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Review

New approaches for risk assessment and management of bovine protothecosis

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ABSTRACT

Protothecosis is a potential zoonosis related to bovine mastitis. In several countries, a higher incidence of protothecal bovine mastitis that is being recorded and the resistance of *Prototheca* species to various factors (chlorine, high temperatures, antimicrobial and antiseptic treatments, pH variations), make it difficult to control its spread among farms. The authors aim to describe the infection caused by microalgae, focusing on the problems within cattle farms and proposing new approaches to farm management, based on Regulation (EU) No 2016/429 on transmissible animal diseases. This new flexible approach, based on risk analysis, is a further tool in protecting against *Prototheca* species. The list of transmissible animal diseases under Regulation (EU) No 2016/429 includes those caused by microorganisms resistant to antimicrobials, which can have important implications for human and animal health, feed and food safety. This approach would involve a series of changes to the rules used for Official Controls (Regulation (EU) No 2017/625) moving from the concept of the food chain to that of the agri-food chain.

Contents

1. Introduction

The genus *Prototheca* consists of achlorophyllic algae that are ubiquitous in the environment and animal gut and can have a parasitic behaviour (Kano, 2020). The genus is now placed in the class Trebouxiophyceae, order Chlorellales, and family *Chlorellaceae*, the same family of *Chlorella* sp., a green alga related to Prototheca sp. It includes species such as (i) *P. wickerhamii*, (ii) *P. zopfii* (iii) *P. blaschkeae*, (iv) *P. miyajii*, (v) *P. ulmea*, (vi) *P. cutis* and (vii) *P. stagnora* (Milanov et al., 2016).

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In particular, *P. zopfii*, *P. miyajii*, *P. cutis* and *P. wickerhamii* can cause human disease, while *P. blaschkeae*, *P. zopfii,* and *P. wickerhamii* can also infect pets. Lastly, *P. blaschkeae* and *P. zopfii* are also responsible for bovine mastitis, a condition that leads to a reduction in milk production and milk quality (Kano, 2020).

Prototheca spp. multiply asexually with multiple divisions, forming endospores that lie within the mother cell (Marques et al., 2010). The endospores are organized in the typical morula conformation (Asfour and El-Metwally, 2010). At the final stage of maturation, the endospores break through the double cellulose wall of the mother cell and shed their envelope, increasing in volume, reaching the adult *Prototheca* spp stage. Spore release is passive, occurring every 5–6 h and their number and size (4–30 μm in diameter) vary among *Prototheca* spp. (Figs. 1-2) (Pore et al., 1984; Lass-Flörl and Mayr, 2007). *Prototheca* spp. multiply using ammonium salts, but not nitrates. They assimilate glucose, fructose and galactose, but are not able to use disaccharides. Their multiplication also requires the presence of thiamine and oxygen while, being devoid of chlorophyll, sunlight does not affect their survival and multiplication (Lass-Flörl and Mayr, 2007).

Therefore, the aim of the present review is to describe the infection caused by microalgae, focusing on the problems within cattle farms and proposing new approaches to farm management, based on Regulation (EU) No 2016/429 on transmissible animal diseases.

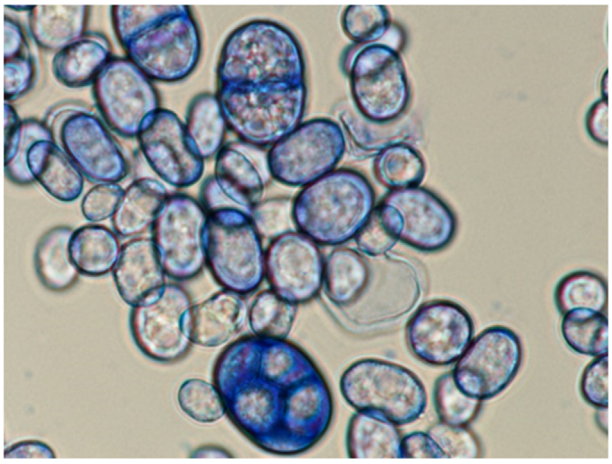

Fig. 1. *Prototheca* spp. isolated from milk samples. Cells in different phases of development: (i) the stage of dividing septum and (ii) during endospores forming (light microscopy - Methylene Blue × 40).

