Eesti Maaülikool
Estonian University of Life Sciences

CRYPTOSPORIDIUM SPP. PREVALENCE, RELATIONSHIP WITH THE GENERAL INFLAMMATORY RESPONSE, FAECAL MICROBIOTA AND HALOFUGINONE LACTATE TREATMENT IN CALVES

KRÜPTOSPORIIDIDE LEVIMUS, SEOS ÜLDISE PÕLETIKUVASTUSE, ROOJA MIKROBIOOTA JA HALOFUGINOON LAKTAADI RAVIGA VASIKATEL

ELISABETH DORBEK-KOLIN

A Thesis
for applying for the degree of Doctor of Philosophy
in Veterinary Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks loomaarstiteaduse erialal

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Institute of Veterinary Medicine and Animal Sciences,
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According to verdict N° 2 of November 18, 2022, the Doctoral Committee of Veterinary and Food Science of Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor Philosophy in Veterinary Medicine and Food Science.

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To my parents

Nutikas nagu lutikas!

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following papers, which are cited in the text by Roman numerals.

I Santoro, A., **Dorbek-Kolin, E.**, Jeremejeva, J., Tummeleht, L., Orro, T., Jokelainen, P., Lassen, B. (2018). Molecular epidemiology of *Cryptosporidium* spp. in calves in Estonia: high prevalence of *Cryptosporidium parvum* shedding and 10 subtypes identified. Parasitology 146, 261–267. DOI: 10.1017/S0031182018001348

II Niine, T., **Dorbek-Kolin, E.**, Lassen, B., Orro, T. (2018). *Cryptosporidium* outbreak in calves on a large dairy farm: effect of treatment and the association with the inflammatory response and short-term weight gain. Research in Veterinary Science 117, 200–208. DOI: 10.1016/j.rvsc.2017.12.015

III **Dorbek-Kolin, E.**, Husso, A., Niku, M., Loch, M., Pessa-Morikawa, T., Niine, T., Kaart, T., Iivanainen, A., Orro, T. (2022) Faecal microbiota in two-week-old female dairy calves during acute cryptosporidiosis outbreak – Association with systemic inflammatory response. Research in Veterinary Science 151, 116–127. Advance online publication. DOI: 10.1016/j.rvsc.2022.07.008

The contribution of author's to the research papers

Paper	Original idea, study design	Data Collection, sample analysis	Data analysis	Manuscript preparation
I	BL, PJ	AS, **ED**, JJ, LT, BL	**ED**, TO	All
II	TO, TN, BL	TN, TO, **ED**, BL	TN, TO	All
III	TO, **ED**, MN	**ED**, ML, TO	**ED**, AH, MN, ML, TP, TN, TK, AI, TO	All

ED – Elisabeth Dorbek-Kolin, TN – Tarmo Niine, BL – Brian Lassen, PJ – Pikka Jokelainen, JJ – Julia Jeremejeva, LT – Lea Tummeleht, AS – Azzurra Santoro, TO – Toomas Orro, AH – Aleksi Husso, MN – Mikael Niku, ML – Marina Loch, TP – Tiina Pessa-Morikawa, TK – Tanel Kaart, AI – Antti Iivanainen, All – all authors of the paper

ABBREVIATIONS

ADWG – average daily weight gain

AIDS – acquired immunodeficiency syndrome

APP(s) – acute phase protein(s)

APR – acute phase response

ASVs – amplicon sequence variants

CI – confidence intervals

EIA – enzyme immunoassay

ELISA – enzyme-linked immunosorbent assay

FDA – Food and Drug Administration of United States of America

gp60 – 60-kDa glycoprotein

HL – halofuginone lactate

Hp – haptoglobin

IFA – immunofluorescence antibody assay

I-FABP – intestinal fatty acid binding protein

IFN- γ – interferon gamma

Ig – immunoglobulin

IL – interleukin

kDa – kilo Dalton (1,000 Dalton)

LBP – lipopolysaccharide-binding protein

opg – oocysts per gram (of faeces)

PCM – phase-contrast microscopy

PCR – polymerase chain reaction

rRNA – ribosomal RNA

SAA – serum amyloid A

Th – T helper lymphocytes

TNF- α – tumour necrosis factor alpha

1. INTRODUCTION

Zoonotic protozoan parasites of the genus *Cryptosporidium* have a global distribution, and their prevalence in cattle ranges from 3.4–96.6% (Thomson *et al*., 2017). They are one of the causative agents of calf diarrhoea, along with rotavirus, coronavirus, *Escherichia coli*, *Giardia*, and others. Many species of *Cryptosporidium* can infect cattle, the most dominant one in pre-weaned calves being *Cryptosporidium parvum* (Chako *et al*., 2010). Only one prevalence study of *Cryptosporidium* spp. has been conducted in Estonia, which found a herd level prevalence of 84% and an animal level prevalence of 30% (Lassen *et al*., 2009). Two species of *Cryptosporidium* were identified in that study – *C. parvum* and *Cryptosporidium ryanae*. This thesis investigated the current prevalence of *Cryptosporidium* spp. in Estonian calves, the *Cryptosporidium* species involved, and the subtypes of *C. parvum* present (**I**).

As newborn calves are still dependent on passive and innate immunity, their immune response towards *Cryptosporidium* spp. should be studied in more detail. The acute phase response (APR) is a sequence of well-orchestrated processes that aims to re-establish homeostasis in an organism after tissue damage or invasion by pathogens (Baumann and Gauldie, 1994). During APR, mainly the liver, along with local tissues to some extent, synthesises acute phase proteins (APPs), whose roles and extents vary between species (Eckersall and Bell, 2010). In cattle, the two major APPs are serum amyloid A (SAA) and haptoglobin (Hp) (Eckersall and Bell, 2010). These and other APPs can be used as non-specific biomarkers of general inflammatory response (Petersen *et al*., 2004). The concentrations of APPs also undergo age-related fluctuations (Orro *et al*., 2006, 2008; Tóthová *et al*., 2015) that seem to be related not only to pathogens or tissue injury. Very few small-scale studies (Pourjafar *et al*., 2011) have been conducted on APPs during *Cryptosporidium* spp. infections in neonatal calves. This thesis aims to shed more light on this subject (**II** and **III**).

Enteric pathogens, such as *Cryptosporidium* spp., are known to alter the composition of the intestinal microbiota (Laurent and Lacroix-Lamandé, 2017). As *Cryptosporidium* spp. infect the epithelial cells of the intestines, it is logical to conclude that they might also influence the microbiota composition. The same can be deduced for halofuginone lactate treatment, as the effects of medications such as antibiotics on the

microbial composition and diversity have been demonstrated in previous studies (Grønvold *et al.*, 2011; Oultram *et al.*, 2015). As the microbiota has an important role in the overall health and welfare of an animal, the interactions with pathogens like *Cryptosporidium* spp. should be studied in more detail to develop more precise control measures against diseases and to find possibilities to improve health and welfare of the animal. Thus, possible associations between *Cryptosporidium* spp. and faecal microbiota composition and diversity were studied (**III**).

2. REVIEW OF THE LITERATURE

2.1. *Cryptosporidium* spp.

It was the year 1907 when Ernest Edward Tyzzer first discovered the protozoan parasite genus later known as *Cryptosporidium* (Tyzzer, 1907). Currently, at least 44 species in this genus have been named, and more than 120 genotypes have been recognised (Ryan *et al.*, 2021). These species all cause a disease called cryptosporidiosis. Cryptosporidiosis has been recorded in vertebrates, including mammals, marsupials, birds, reptiles, amphibians, and fishes (Spickler, 2018). *Cryptosporidium* spp. were first reported in cattle, more specifically in calves, in 1971 (Panciera *et al.*, 1971), and it was mentioned as a sole infective agent for the first time more than a decade later (Tzipori *et al.*, 1983). Four species of *Cryptosporidium* are commonly found in cattle, namely *C. parvum*, *Cryptosporidium bovis*, *C. ryanae*, and *Cryptosporidium andersoni* (Thomson *et al.*, 2017). *C. parvum* has been observed to be the dominant species in pre-weaned calves in many countries (Chako *et al.*, 2010), and it seems to be more prevalent in dairy cattle than in beef cattle (Abeywardena *et al.*, 2015). *C. parvum* is common in animals and humans and is globally distributed (Abeywardena *et al.*, 2015).

2.1.1. Life cycle

Cryptosporidium is considered a minimally invasive intracellular, but extracytoplasmic, parasite that completes its life cycle apically inside the epithelial cells of the small intestine (Petry *et al.*, 2010; Laurent and Lacroix-Lamandé, 2017; Delling and Daugschies, 2022). All members of this genus complete all developmental stages within a single host (O'Hara and Chen, 2011). *Cryptosporidium* spp. have two reproductive phases, asexual and sexual. First, it goes through two cycles of merogony (asexual phase), followed by one cycle of gamogony (sexual phase) (Delling and Daugschies, 2022). The protozoan has one infectious stage, called the sporozoite, which is present in an oocyst (4–6 μm). There are two types of oocysts: thick-walled oocysts (80% of the oocysts) that are excreted from the body with faeces and thin-walled oocysts (20% of the oocysts) that cause autoinfection within the intestines (Kosek *et al.*, 2001).

2.1.2. Cryptosporidiosis in calves

2.1.2.1. Clinical signs

The severity of cryptosporidiosis is strongly related to the immune status of the host. The primary target hosts of this parasite are immunocompromised young individuals (Lantier *et al.*, 2014), particularly pre-weaned calves (<3 months of age) (Santín *et al.*, 2004, 2008).

Cryptosporidium is an intracellular parasite that infects the intestinal epithelial cells and causes debilitating diarrhoea (McDonald *et al.*, 2000). Dairy calves with cryptosporidiosis can eventually present villous atrophy of the small intestinal mucosa and increased intestinal permeability (Wyatt *et al.*, 2010). The main clinical signs in calves affected by cryptosporidiosis are watery (sometimes bloody) diarrhoea, depression or lethargy, anorexia, nutrient malabsorption, and abdominal pain (Naciri *et al.*, 1999; Olson *et al.*, 2004). Usually, the disease is self-limiting (Petry *et al.*, 2010); however, in severe cases, calves can die from dehydration and cardiovascular collapse due to the diarrhoea (Olson *et al.*, 2004).

