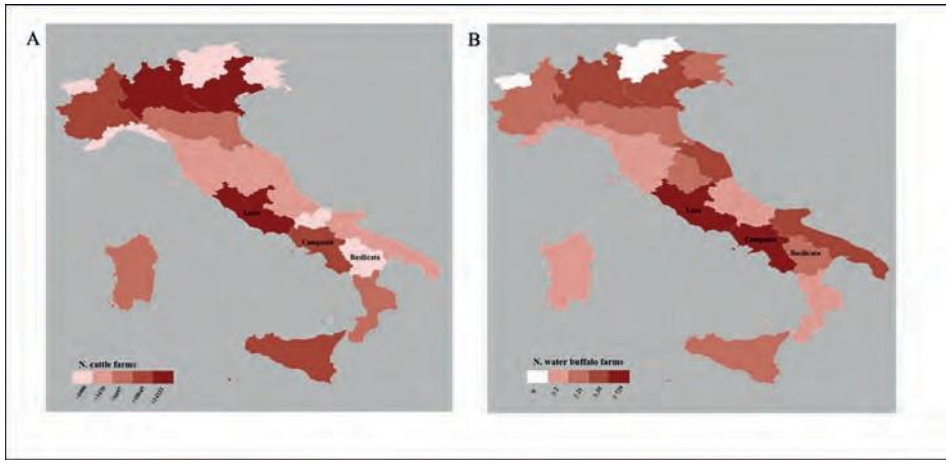

Figure 3.2 Maps of distribution of cattle (**A**) and buffalo (**B**) farms in Italy at 31th December 2019 (data by National Data Bank).

3.3.4 Statistical Analysis

Chi-square (2-test) was employed to verify the association between prevalence and age group of animals and between prevalence of different Eimeria species and regions for both hosts. One-way ANOVA test was performed to detect OPG variability between seasons through the years. Difference was considered significant at $P < 0.05$. These statistical analyses were performed with SPSS 23.0 software (IBM, Armonk, NY, USA).

3.4 Results

3.4.1 Prevalence of Eimeriosis

Eimeria spp. was found in both cattle and water buffaloes showing a prevalence of 91.7% (95% confidence interval, 95%CI = 90.2–93.1) in cattle farms and 81.5% (95% CI = 79.8–83.1) in water buffalo farms with statistically significant difference ($P < 0.05$). In buffaloes from Lazio, the prevalence was higher than in the Campania region with a statistically significant difference ($P < 0.05$). Regarding OPG, the overall mean value was 66.8 in cattle and 55.9 in water buffaloes, but this difference was not statistically significant ($P > 0.05$). These results were represented in Figures 3.3 A, B. The highest prevalence rate and OPG mean values were recorded in young animals (Table 3.1). The one-way ANOVA test showed that calves had OPG values significantly higher ($P < 0.05$) in both cattle and buffalo farms.

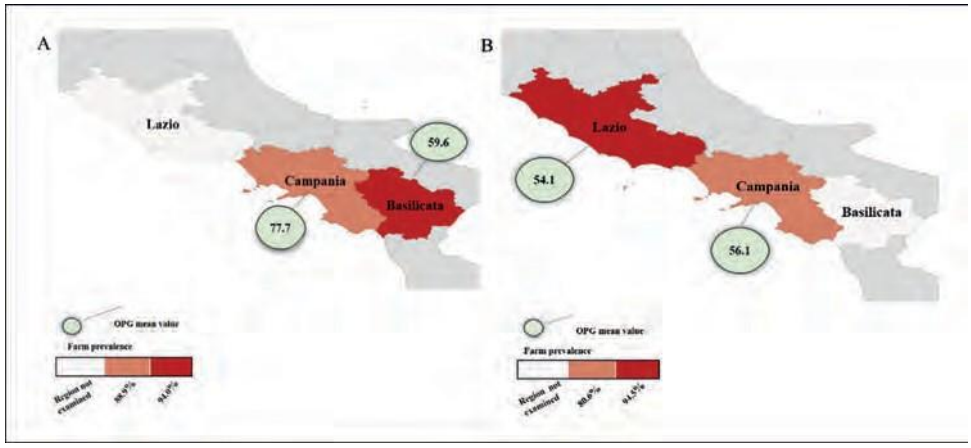

Figure 3.3 Maps of farm prevalence and OPG mean value of cattle (**A**) in Basilicata and Campania and water buffalo (**B**) farms in Lazio and Campania.

Table 3.1 Farm prevalence (95% CI) of *Eimeria* spp., age-group mean OPG, minimum and maximum OPG values, in cattle farms in Campania and Basilicata regions and in buffalo farms in Lazio and Campania regions.

Region	Host	Farm prevalence% (95% CI)	Oocysts per gram of feces (farm prevalence%)				
			Mean (prevalence%)			Minimum	Maximum
			Calves	Heifers	Adults		
Campania	Cattle	88.9 (86.1-97.1)	167.2 (61.0)	65.6 (67.8)	40.1 (38.8)	2	8,065
Basilicata	Cattle	94.0 (92.2-95.4)	188.3 (89.9)	47.4 (87.7)	45.6 (39.8)	2	8,005
Lazio	Water buffalo	94.5 (88.6-97.6)	145.0 (83.3)	46.8 (83.3)	17.0 (74.2)	2	2,250
Campania	Water buffalo	80.6 (78.9-82.3)	256.1 (80.3)	39.7 (77.7)	24.8 (51.8)	2	15,415

3.4.2 Yearly Prevalence and Seasonal Dynamics of Infection

Yearly prevalence of positive farms to Eimeria infection showed a mean of 86.4%in cattle farms and 82.1%in water buffalo farms. A higher coccidiosis prevalence (100%) was reported in cattle farms from 2012 to 2013, in water buffalo farms from 2012 to 2014. Despite the high prevalence of eimeriosis registered every year, a trend of decrease was recorded in the last three years (from 2017 to 2019) in both hosts. The general pattern of the excreted mean OPG was very irregular in both hosts. From 2010 to 2013, the values recorded in cattle and water buffaloes were similar, while the highest OPG values were reported in cattle in 2017 and in water buffaloes in 2016.

Although the annual mean prevalence was highest in spring (86.9%) in cattle farms while in water buffalo farms was highest in summer (82.4%) and winter (82.3%), no statistically significant differences ($P > 0.05$) between seasons were found in either hosts.

3.4.3 Identification of *Eimeria* Species

Nine species of *Eimeria* were found in cattle and eight in water buffaloes (Table 3.2). *E. bovis* and *E. zuernii*, the most pathogenic species in cattle, were present in both hosts and in all the three studied regions. *E. bareillyi*, host-specific and pathogenic for buffalo, was found in Lazio and Campania regions with a prevalence of 13.0 and 11.0%, respectively. Mixed infections were common in both livestock species; in particular, 71.2% of cattle and 39.4% of water buffalo farms were infected with more than one *Eimeria* species. In cattle, the prevalence of *E. subspherica*, *E. zuernii*, *E. bovis*, *E. canadensis*, and *E. alabamensis* was higher ($P < 0.05$) in Basilicata than in the Campania region.

Table 3.2 Prevalence of *Eimeria* species identified in cattle farms in Campania and Basilicata regions and in water buffalo farms in Lazio and Campania regions.

Region	Basilicata	Campania		Lazio
Host	Cattle	Cattle	Buffalo	Buffalo
		Prevalence % (95% CI)		
***Eimeria* species**				
*E. subspherica**	26.2 (20.2-33.1)	12.5 (7.1-20.8)	18.7 (13.1-25.9)	17.2 (10.6-26.4)
*E. zuernii**	40.3 (33.4-47.7)	20.2 (13.2-29.4)	18.1 (12.5-25.2)	18.2 (11.4-27.5)
*E. ellipsoidalis**	43.5 (36.4-50.8)	34.6 (25.7-44.7)	36.1 (28.7-44.3)	36.4 (27.1-46.7)
*E. cylindrica**	36.1 (29.4-43.4)	26.9 (18.9-36.7)	0.0	0.0
*E. alabamensis**	6.3 (3.4-11.0)	1.0 (0.1-6.0)	0.0	0.0
*E. bovis**	78.0 (71.3-83.5)	57.7 (47.6-67.2)	21.3 (15.3-28.7)	23.2 (15.6-33.0)
*E. canadensis**	12.0 (7.9-17.7)	2.9 (0.8-8.8)	0.0	0.0
*E. wyomingensis**	7.9 (4.6-12.9)	7.7 (3.6-15.0)	9.7 (5.7-15.7)	0.0
*E. auburnensis**	8.9 (5.4-14.1)	10.6 (5.6-18.5)	27.1 (20.4-34.9)	26.3 (18.2-36.2)
*E. brasiliensis**	0.0	0.0	0.0	0.0
E. pellita	0.0	0.0	3.2 (1.2-7.8)	0.0
*E. bukidnonensis**	0.0	0.0	0.0	0.0
E. barretlyi	-	-	11.6 (7.2-18.0)	13.0 (10.8-15.9)

**Eimeria* species common to cattle and buffalo

3.5 Discussion

The 10-year surveillance program indicates that eimeriosis is common (up to 100%) in cattle and water buffaloes in the Mediterranean area studied as in different parts of the world (Tomczuk et al., 2015; Gupta et al., 2016; Makau et al., 2017; Dubey, 2018b; Gebeyehu et al., 2018; Alcala-Canto et al., 2019; Bangoura and Bardsley, 2020; Lopez-Osorio et al., 2020). The overall prevalence of *Eimeria* spp. was higher in cattle farms (91.7%) than in water buffalo farms (81.5%). These findings could be explained by the best management practices of modern intensive water buffalo breeding. In particular, the mean coccidiosis prevalence in cattle farms reported in the Campania region in our study (88.3%) was lower than the value of 100% detected in a previous study performed in extensive farms in southern Italy (Rinaldi et al., 2004). For water buffalo farms, the mean prevalence (80.6%), in the decade 2010–2019, showed a small reduction compared to 97.7% reported in the previous decade (2000–2009) in the Campania region (Ghanem et al., 2008; Bosco et al., 2017). Therefore, these results are in agreement with the earlier findings of the 10-year analysis, showing that the epidemiology of *Eimeria* spp. in this study area has changed over time with a slight reduction in the last three years. This decrease may be due to a control plan implemented by CREMOPAR which started in 2014 through the Rural Development Programme (The European Network for Rural

Development (ENRD): https://enrd.ec.europa.eu/country/italy_en) of Campania Region aimed to promote regular and accurate parasitological diagnosis, treatment strategy, and dissemination of best practices of management to cattle and water buffalo farmers. Nonetheless, *Eimeria* is still widespread in the cattle and water buffalo farms. The mean OPG value was 66.8 (min = 2; max = 8,065) in cattle and 55.9 (min = 2; max = 15,415) in water buffaloes, but this difference was not statistically significant ($P > 0.05$). The mean OPG levels were statistically higher in calves (174.3) than in adult animals (43.2), in both livestock hosts, in agreement with other studies performed in cattle in different countries as Pakistan (Rehman et al., 2011), Germany (Bangoura et al., 2012), Kenya (Makau et al., 2017), and Mexico (Alcala-Canto et al., 2019) and in water buffaloes in Brazil (de Noronha et al., 2009) and in Pakistan (Khan et al., 2013). The results of seasonality showed there were no significant differences between the seasons. Some authors found statistically significant differences between seasons and prevalence in animals (Saratsis et al., 2011; Khan et al., 2013; Das et al., 2015; Gupta et al., 2016; Alcala-Canto et al., 2019; Lopez-Osorio et al., 2020), but in the Mediterranean area the large ruminant farming system is mainly intensive and so the presence of *Eimeria* might not be influenced by the weather or by grazing, but rather by overcrowding and herd management (e.g., hygiene of pens).