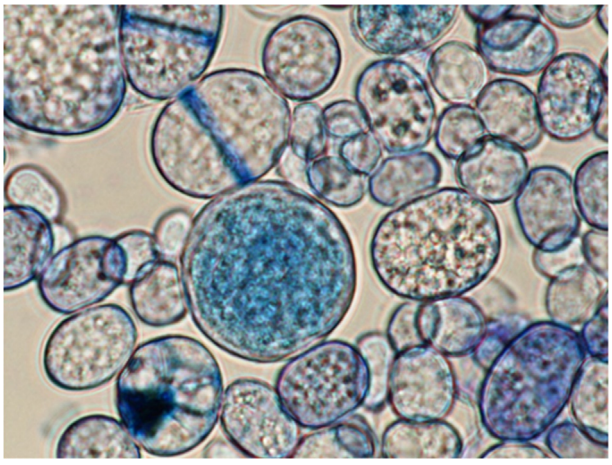

Fig. 2. *Prototheca* spp. isolated from milk samples. Cells with granular content and dividing septum (light microscopy - Methylene Blue × 40).

2. Susceptibility of *Prototheca* spp.

Prototheca species are resistant to several factors: chlorine, high temperatures, antimicrobial and antiseptic treatments, pH variations and different salt concentrations (Marques et al., 2010; Lassa et al., 2010). The resistance of *Prototheca* spp. comes from the presence of sporopollenin, a complex polymer that is able to withstand chemical and enzymatic degradation in the cell wall. This allows it to be highly resistant in the environment and consequently favours its propagation (Marques et al., 2010; Lassa et al., 2010).

Gonçalves et al. (2015) showed that by performing the Minimum Inhibitory Concentration (MIC) technique, *P. zopfii* isolates are able to produce biofilms in stainless steel coupon assays subjected to the action of the following sanitizers: sodium hypochlorite, peracetic acid and iodine solution. Of the 3 sanitizers examined, peracetic acid had the highest effectiveness against *P. zopfii*. The resistance of *P. zopfii* to the sanitizing agents evaluated may concur to determine the persistence of this species in milking and milk-processing areas.

Investigations into *P. zopfii*'s resistance to pasteurization in milk showed that the alga does not suffer any damage at the time/temperature ratio of 72 °C for 15′′ (Regulation (EU) No 2004/853), while it is destroyed at a time/temperature ratio of 65° C for 30′ (Melville et al., 1999). A similar study was conducted by Marques et al. (2010) in which it was tested the resistance of *P. zopfii* and *P. blaschkeae* to the various time/temperature ratios commonly used in the dairy industry (62 °C/15′; 70 °C/20′; 75 °C/20′′; 90 °C/1′′ and 100 °C/1′′). Their findings pointed out a reduction in multiplication of all strains used at high temperatures and the complete destruction of the alga was only observed at 100 °C.

P. zopfii and *P. blaschkeae*, the two species most frequently implicated in the etiopathogenesis of bovine mastitis were also tested at different pH values. Thus, *P. blaschkeae* and *P. zopfii* were able to survive at pH values of between 5 and 12 and 5–9 and up to NaCl concentrations of 4.5% and 18%, respectively (Marques et al., 2010).

Prototheca species have shown high levels of resistance to antifungal and antibacterial drugs. Indeed, the alga is resistant to fluconazole, caspofungin, amoxicillin, penicillin, ampicillin, itraconazole, streptomycin, clotrimazole, neomycin, miconazole and econazole. By contrast, kanamycin, gentamicin, ketoconazole and posaconazole and amphotericin B have been shown to be active in vitro. In addition, natural essences such as tea tree and bergamot oil have shown a significant in vitro efficacy against yeast-like alga (Lopes et al., 2008; Tortorano et al., 2008).