2.1.2.2. Treatment and prevention

In the European Union, there are two drugs currently licenced for the treatment of cryptosporidiosis in calves. One that has been on the market for more than two decades and can be used as prophylaxis. Its active substance is halofuginone lactate (HL), which is marketed under the name Halocur (EMEA/V/C/000040), among others. The indications for its use are the prevention of diarrhoea following a diagnosis of *C. parvum* in farms with a history of cryptosporidiosis and a reduction of diarrhoea due to *C. parvum*. For prevention, its administration should start within the first 24 to 48 h of age, and for reduction of diarrhoea, it should start within 24 h after the onset of diarrhoea. In both cases, the treatment course should last seven days, with the drug administered once daily (Intervet International BV, 2019). Recently, a second drug became available for calves. It uses paromomycin as the active substance, and the aim is to reduce the diarrhoea symptoms and oocyst shedding (Huvepharma NV, 2022). Unfortunately, the pharmacodynamics of these two active substances

are not quite clear but they both have antiprotozoal properties (Intervet International BV, 2019; Huvepharma NV, 2022). For HL, it is known that its main activity is towards the free stages (sporozoite, merozoite) of the parasite (Intervet International BV, 2019).

Good farm management and husbandry practices are essential for preventing cryptosporidiosis. Potential risk factors for calf cryptosporidiosis include, but are not limited to, large herd size, maternity facilities used for many cows, early separation of the calf from its dam, lack of cleanliness and flooring of calf pens, high stocking density of calves, inadequate nutrition, and suboptimal consumption of colostrum (Olson *et al.*, 2004; Robertson *et al.*, 2014; Abeywardena *et al.*, 2015).

2.1.2.3. Epidemiology

Cryptosporidiosis has an average incubation period of five to seven days (Abeywardena *et al.*, 2015). The first shedding is usually observed four days post-infection with a duration of up to two weeks (Innes *et al.*, 2020). It has been reported that oocyst shedding in calves begins as early as the second day after birth (Olson *et al.*, 2004). The onset of shedding of different *Cryptosporidium* species varies from five (*C. bovis*) to 15 days of age (*C. ryanae*) (Åberg *et al.*, 2019), peaking at 14 days (Olson *et al.*, 2004). Notably, oocyst shedding is not always associated with diarrhoea (Thomson *et al.*, 2017). During infection, a very high number of oocysts can be shed in calf faeces, with up to 10^6 to 10^{10} oocysts per gram of faeces (opg) (Nydam *et al.*, 2001; Armon *et al.*, 2016). Oocysts are infectious when they are excreted by the host (Kosek *et al.*, 2001).

Cryptosporidium spp. oocysts are transmitted via the faecal-oral route (Ryan *et al.*, 2016), which is an umbrella term for all the following transmission routes: person-to-person, animal-to-person, person-to-animal, animal-to-animal, waterborne, foodborne, and airborne transmission (Cacciò, 2005). Contaminated water and contact with infected individuals (animals or humans) are considered major sources of infection (Laurent and Lacroix-Lamandé, 2017).

In a previous study of *Cryptosporidium* spp. in Estonia, researchers found that 84% of the cattle farms were infected, with a sample prevalence of 30% (Lassen *et al.*, 2009). In the same study, two species were identified:

C. parvum and *C. andersoni*. In Sweden, *C. parvum* was not detected, but the farms were infected with either *C. bovis, C. ryanae*, or both at the same time (Åberg *et al.*, 2019). It is important to note that not all *Cryptosporidium* species are zoonotic; for example, while *C. parvum* is zoonotic, *C. hominis* is anthroponotic (Cacciò and Chalmers, 2016).

To show the zoonotic potential and characterise the overall transmission dynamics of *Cryptosporidium* spp., many studies have used the 60-kDa glycoprotein (*gp60*) gene sequence analysis (Xiao, 2010). The *gp60* gene can be used for subtyping within a species (Strong *et al.*, 2000). The nomenclature system for subtypes is based on *gp60*-based typing, and it starts with a Roman numeral and lower-case letter for genotypes of *Cryptosporidium*, followed by uppercase letters denoting the number of tandem serine-coding trinucleotide repeats (TCA (A), TCG (G), and AAG (R)) (Ryan *et al.*, 2021). Example of this can be seen in figure 1. Calves are mostly infected with subtypes belonging to the IIa subtype family, of which subtype IIaA15G2R1 is the most common (Xiao, 2010). In Sweden, two *C. parvum* subtypes have been identified in beef suckler calves, IIaA16G1R1 and IIdA27G1 (Björkman *et al.*, 2015); IIaA15G2R1 is the most prevalent subtype in Latvia (Deksne *et al.*, 2022), and IIaA15G2R1 and IIaA16G3R1 are the most prevalent subtypes in Sardinia (Díaz *et al.*, 2018).

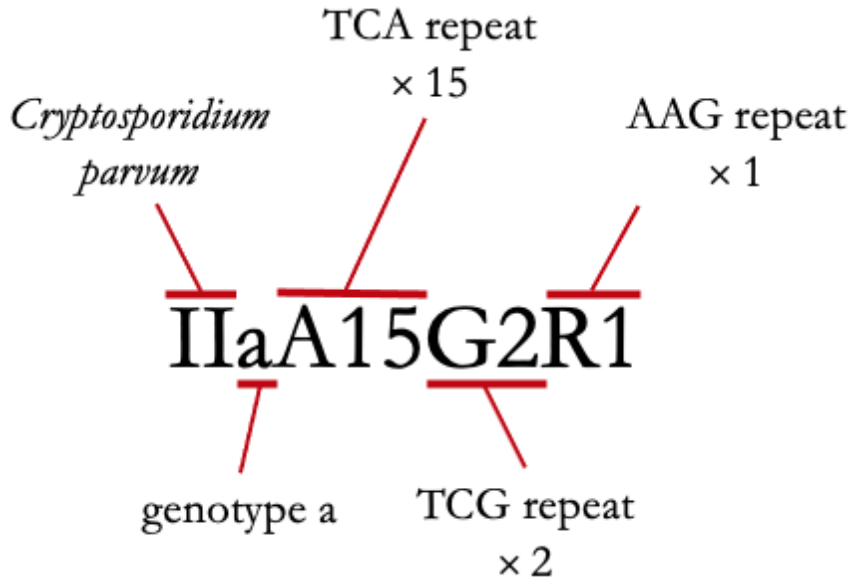

Figure 1. Nomenclature of *Cryptosporidium* spp. subtypes (based on Ryan *et al.,* 2021).

2.1.2.4. Effect on future performance

It has been suggested that cryptosporidiosis has long-lasting negative effects on animal performance and production efficiency (Klein *et al.*, 2008; Shaw *et al.*, 2020). *Cryptosporidium* spp. infections have been linked to lower weight gain in calves (da Silva Abreu *et al.*, 2019; Shaw *et al.*, 2020; Renaud *et al.*, 2021), lambs (Jacobson *et al.*, 2016), goats (Jacobson *et al.*, 2018), and humans (Khalil *et al.*, 2018). Infected calves were also found to have an intestinal permeability elevated by 100% and a reduced intestinal nutrient absorptive capacity, which can lead to lower weight gain (Klein *et al.*, 2008). A loss of barrier function is caused by the synergistic effect of both the parasite and inflammatory monocytes (de Sablet *et al.*, 2016).

2.1.3. Cryptosporidiosis in humans

Cryptosporidiosis is a zoonotic disease that poses a risk, especially to people working with calves (Abeywardena *et al.*, 2015). The first human case of cryptosporidiosis was reported in 1976 in a three-year-old child in the United States of America (Nime *et al.*, 1976), which was five years after the first reported case in calves (Panciera *et al.*, 1971). Most cases of human cryptosporidiosis are caused by *C. hominis* and *C. parvum* (Cacciò and Chalmers, 2016), but *C. meleagridis*, *C. felis*, and *C. canis* can also be zoonotic (Xiao and Feng, 2008). It seems that *C. hominis* is more common in urban settings, especially during the late summer and autumn, and is associated with young children, while *C. parvum* is more common among rural populations during spring and is associated with animal exposure (Elwin *et al.*, 2007). Human infections have been recorded with more than 15 different *Cryptosporidium* species (Checkley *et al.*, 2015).

The clinical signs in humans are similar to those in calves, including diarrhoea, abdominal pain, vomiting, fever, and malabsorption (Ryan *et al.*, 2016). *Cryptosporidium* spp. can cause intestinal damage, which can then lead to long-term cognitive deficits, impaired immune responses, and reduced vaccine efficacy in humans (Guerrant *et al.*, 1999). The host's immune status is known to have a major impact on disease severity and prognosis; immunocompetent patients are most likely to have self-limiting diarrhoea and transient gastroenteritis that can last for up to two weeks (Ryan *et al.*, 2016). For decades, *C. parvum* has been seen

as an opportunistic human pathogen that only causes significant consequences in immunocompromised individuals. This is supported by the fact that in patients with acquired immunodeficiency syndrome (AIDS), *Cryptosporidium* spp. infections are often chronic and life-threatening (O'Hara and Chen, 2011). However, one study suggested that there may be a possibility of respiratory transmission in immunocompetent children (Checkley *et al.*, 2015). It should be noted that only 10 or fewer oocysts are sufficient to cause cryptosporidiosis, even in clinically healthy adults (Okhuysen *et al.*, 1999; Chappell *et al.*, 2006).

Contaminated food and water (both drinking and recreational water) have been found to be sources of cryptosporidiosis outbreaks worldwide (Ryan *et al.*, 2016). Seroprevalence rates for human cryptosporidiosis have been reported to be 33.0–88.0% in Southern and Eastern Europe, 16.9–54.0% in the United States of America, and 64.0–94.6% in lower income countries (Collinet-Adler and Ward, 2010). In many countries, cryptosporidiosis is both under-diagnosed and under-reported (Cacciò and Chalmers, 2016). In Estonia, only a few cases in humans have been reported annually (Health Board, 2020).

Contact with calves is a risk factor for sporadic cryptosporidiosis in humans (Abeywardena *et al.*, 2015). Cryptosporidiosis is also an occupational hazard for people working with animals, especially young calves, such as farm workers (Autio *et al.*, 2012; Robertson *et al.*, 2019), scientists (Lassen *et al.*, 2014), and veterinary students (Pohjola *et al.*, 1986; Kiang *et al.*, 2006; Gait *et al.*, 2008; Kinross *et al.*, 2015; Galuppi *et al.*, 2016; Benschop *et al.*, 2017). In the European Union, there is no licenced medication for the treatment of human cryptosporidiosis, and in the United States of America, nitazoxadine is the only drug that has been approved by the Food and Drug Administration (FDA) (Sparks *et al.*, 2015).