The most prevalent species of *Eimeria* found in this study were *E. bovis* (67.9%), *E. ellipsoidalis* (39.1%), *E. cylindrica* (31.8%), and *E. zuernii* (30.3%) in cattle. These species were widespread also in other countries (Koutny et al., 2012; Enemark et al., 2013; Cruvinel et al., 2018; Gebeyehu et al., 2018; Lopez-Osorio et al., 2020), while some species, such as *E. pellita*, *E. bukidonensis*, and *E. brasiliensis* (Koutny et al., 2012; Enemark et al., 2013; Cruvinel et al., 2018; Alcala-Canto et al., 2019; Lopez-Osorio et al., 2020), were not found in our study. In water buffaloes, *E. ellipsoidalis* was the most prevalent (36.3%) species, followed by *E. auburnensis* (26.7%), *E. bovis* (22.3%), and *E. zuernii* (18.2%); in addition, *E. bareillyi*, the buffalo hostspecific species, showed a prevalence of 12.3%. These *Eimeria* species were found also in other countries, such as Netherlands, Egypt, Turkey, Iran, Pakistan, India, and Brazil (Dubey, 2018b; Gupta et al., 2016; El-Alfy et al., 2019), while *E. cylindrica*, *E. alabamensis*, *E. canadensis*, *E. brasiliensis*, and *E. bukidnonensis* found by several authors (Dubey, 2018b; Gupta et al., 2016; El-Alfy et al., 2019) were not found in our study. Mixed infections with more than one species were common in cattle and buffalo farms with values of 71.2 and 39.4%, respectively. Of the

Eimeria species detected in this study, only *E. bovis*, *E. zuernii*, *E. auburnensis*, and *E. bareillyi* are responsible of severe clinical disease due to intestinal lesions with effects on the digestive process and overall homeostasis (Daugschies and Najdrowski, 2005). However, the presence of clinical eimeriosis was not assessed in this study and further research is needed to investigate the effects of different species and OPG level on disease development in cattle and buffaloes. *Eimeria* species in cattle and water buffalo are identified only through morphological characteristics, but to date there are no studies showing that species in cattle are genetically identical to the ones in water buffaloes. For this reason, molecular techniques using the 18S ribosomal RNA (rRNA) region can be used, not only to identify *Eimeria* species but also to study intra- and inter-genetic variations in cattle and water buffalo species (Carvalho et al., 2011a; Koreeda et al., 2017). The accurate identification of *Eimeria* species has important implications for disease control (Carvalho et al., 2011b), selection of treatment strategies [e.g., metaphylactic treatments; (Zechner et al., 2015)], and identification of alternative therapeutic approaches [e.g., ozone and intestinal microbiome; (Đuričić et al., 2015; Burgess et al., 2017)]. Metaphylactic treatments with toltrazuril was very useful against *Eimeria* infections in cattle (Veronesi et al., 2013; Philippe et al., 2014; Zechner et al., 2015), as well as in water buffaloes (Bosco et al., 2015), showing improved performances in animals (e.g., faster body weight gain, positive influence on the average age at the first birth, increased overall percentage of pregnancies). Moreover, a reduction in oocyst excretion was demonstrated, with particular reference to the two species considered to be mainly responsible for clinical coccidiosis (*E. zuernii* and *E. bovis*) (Veronesi et al., 2013). Therefore, the metaphylactic approach should also contribute to the reduction in environmental contamination with oocysts, limiting the infection pressure (Daugschies et al., 2007; Veronesi et al., 2013). However, the efficacy of toltrazuril could be increasingly reduced by the development of *Eimeria* resistance in ruminants (Odden et al., 2018). Thus, new lowcost and eco-friendly anti-*Eimeria* strategies are urgently required. Alternative therapeutic approaches based on ozone in ruminants (Đuričić et al., 2015) could be useful to control *Eimeria* infections as demonstrated in poultry (Liou et al., 2002). Moreover, recent studies have highlighted the complex network of interactions occurring between protozoa and the gut commensal flora, showing the potential contribution of the intestinalmicrobiome in the control of parasitic infections (Burgess et al., 2017). In conclusion, the findings obtained showed that the coccidiosis is a

persistent and complex problem, so a combination of good management practice, affordable diagnostic techniques, and strategic treatments (traditional and/or alternative) could be useful to plan an effective control of *Eimeria* infections in large ruminants.

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Chapter 4

Comparative cost-effectiveness of immunoassays and FLOTAC for diagnosing *Giardia* spp. infection.

*Based on the manuscript: Pepe P, Ianniello D, Alves LC, **Morgoglione ME**, Maurelli MP, Bosco A, Cringoli G, Rinaldi L. 2019. Comparative cost-effectiveness of immunoassays and FLOTAC for diagnosing *Giardia* spp. infection Parasit Vectors. 12(1):158

4.1 Abstract

Giardia spp. is a protozoan pathogen and is the most common enteric parasite of domestic animals and humans. Assays for detecting infection in fecal samples using direct or indirect examinations are important tools for diagnosing the disease. The objective of the present study was to compare the cost-effectiveness of immunoassays and FLOTAC technique for diagnosing *Giardia* spp. infection in dogs.
Fecal samples from 80 positive stray dogs were tested for the presence of copro-antigens of *Giardia* spp. using the direct immunofluorescence assay (IFA), a rapid enzyme-linked immunosorbent assay (ELISA) and the FLOTAC double technique. All methods were performed in accordance with the instructions reported in the original description for each technique. The results showed that ELISA can be run in less time than IFA and almost at the same time of the FLOTAC technique. Among the tests used in this study, FLOTAC had the lowest cost per correct diagnosis, compared with immunoassays.
The results from this cost-effectiveness analysis, in combination with the sensitivity and specificity of the FLOTAC technique, suggest that the FLOTAC technique can be use in the routine diagnosis of *Giardia* spp. Infection in dogs.

4.2 Introduction

Among protozoal infections, giardiasis is the most common disease in a wide variety of animals, including humans (Robertson, 2014). Once a person or animal has become infected with *Giardia* spp., zoonotic transmission cycles may occur (Esch, 2013). In fact, new evidence has shown that there is a strict genetic relationship between some *G. duodenalis* genotypes isolated from infected humans and dogs (Marangi et al., 2010). In particular, assemblages A (subtypes I and II) and B (subtypes I and IV) have been associated with human infections (Zheng et al., 2014) but are also found in a number of other mammalian hosts (Vanni et al., 2012), and assemblage C from dogs was found in humans in Europe (Štrkolcová et al., 2015), thus suggesting the possibility of interspecies transmission (Capelli et al., 2006). *Giardia* infection in dogs is an important disease in veterinary medicine (Bouzid et al., 2015) and infected animals show clinical signs of disease two to three weeks after infection (Serradell et al., 2016), characterized by diarrhea, vomiting, weight loss and lethargy. However, some animals do not present any clinical signs (Volkamann et al., 2017). Surveys on a variety of

canine populations have revealed a prevalence of *Giardia* infection ranging from 10% among well-cared-for dogs to 36–50% among puppies and up to 100% among kennel dogs, which are at highest risk of disease transmission (Uchôa et al., 2018). Many factors appear to affect the prevalence of the infection including the animal's characteristics (age, living conditions, animal density, nutritional status and immune status) and the diagnostic test used (Thompson et al., 2007).

Assays for detecting infection in fecal samples using direct or indirect examinations are important tools for diagnosing the disease (Al-Saeed et al., 2010; Weitzel et al., 2006; Salman, 2014). In general, the diagnosis is based on the detection of *Giardia* cysts (and occasionally trophozoites) in the feces of infected dogs (Olson et al., 2010). The traditional approaches, such as use of fecal smears and flotation in tubes, have significant limitations due to the small size of the cysts. Moreover, shedding of cysts is intermittent, even in chronically infected individuals, thus requiring multi-day fecal examination (Rishniw et al., 2010). Therefore, more sensitive diagnostic immunoassays such as the immunofluorescence assay (IFA; regarded as the "gold standard"), immunochromatography and the enzyme-linked immunosorbent assay (ELISA) (Jahan et al., 2014) have been recognized as important tools for detecting *Giardia* spp. in fecal samples from dogs. A new technique known as FLOTAC has been developed and proposed for diagnosing enteric parasites in animals and humans. In several studies, it has been shown to have high sensitivity, specificity and accuracy (Cringoli et al., 2010). The aim of this study was to compare the cost effectiveness of immunoassays and FLOTAC technique for diagnosing *Giardia* spp. infection in dogs.

4.3 Materials and Methods

The objective of the present study was to compare the cost-effectiveness of immunoassays and the FLOTAC technique for diagnosing *Giardia* spp. infection in dogs. A total of 80 positive fecal samples according to the gold standard IFA test, were included in this study. All samples were from stray dogs living in the city of Naples (Campania region, southern Italy) that had been brought to the veterinary Hospital of the School of Veterinary Medicine. The technicians were blinded to patient history and results of tests. Three methods were used: the IFA test using a MeriFluor ® *Cryptosporidium/Giardia*, (Meridian Bioscience Diagnostic, Cincinnati, OH, USA), a rapid ELISA using the IDEXX SNAP® test (Idexx Laboratories Inc., Schiphol-Rijk, Netherlands), and the FLOTAC double technique (Cringoli et al., 2010) in which zinc sulfate (specific gravity = 1.350) was used as the flotation solution. Magnifications of 100× and 400×

were used to identify protozoan cysts. The results were expressed as the arithmetic mean of the number of cysts per gram (CPG) of feces. In order to evaluate cost-effectiveness, the IFA test was used as the gold-standard test. In using IFA, the numbers of Giardia spp. cysts found were ranked into the following three levels: 1 (1 cyst); 2 (1–2 cysts); and 3 (3–4 cysts) per reading area. All methods were performed in accordance with the manufacturer's instructions. The sensitivity, specificity, positive predictive value (+PV), negative predictive value (-PV), accuracy, true estimated prevalence and incorrect classification were determined in comparison to the IFA technique as the gold standard. The InStat software 3.01 (GraphPad Software, Inc., San Diego, California, USA) was used to calculate all parameters. To assess the cost effectiveness of the immunoassays and the FLOTAC it was considered that laboratories would have all the necessary equipment to undertake the tests. To calculate a measure of agreement between IFA, ELISA and FLOTAC, the results were assessed using Cohn's Kappa coefficient with 95% confidence interval.

4.4 Results

Among the 80 samples examined, all (100%) were found to be positive by the FLOTAC test. The only test that revealed a negative sample was ELISA. The costs of all the kits were ascertained based on an internet survey of the commercial kits available for diagnosing Giardia spp. (Table 4.1). The time taken to analyze the samples using each of the techniques and the sensitivity and specificity of each diagnostic test kit for *Giardia* spp. are shown in Tables 4.2 and 4.3, respectively. Comparing the sensitivity and specificity of these tests, FLOTAC and IFA have the same capability to diagnose *Giardia* spp. infection in dogs but the FLOTAC technique showed higher sensitivity than ELISA. The Kappa test showed a good and a very good agreement of 1.00 (IFA/FLOTAC) and 0.98 (IFA/ELISA), respectively.

Table 4.1 Direct costs of diagnosis kits of Giardia infection based on an internet survey of commercial kits.

Test	Mean (US$)	Minimum (US$)	Maximum (US$)
ELISA	11.4	8.71	16.3
IFA	9.8	7.20	14.6
FLOTAC	1	0.50	1.5

Table 4.2 *Giardia* infection according to target of test, time and cost by sample.

Test	Target	Result (min)	Cost/sample
ELISA	Antigen	11-12	+++
IFA	Antigen	40-50	++
FLOTAC	Parasite	12-15	+

Note: One sample is required for all tests

Table 4.3 Evaluation of immunoassay tests and FLOTAC technique compared to the immunofluorescence antibody test as a gold test in diagnosis of *Giardia* spp. infection in dogs.