3. *Prototheca* species isolation and identification

Prototheca spp. identification relies on colony morphology and biochemical activity, which is being evaluated by auxanographic carbohydrate assimilation assays (Pore et al., 1984; Lass-Flörl and Mayr, 2007; Masuda et al., 2016). Isolation of *Prototheca* spp. in milk from individual cows is carried out by microbiological testing of both individual cows' milk and bulk milk, as well as from different types of environmental samples: drinking water, milking plant washing water, bedding, faeces, surfaces, fodder and other types of feed, etc. (Arrigoni et al., 2010). The most commonly used culture media for isolating *Prototheca* spp. are: (i) Glucose broth, (ii) Prototheca Isolation Medium (PIM), (iii) Glucose agar, (iv) Potato dextrose agar, (v) Blood agar and (vi) Sabouraud-dextrose agar.

Răpuntean et al. (2006) demonstrated that *Prototheca* spp. multiply rapidly on both liquid and solid media, such as glucose media, potato agar, blood agar and others. Plates are incubated in aerobic conditions at 37 °C and examined at 48 and 72 h after incubation.

The cultural characteristics of *P.* spp. vary greatly over time, which is why Răpuntean et al. (2006) suggest that plates incubated at 37 °C should be examined under a stereomicroscope at two different times: at 48–72 h and after 5–7 days.

After culture, the final diagnosis is performed using carbohydrate assimilation tests (API 20C Bio Merieux; VITEK® Yeast Biochemical Card; RapID Yeast Plus System-Remel). All *Prototheca* species assimilate glucose and carbon sources (Milanov *et al.,* 2006).

Another important contribution from the laboratory is the option of typing strains isolated from different matrices using biomolecular techniques in order to interpret the environmental isolates of *Prototheca* spp., identify sources of infection and adopt appropriate measures to manage hazards in the farm environment. The techniques currently available are genotype-specific PCR, Restriction Fragment Length Polymorphism (RFLP) or Real Time PCR associated with melting resolution analysis. These methods enable the various Prototheca species to be differentiated (Arrigoni et al., 2010). Molecular analyses such as nucleotide sequencing of the large subunit D1/D2 region and the small subunit of rDNA have been carried out to classify *Prototheca* spp. (Pore et al., 1984; Satoh et al., 2010; Masuda et al., 2016). These target regions are preserved between fungi and *Prototheca* spp. and can be successfully amplified using the same primer sets (Masuda et al., 2016).

Recently, a new speed method for phenotypic characterization of *P.* has been developed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry proteomic analysis and used to identify isolates from clinical specimens taken from human and animal protothecoses (Masuda et al., 2016; Ahrholdt et al., 2012; Irrgang et al., 2015; Fernández et al., 2019). The above method is a useful tool for differentiating among all *Prototheca* spp., and to compare the sequences of the amplicons obtained with those deposited in the GenBank database.

4. Human protothecosis

Protothecosis is a sporadic human disease occurring worldwide (Lass-Flörl and Mayr, 2007). Although human infections with this genus of algae are commonly considered unusual, an increasing number of cases are being diagnosed, especially among immunocompromised patients, those on corticosteroid treatment, or both; this is one of the reasons why this disease currently raises interest in human and veterinary medicine (Todd et al., 2018). The pathogenesis of protothecosis is largely unknown (Lass-Flörl and Mayr, 2007) but certain occupational groups are particularly at risk of infection with *Prototheca* species infections, such as: workers in rice fields, fishermen (Tejada et al., 1994), farmers (Chao et al., 2002), raw seafood handlers and aquarium staff (Boyd et al., 1995). The first reported human case of protothecosis was diagnosed in 1964, in Sierra Leone, on the foot of a rice farmer. The lesion started as a depigmented area, injured several times by the fact that the patient walked barefoot, until it became a papule with a raised margin (Davies et al., 1964). The skin is the organ most frequently exposed to protothecal infection, accounting for 54% of the 211 cases reported up to 2017 (Todd et al., 2018), through contact with contaminated soils or water, insect bites, or through traumatic inoculation with the alga (Todd et al., 2018).