2.1.4. Diagnostic methods

Several laboratory methods have been used to diagnose cryptosporidiosis. Each of these tests has its limitations, such as cost, performance, differentiation of clinical significance, and assessment of co-infections with other pathogens (Checkley *et al.*, 2015). In clinical settings, the detection of *Cryptosporidium* spp. is still mainly based on

microscopy using stains or immunofluorescence antibodies, as well as other antigenic detection methods (Ryan *et al.*, 2016). Microscopy and antigen detection assays are useful for clinical diagnoses at the genus level (Checkley *et al.*, 2015), although it should be noted that *Cryptosporidium* spp. oocysts are so small that detection by microscopy is unreliable (Cacciò and Chalmers, 2016) and lacks both sensitivity and specificity (Chalmers and Katzer, 2013).

Other laboratory methods include, but are not limited to, fluorescence microscopy using Auramine O or acid-fast modified Ziehl-Neelsen staining, enzyme-linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), immunochromatographic lateral flow assays, phase-contrast microscopy (PCM), and molecular methods such as multiplex polymerase chain reaction (PCR) (Chalmers and Katzer, 2013; Fournet *et al.*, 2013; Ryan *et al.*, 2016). Depending on the source of information, the most reliable diagnostic method known is either staining with malachite green (Thompson *et al.*, 2016) or immunofluorescence antibody assays (IFA), with a specificity up to 97% (Ryan *et al.*, 2016).

Many studies have reported the presence of *Cryptosporidium*-specific serum immunoglobulins (IgG, IgM, and IgA) and faecal IgA antibodies during human cryptosporidiosis (Borad and Ward, 2011). According to Collinet-Adler and Ward (2010), serology should not be considered a good diagnostic tool because it does not always reflect an active infection.

The 18S ribosomal RNA (rRNA) and *gp60* genes are the most widely used molecular markers for typing *Cryptosporidium* isolates and their subtypes (Strong *et al.*, 2000; Xiao, 2010; Zahedi *et al.*, 2016).

2.2. Host response to *Cryptosporidium* spp.

2.2.1. General immunological aspects

Cryptosporidiosis is usually self-limiting and lasts up to two weeks, suggesting an efficient host antiparasitic immune response (Ryan *et al.*, 2016). There is no consensus on whether calves are resistant to reinfection with cryptosporidiosis (Harp *et al.*, 1990; Thomson *et al.*, 2019).

Although newborns are immunocompetent from birth, their adaptive immune systems are still immature (Kovarik and Siegrist, 1998; Morein *et al.*, 2002). Both innate and adaptive immunity play a role in the fight against *Cryptosporidium* spp. infections (Laurent and Lacroix-Lamandé, 2017). During cryptosporidiosis, infected epithelial cells produce pro-inflammatory cytokines, chemokines, and antimicrobial peptides (Thomson *et al.*, 2017). Interferon gamma (IFN-γ) is one of the key pro-inflammatory cytokines that control the infection with *C. parvum*; others include interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumour necrosis factor-alpha (TNF-α) (Laurent and Lacroix-Lamandé, 2017). Infection with *Cryptosporidium* spp. stimulates the production of IL-2, IL-12, IL-15, and IL-18, which then activate the natural killer cells to produce more IFN-γ (Ivanova *et al.*, 2019). Some studies have indicated that IL-17A plays a role in reducing *C. parvum*-dependent damage to the host cells (Drinkall *et al.*, 2017).

$CD4^+$ T-cells (T lymphocytes) play an important role in the immune response to *Cryptosporidium* spp. (Petry *et al.*, 2010). Distinct types of T helper lymphocytes are responsible for inducing and regulating cellular (T helper 1, Th1) and humoral (T helper 2, Th2) responses (Arredouani *et al.*, 2003). The differentiation of these two T cells is partly dictated by the cytokine milieu present at the time of differentiation (Thomson *et al.*, 2017). Some Th2 cytokines (such as IL-4) are produced or upregulated by *C. parvum* (Partida-Rodríguez *et al.*, 2017). The production of IL-4 in response to a *C. parvum* infection may be a driver of protective Th1 responses (Thomson *et al.*, 2017). It has been suggested that the most effective response against *Cryptosporidium* spp. is a dynamic response, with an initial strong Th1 response followed by an increase in the Th2 response (McDonald, 2000; Tessema *et al.*, 2009).

Some studies have suggested that calves could be (partially) protected from cryptosporidiosis by colostral anti-*C. parvum* IgG antibodies during the first month of their life (Wang *et al.*, 2003; Lefkaditis *et al.*, 2020). However, calves up to one month of age have shown a weaker antibody response to *C. parvum* (with up to only a twofold increase in serum antibody titre), than calves one to three months of age, whose serum antibody levels increased approximately tenfold (Harp *et al.*, 1990). The same phenomenon has been reported in healthy humans (Chappell *et al.*, 1999).

2.2.2. Acute phase proteins

Following tissue injury or infection of the host, acute phase proteins (APPs) are produced mainly by the liver in a process called acute phase response (APR) (Baumann and Gauldie, 1994). The concentration and kinetics of systemic APPs during the inflammatory response appear to be related to the severity of tissue damage and the time course of the inflammation process (Kent, 1992). During this process, APP concentrations in the blood serum change by >25% from their normal levels in response to pro-inflammatory cytokines. An APP is considered a major responder when its concentration rises 100–1000-fold after stimulation (peak at 24–48 h), and a minor responder if the rise is 5–10-fold (peak at 2–3 days) (Eckersall and Bell, 2010). Thus, measuring the circulating levels of APPs provides valuable and quantifiable information about the ongoing APR, and they can be used as highly sensitive, non-specific disease markers (Thompson *et al.*, 1992; Van Leeuwen and Van Rijswijk, 1994; Petersen *et al.*, 2004). It is worth noting that there can be great individual- and species-related differences in the APR; in cattle, two major APPs have been identified – Hp and SAA (Eckersall and Bell, 2010). Only few small-scale studies have been conducted on APPs during *Cryptosporidium* spp. infections in dairy calves, showing an increase of APPs in calves with cryptosporidiosis (Pourjafar *et al.*, 2011).

2.2.2.1. Serum amyloid A

Although its main origin is the liver, SAA is also produced by various extrahepatic tissues such as the intestines, lungs, and mammary glands (Urieli-Shoval *et al.*, 1998; Berg *et al.*, 2011). SAA functions as a binder for cholesterol, an immunomodulator, and can act as a pattern recognition protein by opsonising bacteria (Ceciliani *et al.*, 2012). In cattle, SAA concentrations are elevated more by acute inflammations than by chronic conditions (Horadagoda *et al.*, 1999). The synthesis of SAA is mediated by the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Uhlar *et al.*, 1997). SAA is now known to be a soluble pattern-recognition receptor that induces Th2 immunity (Smole *et al.*, 2020).

The concentration of SAA in the blood serum undergoes age-related fluctuations after birth. It is the lowest at birth, rises throughout the first

week, and peaks by the seventh day, after which it decreases to adult levels (Alsemgeest *et al.*, 1995; Orro *et al.*, 2008; Tóthová *et al.*, 2015).

2.2.2.2. Haptoglobin

Hepatocytes are the main producers of Hp (Theilgaard-Mönch *et al.*, 2006), which is induced by pro-inflammatory cytokines (such as IL-6) (Oliviero and Cortese, 1989). The main function of Hp is to bind haemoglobin, which prevents oxidative damage caused by free haemoglobin. Other biological functions of Hp include activating an anti-inflammatory response, exerting a bacteriostatic effect, enhancing angiogenesis, and acting as an extracellular chaperone (Ceciliani *et al.*, 2012). Hp has been found to strongly shift the balance of the Th1/Th2 immune response towards Th1, thus promoting cellular immunity (Arredouani *et al.*, 2003).

In calves, the serum concentrations of Hp are the lowest at birth (Alsemgeest *et al.*, 1995; Tóthová *et al.*, 2015) and highest at 7–13 days after birth (Orro *et al.*, 2008; Ceciliani *et al.*, 2012; Tóthová *et al.*, 2015; Ramos *et al.*, 2021).

2.3. Microbiota of calves

2.3.1. Intestinal microbiota of newborn calves

Previously, it was thought that the foetus is sterile until parturition, which is when it makes first contact with the outside world (Malmuthuge *et al.*, 2015); however, recent studies such as those by Alipour *et al.* (2018) and Guzman *et al.* (2020) have shown that the foetal gastrointestinal tract is colonised by a diverse microbiota even before birth. The microbiota is the overall composition of the microbial community, which is usually identified through targeted 16S rRNA gene amplicon sequencing (Malmuthuge and Guan, 2017).

The rectal microbiota of calves rapidly changes during the first week of life (Alipour *et al.*, 2018). The microbial diversity increases with age (Dill-McFarland *et al.*, 2017), with the highest species richness and diversity detected during weaning (Klein-Jöbstl *et al.*, 2014).

Depending on the breed, feeding or diet, management practices, and microbial profiling methods, the microbial compositions have differed among studies, with the highest abundance of either the phyla *Bacteroidetes* or *Firmicutes* (Malmuthuge and Guan, 2017). *Firmicutes* and *Bacteroidetes* were found to be dominant throughout the gastrointestinal tract, especially in the colon (Jami *et al.*, 2013; Myer *et al.*, 2017).

The diversity of faecal microbiota is associated not only with the age of the calf but also with the disease status and growth rates (Oikonomou *et al.*, 2013). Many factors can alter calves' microbial composition, such as antibiotic treatment (Grønvold *et al.*, 2011; Oultram *et al.*, 2015), feeding of milk with antimicrobial drug residues (van Vleck Pereira *et al.*, 2016), heat stress (Chen *et al.*, 2018), weaning (Meale *et al.*, 2016), and diarrhoea (Gomez *et al.*, 2017). Changes in the microbiome of diarrhoeic calves can be reverted to the pre-diarrhoeic stage within one week, regardless the age of the calf (Kim *et al.*, 2021). Age-related changes are not influenced by the possible continued vertical transmission of microbes from their dams (Barden *et al.*, 2020).