Parameter (%)/technique	ELISA	FLOTAC
Sensitivity	98.75	100
Specificity	100	100
True prevalence	100	100
Estimated prevalence	98.00	100
Predictive value (+)	100	100
Predictive value (-)	0	0
Accuracy	98.75	100
Incorrect classification	1.25	0

4.5 Discussion

The coproparasitological diagnostic tests for *Giardia* spp. in dogs that have been used include direct smears, fecal flotation, centrifugal fecal flotation, IFA, ELISA and polymerase chain reaction (PCR) assay. These tests can be used either alone or in combinations in order to improve the sensitivity (Tangtrongsup and Scorza, 2010). Moreover, it has been reported that to make a true diagnosis of *Giardia* spp. infection in dogs, immunoassays need to be used because the sensitivity and specificity of these tests are higher (Rishniw et al., 2010). One step ELISA and immunofluorescence assays

have been recognized as important tools for detecting *Giardia* spp. in fecal samples from dogs (Cerak and Bauer, 2004; Gundlach et al., 2005; Dryden et al., 2006; Rishniw et al., 2010). Several studies comparing diagnostic tests for *Giardia* spp. infection in dogs have shown that parasitological tests and immunoassays have similar performance (Uehlinger et al., 2017) and that they need to be used together (Costa et al., 2016). On the other hand, almost all studies have shown that immunoassays were more sensitive and that they improved the accuracy of diagnosing *Giardia* spp. infection in dogs (Zimmer and Burrington, 1986; Decock et al., 2003; Cerak and Bauer, 2004; Gundlach et al., 2005; Dryden et al., 2006; Geurden et al., 2008; Rishniw et al., 2010). IFA is the serological test that is most used for diagnosing *Giardia* spp. infection, given that it is regarded as the gold-standard test. ELISA is also widely used, not only because it is a highly sensitive and specific test, but also because it is very easy to use. Since immunoassays detect antigens, it can be expected that both ELISA and IFA would detect more dogs as positive than would tests based on cysts, such as the FLOTAC technique. However, in the present study, it was observed that the FLOTAC technique showed the same sensitivity and specificity as IFA and a higher sensitivity than ELISA. Dog owners generally associate giardiasis when their pets presenting the symptom of diarrhea. However, some animals remain asymptomatic (Olson et al., 2010) and sometimes they are erroneously treated. These animals present lower numbers of cysts in stool samples and false-negative test results may occur. In this regard, antigen tests are not indicated for the follow-up of patients with persistent symptoms after being treated for giardiasis, because the test sensitivity is compromised (Strand et al., 2008). For detection of intestinal protozoa, the FLOTAC technique has been reported as a promising test in comparison with other parasitological techniques (Becker et al., 2011). Furthermore, Speich et al. (2010) made a comparative cost assessment of the FLOTAC and Kato-Katz techniques for diagnosing soil-transmitted helminths, and they found that the cost of the FLOTAC technique was higher than the cost of Kato-Katz, when salaries and costs due to materials and infrastructure were included. However, to our knowledge, the present study is the first to compare FLOTAC with immunoassays. To make a diagnosis of dog's giardiasis, the laboratories should have the necessary equipment for the accomplishment of the tests, particularly for immunoassays. All three techniques, IFA and ELISA and FLOTAC, can be performed at an unsophisticated laboratory, but FLOTAC is a diagnostic tool that is easy to apply for routine diagnosis of *Giardia* spp. infection in dogs. Analysis on the time taken and the samples required for making the diagnosis using each of the techniques showed that

ELISA could be run in a shorter time than IFA and that this time was closer to that required for the FLOTAC technique (Table 4.2). The time that has elapsed between the onset of clinical signs and making the diagnosis of giardiasis in dogs is an important point because some animals show severe diarrhea that may be fatal if left untreated. Among the tests used in this study, FLOTAC had the lowest cost per correct diagnosis, in comparison with the immunoassays (Table 4.1). In this study there was very good agreement between results obtained with IFA and FLOTAC. The discordance between IFA and ELISA assay can be explained by the detection limits for this test, which detects cyst wall proteins (Uiterwijk et al., 2018).

4.6 Conclusions

The results from this cost-effectiveness analysis, in combination with the sensitivity and specificity of the FLOTAC technique, suggest that the FLOTAC technique can be used in making routine diagnoses of *Giardia* spp. infection in dogs.

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Chapter 5

Innovative ozonated water generators: industrial activities

5.1 Industrial company

DraperBIOTECH is the industrial company partner involved in the PhD programme.
This company is the last-born section (2016) in the largest "family" of draperVENT specialised in selling and installing package ventilation systems in poultry broiler and free-range layer houses throughout the United Kingdom (UK). DraperBIOTECH (Figure 5.1) specialises in the purification of both air and water utilising ozone as eco-friendly and alternative sanitation compound to prevent oral-faecal transmission of *Campylobacter* and *Necrotic Enteritis* in poultry. Ozone treatment was associated with increased weight gain and this trend was evident at all poultry ages. There was no difference in feed consumption between groups, meaning than feed conversion rate (FCR) was improved with ozone treatment.
The ozone applications bias the birds gut flora towards aerobic respiration. Odour from the litter and the occurrence of *Pododermatitis* are reduced. FCR significantly improves.
The company has been employed and experimented two separate sanitation lines, air and water, to control bacterial infections for chicken houses.
The water ozonation system has been developed within the use the boron doped diamond electrode in order to generate ozone directly in drinking water for animals. The ozonated water produced is injected directly into the continuous flow of drinking water for animals.

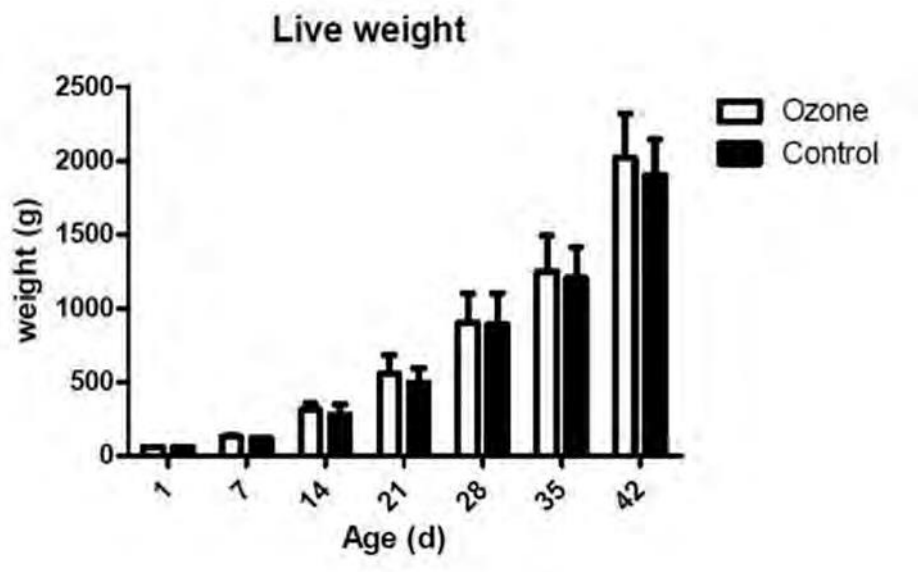
Fig. 5.1 – The DraperBIOTECH Chief Executive Officer.

5.2 Industrial activities

The general objective of the industrial PhD programme is to support the promotion and strengthening of higher education of student specialization in industrial field.
To gain technical specific knowledge on ozone to implement the OZO-PAR system, I spent a period of seven months at the Research and Development Sector and the Production Sector of the DraperBiotech Limited (DBL), Somerset, United Kingdom (UK).
Firstly, the training was spent at the company headquarter and focused on background information on physical and chemical properties of ozone (O_3), its oxidative capacity, chemical reactions in water, etc. Practical training on the use of the electrolytic ozone generator prototype, related equipment and operating parameters (e.g., temperature, pH value, inorganic and organic compound, etc.) for the purpose of *in vitro* and *in vivo* was carried out.
Moreover, the training in UK included the visits in poultry and cattle farms. The poultry farm, located in the Somerset, South England, is an intensive broiler livestock farm, consists of seven chicken houses with full/empty cycle. Here, in one chicken house, ozone is used to sanitize the air by Corona discharge plant; however, ozonated water generated by the electrolytic cell system in drinking water flow was used as an alternative methaphylatic intervention (Figures 5.2 a, b). The effectiveness of ozone is assessed on the weight gain of chickens at the slaughter. Studies conducted in collaboration with University

Figure. 5.2 The poultry farm, located in the Somerset, South England. a) House chicken indoor view; b) Drinking water pipefitting system for chickens

of Bristol showed that ozone has an effect on the weight gain of treated chickens compared to non-treated chicken as control (Graph 5.1).



Graph 5.1. Excerpts from trials report conducted by University of Bristol (http://www.drapervent.com/solutions/biotechsolutions)

The second study site was a fattening Aberdeen Angus farm located in Salisbury, Wiltshire County, UK (Figure 5.3).

Figure 5.3 Aberdeen Angus calves

The ozonated water was given continuously in drinking water to young calves (Figure 5.4) at about 0.3 mg/l. Preliminary results showed ozone scouring stopped calves grew faster than untreated animals and less antibiotics were used (Morgoglione et al., 2019). The mechanism of this effects is not well understood. Unfortunately, there is a lack of studies in ruminant livestock regarding ozonated drinking water as alternative drug to control parasitic infections *in vivo*. In veterinary medicine, the ozone is still used as local therapy (Sciorsci et al., 2020). Whilst, Remondino et al. (2018) referred the use of 0.2-0.5 mg/l ozonated water in swine animals. Introduced in the intestine, ozone restores a suitable *eubiosis* by reducing the importance of pre/probiotic additions or enhancing them (synergistic effect). The ozonated water used in the Aberdeen Angus farm (Salisbury, UK) was generated in the point of use by a farm box (figure 5.5).

Figure 5.4 Aberdeen Angus calves and free access watering hole (Salisbury, UK)

Figure 5.5 Ozone farm box system installed in the Aberdeen Angus farm (Salisbury, UK).

The farm box was composed by 3 main parts: electrodes, cells and pipefitting.

Electrodes. The electrodes are constructed of a chemically resistant, electrically conductive, fluoropolymer that has an active surface of electrically conductive sp3 monocrystalline carbon on one side and an embedded current distributor on the opposite side. The current distributor is a fine copper mesh embedded in the surface of the fluoropolymer and insulated by a further layer of polymer to prevent electrolytic corrosion during operation. The electrodes are designed to optimise the edge length to surface area ratio in order to maximise current efficiency and ozone generation. The maximum recommended current density of the electrodes installed is 1 Amp/cm^2. The electrodes are designed to fit inside standard metric pipe sizes, in this case 25mm OD (outside diameter). The maximum current that can be applied to the electrodes installed is 2 Amps.

Cells. The electrolytic cell is constructed of a pair of identical electrodes, described above, separated by a proton exchange membrane. This may also be described as a cation exchange membrane as it permits the passage of calcium ions and other positively charged metal ions as well as protons (hydrogen ions) to pass through the membrane to the cathode. The proton exchange membrane is a copolymer of perfluorosulphonic acid and polytetrafluoroethylene (PTFE). This membrane prevents the passage of negatively charged anions, such as chloride, to pass through to the anode. This specific Nafion membrane permits polarity reversal thereby preventing the electrodeposition of calcium and other metals on the cathode. Polarity reversal of the electrodes is required to prevent passivation that ultimately results in the insulation of the electrodes and the failure of the cell to generate ozone. The special membrane is described as a solid polymer electrolyte and is extremely hygroscopic. The electrodes are clamped together, either side of the membrane, by two PEEK (polyether ether ketone) bolts. These bolts to not conduct electricity. The membrane extends all around the electrodes by at least 3mm.

Pipefittings. The assembled electrolytic cell is slotted into the pipefitting and the two cables (one from each electrode) exit through a special, watertight, cable seal as shown on the drawing. The cell is held in the centre of the pipe by the extended PEEK bolts. The supporting pipe is transparent (i.e., contains no pigment) so that the operation of the cell may be observed. A flow switch is located in front of the cell. When flow is detected, an electrical signal is sent to the control box which immediately turns the electrolytic cell

on immediately. When flow ceases, the cell is turned off. A simple sieve or filter should always be installed in front of the cell to ensure that water flows freely through both the flow sensor and the cell (Figure 5.6).

Figure 5.6 Schematic diagram showing pipefitting with flow sensor

During my PhD, thanks to the EU COST Action COMBAR (CA16230-43831), I also had the possibility to perform a Short-Term Scientific Mission (STSM) at the Bristol Veterinary School, Faculty of Health Science, Langford, Bristol, UK.
The topic was: "Comparison between conventional microscopy (Mini-FLOTAC) with DNA-based technology for helminths, protozoa and microbiome in large ruminants."