However, *Prototheca* spp. is also capable of infecting other areas of the human body: fingernails (Galan et al., 1997), the olecranon bursa (Ahbel et al., 1980), the respiratory tract (Iacoviello et al., 1992) and the digestive system (Raz et al., 1998; Sands et al.,1991). As the report by Todd et al. (2018) clearly illustrates, all human cases have been caused by *P. zopfii, P. wickerhamii,* or *P. blaschkeae*. *P. wickerhamii* is the most frequently isolated etiologic agent of human protothecosis, regardless of geographic region (Inoue et al., 2018). Human-human transmission has been ruled out (Milanov et al., 2006).

The onset of protothecosis can be either localized or disseminated, either acute or chronic, and infections can involve both immune-competent and immunocompromised individuals (Leimann et al., 2004). In addition, 3 clinical forms of protothecosis has been recognized, that are: (i) cutaneous lesions, (ii) olecranon bursitis, and (iii) disseminated or systemic infections (Leimann et al., 2004), with the latter being more common in patients with a long-term course of primary disease or immune dysfunction (diabetes mellitus, malignancy, chemotherapy and HIV infection) (Kunova et al., 1996); in many cases, patients with "defects in cell-mediated immunity" have the worst prognosis (Lass-Flörl and Mayr, 2007). Average age of diagnosed patients is 30 years of age or older, but cases in children and infants have also been described (Torres et al., 2003; Sari et al., 2018). As regards sources of human protothecal infections, these ubiquitous microalgae (Pore et al., 1983) can be isolated from many reservoirs, such as the environment, animals (cattle, deer, dogs), and food, such as bananas, potato peel, cow's milk and some of its derivatives (butter and cheese) (Pore, 1985a,1986b; Nelson et al., 1987; Huerre et al., 1993). A major source of human exposure to *P.* spp. appears to be raw milk (Lass- Flörl and Mayr, 2007). According to Abdelhameed's 2016 study, in which 300, randomly selected, raw milk and cheese samplings were collected from Qena city markets (Egypt), a high prevalence of *Prototheca* species was found in 55 raw milk and 3 cheese samples (Damietta and Kareish cheese), respectively. This confirms that where raw milk and cheese are contaminated, they represent a human source of exposure and a health hazard to consumers, as already suspected by Costa et al., 1998 who reported a case of enteritis caused by cheese made from raw milk. In agreement with this study, Sarale et al. (2014) performed experimental tests on: (i) raw milk intended for industrial pasteurization (72 °C/15 sec.) contaminated with 1200 CFU/ml of *Prototheca* spp., cheese prepared with raw milk intended for refrigeration and seasoning contaminated with 1800 CFU/ml of *Prototheca* spp., (iii) yogurt prepared with a mixture of contaminated raw milk (1200 CFU/ml of *P.* spp.), boiled milk and a starter. These trials demonstrated the efficacy of pasteurization in reducing or even eliminating *Prototheca* spp. from milk and also the effectiveness of refrigeration in limiting *Prototheca* spp. multiplication both in cheese and yogurt. However, during the seasoning process at room temperature, an increase in *Prototheca* spp. concentration was recorded (Sarale and Midulla, 2014). Therefore, the implementation of sanitary procedures during production, processing and storage, as well as sufficient thermic treatment of milk, is recommended (Abdelhameed, 2016).

5. Bovine mastitis

Bovine mastitis caused by *Prototheca* spp. usually presents as an asymptomatic and chronic form, with a somatic cell count that can even surpass 10^6 cells/ml, but in some cases acute forms with clinical symptoms can be observed (Janosi et al., 2001). Indeed, *P. blaschkeae* and *P. zopfii* are the species associated with bovine mastitis, which leads to a peculiar thin secretion of watery milk containing white flakes and to a reduction in milk amount. Furthermore, increased parenchymal thickness and a progressive decrease in milk production are associated with atrophy of the affected quarter (Fig. 3).

Economic losses result directly from lower milk production and premature culling of affected animals, while indirectly from veterinary care costs (Jagielski et al., 2019).