The intestinal microbiota plays an important role in regulating innate and adaptive immune responses, and helps maintain immune homeostasis (Kayama and Takeda, 2015). It has been suggested that the gut microbiota is linked to the productivity of farm animals (Kraimi *et al.*, 2019), and that bacteria specifically belonging to the genus *Faecalibacterium* could be beneficial for the growth and health of calves (Oikonomou *et al.*, 2013).

2.3.2. *Cryptosporidium* spp. in the intestinal tract and effects on microbiota

Infection with enteric pathogens can change microbiota composition which, in turn, can either promote or resist the infection (Laurent and Lacroix-Lamandé, 2017). Klein *et al.* (2008) found that a *C. parvum* infection causes the intestinal permeability to be elevated by more than 100% seven days post-infection. They also found that the permeability index returned to the normal range by 21 days post-infection. The perturbation of the microbiota induced by *C. parvum* appears to be reversible (Ras *et al.*, 2015). The permeability of the intestine (i.e., intestinal epithelial damage) can be measured using biomarkers such as intestinal fatty acid-binding protein (I-FABP) (Ok *et al.*, 2020).

Dietary or age-related changes in the gut microbiota may affect the ability of *Cryptosporidium* spp. to infect the gut (Thomson *et al.*, 2017). Indole, an intracellular signalling molecule (Lee and Lee, 2010), or indole-producing bacteria, can affect the ability of *Cryptosporidium* spp. to establish an infection (Chappell *et al.*, 2016). Curcumin supplementation can modulate the microbial composition of the gut and stop oocyst shedding in mice infected with *C. parvum* (Rahman *et al.*, 2022). In another study with mice, researchers discovered that the intestinal microbiota of infected animals differed from that of uninfected animals, regardless of the *C. parvum* isolate used (Ras *et al.*, 2015). In mice, infection with *Cryptosporidium* spp. was associated with a decrease in the relative abundance of *Firmicutes* and an increase in *Bacteroidetes* (Mammeri *et al.*, 2019a).

The microbial composition of the gut affects the control and protection against *Cryptosporidium* spp. infection in neonatal mice (Lantier *et al.*, 2014). In humans, a lower abundance of the genus *Megasphaera* is associated with cryptosporidial diarrhoea, and it has been suggested that the overall gut microbiota composition may play a role in the severity of cryptosporidiosis (Carey *et al.*, 2021). There may be a synergistic effect between the bacterial genus *Fusobacterium* in faeces and *C. parvum* in calves (Ichikawa-Seki *et al.*, 2019). It has also been shown that probiotic supplementation can reduce oocyst shedding or the duration of shedding but does not prevent infections (Laurent and Lacroix-Lamandé, 2017).

3. AIMS OF THE STUDY

The main aim was to study the prevalence of the gastrointestinal protozoan parasite *Cryptosporidium* spp. in Estonian calves (**I**), their association with general inflammatory response (**II** and **III**) and faecal microbiota (**III**), and the effect of treatment of cryptosporidiosis (**II** and **III**).

The specific aims of this dissertation were:

1. To study the prevalence, the *Cryptosporidium* species shed, and the subtypes of *C. parvum* in Estonian calves up to two months of age (**I**).

2. To study the association of *Cryptosporidium* spp. with general inflammatory response (APPs) in calves up to six weeks of age (**II**).

3. To study the effects and associations of HL treatment on *Cryptosporidium* spp. infection dynamics in calves up to six weeks of age (**II**).

4. To study the possible relationships of faecal microbiota composition and diversity with *Cryptosporidium* spp. infections and general inflammatory response (APPs) in two-week-old calves (**III**).

4. MATERIALS AND METHODS

A brief overview of these methods is presented in this section. More detailed descriptions of the methods can be found in the corresponding sections in the articles **I**, **II**, and **III**.

4.1. Study population

Calves (**I**): 486 calves aged up to two months of age from 53 cattle farms all over Estonia. Sampling was conducted from April 2013 to May 2014 and from January to March 2015.

Dairy calves (**II**): 144 female dairy calves born from the 21st of January to the 16th of March 2015 on a large-scale Central Estonian dairy farm (>1800 cows). Rotavirus, coronavirus, and *E. coli* were present in the farm before the cryptosporidiosis outbreak. Most likely, also *Cryptosporidium* spp. were present in the farm before the acute outbreak. The herd tested positive for bovine viral diarrhoea virus at the time of the study.

Dairy calves (**III**): 112 female dairy calves born from the 21st of January to the 16th of March 2015 on a large-scale Central-Estonian dairy farm (>1800 cows). These calves were derived from study **II**.

4.2. Sample collection

In calf study (**I**), 486 faecal samples were collected from calves up to two months of age from 53 farms. OpenEpi was used to calculate the minimum sample size of 35 farms for study **I** (Dean *et al.*, 2015). The calculation was based on population size of 5582 cattle herds, absolute precision of 10% and an expected proportion of farms with calves shedding *C. parvum* of 10%. The aim was to sample at least 50 farms to ensure sampling from all counties. The sampling was proportionally stratified to all the 15 counties of Estonia according to the number of farms listed in each county. Expecting that at a given moment, at least 30% of calves would be shedding oocysts on a farm where *Cryptosporidium* spp. is present (Lassen *et al.*, 2009), it was evaluated that sampling 10 calves per farm would be sufficient to find at least one calf shedding oocysts if *Cryptosporidium* spp. was present on the farm. The farms were included in the study pool if they were officially registered,

and their herd size was at least 50 cattle; from this pool, farms were selected for sampling using the random number generator (Microsoft Excel, Microsoft Corporation).

In the dairy calf study (**II**), 901 serum and 767 faecal samples were collected from female calves up to three months of age. The weights of the calves were recorded immediately after birth and at one and three months of age.

In the second dairy calf study (**III**), 112 serum and 112 faecal samples were collected from two-week-old female calves.

In studies **II** and **III**, blood samples were collected directly from the jugular vein into sterile evacuated test tubes using 18 G sterile needles. The blood samples were transported to the laboratory and centrifuged at 1,800 × *g* for 10 min to separate the serum. Serum samples were frozen and stored at −20°C until further analysis.

In all studies (**I**, **II**, and **III**), faecal samples were collected directly from the rectum with a clean, disposable latex glove, and placed in a clean plastic cup. Faecal samples were either frozen and stored at −18°C until analysis (**I**), or stored at 4°C for up to 48 h before analysis (**II** and **III**).

The total number of animals included and samples collected in the studies are presented in Table 1.

Table 1. Samples collected for the studies.

Animals	Animals (n)	Faecal samples (n)	Blood samples (n)
Calves (**I**)	486	486	–
Dairy calves (**II**)	144	767	901
Dairy calves (**III**)	112	112	112
Total	**742**	**1,401**	**1,013**

4.3. Analysis of blood samples

In dairy calf studies (**II** and **III**), serum SAA concentrations were measured using a commercially available sandwich ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland). Hp serum concentrations were measured using the haemoglobin binding assay

method described by Makimura and Suzuki (1982), with a small modification of tetramethylbenzidine (0.06 mg/ml) used as a substrate (Alsemgeest *et al.*, 1994).

In study **III**, the serum concentrations of IL-1β, IL-6, and TNF-α were measured using commercially available ELISA kits from Cusabio Biotech (Wuhan, Hubei, China). The serum concentration of I-FABP was measured using a commercial ELISA kit (bovine I-FABP ELISA kit (MBS035016); MyBiosource Inc., San Diego, CA, USA). All measurements were recorded using a spectrophotometer (Magellan Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The inter- and intra-assay coefficients of variability for all methods were <15%.

In calf study (**I**), no blood samples were collected.

4.4. Faecal sample analysis

For calf study (**I**), molecular methods were used to detect, identify, and characterise *Cryptosporidium* spp. in faecal samples. DNA was extracted using a PSP® Spin Tool DNA kit (STRATEC Biomedical AG, Birkenfeld, Germany). The samples were then subjected to PCR amplification of the 18S rRNA gene (Xiao *et al.*, 1999). Samples that tested positive for gene targeting were further targeted for the *gp60* gene for *Cryptosporidium* subtype identification (Peng *et al.*, 2001).

In both dairy calf studies (**II** and **III**), *Cryptosporidium* spp. oocysts were detected using an immunofluorescent staining method (Crypto/Giardia Cel, Cellabs Pty Ltd., Sydney, Australia). A light microscope was used to count the oocysts in the visual field at 200× magnification. The approximate count of the oocysts was calculated as the opg (de Waele *et al.*, 2010).

In study **III**, faecal DNA was extracted using the same kit as for the calf study (**I**); however, an additional bead beating process was used.

4.5. Microbiota analysis

In study **III**, extracted faecal DNA samples were sequenced for the V3−V4 region of the 16S rRNA genes using the Illumina MiSeq platform (Husso *et al.*, 2020) to analyse the microbial composition.

Briefly, the bioinformatics pipeline was as follows: the quality of the reads was inspected using FastQC and MultiQC (Andrews *et al.*, 2015; Ewels *et al.*, 2016). Leftover primes and spacers were trimmed using CutAdapt (version 1.10) (Martin, 2011). For QIIME2, a mapping file was created and validated using the Keemei software (Rideout *et al.*, 2016). The FASTQ files were then imported into QIIME2 (version 2018.4), and the DADA2 plugin was used to denoise and filter the reads, call amplicon sequence variants (ASVs), and generate a feature table (Callahan *et al.*, 2016; Bolyen *et al.*, 2019). A naïve Bayes classifier was trained in QIIME2 against the SILVA 132 97% database, extracted to only include the V3–V4 reference region, and used to assign taxonomy to ASVs (Quast *et al.*, 2013; Bokulich *et al.*, 2018). Sequences derived from chloroplasts or mitochondria were removed and singletons were filtered out.

4.6. Halofuginone lactate treatment

In studies **II** and **III**, the calves received HL treatment (Halocur, Intervet International B.V., Boxmeer, Netherlands) because of an acute cryptosporidiosis outbreak on the farm. It was started as a mass treatment and included all calves younger than 14 days. As per to the manufacturer's instructions, the initiation of treatment was less than 48 h from birth, lasted for seven days (to prevent cryptosporidial diarrhoea) and was administered orally once per day. The dosage of Halocur is 100 µg of halofuginone base per kg of body weight once a day for seven consecutive days (i.e., 2 ml per 10 kg of body weight). For convenience, the simplified dosage scheme by the manufacturer was used: for calves weighing 35–45 kg 8 ml of Halocur, and for calves 45–60 kg 12 ml of Halocur. For smaller or larger weights, 2 ml of Halocur per 10 kg body weight was used for calculating the exact dosage. The weight of the older calves was estimated by the farm veterinarians on-site, the newborns were weighed with a digital scale. As sampling for these studies started before HL treatment was initiated, the calves were exposed to different treatment regimens depending on their age.