After the experience in UK, once in Italy, the activities related to *in vitro* tests were performed at the Regional Center for Monitoring of Parasitosis (CREMOPAR), Department of Veterinary Medicine and Animal Production, University of Napoli Federico II.
The ozone generator employed for the in vitro experiments was a bench prototype that uses an electrode and an electrochemical cell provided by Mr Patrick S. Bray, the inventor of electrolytic ozone generators

5.3 Electrolytic ozone generator prototype

The ozone generator employed for the *in vitro studies* uses electrochemical cells within an electrode to produce ozone through the electrolysis of water. Electrochemical cells for the production of ozone from water generally comprise an anode and a cathode, with the anode and cathode being separated by a semi-permeable membrane, also referred to as a proton exchange membrane. The electrochemical production of ozone from water may be represented generally by the following formula:

$$3H20 \rightarrow 03 + 3H2 \quad \Delta H°298 = 207.5 \text{ kcal}$$

The reaction at the anode of the electrochemical cell may be represented by the following formula:

$$3H20 \rightarrow 03 + 6H+ + 6e-$$

In the specific case the electrochemical cell included. the electrode (Figure 5.7)

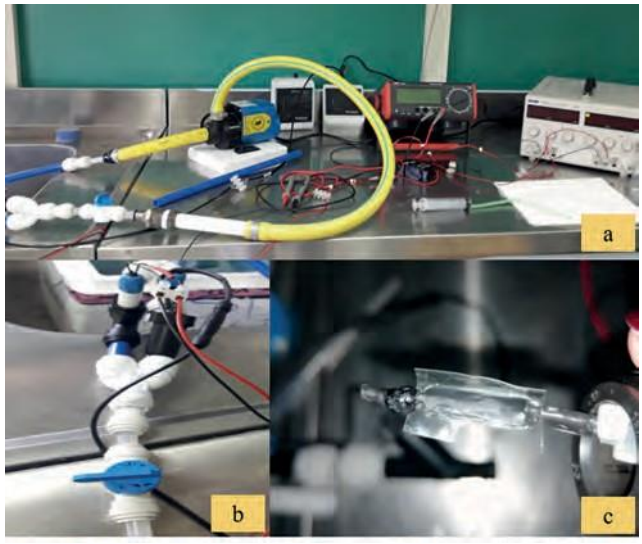


Figure 5.7 Laboratory ozonated water generator apparatus assembled with a water pump, an electric power generator, a series of pipelines made by PMMA (polymethylmethacrylate and voltage meter (a); detail of the water flow joint with electric wires inserted (b); single electrode generating ozone in water.

5.4 Equipment

Below a description of the various components of the bench ozone generator.

a—Electrolytic cell
The electrode assembly comprises an electrode body. The electrode body is formed by polycrystalline diamond.
The polycrystalline diamond may be formed using any suitable technique. More particularly, the electrode body is cut from a diamond wafer, for example by means of a laser. A particularly preferred diamond material is a doped diamond material, more preferably boron-doped diamond.
The electrode body comprising first and second opposing contact surfaces, the first contact surface for contacting a semi-permeable membrane; wherein the electrode assembly further comprises a first layer comprising an electrically conductive material, the first layer extending across at least a portion of the second contact surface of the electrode body.

b-Electricity generator
It is a commercial device to furnish electricity for electrolytic cell.

c- Alternator
It is a clock switch to generate alternate current flow.

d-Hydraulic pump
The hydraulic pump is a mechanical source of power that converts mechanical power into hydraulic energy (hydrostatic energy i.e., flow, pressure). It generates flow at rate of 1 L/min. When a hydraulic pump operates, it creates a vacuum at the pump inlet, which forces liquid from the reservoir into the inlet line to the pump and by mechanical action delivers this liquid to the pump outlet and forces it into the hydraulic system.

e- Control Box
This device was used to signal proper functioning of electrodes depending on voltage modification.
Basic setting: (i) the water flow should be more than 1 L/minute if it is less, the flow switch will turn the current off and the Red light will come on; (ii) the flow switch (the Y fitting) is facing vertical. If it is lying on its side, the pressure sensor will stick (and will not work properly).

The control box has coloured lights to signal any variation of. The scale colour lights set a range from green, passing through orange up to red.

1. Green light is “on”, means that the Cell is operating at normal voltage (17 and 24 Volts) for tap water.

This is normally the case for mains (tap) water where the conductivity is around 700µS/cm.

2. Orange light is “on”, means that the voltage of the Cell has risen above 24 Volts, but the cell is still working correctly and delivering the required current (1.0 Amp).
3. Red light is “on”, means that the voltage has increased to 26 Volts or more. The Red light will remain “on” until 32 Volts.
   In case of the Cell voltage exceed 32 Volts, the Control Box turns the current “off”.

If the single RED light is turned “on” whilst the water flow is greater than 1 L/minute, this signifies that the electrical resistance of the Cell has increases and the maximum permissible voltage of the Cell has been reached.

f- Flow Switch

This component is integrated in electrolytic cell by detecting any variation on electric current flow.

g-Voltmeter is an instrument used for measuring electric potential difference between two points in an electric circuit. It is connected in parallel. It usually has a high resistance so that it takes negligible current from the circuit.

h-Pipes Polytetrafluoroethylene (PTFE) is a synthetic fluoropolymer of tetrafluoroethylene that has numerous applications. PTFE is a thermoplastic polymer, which is a white solid at room temperature. It is better known as Teflon.

i-Glassware (the bottle or beaker) rather than plastic. This is because plastics can attract ions to their surfaces. When the ozone concentration is relatively low, this could deplete the various oxygen radicals. It is good practice to use glassware wherever possible.

l- Compact Ozone Meter (Palintest©)

This instrument provides rapid analysis of ozone. It is based on a colorimeter method. DPD (N, N-diethyl-p-phenylenediamine) colorimetric glycine

method for residual chlorine was employed to measure the ozone concentration.

m-Ph meter (CP-105)
This instrument provides pH measure**.**

n-Thermometer (CRISON TM 65)
This instrument provides measure of temperature

5.5 Inactivation (disinfection) process – Background

Ozone in an aqueous solution may react with microbes either by direct reaction with molecular ozone or by indirect reaction with the radical species formed when ozone decomposes. Ozone is known to attack unsaturated bonds, forming aldehydes, ketones or carbonyl compounds (Langlais et al., 1991). It is likely, therefore, that microbes become inactivated through ozone acting on the cytoplasmic membrane (due to the large number of functional proteins), the protein structure of a virus capsid, or nucleic acids of microorganisms.
Generally, the principal factors that influence disinfection efficiency are concentration, contact time, temperature and pH. Disinfectant concentration and contact time are integral to disinfection kinetics and the practical application of the CT concept (CT being the disinfectant concentration multiplied by the contact time). Temperature, over the range appropriate for drinking-water, affects the rate of disinfection reactions according to the Arrhenius equation, although this may not hold for certain disinfectants at low temperatures. The pH of the disinfectant solution affects the reaction kinetics. (WHO, 2004). Table 5.1

Table 5.1 CT values (mg/min l–1) for 99% inactivation at 5°C

	Free chlorine	Preformed chloramines	Chlorine dioxide	Ozone
Microorganism	(pH 6–7)	(pH 8– 9)	(pH 6–7)	(pH 6–7)
E. coli	0.034–0.05	95–180	0.4–0.75	0.02
Poliovirus	1 1.1–2.5	770–3740	0.2–6.7	0.1–0.2
Rotavirus	0.01–0.05	3810–6480	0.2–2.1	0.006–0.06
Phage f2	0.08–0.18	–	–	–
G. lamblia cysts	47–>150	–	–	0.5–0.6
G. muris cysts	30–630	1400	7.2–18.5	1.8–2.0

Adapted from Hoff (1986)

5.6 Preliminary *in vitro* and *in vivo* tests

In order to determine the concentration of O_3 and the contact times needed to inactivate *Eimeria* oocysts and *Giardia* cysts in well water a preliminary *in vitro* test was performed as described below.

5.6.1 Sampling and recovery of *Eimeria* oocysts and *Giardia* cysts.

Eimeria oocysts and *Giardia* cysts were recovered from faecal samples collected from naturally infected water buffalo calves (1-4 months) using the FLOTAC dual technique with an analytic sensitivity of two oocyst and cysts per gram (OPG and CPG, respectively) of faeces using two flotation solutions, namely sodium chloride (specific gravity, s.g.=1.2) to detect *Eimeria* oocysts (Cringoli et al., 2010) and zinc sulphate (s.g.=1.35) to detect *G. duodenalis* cysts (Pepe et al., 2019). Magnifications of 100× and 400× were used to identify protozoan (oo)cysts. The faecal samples were processed within two hours of collection using the egg recovery technique (Bosco et al., 2018) with some modifications used to recover protozoan (oo)cysts. Firstly, faecal samples were homogenized and filtered under running water through sieves with a mesh size of 1 mm, 250 µm, 100 µm and 50 µm to separate the (oo)cysts from the faeces; moreover, one other sieve of 25 µm was employed for *G. duodenalis*. Next, faecal suspension

filtered were centrifuged for three minutes at 170g, after which the supernatant was discarded. In the end, the pellets were resuspended with 40% sucrose solution to float the (oo)cysts which were then transfer in new tubes, mixed with distilled water and then centrifuged two more times to remove pellets and to get a clear aqueous solution with (oo)cysts. Then, ten aliquots of 10 µl each were taken, after a through homogenization of (oo)cysts preparation into two tubes for ten times (avoiding foam formation) for each aliquot to provide a count of (oo)cysts at 100X and 400X magnifications.

5.6.2 *In vitro* test for *Eimeria* spp. (study no. 1)

A total of 510,000 oocysts were collected and divided into 17 aliquots consisting of 30,000 oocysts each. Sixteen groups (GE) of 4 aliquots each were treated with ozone concentration of 0.5, 1, 2 and 3 mg/l for 15, 30, 45 and 60 minutes and one aliquot (C) contained non-treated water. The figure 5.8 shows the study design of the preliminary *in vitro* test for *Eimeria.*

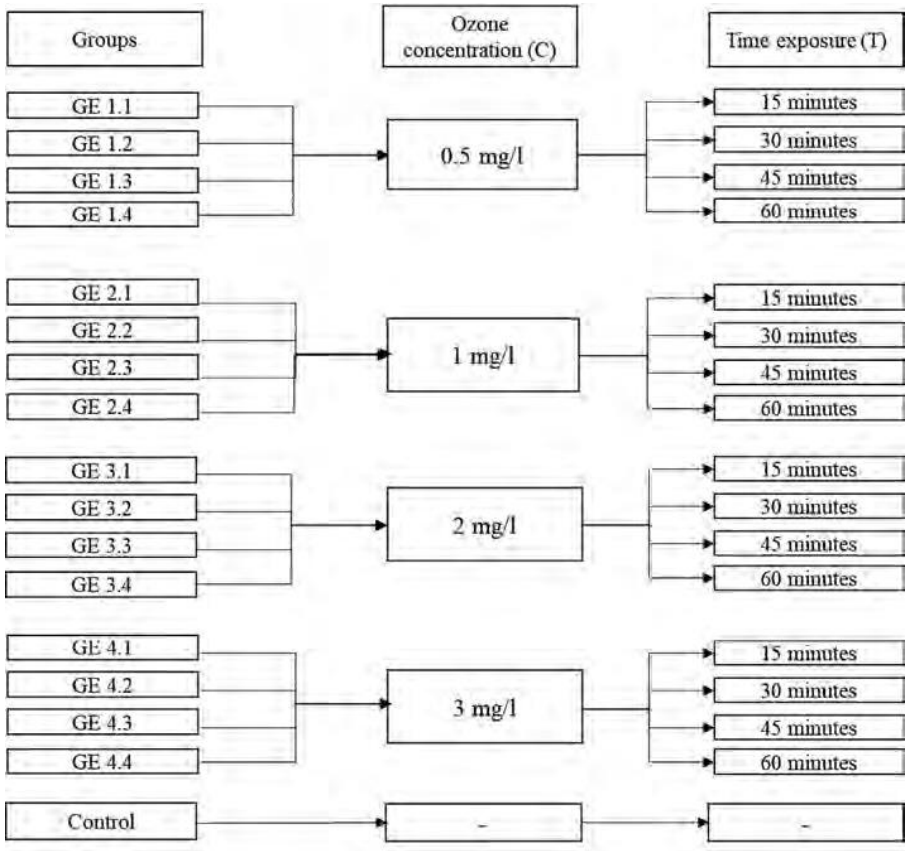


Figure 5.8 Study design of the *in vitro* effect of ozonated water treatment on *Eimeria* spp. oocysts isolated from naturally infected water

After ozone treatment the oocysts suspension were centrifuged at 170 g for 3 minutes and the pellet suspended in 2.5% of potassium dichromate to allow sporulation by incubating at 25 °C for 6 days. At the end of the incubation, each aliquot was centrifuged for three min at 170g, the supernatant was discharged in order to remove potassium dichromate from the oocyst suspension by repeated dilution with distilled water. Then, the aliquots were stored at 4°C until being counted. Each aliquot was examined by light microscopy at 100x, 400x and 1000x magnification. In order to evaluate the effect on oocysts the number of sporulated and non-sporulated or lysed oocysts were counted, and the percent sporulation was estimated by counting the number of sporulated oocysts by a total of 100 oocysts (Figure 5.9).