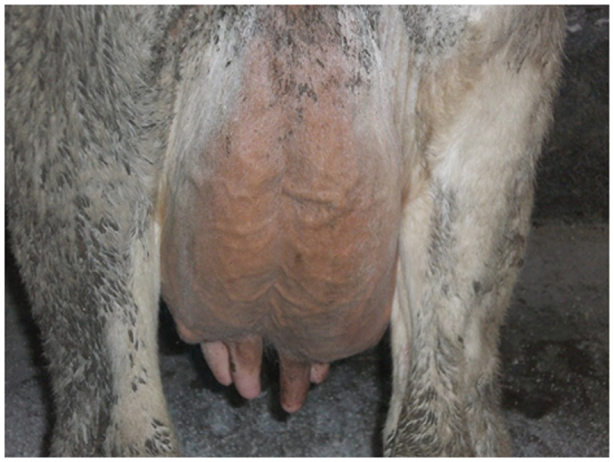

Fig. 3. Dairy cattle. Slight atrophy of the quarter of the mammary gland affected by *Prototheca* spp.

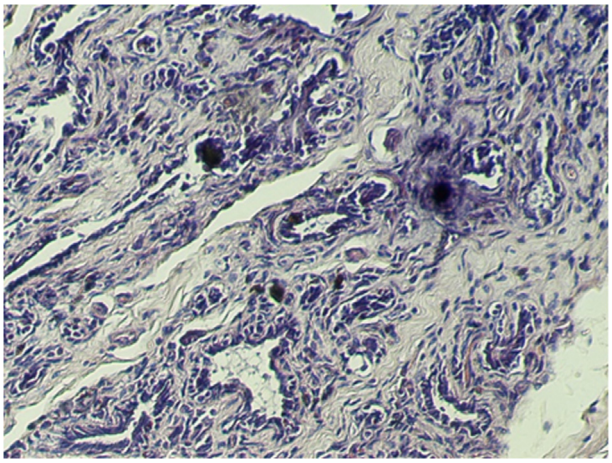

Fig. 5. *Prototheca* organisms in bovine mammary gland. Damage of mammary alveoli with *Prototheca* in dark clusters (light microscopy - Hematoxylin Eosin × 40).

P. zopfii was first identified as an etiologic agent of bovine mastitis in 1952 in Germany. To date, *P. zopfii* has been isolated all over the world from the milk of cows with clinical and subclinical mastitis (Milanov et al., 2016). In recent years, an increase in the incidence of *P. zopfii* mastitis has been reported worldwide (Milanov et al., 2016; Zecconi, 2011). It has also been shown that the larger the size of livestock herds, the greater the presence of *Prototheca* spp. This must be related to the use of cooling systems which increase the amount of water in the courses and litter, thus favouring multiplication, or lack of hygiene adaptation. Indeed, *Prototheca* spp. infections among animals occur via the oral-faecal route. This pathogen can be isolated from 20% to 70% of evaluated faeces samples from healthy animals (Rakesh et al., 2006). *Prototheca* spp. are ubiquitous and can be isolated from different environmental reservoirs. Given that a dairy cow diet is mainly composed of forages and grains, these algae may be temporarily part of a cow's gastrointestinal tract and as such be excreted intact (Milanov et al., 2016).

Prototheca spp. can also penetrate the mammary gland through teat sores. There they are engulfed by cells, macrophages and sometimes neutrophils from the alveolar lumen and interstitium of the infected mammary gland, where they multiply (Rakesh et al., 2006). The first inflammatory reaction is relatively mild and then progressively escalates. Histological findings, as such mammary gland infections progress, show interstitial mastitis associated with enlarged mammary acini and severe epithelial damage (Fig. 4).

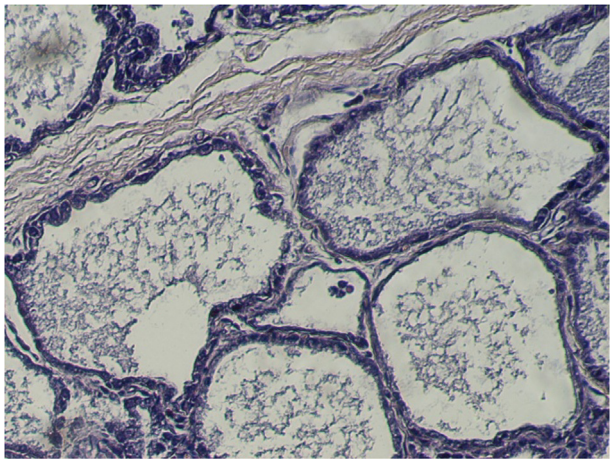

Fig. 4. *Prototheca* organisms in bovine mammary gland. Dilated mammary acini associated with destruction of their epithelium (light microscopy - Hematoxylin Eosin × 20).