The calves were retrospectively divided into three groups based on their HL treatment regimen: Group 1) was treated correctly (initiation <48 h from birth which lasted ≥ seven days); 2) was treated incorrectly (delayed start (>48 h from birth) and lasted < seven days); and 3) did not receive

any treatment. Table 2 shows the number of calves in each treatment group in studies **II** and **III**.

Unfortunately, in study **I**, no information was available about whether the calves on the farms included were treated with HL or not. On farm level, the proportion of calves that had severe diarrhoea and died within their first month of life was recorded for both those that did receive veterinary treatment and those that did not.

Table 2. Halofuginone lactate treatment groups by studies.

Animals	Not treated (n)	Treated incorrectly (n)	Treated correctly (n)	Total (n)
Dairy calves (**II**)	34	45	65	144
Dairy calves (**III**)	21	39	52	112
Total	**55**	**84**	**117**	**256**

4.7. Statistical analysis

In study **I**, a calf was considered *Cryptosporidium* spp.-positive if its sample tested positive for *Cryptosporidium* spp. 18S rDNA. A farm was considered positive if at least one among the investigated calves tested positive for *Cryptosporidium* spp. infection. Multivariable logistic regression models were used for dichotomous animal-level outcomes: calf testing positive for *Cryptosporidium* spp., calf testing positive for *C. parvum*, and calf testing positive for the *C. parvum* subtype IIa18G1R1. The farm was used as random factor to account for clustering. First, variables with a p-value ≤ 0.20 in univariable analysis were included in the model, followed by a stepwise backward elimination procedure. Biologically meaningful interactions and possible confounding effects were assessed. Statistical significance was set at $p < 0.05$.

For study **II**, multiple linear regression models were used for associations of the calf serum concentrations of SAA and Hp with different *Cryptosporidium* opg categories at different weeks of age and for the association of average daily weight gain (ADWG) with different HL treatment groups.

For study **III**, linear regression models were used to evaluate the associations between microbial diversity (Shannon index) and age, innate

immunity response marker variables (SAA, Hp, IL-1β, IL-6, and TNF-α), *Cryptosporidium* spp. infection, and HL treatment. The Shannon index was a response variable, and age (continuous variable), protein groups, *Cryptosporidium* spp. infection groups, and HL treatment groups were explanatory variables. A stepwise backward elimination procedure was used to select the final models.

To calculate the *Firmicutes*-to-*Bacteroidetes* ratio, relative abundancies of these two phyla were used. Linear regression was used to investigate the association between *Cryptosporidium* spp. and HL groups with the *Firmicutes*-to-*Bacteroidetes* ratio. Age was included as a continuous variable. The Bonferroni p-value was used for pairwise comparisons among categorical variables. Logarithmic transformation was used to achieve normal distribution of the outcome variable.

Variance partitioning analysis was used to investigate how the systemic innate immunity response markers, *Cryptosporidium* spp. infection, and age explain faecal microbiota variability at the genus level. Continuous variables were used (except in the case of HL treatment).

Random forest analysis was used to investigate how well faecal microbiota explain our chosen parameters – cytokines, APPs, *Cryptosporidium* spp. infection, HL treatment, and I-FABP. Continuous variables (except IL-1β) were divided into low, moderate, and high concentration groups of similar size, based on the lowest to highest concentration levels in the serum. Based on the serum concentrations of IL-1β, the calves were divided into two groups: low (below the detection level of the assay) and high (above the detection level of the assay). Similar to previous models, calves were also divided into groups based on the amount of *Cryptosporidium* spp. oocysts shed in their faeces (opg), and for the HL treatment. Genus-level microbiota data were used for statistical analyses. All genera with a prevalence of ≥10% in calves were included.

The 30 most influential genera found through random forest analysis were further analysed using negative binomial regression models to investigate the direction of the associations of microbial genera with *Cryptosporidium* spp. and marker variables. Each genus was included as a response variable. Age at sampling and *Cryptosporidium* spp. oocyst counts were included as categorical explanatory variables in all the

models. The total abundance of all the bacterial genera in the sample was included as an exposure variable. Wald test p-values were calculated for categorical variables and Holm-Šidák adjusted p-values were calculated out of 30 Wald test p-values to account for multiple comparisons. Bonferroni correction was used for pairwise comparisons between three-level categorical variables after a significant Holm-Šidák-corrected Wald test. Graphs of the observed proportions of negative binomial probabilities along with a Poisson model were used to evaluate the suitability of a negative binomial distribution to the models used. The association with ADWG was investigated for all genera that were significantly associated with I-FABP or any systemic innate immunity response marker. ADWG at nine months of age (low, moderate, and high) was used as an explanatory categorical variable. To control for possible confounding factors, age at sampling and *Cryptosporidium* spp. groups were included as categorical explanatory variables, and calf birth weight was included as a continuous explanatory variable. The Bonferroni correction was used for pairwise comparisons of p-values for categorical variables.

Initial data management was performed using Microsoft Excel for Mac (version 15.0) (**I**) and Microsoft Excel 2016 (**II** and **III**) (Microsoft, Redmond, WA, USA). Statistical analyses were performed using STATA IC (version 14.2 for Mac) (**I**) and STATA IC (versions 14.1 (**II**) and 14.2 (**III**)) for Windows (StataCorp LP, College Station, TX, USA). R (version 4.0.1) was used for variance partitioning and random forest analyses (**III**).

Additional details regarding the statistical methods can be found in the materials and methods sections of the respective articles.

5. RESULTS

5.1. Epidemiology of *Cryptosporidium* spp. in calves in Estonia

In study **I**, *Cryptosporidium* spp. was identified in 22.6% of samples (Table 3). Three species were identified: *C. parvum* (95.5%), *C. bovis* (3.6%), and *C. ryanae* (0.9%). Ten subtypes of *C. parvum* were identified, including one novel subtype (IIaA14G1R1, IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1, IIaA16G2R1, and the novel subtype IIlA21R2). The most common subtype was IIaA18G1R1 (35.8%), which was found in almost half of the farms positive for *C. parvum* (45.5%). Of the 53 study farms, 35 (66.0%) were positive for *Cryptosporidium* spp. (Figure 2). Oocyst shedding was most prevalent during the second week of life (Figure 3). The odds of a calf aged 8–14 days being *Cryptosporidium* spp.- or *C. parvum*-positive were 10.1 (CI 4.53–22.36) and 10.4 (CI 4.58–23.74) times higher than the odds of a calf aged up to 7 days, respectively.

Table 3. Prevalence of *Cryptosporidium* species on animal level (n = 486) and on herd level (n = 53).

Species	**Positive animals (n)**	**% of positive animals (95% CI*)**	**Positive farms (n)**	**% of positive farms (95% CI*)**
C. parvum	105	21.6 (18.12 – 25.43)	33	62.3 (48.72 – 74.50)
C. bovis	4	0.8 (0.26 – 1.97)	2	3.8 (0.64 – 11.91)
C. ryanae	1	0.2 (0.01 – 1.01)	1	1.9 (0.09 – 8.95)
Total	**110**	**22.6 19.08 – 26.51)**	**35**	**66.0 (52.57 –77.79)**

* CI – confidence interval (Mid-P exact)

Figure 2. Map of Estonia showing 18 farms negative for *Cryptosporidium* spp. (grey circles), three farms positive for *Cryptosporidium* spp. other than *C. parvum* (grey diamonds), and 33 farms positive for *C. parvum* (black circles). Faecal samples from three to 14 (median 10) calves per farm were tested, and a farm was considered positive if faecal sample of at least one of the sampled calves tested positive.

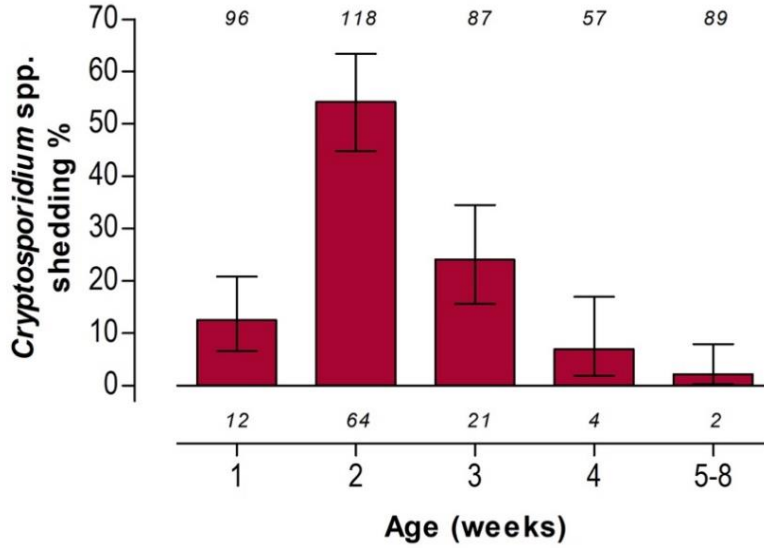

Figure 3. The percentage of calves shedding *Cryptosporidium* spp. (n = 93) out of all the calves in the study (n = 486) by age (weeks) illustrating the dynamics of infection in Estonian calves (**I**). The number on the top represents the number of sampled calves for each week, the number below each bar represents the number of positive samples.

In studies **II** and **III**, faeces were examined for the presence of *Cryptosporidium* spp. oocysts in calves from one farm. In study **II**, one-third of the faecal samples (33.3%; 218/655) were positive for oocysts, with more than four out of five calves being positive (84.7%; 122/144) for oocyst shedding. The *C. parvum* subtype IIaA18G1R1 was identified. After the first week of life, shedding of *Cryptosporidium* spp. oocysts was initiated and opg levels increased up to two weeks of age. The opg levels of calves by age in study **II** are shown in Figure 4. In study **III**, which only included the two-week-old calves (n = 112), approximately half (51.8%) were *Cryptosporidium* spp.-positive.