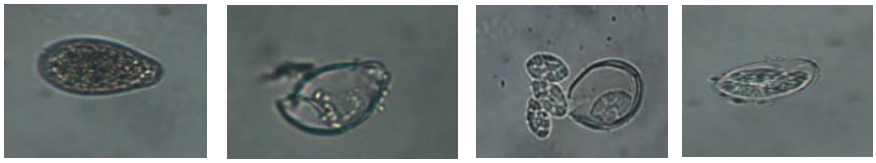


Figure 5.9. Effect on viability of *Eimeria* oocysts in the ozone treated groups. From left to right: a. Non-sporulated oocyst; b. Damaged oocyst in group GE3 (3 m/l*60 minutes); c. sporulated oocyst in group GE3 (3 m/l*15 minutes); d. sporulated oocyst in group GE2 (2 m/l*15 minutes).

Unfortunately, the results didn't show any effect on the viability of *Eimeria* spp. oocysts (Table 5.2). The presence of organic and inorganic compounds associated to parasites in well water may require the use of higher ozone doses to achieve parasite inactivation (Dumètre et al., 2012).

Table 5.2 Results of the preliminary *in vitro* test (study no. 1)

Table results

Eimeria groups	Ozone concentration C (mg/l)	TIME (minutes)	CT	Sporulated (%)	Not sporulated (%)	Degenerate (%)
GE1.1	0.5	15	7.5	99	1	0
GE1.2		30	15	99	1	0
GE1.3		45	22.5	97	3	0
GE1.4		60	30	99	1	0
GE2.1	1	15	15	98	2	0
GE2.2		30	30	99	1	0
GE2.3		45	45	97	3	0
GE2.4		60	60	96	4	0
GE3.1	2	15	30	100	0	0
GE3.2		30	60	95	5	0
GE3.3		45	90	96	4	0
GE3.4		60	120	94	3	3
GE4.1	3	15	45	100	0	0
GE4.2		30	90	95	0	0
GE4.3		45	135	99	1	0
GE4.4		60	180	93	1	6
GE5	—	—	—	96	3	1

5.6.3 *In vitro* test for *Eimeria* spp. (study no. 2)

In this *in vitro* test 70,000 *Eimeria* oocysts, recovered as described in section 6.7.1, circulated free into the bench ozone generator in direct contact with electrolytic cell. Ozone concentration used was 0.5 mg/l for 15 minutes.
Next, after treatment, the oocysts suspension was filtered with microfilter (0.4 µm) to separate the oocysts from the water, then the oocysts filtered were centrifuged at 700g for 3 minutes and suspended in 2% of potassium dichromate for sporulation by incubating at 25 °C for 5 days.
Each aliquot was examined by light microscope, counting up to 100 oocysts and morphology was evaluated (percentage of unsporulated, sporulated and degenerated oocysts).

Table 5.3 Results of the preliminary *in vitro* test (study no. 2): *Eimeria* viability at different ozone concentrations and contact times.

Group	Concentration (mg/l)	Time exposure (minutes)	Oocysts sporulated (%)	Oocysts unsporulated (%)	Oocysts degenerated (%)
GE1	0.5	15	46.3	42.2	49.5
Control	-	-	69.1	0.9	30.0

*degenerated shell

Figure 5.10 Effect on viability of *Eimeria* oocysts in the ozone treated groups. Coclkwise from the left to the right: a. Non-sporulated oocyst; b. Damaged oocyst in group GE1 0.5 m/l*15 minutes); c. sporulated oocyst in group GE1 (0.5 m/l*15 minutes); d. sporulated oocyst in group Control.

5.6.4 In vitro test for *Giardia duodenalis*

A total number of 120,000 *Giardia* cysts were divided into 10 aliquots with 12,000 cysts each.
This *in vitro* test was performed to assess the effect of ozone simulating acidic gastric ambient using a medium with pepsin and hydrochloric acid (HCl).
Giardia cysts were treated as described in figure 5.11. Two groups (GG1, GG2) were treated with ozone at the concentrations of 1 mg/l for 15 and 10 minutes, respectively. One group (GG3) was non -treated group (medium); one group (GG4) was treated with ozone at concentration of 1 mg/l in medium solution for 15 minutes contact time and one group (GG5) were control group.

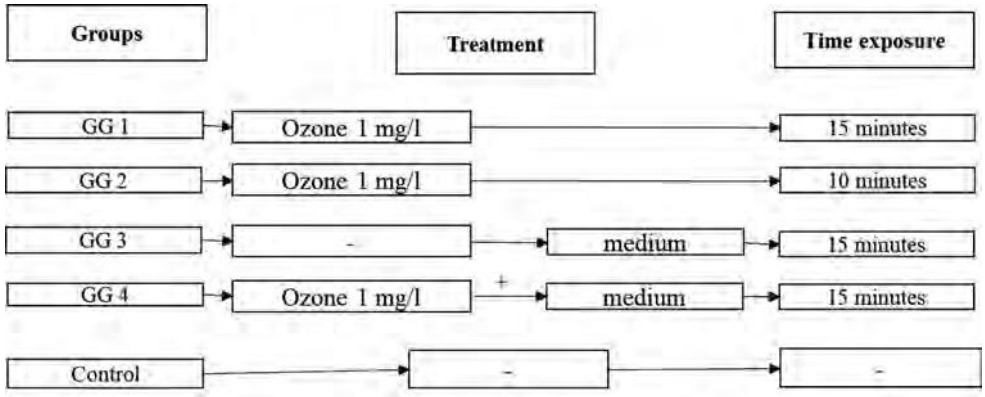


Figure 5.11 Study design for the *in vitro* test for *G. duodenalis*

The results showed a slight effect on *Giardia* cysts. The highest percentage of degenerate cysts was observed in the group treated with acid medium for 15 minutes (GG4), confirming that survival of the parasite in the stomach is possible only in the absence of HCl (Martinsen et al., 2005).

Table 5.4 Effect of ozone on the *Giardia duodenalis* cysts viability.

Group	Ozone concentration (mg/l)	Time exposure (minutes)	Intact cysts (%)	Degenerate cysts (%)
GG1	1	15	71.6	28.4
GG2	1	10	69.9	30.1
GG3	1	15	73.6	26.4
GG4	-	15	67.7	32.4
GG5	-	-	97.4	2.7

5.6.5 *In vivo* test for *Eimeria* spp.

A preliminary *in vivo* test was performed in water buffalo calves to evaluate the effect of ozonated drinking water on the *Eimeria* oocyst output reduction as well as the effect on animal performance (e.g. weight gain). A controlled field trial was conducted in a water buffalo farms located in the Salerno province of southern Italy; in this farm, with a known history of coccidiosis (mean values = 150–10,000 OPG), the buffalo calves were bred in individual boxes from the birth to the 7th/8th week of age and then transferred to concrete based pens. The study was conducted in accordance with national animal welfare requirements and approved by the Ethical Committee of the Department of Veterinary Medicine and Animal Production, UNINA.
Thirty-six calves (aged 7 weeks) were divided randomly into three groups of 12 calves each with similar age, weight, number of *Eimeria* OPG (clinical signs were not present in any buffalo). One group was treated with toltrazuril (group TOL) at 15 mg/kg, the second group was treated with ozonated drinking water (group OZO) at 0.5 mg/l, and the third group was remained as untreated controls (group CONT). The drug, namely toltrazuril (Baycox® Bovis, oral suspension, 50 mg/ml) were administered according to the manufacturer's instruction whilst the ozonated water (4 litres/day) was given ad libitum for 21 days.

Oocysts were counted every three days on each buffalo from Day 0 to Day 27. The FLOTAC double technique was used with an analytical sensitivity of 2 oocysts per gram of faeces (OPG), using the Sheather's sugar flotation solution (specific gravity = 1.200) (Cringoli et al., 2010). In order to sporulate and identify the *Eimeria* species, the faecal samples from each farm were pooled and diluted using a 2.5% potassium dichromate solution, then stored in wide-surfaced containers and kept at 26–28 ◦C for one week and the samples were oxygenated several times a day (de Noronha et al., 2009). The *Eimeria* oocysts were identified using the morphometric keys proposed by de Noronha et al. (2009).

The body weight of each buffalo calf was recorded fortnightly, starting from the day of treatment and continued weekly until the 21st day of the trial. In addition, each buffalo calf was examined clinically every week for the duration of the study.

Statistical analysis was performed using GraphPad Software© (Prism company) for Windows. The quantitative data (OPG and body weight) were tested using a one-way ANOVA in conjunction with the Dunn's for post hoc comparison. Significance testing was set at $p < 0.05$.

The average oocyst excretion group decreased significantly ($p < 0.05$) in the treated groups (TOL) with 117.6 OPG compared to the CONT with 168.4 OPG; whilst OZO treated group showed any statistical difference ($p>0.05$) in mean oocysts excretion (164 OPG) compared to CONT group (Figure 5.12).

The species of *Eimeria* found in the animals were *E. ellipsoidalis*, *E. bovis*, *E. zuernii* and *E. subspherica*. The body-weight gains recorded fortnightly were significantly higher in the TOL groups compared to the OZO and the CONT groups ($p < 0.05$).

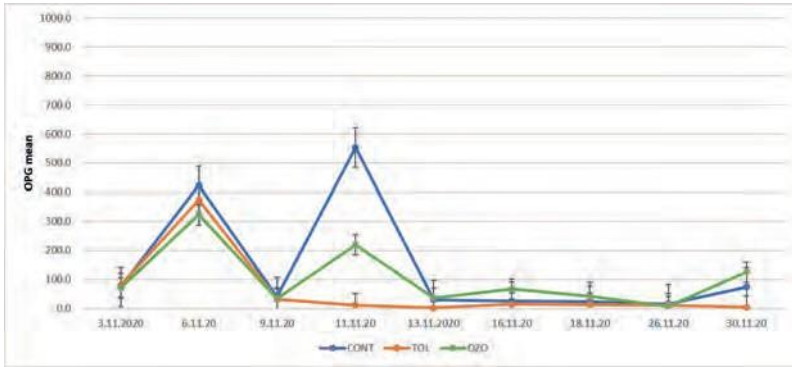


Figure 5.12 Dynamic of the elimination of *Eimeria* oocysts in three experimental groups during the course of the in vivo trial in water buffalo farm.

5.7 Conclusion

The results of the preliminary *in vitro* and *in vivo* studies on the effect of ozonated water on *Eimeria* and *Giardia* suggested that ozone could be a promising eco-friendly tool to control protozoa infections in water buffalo farms. However further *in vitro* and *in vivo* studies are required to assess the ozone parameters (e.g. concentration, time etc.) to be used in laboratory and field settings.

5.8 References

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Chapter 6

In vitro evaluation of ozonated water treatment on the viability of *Eimeria* oocysts and *Giardia* cysts from water buffaloes: a proof-of-concept study.

*Based on the manuscript In vitro evaluation of ozonated water treatment on the viability of *Eimeria* oocysts and *Giardia* cysts from water buffaloes: a proof-of-concept study. **Morgoglione ME**., Bosco A, Ciuca Lavinia C, Pepe P, Coles G, Cringoli G, Rinaldi L. Submitted to Veterinary Science

6.1 Abstract

The aim of this proof-of-concept study was to evaluate the *in vitro* effect of ozonated water treatment on the viability of *Eimeria* oocysts and *Giardia* cysts isolated from naturally infected water buffaloes. *Eimeria* oocysts were divided into seven groups of six replicates that were treated with ozonated water at three ozone concentrations (0.5, 1 and 2 mg/L) and two contact times (five and ten minutes) and one group (negative control) was exposed to non-treated water. *Giardia* cysts were divided into nine groups of six replicates and were treated with ozonated water at four ozone concentrations (0.1, 0.3, 0.5 and 1 mg/l) and two contact times (one and two minutes) and one group (negative control) was exposed to non-treated water. The results of ozonated water treatment gave a 33% inhibition of sporulation of *Eimeria* oocysts and rendered 96.3% of *Giardia* cysts non-viable, suggesting that ozonated water treatment could be a promising alternative therapy for controlling intestinal protozoa infections in water buffaloes.