In addition, in the lumen of damaged mammary alveoli, *Prototheca* microalgae appear in the form of dark clusters (Fig. 5) (Bozzo et al., 2014).

Infection is confined to the mammary gland and to regional lymph nodes and its spread from the initial lesions to other visceral organs is rarely observed (Migaki et al., 1982; Zecconi, 2011).

It is essential to remember that calves fed contaminated milk can harbour the microalgae in their gastrointestinal tract and become a source of faecal spread themselves. Once the infection is established, it is maintained in the herd through excretion by clinically stable individuals (Roesler and Hensel, 2003).

Somatic cell content may vary between infected animals depending on the inflammatory response of the animals and the pathogenic characteristics of the *Prototheca* (Zecconi, 2011). Somatic cell counts are without doubt one of the most useful tools for assessing dairy cow welfare and, especially, udder health. This information must be considered on an ongoing basis, not just when a problem arises. In addition, somatic cell count (SCC) assessment is most valuable in cases of environmental or sub-clinical mastitis (Bolzoni et al., 2006).

6. New strategies for the control of bovine mastitis

The control of *Prototheca* spp. infections is hampered by the lack of effective therapy. Despite the in vitro efficacy of several antifungals, treating animals with these conventional drugs is fraught with difficulties (Lagneau, 1996; Sato et al., 1998; Rakesh et al., 2006; Tortorano et al., 2008).

Therefore, since the presence of *Prototheca* spp. on the farm cannot be prevented, as it is an environmental pathogen, the only solution is to avoid increasing concentrations of this microalga and prevent it from entering the mammary gland. This can be achieved by maintaining hygiene both of bedding and during milking activities, by making correct use of water on dirty teats and udders (Bozzo et al., 2014). Together with these common issues with environmental mastitis (hygiene of bedding and milking procedures) an epidemiologically important factor that must be taken into account to control the spread of *Prototheca* spp. is the connection between its presence in cows' milk and its ability to colonise the gastrointestinal tract. Taking this into account, feeding of milk unfit for human consumption to heifers should be prohibited. Indeed, heifers can themselves become intermittent shedders of

the microalga as a result of intestinal contamination (Bozzo et al., 2014).

New approaches for herd and disease management are now available, based on EU Regulation 2016/429 of the European Parliament and of the Council on transmissible animal diseases amending and repealing certain acts in the field of animal health (Animal Health Act). The Regulation introduces the obligation to assess transmissible animal diseases in prevention through the biosecurity system and risk analysis. Furthermore, to facilitate so-called safe trade, the regulation introduces the responsibility of animal keepers, breeders, veterinarians and the competent authority. Indeed, operators are responsible for animal health; for the responsible and prudent use of drugs, without affecting the responsibility and role of veterinarians; for reducing the risk of spreading diseases and for proper livestock breeding (Article 10 of Regulation (EU) 2016/429 of the European Parliament and of the Council). For this reason, farm staff and veterinarians must have proper knowledge of: diseases, that include zoonosis; biosecurity principles; the linking between human and animal health; correct livestock farming practices; antimicrobial resistance and its consequences (Article 11 of Regulation (EU) 2016/429 of the European Parliament and of the Council).

For external biosecurity purposes, the position of farm, vehicle and passenger traffic, as well as sanitary facilities and the separation of clean and dirty areas should all be assessed (Regulation (EU) 2016/429 of the European Parliament and of the Council). According to biosecurity measures customized to fit individual farm characteristics, the farm should be demarcated from appropriate fencing and warning signs (Lewerin et al., 2015). Parking spaces should be located off the premises and a disinfection area for vehicles should be arranged. Staff and visitors should be provided with plant-specific clothing and boots or disposable overalls and boot covers. Visitors should only be admitted by date and visitor rules and a visitors' report should be made available. As far as possible, contaminated work paths (slurry collection, carcass transport) should not cross those of clean work processes (feed transport, milk collection) (Regulation (EU) 2016/429 of the European Parliament and of the Council).