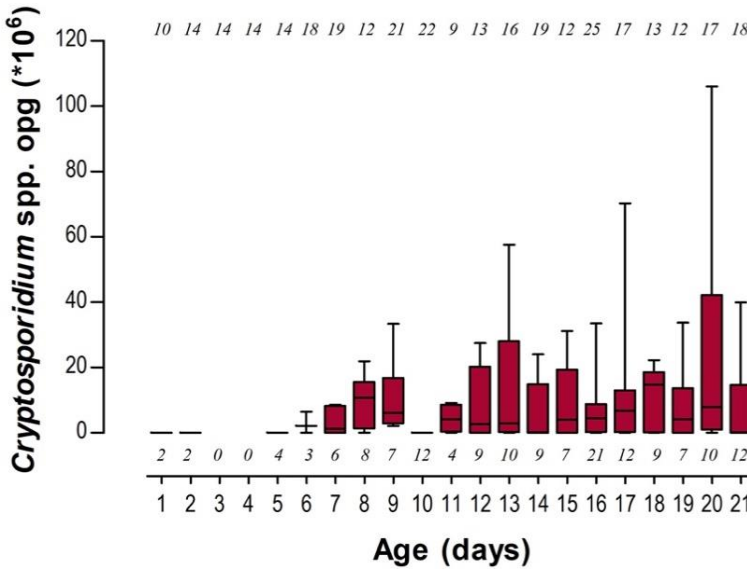

Figure 4. A box-and-whiskers plot of the faecal samples positive for *Cryptosporidium* spp. (n = 218) and their oocysts count (opg – oocysts per gram of faeces) illustrating the dynamics of infection in female dairy calves (**II**). The median level is marked with a horizontal line on the box. The upper level of box is the upper quartile, and the lower level of box is the lower quartile. The number on the top represents the number of samples from that day, the number below each bar represents the number of positive samples. Only samples that tested positive are represented.

5.2. Treatment with halofuginone lactate

In the study **II**, we observed that during the first three months, 21 calves (14.6%) died or were euthanised, most due diarrhoea. We also observed that the group of calves that did not receive HL treatment had the most deaths. In total, 66.7% (14/21) of the calves died in that group. Overall, the death rate of calves was lower in both treatment groups when compared to the not treated group (Figure 5).

During the second week of life, serum Hp concentrations were significantly higher in the untreated group than in the other treatment groups and study weeks (**II**).

The average birth weight of the calves (n = 144) was 41.2 ± 5.1 kg (range 27 to 52 kg) and the average weight at three months of age (n = 120; average age at weighing 103 days ± 13.3 days) was 123.5 ± 17.9 kg (range 61 to 192 kg) (**II**). Different HL treatment regimens of dairy calves resulted in differences in ADWG at three months of age. According to

linear regression model, the calves treated with HL according to the manufacturer's instructions had lower ADWG (average –107 g; 95% CI −176.37 − −38.06; p = 0.003) than those that were not treated with HL.

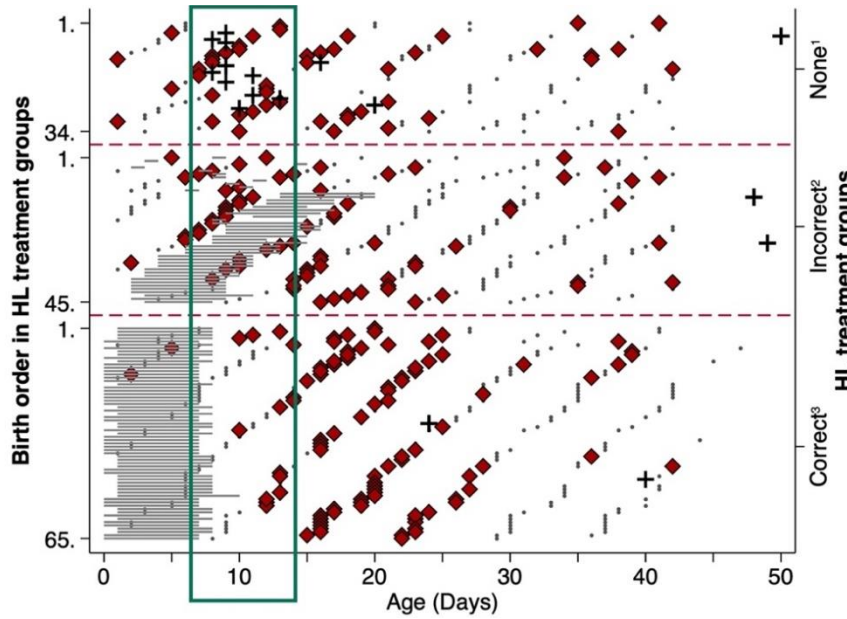

Figure 5. *Cryptosporidium* spp. infection patterns differentiated by halofuginone lactate (HL) treatments (**II** – the full figure, and **III** – the time period (second week of life) showed in the green rectangle). Female dairy calves (n = 144) were retrospectively assigned into groups based on the received HL treatment regimen (separated by dashed lines in the figure): 1) not treated (n = 34), 2) treated incorrectly (treatment started >48 hours after birth or lasted <7 days) (n = 45), and 3) treated according to manufacturer's instructions (started <48 hours after birth and lasted ≥7 days) (n = 65). (◆) *Cryptosporidium* spp. positive (n = 122)); (·) *Cryptosporidium* spp. negative (n = 22); (+) death (n = 17); horizontal lines represent HL treatment, and the length represents the treatment in days. The y-axis represents the birth order of calves in different HL treatment groups, starting with the oldest and ending with the youngest; x-axis represent the age of the calf.

5.3. Acute phase proteins associated with *Cryptosporidium* spp. and microbiota in calves

In female dairy calves (**II** and **III**), we found that if the calves had a high number of *Cryptosporidium* spp. oocysts in their faeces during the second week of life, they would also have higher serum concentrations of SAA and Hp (Figure 6). At the same time, Hp serum concentrations were also higher in the group of calves that were not treated with HL.

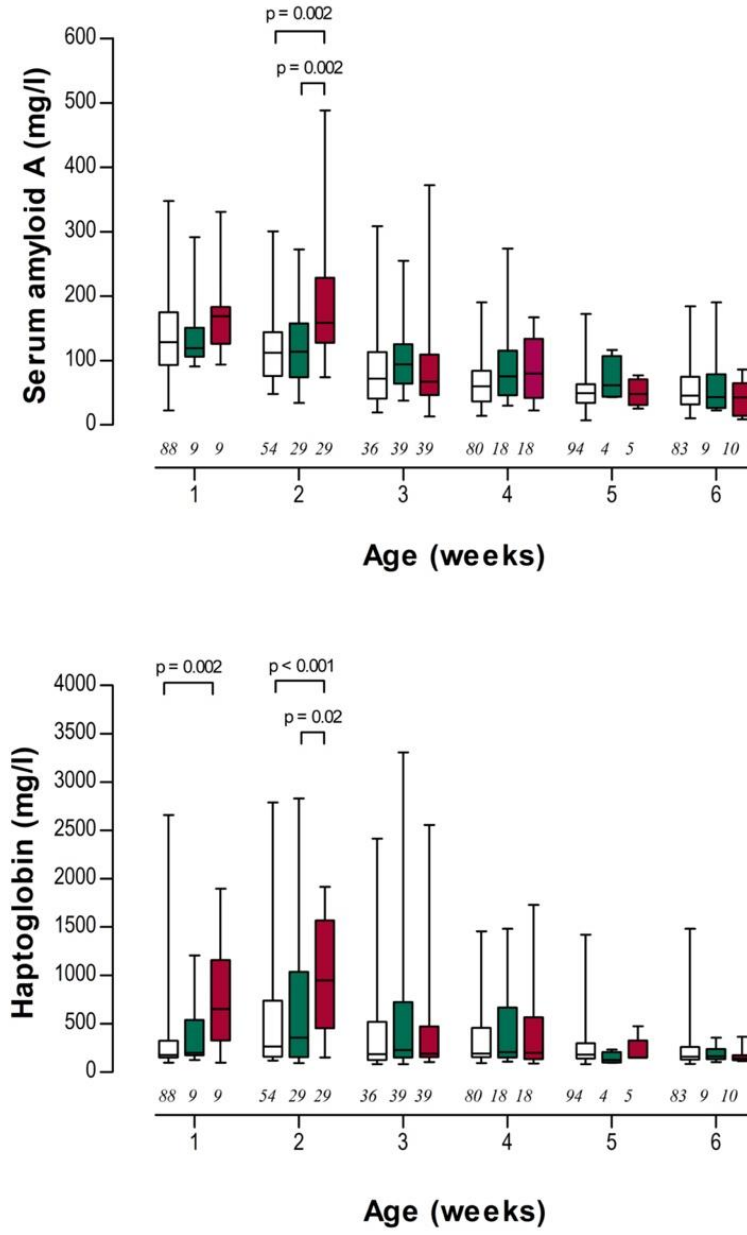

Figure 6. Serum amyloid A (SAA) and haptoglobin (Hp) concentrations in calf serum by week of age and *Cryptosporidium* oocyst count category in faecal samples. White = *Cryptosporidium* negative (no oocysts found); blue = low oocyst count (oocyst count below the median of the positive samples of the week in question); red = high oocyst count (oocyst count above the median of the positive samples of the week in question). The number of calves in each group is marked in italics above the x-axis.

Acute phase proteins were associated with a relative abundance of some bacterial phyla in the faeces of two-week-old female dairy calves (**III**). The relative abundance of *Fusobacteria* was higher and that of *Bacteroidetes* was lower when high concentrations of SAA and Hp were present in serum. SAA and Hp can explain 1.16% of the variation in faecal microbiota composition (Figure 7).