6.2 Introduction

Dairy water buffalo (*Bubalus bubalis*) farming plays an important role in the economy of several countries, including Italy, as their milk is almost exclusively used for the production of-mozzarella-cheese (Masucci et a., 2016; Minervino et al., 2020). In intensive farming systems, the infection of water buffaloes with intestinal protozoa, such as *Giardia*, and *Eimeria*, threatens the profitability and sustainability of milk production (Rinaldi et al., 2007; Cringoli et al., 2009; Bosco et al., 2017; Morgoglione et al., 2020). These parasites are the leading cause of neonatal diarrhoea, with negative impact on the growth performance of buffalo calves, resulting in economic losses (de Aquino et al., 2020). Water buffaloes acquire *Eimeria* infections soon after birth, irrespective of the management systems and severe outbreaks can occur resulting in morbidity and mortality (Dubey, 2018). Moreover, infection by *G. duodenalis* is a public health concern because of the potential zoonotic transmission to human (Keeton and Navarre, 2018; Santin, 2020). Therefore, the adoption of appropriate control strategies against intestinal protozoa are a considerable challenge for water buffalo farms (Bosco et al., 2017; El Debaky et al., 2019). Although metaphylactic approaches have been used successfully to control *Eimeria* (e.g., toltrazuril and diclazuril) (Daugschies et al., 2007; Bosco et al., 2015) and *Giardia* (e.g., fenbendazole and albendazole) (Thompson, 2004; Santin, 2020)

infections in ruminant farms, other prophylactic measures are needed to reduce environmental contamination in order to limit the infection pressure (Daugschies et al., 2002). Currently, prophylactic measures include complete cleaning and disinfection of housing facilities, sanitation of drinking water for animals using products such as ammonia, chlorine dioxide, hydrogen peroxide and ozone (Collignarelli, 2018; Keeton and Navarre, 2018; de Aquino et al., 2020). Ozone, an allotropic form of oxygen constituted by three oxygen atoms, is produced in three different ways: by electrical discharges, through ultraviolet radiation, and by some chemical processes (Eliasson et al., 1987; Garamoon et al., 2002). Being a very powerful oxidant, it is well known for its bactericidal, virucidal, and fungicidal actions, which are used for water treatment and medical applications (Knowbler, 2004). Currently, in Italy, ozone can be used exclusively as a sanitizer. It is presently being reviewed by the European Environmental Agency and for use as a biocide, in disinfection, food and animal's feeds, drinking water, and as a preservative for liquid systems, under the Biocidal Products Regulation (BPR) of European Chemical Agency (ECHA).

In the last few years, scientific and commercial interest in ozone-therapy, both in human and veterinary medicine has been increasing (Elvis and Ekta, 2011; Đuričić et al., 2015; Sciorsci et al., 2020). The main advantage of using ozonated water treatment in livestock and animal husbandry sectors is the ability of reducing or destroying the microbial pathogens, thus resulting in an improvement of the general health of the animals (Loeb et al., 2012; Heacox, 2013; Ozone Systems, 2014).

Given the potential applications in veterinary medicine, the aim of this proof-of-concept study was to evaluate the *in vitro* effect of ozonated water on *Eimeria* oocysts and *Giardia* cysts isolated from naturally infected water buffaloes.

6.3 Materials and Methods

6.3.1 Sampling and coprological analysis

Eimeria oocysts and *Giardia* cysts were recovered from faecal samples collected from naturally infected water buffalo calves (1-4 months), in a farm located in southern Italy with a known history of protozoa infections. Thirty individual fresh faecal samples were collected directly from the rectal ampulla of the animals. Each faecal sample was analysed by the FLOTAC dual technique with an analytic sensitivity of two oocyst/cysts per gram (OPG and CPG, respectively) of faeces using two flotation solutions,

namely sodium chloride (specific gravity, s.g.=1.2) to detect *Eimeria* oocysts (Cringoli et al., 2010) and zinc sulphate (s.g.=1.35) to detect *G. duodenalis* cysts (Pepe et al., 2019). Magnifications of 100× and 400× were used to identify protozoan (oo)cysts. The positive samples with a mean value of 30,000 OPG for *Eimeria* and with a mean value of 15,000 CPG for *G. duodenalis* were processed to purify the (oo)cysts for the *in vitro* tests. The tests were organized as described in Figure 6.1 and in the following sections.

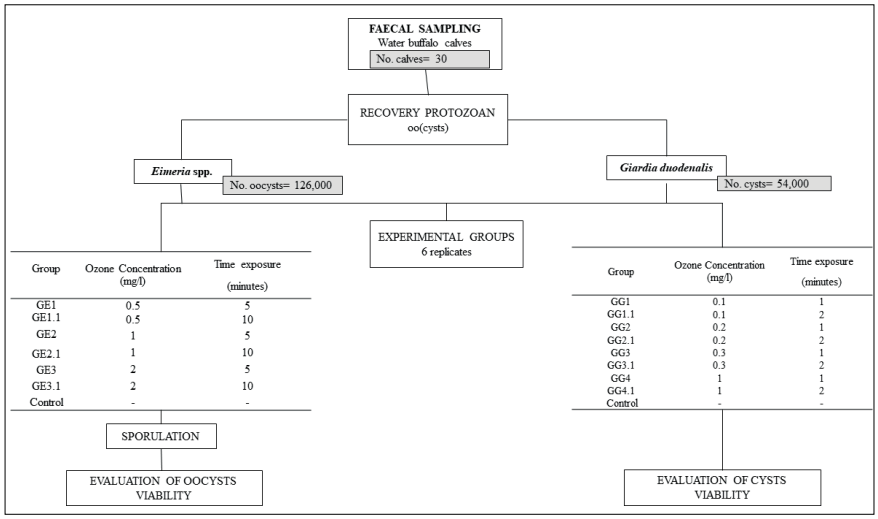

Figure 6.1 Study design of the *in vitro* effect of ozonated water treatment on *Eimeria* spp. oocysts and *Giardia duodenalis* cysts viability isolated from naturally infected water buffaloes.

6.3.2 Recovery of *Eimeria* spp. oocysts and *Giardia duodenalis* cysts

The faecal samples were processed within two hours of collection using the egg recovery technique by Bosco et al. (2018) with some modifications. Briefly, the faecal samples were homogenized and filtered under running water through sieves with different mesh sizes as follow: of 1 mm, 250 µm, 100 µm and 50 µm to separate the (oo)cysts from the faeces; with a sieve of 25 µm for *G. duodenalis*. The faecal suspension obtained was centrifuged for three minutes at 170g, and the supernatant was discarded. Finally, the pellets were resuspended with 40% sucrose solution and transferred to new tubes. To obtain a clear aqueous solution with *Eimeria* spp. oocysts and *Giardia* cysts two successive rounds of centrifugation with distilled water

were performed. After a thorough homogenization (avoiding foam formation) of the suspension into the tubes, ten aliquots of 10 µl each were taken in order to count the number of *Eimeria* oocysts or *Giardia* cysts at 100X and 400X magnifications.

6.3.3 Water ozonisation

The ozonated water was generated in a small-scale circuit by passing the distilled water through an electrolytic cell with a current of 1A (amps). The water was pumped past the electrode at rate of 1 L/min, continuously to produce ozone (Okada and Naya, 2012). A DPD (N, N-diethyl-p-phenylenediamine) colorimetric glycine method for residual chlorine using a compact ozone meter (Palintest©) was employed to measure the ozone concentration (Palin, 1974; Wickramanayake et al., 1984). In a preliminary experiment, it was observed that the concentration of aqueous ozone varied with temperature. However, the treated solution showed near-saturation with ozone after the first minute of treatment. Ozone treatment in subsequent tests was set at an average temperature of 25 °C with pH=7 (Wickramanayake et al., 1984; Elovitz et al., 2000; Gardoni et al., 2012; Galdeano et al., 2018). To evaluate sanitation kinetics, the concentration-time concept was applied, i.e., ozone concentration (C) in mg/l was multiplied by contact-time (*t*) in minutes (C*t*) (WHO, 2012).

6.3.4 *In vitro* test for *Eimeria* spp.

Eimeria oocysts (total count=126,000) were divided into seven groups (Group *Eimeria*-GE) of six replicates each in 42 glass vials, randomly as follows: six groups (GE1; GE1.1; GE2; GE2.1; GE3; GE3.1) were treated with ozonated water at three ozone concentrations (0.5, 1 and 2 mg/L) and two contact times (5 and 10 minutes). One group was exposed to non-treated water (negative control) as shown in Table 6.1. The aliquots of all the groups were centrifuged for 3 min at 170g and the pellet resuspended in an aqueous solution of potassium dichromate 2.5% and incubated in a wide-surfaced container at 26–28°C for 60-72 hr., oxygenating the samples several times a day to preserve the oocysts (de Noronha et al., 2009). At the end of the incubation, each aliquot was centrifuged for three min at 170g, the supernatant was discharged to remove potassium dichromate from the oocyst suspension by repeated dilution with distilled water. Then, the aliquots were stored at 4°C until being counted. Each aliquot was examined by light microscopy at 100x, 400x and 1000x magnification. In order to evaluate the effect of ozonated water treatment the number of sporulated and

non-sporulated, deformed or lysed oocysts were counted, and the percent sporulation was estimated by counting the number of sporulated oocysts in a total of 100 oocysts (Daugschies et al., 2002; Liou et al., 2002; Molan and Liu, 2009).

Table 6.1 *In vitro* tests for *Eimeria* oocysts using different ozone concentrations and time exposure (minutes) in the GE.

Group-*Eimeria* (6 replicates)	**Ozone-concentration (mg/l)**	**Time exposure (minutes)**	***Ct** value**
GE1	0.5	5	2.5
GE1.1	0.5	10	5
GE2	1	5	5
GE2.1	1	10	10
GE3	2	5	10
GE3.1	2	10	20
Control	--	--	--

***** *Ct* is expressed by ozone concentration (C) in mg/l multiplied by contact-time (t) in minutes.

6.3.5 In vitro test for *Giardia duodenalis*

G. duodenalis cysts (total count=54,000) were divided into nine groups (Group Giardia-GG) of 6 replicates each (54 glass vials) as follows: eight groups (GG1; GG1.1; GG2; GG2.1; GG3; GG3.1) were treated with ozonated water at four ozone concentrations (0.1, 0.3, 0.5 and 1 mg/l) and two contact times (one and two minutes) (Table 6.2). One group was exposed to non-treated water (negative control). *Giardia* cyst viability was evaluated by non-fluorogenic dye exclusion method with trypan blue (Rousseau et al., 2018; Hamdy et al., 2019; Samarro Silva and Sabogal-Paz, 2021). The percentage of non-viable cysts was calculated using the formula: [1- (total number of viable cysts per ml of aliquot /total number of cysts per ml of aliquot)] × 100.

Table 6.2 *In vitro* tests for *Giardia* oocysts using different ozone concentrations and time exposure (minutes) in the GG.

Group-*Giardia* (6 replicates)	Ozone-concentration (mg/l)	Time exposure (minutes)	*Ct** value
GG1	0.1	1	0.1
GG1.1	0.1	2	0.2
GG2	0.2	1	0.2
GG2.1	0.2	2	0.4
GG3	0.3	1	0.3
GG3.1	0.3	2	0.6
GG4	1	1	1
GG4.1	1	2	2
Control	-	-	-

***** *Ct* is expressed by ozone concentration (C) in mg/l multiplied for contact-time (t) in minutes.

6.3.6 Statistical analysis

One-way ANOVA was performed to detect the significant difference between the treated and non-treated groups of GE and GG with Post hoc Turkey's tests. For all comparisons, a level of α =0.05 was assumed, and the obtained *P*-values were rounded to two decimal places. Statistical analysis were performed using SPSS Statistics v.23 (IBM, Armonk, NY, USA).

6.4 Results

6.4.1 *Eimeria* oocysts viability

Based on sporulation, four species of *Eimeria* were identified in the positive samples, i.e., *E. ellipsoidalis*, *E. bovis*, *E. subspherica* and *E. bareillyi* with the following prevalence: 42%, 29%, 18% and 11%, respectively. The sporulation rate of the oocysts treated with ozone was significantly ($P<0.001$) lower than that in the non-treated group (Table 6.3). The best results were obtained in the GE3 treated group with *Ct* value of ten (2 mg/l*5minutes) that revealed a rate of 33.0% of non-sporulated oocysts. There was no significant difference ($P>0.005$) between the prevalence of the four *Eimeria* species identified in both treated and non-treated groups. In subsequent incubation, bacteria were seen to attach to the surface of the ozone-treated oocysts (Fig. 6.2a, b). In contrast, attachment by bacteria to the non-treated control oocysts was minimal (Fig. 6.2c). Many oocysts did not complete the sporulation process after ozone treatment and remained at

the early cytoplasmic contraction stage or showed internal structure degeneration (Fig. 6.2d).