In addition, a biosecurity plan requires strict control of the disinfection baths for vehicles and facilities for cleaning and disinfecting hands, footwear and transport vehicles, as well as for the storage of cleaning and disinfection agents (Regulation (EU) 2016/429 of the European Parliament and of the Council). Particular attention must be paid to how feed and drinking water are managed. As regards water, drinking-related parameters should be measured, such as smell, opacity and faecal contamination and regular controls must be conducted on the water supply system, distribution systems, and drinkers used (Hartung et al., 2000; Van Eerdenburg, et al., 2021). In the case of feed hygiene, the focus should be on minimizing the risk of contamination during storage, transport and distribution of feed. Feed storage facilities should be regularly inspected and cleaned. Housing hygiene should be evaluated separately for adult dairy cattle, calves, and young cattle, while feeding areas should be examined for dimensional accuracy and cleanliness (Moore et al., 2012). In agreement with Väärikkälä et al., (2019), inspections of livestock welfare should be especially conducted during the cold and rainy seasons and in holdings with limited average herd size. As regards maternity pen, a well bedded and well ventilated area designated specifically for calving, hygiene measures should be based on the all-out-all-in cleaning principle to prevent the circulation of pathogens and antibiotic-resistant microorganisms, to reduce calves mortality rates and to prevent injuries among veterinary staff and livestock (Moore et al., 2012).

Purchased animals should only come from herds that have been inspected by a veterinarian and should initially be kept separate from their own stock (quarantine). Quarantine is indicated when animals are brought in from other farms. Even on return from shows, animal markets or even veterinary practices, animals should not be reintroduced to the herd before an appropriate separation period. The quarantine barn should be run according to the all-in, all-out principle and should be managed completely separately from the herd.

Sick animals should also be housed separately, but in visual contact with other calves. Proper preparation of milk replacer and thorough cleaning and disinfection of milk-carrying parts (buckets, teats, milk lines) are essential to maintain calf health (Moore et al., 2012; Regulation (EU) No 2016/429).

Therefore, the most effective tools for limiting the spread of these microalgae on farms are: (i) identify infected cows through cyto-bacteriological analysis with selective medium for *P.* spp.; (ii) separate healthy animals from infected ones (milked last), particularly during the lactation period; (iii) cull infected animals if low in number; (iv) provide excellent hygiene of both bedding and of aisles/runs**;** (v) adopt a proper milking routine and good hygiene during milking procedures; (vi) prohibit the use of milk from infected animals to calves, even if pasteurized; (vii) separate infected animals from healthy ones in calving areas; (viii) postpartum control of cow's milk samples; (ix) analyse bulk milk from healthy individuals weekly for the presence of *P.* spp. If positive, repeat the analysis immediately and, if confirmed, individually monitor cows, belonging to the healthy group (Bozzo et al., 2014; Zecconi, 2011).

7. Conclusion

The new approaches for the proper management of farms indicated by Regulation (EU) No 429/2016 on transmissible animal diseases, represent major innovations that will bring objective benefits in the management of diseases such as Bovine Protothecosis. Indeed, the Regulation establishes: (i) increased prevention and better surveillance, (ii) a flexible approach based on risk analysis and objective scientific data, (iii) greater responsibility for farmers (iv) greater responsibility for EU Member States.

Taking into account that transmissible diseases, including those caused by antimicrobial resistant microorganisms, can have important implications for human and animal health, feed and food safety, the Regulation resulted in a number of changes to the rules on Official Controls (Regulation (EU) No 2017/625). These are to be conducted in a harmonized manner across the European Union, covering the entire supply chain and effectively replacing previous regulations on food of animal and plant origin. This is thus a move away from the concept of the food chain and towards that of the agri-food chain.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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