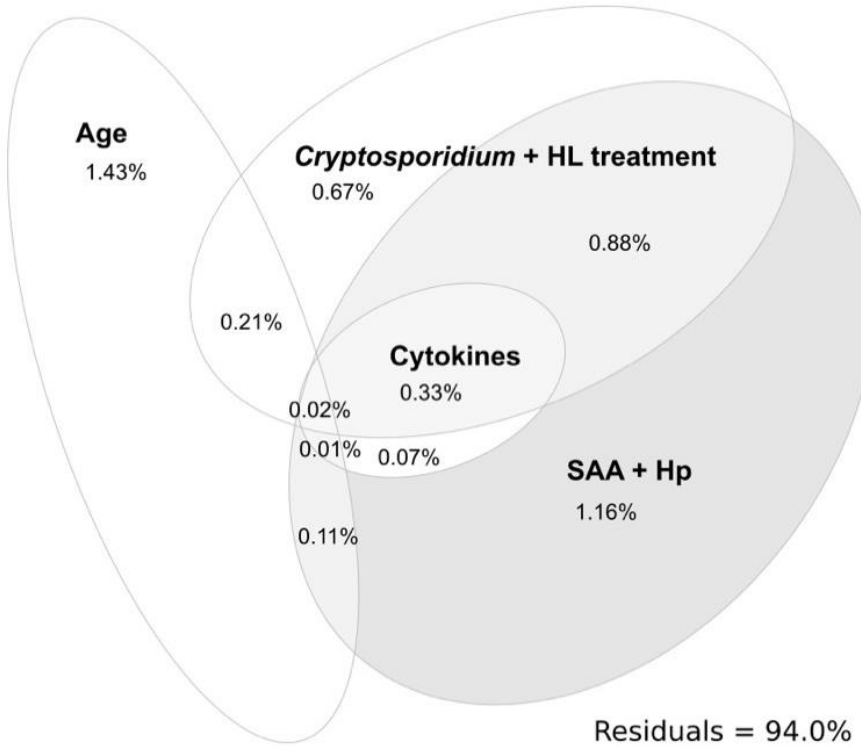

Figure 7. Variance partitioning analysis of the calves' (n = 112) intestinal microbiota at the genus level (n=102). Results are represented as percentiles of the 6.0% of microbial variance explained by these variables. The continuous variables included in the analysis were age at sampling in days ('Age'), cytokines in ng/l (including interleukin-1β, interleukin-6, tumour necrosis factor-α) ('Cytokines'), serum amyloid A (SAA) and haptoglobin (Hp) in mg/l together ('SAA + Hp'), and *Cryptosporidium* spp. infection (opg) and halofuginone lactate (HL) treatment groups together ('*Cryptosporidium* + HL treatment').

Using random forest analysis (Figure 8), we found that *Fusobacterium* was the most influential bacterial genus for SAA, and that the same genus was ranked fourth for Hp. *Fusobacterium* was found in 83% of the calves. Its abundance was the lowest in the low SAA serum concentration group. The abundance of the genus *Collinsella* in faeces showed a

nonlinear association with serum SAA concentration (moderate SAA group had lower *Collinsella* abundance than the low and high SAA groups).

For Hp, the most influential genus was *[Ruminococcus] torques* group (Figure 8). A high Hp concentration in serum was positively associated with an abundance of this genus and negatively associated with *Flavonifractor* abundance in faeces.

The abundance of faecal *Peptostreptococcus* was positively associated with both high SAA and Hp serum concentrations ($p < 0.001$ for both).

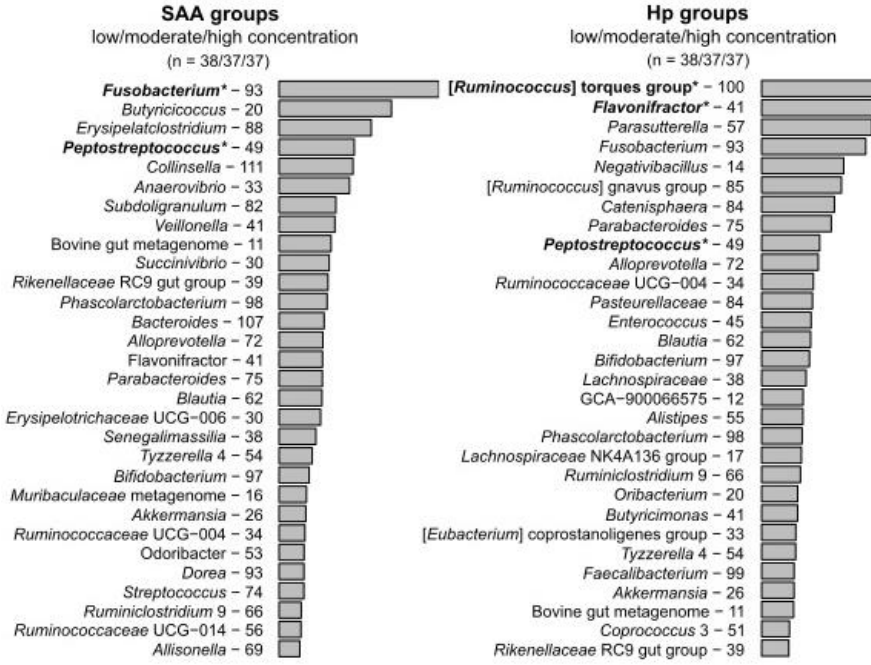

Figure 8. Random forest analysis results of the 30 most influential faecal bacterial genera (out of 102) by groups of calves (n = 112). Lengths of the bars (mean decrease in accuracy) show the relative importance of genera. Serum amyloid A (SAA) and haptoglobin (Hp) groups were defined according to serum concentrations, from lowest to highest. The number after the genus name represents the number of calves in which this genus was found.

* Genera with significant association with variable group (Holm-Šidák corrected Wald test), as analysed using negative binomial models.

5.4. Microbiota composition and cryptosporidiosis

In study **III**, the composition of the microbiota was determined using 16S rRNA sequencing. The faecal microbiota of two-week-old female dairy calves was composed of the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria*, in order of decreasing relative abundance (Figure 9A).

Microbial diversity, measured by the Shannon index, increased with calves' age and decreased if high levels of *Cryptosporidium* spp. oocysts were found in the faeces ($p = 0.001$ and $p = 0.007$, respectively) (Figure 10A and B). HL treatment was associated with increased microbial diversity. The relative abundance of faecal *Actinobacteria* was the highest in the two groups of calves that received treatment (correct and incorrect administration), and the relative abundance of faecal *Fusobacteria* was lower (Figure 9B). The higher the SAA and Hp concentrations in serum, the greater the relative abundance of *Fusobacteria*, and the lower the relative abundance of *Bacteroidetes* in faeces (Figure 9C). The *Firmicutes*-to-*Bacteroidetes* ratio increased in calves with *Cryptosporidium* spp. infection and decreased with HL treatment (Figure 11) ($p = 0.06$) (previously unpublished data).

The chosen markers – age, cytokines, combined SAA and Hp serum concentrations, and combined *Cryptosporidium* spp. infection and HL treatment – could explain only 6% of the microbial variation in the samples (Figure 7). Infection with *Cryptosporidium* spp. by itself could only account for 0.68% of the microbial diversity in the faecal samples. HL does not explain any of the microbial variations on its own; it completely overlaps with the *Cryptosporidium* spp. explanation. Of the APPs, only Hp had the ability to explain some (1.9%) of the microbial variation on its own, and SAA fully overlapped with it.

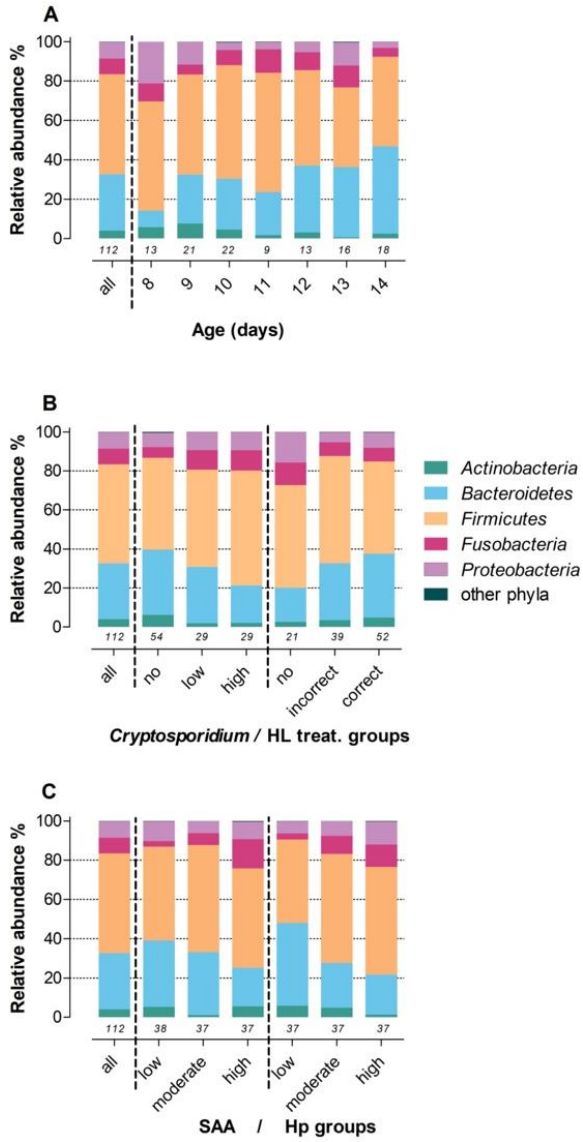

Figure 9. Faecal microbiota composition at phylum level in calves (n = 112) based on days of age (A), *Cryptosporidium* spp. infection status and halofuginone lactate (HL) treatment groups (B), and serum amyloid A (SAA) and haptoglobin (Hp) serum concentrations groups (C). The small numbers given in italics under the columns indicate the number of calves in that group. Column 'all' shows the composition of microbiota in all calves included in this study. The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – oocyst count below the median of the positive samples of the week in question, and high – oocyst count above the median of the positive samples of the week in question. HL treatment groups were: no – no treatment received, incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d.