Table 6.3 The sporulation rate of the *Eimeria* spp. oocysts treated with ozonated water.

Group-*Eimeria* (6 replicates)	**Sporulated oocysts (%)**
GE1	83.2
GE1.1	82.6
GE2	77.2
GE2.1	76.5
GE3	77.0
GE3.1	76.3
Control	89.0

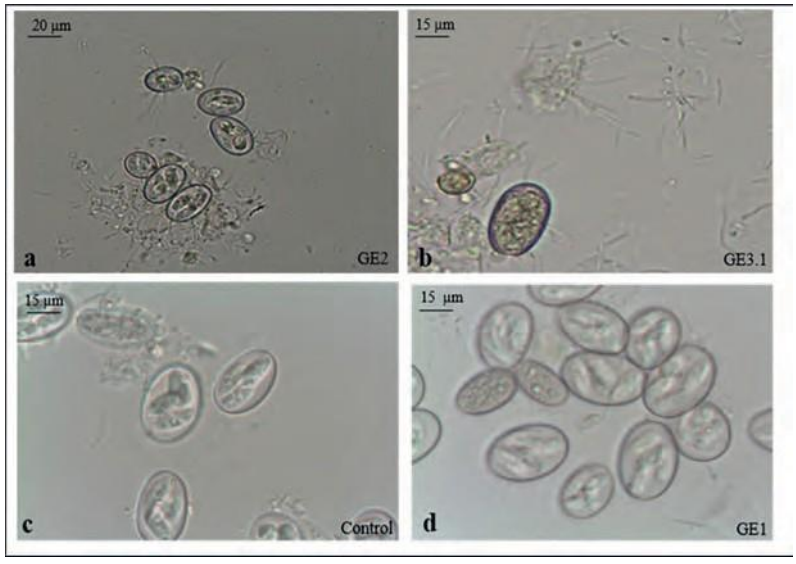

Figure 6.2 Presence of bacteria in GE2, in GE3.1 treated group (a,b) and in control group (c); Oocysts at early cytoplasmatic stage (d).

6.4.2 *Giardia duodenalis* cysts viability

The control group showed 99% of intact *Giardia* cysts. There was a significant difference (P <0.0001) of the mean viable cysts and the mean of non-viable cysts in the GG.1 treated group and non-treated group. Specifically, the highest percentage of non-viable cysts was obtained in the GG3.1 treated group (96.3%) at *Ct* of 1 (0.5 mg/l*2 minutes), as showed in Table 6.4 and Figure 6.3 (a, b).

Table 6.4 Effect of ozone on the *Giardia duodenalis* cysts viability.

Group-*Giardia* (6 replicates)	Non-viable cysts (%)
GG1	16.2
GG1.1	34.5
GG2	86.3
GG2.1	94.0
GG3	76.8
GG3.1	96.3
GG4	90.8
GG4.1	95.2
Control	2.0

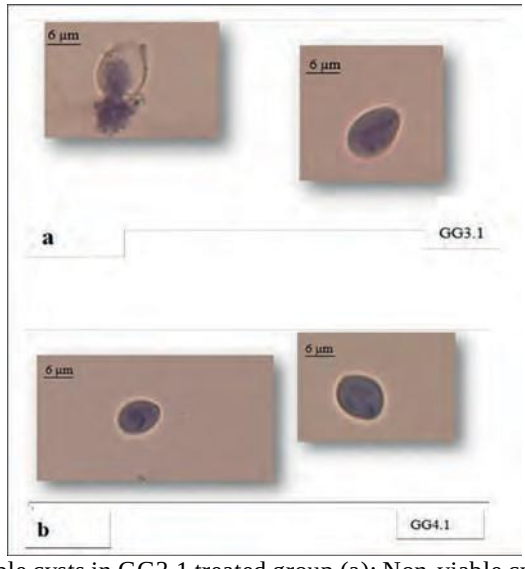

Figure 6.3. Non-viable cysts in GG3.1 treated group (a); Non-viable cysts in GG4.1 treated group (b).

6.5 Discussion

Ozone is one of the most powerful known oxidants, used for inactivation of pathogens including bacteria, fungi, yeasts, protozoa, and viruses (Khalifa et al., 2001; Erickson and Ortega, 2006; Bialka and Demirci, 2007; Elvis and Ekta, 2011; Varga and Szigeti, 2016; Marino et al., 2018; Megahed et al., 2019). Ozone action against protozoa has been demonstrated in vitro with different parasites such as *Leishmania*, *Giardia*, *Cryptosporidium*, *Blastocystis*, *Cyclospora*, etc. using ozonated oil and ozonated water (Khalifa et al., 2001; Erickson and Ortega, 2006; Pereira et al., 2008; Ran et al., 2010; Rajabi et al., 2015). Moreover, the use of ozone is widely applied in drinking water and wastewater treatment (Rodrìguez et al., 2008; Wei et al., 2017; Iakovides et al., 2019; Vitali and Valdenassi, 2019). Owing to these activities, ozone is widely used in human and veterinary medicine through several pharmaceutical forms (Dubey, 2008).

This proof of concept study is the first attempt to investigate the in vitro effectiveness of ozonated water treatment on the viability of *Eimeria* spp. oocysts and *G. duodenalis* cysts isolated from water buffaloes. Our study revealed that the inhibition of sporulation of the *Eimeria* oocysts induced by the ozone treatment was time -and -concentration-dependent. Indeed, the *Eimeria* oocysts suffered a partial inhibition of sporulation in the treated group (GE3) with 2 mg/l ozone concentration and 5 minutes of exposure. This might be due to the alteration of the surface structure of the oocysts by ozone, as it has been shown in other similar studies for *E. colchici*, *E. necatrix*, *E. maxima*, *E. acervuline* oocysts (Liou et al., 2002; Neretti et al., 2018). Coccidian oocysts are extremely resistant to common physical and chemical compounds due to their complex structure (Dumètre et al., 2008; Daugschies et al., 2013; Martinelli et al., 2017). However, in the present study many oocysts after ozone treatment showed deformed shape, incomplete development, and remained at the early cytoplasmic contraction stage. It should be noted, however, that the study by Liou et al. (2002) reported ozone-treated *E. colchici* oocysts being infective after 3 months even if their sporulation was incomplete (Liou et al., 2002). Therefore, our results are not conclusive regarding infectivity and further studies should be carried out for the buffalo *Eimeria* species identified in the present study. Ozone alone or in combination with other chemical (chlorine, chlorine dioxide) or physical processes (e.g., filtration, flocculation, UV, etc.) has been reported to inactivate the cysts of *Giardia* (Erickson and Ortega, 2006; Wickramanayake et al., 1984, 1985; Finch et al., 1993; Betancourt et al., 2004).

In the present study, the highest percentage of non-viable cysts was obtained in the GG3.1 treated group at 0.3 mg/l ozone concentration and 2 minutes time exposure (96.3%). Our results were similar to those obtained by Finch et al., (1993) where the highest percentage of *G. duodenalis* inactivation cysts (99.9%) was obtained with 0.5 mg/l ozone concentration and 5 minutes of time exposure (Finch et al., 1993).

Eimeria spp. and *G. duodenalis* are still common and widespread GI parasites in water buffalo farms in different parts of the world (Rinaldi et al., 2007; Bosco et al., 2015; Gupta et al., 2016; Ojeda-Robertos et al., 2017; Tavassoli et al., 2018; Bangoura and Bardsley, 2020; Morgoglione et al., 2020). In the Mediterranean area, *Eimeria* spp. is still the most prevalent protozoa with an overall rate of 81.5%, according to Morgoglione et al. (2020), whilst *G. duodenalis* was present in buffalo farms in central and southern Italy, with a rate of 30% and 18%, respectively (Bosco et al., 2017; Morgoglione et al., 2020). *Giardia* is present in buffalo farms with a wide range of prevalence from 0.7% to 40.9% worldwide (de Aquino et al., 2020). Furthermore, molecular investigations of *G. duodenalis* isolates showed the presence of zoonotic parasites (*G. duodenalis* assemblage A) and host-specific parasites (*G. duodenalis* assemblage E), suggesting that water buffaloes can contribute to environmental contamination with cysts potentially infectious to humans if their faeces are improperly disposed of (Thompson, 2004).

Recent studies have shown that the water is one of the main sources of infection of *Cryptosporidium* spp. and *G. duodenalis* and its (oo)cysts are unlikely to be inactivated by routine chemical disinfectants or sanitizing water treatments (Giangaspero et al., 2009; Caradonna et al., 2017). Thus, drinking water sanitation play an important role in the complex control of protozoa in water buffalo farms, reducing environmental contamination pressure and protecting animals from infections. Prevention of overcrowding, feeding off the ground, and sanitation of feeding and watering equipment are important. Hence, these approaches represent a relevant challenge in intensive livestock farming (Daugschies et al., 2013). Moreover, the control of intestinal protozoa in buffalo farms is certainly not easy to implement for the considerable spread and resistance of their (oo)cysts in the environment and in the water for months (Keeton and Navarre, 2018; Adeyemo et al., 2019). To date, as vaccine prophylaxis measures to control of GI protozoa are not yet available in ruminants best combination of rational treatments, hygiene and herd management are

indispensable tools to reduce the risk of transmission of infections in water buffalo farms (Thompson, 2004; Santin, 2020).

In recent years the scientific community has had an increased interest in new low-cost and eco-friendly systems to control parasites, thus alternative therapeutic approaches based on ozone in ruminants could be useful to control protozoa infections as demonstrated in poultry against *Eimeria* (Liou et al., 2002; Đuričić et al., 2015). Although the use of ozone in veterinary medicine can be traced back more 30 years, it is still rarely employed and only for the treatments of few diseases such as mastitis, vaginitis, enteritis, etc. to reduce antibiotic administration (Đuričić et al., 2015; Sciorsci et al., 2020). Furthermore, ozone has been successfully used in dairy industries for the cleaning operation in milk processing and for reducing the concentrations of pollutants in dairy wastewaters (Varga and Szigeti, 2016). Despite the advantages of ozone, some limitations are associated with the ozonated water technology, such as the high cost of ozone generators, the need for operating and service infrastructure on a large scale that could determine the limited use of ozone in livestock farms with electrodes, as for example the one used in this study, and with the development of new boron doped diamond electrodes, it is now possible to generate ozone from water at the point of use. The cells required for this purpose are simple, robust, reliable, low voltage and low cost method (Heim et al., 2015; Morgoglione et al., 2019). The outcomes of this proof of concept study suggest ozonated water treatment is a promising alternative therapy for controlling intestinal protozoa infections in water buffaloes, though further *in vitro* and *in vivo* tests are needed.

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Sustainable control of intestinal protozoa in water buffalo farms *via* ozonated water: current status and future perspectives

The profitability and sustainability of water buffalo farming systems are threatened by the infection with intestinal protozoa, such as *Giardia*, *Cryptosporidium* and *Eimeria* (Rinaldi et al., 2007; Cringoli et al., 2009; Bosco et al., 2017; Morgoglione et al., 2020; de Aquino et al., 2020). Among these protozoa, *Eimeria* spp. and *Giardia duodenalis* cause the most common infections in buffaloes affecting mainly young animals and represent persistent and complex problems in water buffaloes that impair health, welfare, and production of this livestock species.

The prevalence of intestinal protozoa in large ruminants reared in intensive farms has constantly increased over the years. Therefore, updated knowledge of their epidemiology, diagnosis and control is of pivotal importance for both scientific and practical purposes.

Infection by *G. duodenalis* is well known in buffalo farms and the prevalence ranges from 0.7% to 40.9% worldwide, whilst in central and southern Italy, *Giardia* is reported in buffalo farms with a rate of 30% and 18%, respectively (Bosco et al., 2017). Furthermore, molecular investigations of *G. duodenalis* isolates showed the presence of zoonotic parasites (*G. duodenalis* assemblage A) and host-specific parasites (*G. duodenalis* assemblage E), suggesting that water buffaloes can contribute to the environmental contamination with cysts potentially infectious to humans if their faeces are improperly disposed of (Cacciò et al., 2005, 2007; Rinaldi et al., 2007).

Due to the lack of recent epidemiological data on eimeriosis in large ruminants, an epidemiological investigation of *Eimeria* spp. in water buffaloes in southern Italy was performed (Chapter 3) to complete the prevalence scenario of intestinal protozoa in this livestock species. The findings indicated that *Eimeria* spp. is widespread in water buffalo farms with high prevalence values (81.5%) despite a slight reduction compared to the previous decade (97.7%) as reported by Bosco et al. (2017). These results are in contrast with other studies performed in Brazil (De Noronha, 2009), Mexico (Ojeda-Robertos et al., 2017), Iran (Tavassoli et al., 2018) that reported a lower prevalence (around 35%). However, in these countries water buffaloes are reared under extensive production systems while in Italy the modern intensive water buffalo breeding has completely replaced the traditional extensive/semi-extensive buffalo farming, so the presence of *Eimeria* might not be influenced by the weather or by grazing, but rather by overcrowding and herd management (e.g., hygiene of pens).

The availability of affordable diagnostic tools is of pivotal importance to obtain accurate measures of infection rates as well as to design and plan appropriate control strategies. To date, while copromicroscopic techniques are well-established methods for the detection of *Eimeria* oocysts, some concerns still apply to the use of copromicroscopic methods for the detection of *Giardia* cysts. For this purpose, the immunoassays (IFA/ELISA) and the FLOTAC techniques were compared for diagnosing *Giardia* spp. infection (Chapter 4). IFA is the immunological test that is most used for diagnosing *Giardia* spp. infection, given that it is regarded as the gold-standard test. ELISA is also widely used, not only because it is a highly sensitive and specific test, but also because it is very easy to use. Comparing the sensitivity and specificity of these tests, FLOTAC and IFA showed the same capability to diagnose *Giardia* spp. infection but the FLOTAC technique showed higher sensitivity than ELISA. Furthermore, a perfect agreement was found between the performance of IFA and FLOTAC. The findings of this study (chapter 4) suggest that the FLOTAC technique can be use in the routine diagnosis of *Giardia* spp. infection in different animal species including pets and livestock.

The use of improved diagnostic tools combined with the application of the best practice of managment and control are fundamental actions to control intestinal protozoa in livestock.

Consistently with previous consideration exposed in chapter 2, the adoption of appropriate control strategies against intestinal protozoa are a considerable challenge for water buffalo farms (Bosco et al., 2017; El Debaky et al., 2019). Although, metaphylactic approaches have been used successfully to control *Eimeria* (e.g., toltrazuril and diclazuril) (Daugschies et al., 2007; Bosco et al., 2015) and *Giardia* (e.g., fenbendazole and albendazole) (Thompson, 2008; Santin, 2020) infections in ruminant farms, prophylactic measures are needed to reduce environmental contamination in order to limit the infection pressure (Daugschies et al., 2002).

Several factors may contribute to the continue reinfections of animals in contaminated environment, such as overcrowding, large numbers of parasitic elements released into the environment by the infected hosts, the role of synanthropes (e.g., insects, rodents, ect) in spreading cysts and oocysts, the rapid transmission of infective stages of intestinal protozoa to susceptible animal hosts, e.g., water buffaloes.

Proper hygiene regime, drinking water sanitation using products such as ammonia, chlorine dioxide, hydrogen dioxide and ozone (de Aquino et al., 2020) play an important role in the complex control of intestinal protozoa infection in water buffalo farms; however, chlorine is associated with

various problems, such as the production of several carcinogenic disinfection by-products (DBP), thus natural sanitizer could represent a valuable alternative, due in particular to the absence of residues or by-products harmful to health.

Ozone is a powerful oxidant, well known for its bactericidal, virucidal, and fungicidal actions, which are used for water treatment and medical applications (chapter 2). Currently, in Italy, ozone can be used exclusively as a sanitizer. It is presently being reviewed by the European Environmental Agency and for its use as a biocide, in disinfection, food and animal's feeds, drinking water, and as a preservative for liquid systems, under the Biocidal Products Regulation (BPR) of the European Chemical Agency (ECHA).

Although the use of ozone in veterinary medicine can be traced back more 30 years, it is still rarely employed in dairy farms for the treatments of few diseases (e.g., mastitis, vaginitis, enteritis) to reduce the use of antibiotics (Chapter 2). The main advantage of using ozonated water treatment in livestock and animal husbandry sectors is the ability of reducing or destroying microbial pathogens, thus resulting in an improvement of the general health conditions of the animals.

Although ozone is effective against the majority of microorganisms, few studies have been conducted so far about its effectiveness against intestinal protozoa, e.g., *Eimeria* and *Giardia* (chapter 6). Based on that, the overall aim of this industrial PhD project was to develop an ozonated water system to improve the control strategies against intestinal protozoa infecting water buffaloes in souther Italy. Specifically, an innovative polycrystalline diamond electrode to generate ozone directly in water was provided by the draperBiotech industrial company (UK).

The preliminary studies of the *in vitro* activity of ozonated water (using well water as medium) on the viability of *Eimeria* oocysts and *Giardia* cysts collected from water buffaloes, didn't show any effect on both protozoa. Likely, the presence of organic and inorganic compounds in well water could have inhibited the effect of ozone on parasite inactivation (Dumètre et al., 2012). To overcome these issues further *in vitro* tests were setted up using distilled water as medium to avoid any interference and standardize the concentration and exposure time needed to inhibit the viability of both protozoa involved in the study (chapter 6).

The results revealed that the inhibition of sporulation of the *Eimeria* oocysts induced by the ozone treatment was time -and -concentration-dependent. Indeed, the *Eimeria* oocysts suffered a partial inhibition of sporulation in the treated group with 2 mg/l ozone concentration and 5 minutes of exposure timing. This might be due to the alteration of the surface structure of the

oocysts by ozone, as it has been already shown in other similar studies for different species of *Eimeria* infecting poultry (Liou et al., 2002; Neretti et al., 2018). Coccidian oocysts are extremely resistant to common physical and chemical compounds due to their complex structure (Dumètre et al., 2008; Daugschies et al., 2013; Martinelli et al., 2017). However, in the present study many oocysts after ozone treatment showed deformed shape, incomplete development, and remained at the early cytoplasmic contraction stage. It should be noted, however, that the study by Liou et al. (2002) reported ozone-treated *E. colchici* oocysts being infective after 3 months even if their sporulation was incomplete (Liou et al., 2002). Therefore, our results are not conclusive regarding the infectivity of *Eimeria* oocysts after the ozone treatment and further studies should be carried out for the buffalo *Eimeria* species identified in the present study. Ozone alone or in combination with other chemical (chlorine, chlorine dioxide) or physical processes (e.g., filtration, flocculation, UV, etc.) has been reported to inactivate the cysts of *Giardia* (Erickson and Ortega, 2006; Wickramanayake et al., 1984, 1985; Finch et al., 1993; Betancourt et al., 2004).

Sanitation of drinking water for animal use was performed using a specific electrolytic ozone generator whitin polycrystalline diamond electrodes designed and engineered in a box (farm box) installed in a commercial farm of Aberdeen Angus cattle located in Salisbury, Wiltshire County, UK.

In UK, a trial *in vivo* was performed in fatting cattle to improve the ozone generator directly on the farm in the point of use, giving continuously ozonated water as therapeutic treatment to animals. The results showed ozone stopped the scouring of calves that grew faster than untreated animals and less antibiotics were used (Morgoglione et al., 2019). The mechanism of this effects is not well understood. Unfortunately, there is a lack of studies in ruminant livestock regarding the use of ozonated drinking water as alternative method to control parasitic infections *in vivo*. In veterinary medicine, the ozone is still used as a local therapy (Sciorsci et al., 2020). Whilst, Remondino et al. (2018) referred the use of 0.2-0.5 mg/l ozonated water in swine animals. Introduced in the intestine, ozone restores a suitable *eubiosis* by reducing the importance of pre/probiotic additions or enhancing them (synergistic effect).

In order to fill the gap of knowledge about the effects of ozonated water on intestinal protozoa, a second proof-of-concept *in vivo* trial was performed in Italy, to evaluate the *Eimeria* oocysts output reduction and the weight gain in water buffalo calves treated with ozonated drinking water (Chapter 5).

The results showed a slightly reduction of *Eimeria* mean OPG in the treated group (OZO), compared to the control group (CONT), although any statistically difference ($p < 0.05$) was found. In addition, the slight reduction of mean OPG resulted in a reduced body-weight gain in the OZO group compared to the TOL group ($p < 0.05$). The reduced efficacy *in vivo* could be due to different factors such as the nominal concentration of ozone may have been reduced by the saliva from calves drinking that was left in the water would and would have reduced the effective ozone concentration and the amount of water drinked by animals.

A further proof-of-concept study was conducted to evaluate the *in vitro* effect of ozonated water treatment on the viability of *Eimeria* oocysts and *Giardia* cysts isolated from naturally infected water buffaloes (Chapter 6). *Eimeria* oocysts were divided into seven groups of six replicates that were treated with ozonated water at three ozone concentrations (0.5, 1 and 2 mg/L) and two contact times (five and ten minutes) and one group (negative control) was exposed to non-treated water. *Giardia* cysts were divided into nine groups of six replicates and were treated with ozonated water at four ozone concentrations (0.1, 0.3, 0.5 and 1 mg/l) and two contact times (one and two minutes) and one group (negative control) was exposed to non-treated water. The results of ozonated water treatment gave a 33% inhibition of sporulation of *Eimeria* oocysts and rendered 96.3% of *Giardia* cysts non-viable, suggesting that ozonated water treatment could be a promising alternative method for controlling intestinal protozoa infections in water buffaloes.

The outcomes of these studies suggest ozonated water treatment could be a promising alternative method to control intestinal protozoa infections in water buffaloes, though further *in vitro* and *in vivo* tests are needed.

The results achieved represent the “first attempts” to fully acknowledge of applicability of ozone as sanitizer in water buffalo farms.

However, for a complete understanding of the mechanisms of action of ozone on the infectivity of *Eimeria* spp. and *G. duodenalis* as therapeutic alternatives, it would be useful to perform testing *in vitro* the efficacy of ozone against reproductive forms, cultivable *in vitro* of sporozoites in the case of *Eimeria* and trophozoites in the case of *Giardia* infectious forms.

A hypothetical higher sensitivity of these forms to the treatment with ozone *in vitro* would allow, after the experimental infection of animals with oo/cystic forms (i.e., *in vivo*), to highlight the ability of ozone to reduce the emission of oo/cysts in the environment, providing data on the “prophylactic” capacity of ozonated water. Scarce are the scientific data on the effect of ozone on the infectious stages of *Eimeria* spp. The few available

are focused on *Cryptosporidium* and *Toxoplasma* in animal bioassay tests (Bukhari et al., 2000; Liou et al., 2002; Dumètre et al., 2008; Pereira et al., 2008). Moreover, the action of ozone against the reproductive stages of protozoa has been demonstrated *in vitro* against *Leishmania* using ozonated oil (Rajabi et al., 2015). However, ozonated oil has demonstrated its cytotoxic activity on the trophozoites of *G. duodenalis* cultivated *in vitro* (Hernández et al., 2008) due to the high concentration of ozonide and peroxide.

Despite the advantages of ozone, some limitations are associated with the produced ozonated water technology, such as the high cost of the ozone generator, the need for operating and service infrastructure on a large scale that could determine the limited use of ozone in livestock farms in practice. With the development of the technology employed in this PhD, the production of ozone at small scale and low cost can be pursued and its possible use should be further explored.

In future perspectives, the role of natural disinfection treatment should be taken into account due to the control of emerging contaminants in dairy farming where the sanitation aspect is limited to the milk line production (i.e., against intestinal protozoa which are not currently regulated in Italy). Being a natural substance, the potential environmental sustainability of the use of ozone in other areas (such as water disinfectant; pesticide action in agriculture; and antibiotic, anti-inflammatory and antiviral actions in animal husbandry and fish farming) are of great interest.

Considering the health implications and the economic relevance of water buffalo farming systems in southern Italy and other parts of the world, the development of sustainable approaches to control pathogens as intestinal protozoa are of extreme importance from both a scientific and a practical point of view.

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