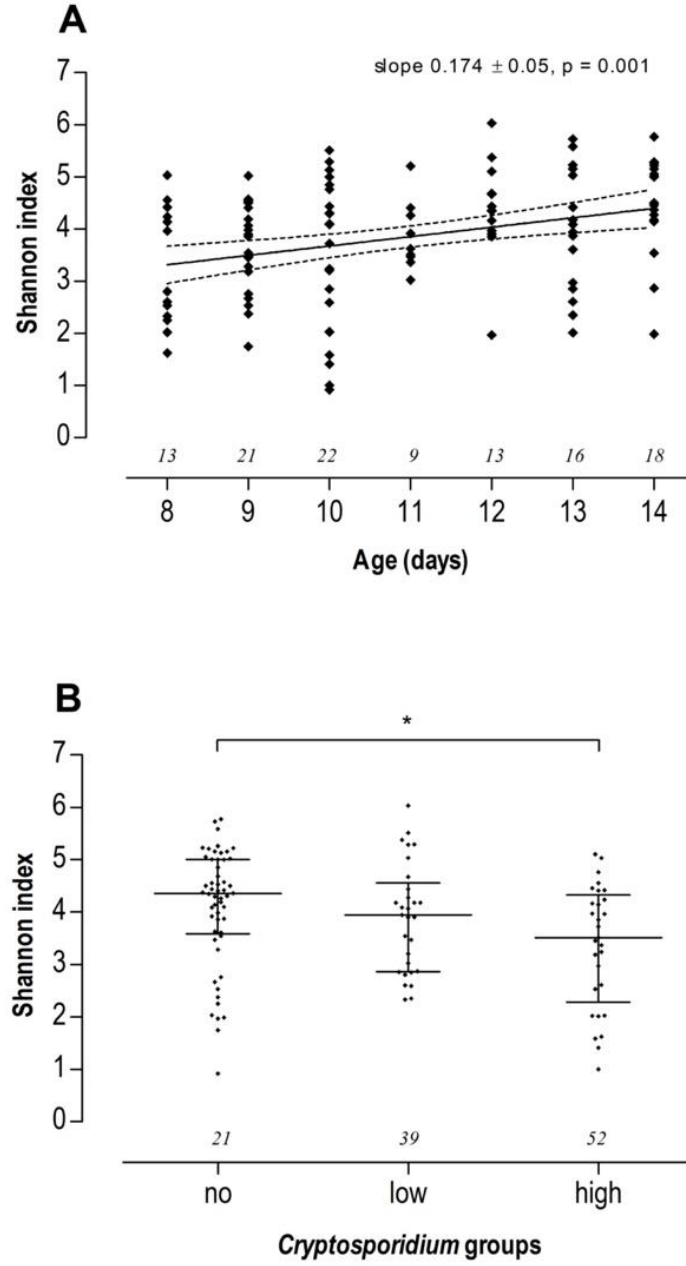

Figure 10. Faecal microbiota diversity by age (days) (A) and *Cryptosporidium* spp. infection group (B), as evaluated using linear regression models. The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – oocyst count below the median of the positive samples of the week in question, and high – oocyst count above the median of the positive samples of the week in question. The small numbers given in italics under the columns indicate the number of calves in that group.

* Significant difference, Bonferroni-corrected $p < 0.05$.

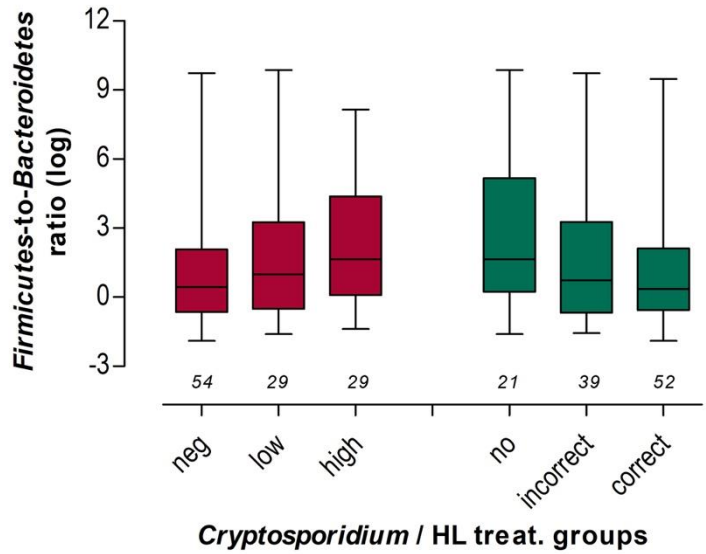

Figure 11. *Firmicutes*-to-*Bacteroidetes* ratio in faeces by *Cryptosporidium* spp. infection groups and halofuginone lactate (HL) treatment groups. Log-transformed ratio is presented. The small numbers given in italics under the columns indicate the number of calves in that group.

The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – oocyst count below the median of the positive samples of the week in question, and high – oocyst count above the median of the positive samples of the week in question. HL treatment groups were: no – no treatment received, incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d.

The most influential bacterial genus (with the highest relative importance) for *Cryptosporidium* spp. was *Ruminiclostridium 9* in the random forest analysis. The most influential bacterial genus for the HL treatment was *Erysipelotrichaceae* UCG-009 (Figure 12).

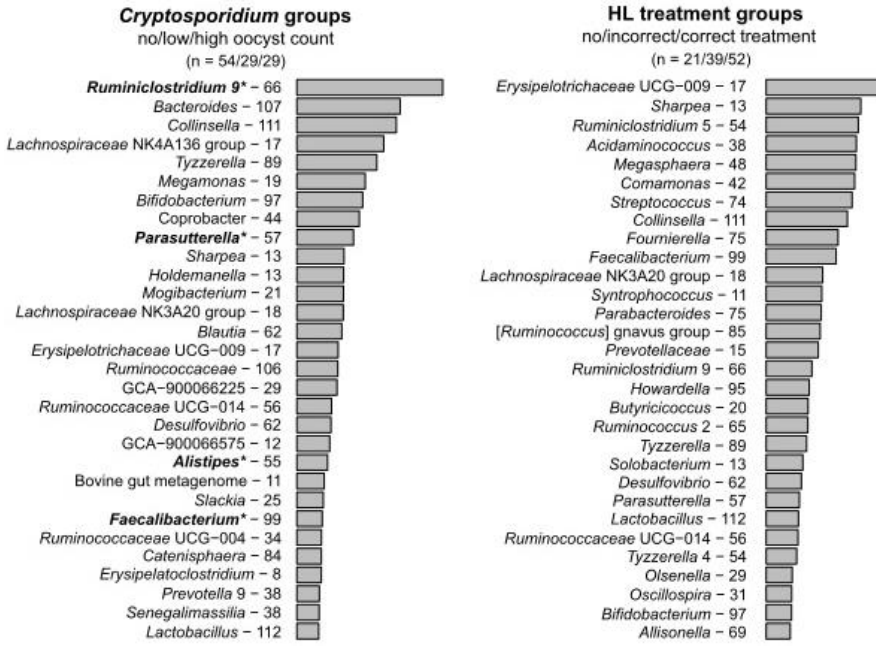

Figure 12. Random forest analysis results of the 30 most influential faecal bacterial genera (out of 102) by groups of calves (n = 112). Lengths of the bars (mean decrease in accuracy) show the relative importance of genera. *Cryptosporidium* spp. infection groups were defined according to faecal oocyst count (no oocyst and below or above the median of the same week), while halofuginone lactate (HL) treatment groups were defined on the basis of the received treatment (incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d). The number after the genus name represents the number of calves in which this genus was found.

* Genera with significant association with variable group (Holm-Šidák corrected Wald test), as analysed using negative binomial models.

The 30 most influential faecal bacterial genera by group identified by random forest analysis were further analysed using negative binomial models to investigate the associations with variable groups (Holm-Šidák-corrected Wald test p-values). Four faecal genera abundancies had statistically significant (Bonferroni corrected) associations with *Cryptosporidium* spp. – *Ruminiclostridium 9*, *Alistipes*, *Parasutterella*, and *Faecalibacterium* (Figure 13).

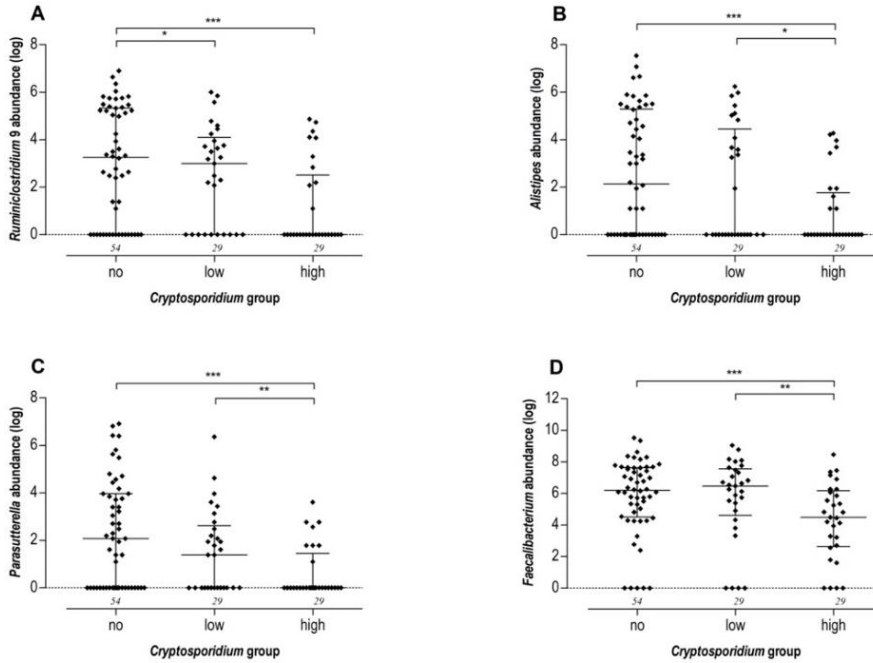

Figure 13. Association of *Cryptosporidium* spp. groups with four faecal genera abundancies, as assessed using negative binomial model. *Cryptosporidium* spp. infection groups (no oocysts found, low oocyst count, and high oocyst count) and *Ruminiclostridium 9* (A), *Alistipes* (B), *Parasutterella* (C), and *Faecalibacterium* (D) abundances in samples are shown. Log-transformed abundance of these genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.

* Significant difference, Bonferroni-corrected $p < 0.05$

** Significant difference, Bonferroni-corrected $p < 0.01$

*** Significant difference, Bonferroni-corrected $p < 0.001$

6. DISCUSSION

6.1. *Cryptosporidium* infection in calves in Estonia

Cryptosporidium spp. are widespread in Estonian farms and calves up to two months of age. A previous study by Lassen *et al.* (2009) found that 84% of studied farms had *Cryptosporidium* spp. shedding calves, with a sample prevalence of 30.0% in calves and cows. Our study found the level of farm prevalence to be lower at 66.0%, with an animal-level prevalence of 22.6% (**I**). The difference in prevalence between our study and the one by Lassen *et al.* (2009) could be explained by the different sampling methods; in our study, we sampled animals up to two months of age, while Lassen *et al.* sampled animals of all ages. They found that in calves up to three months of age, the sample prevalence was 24.0%, with a herd prevalence of 68%, which was very similar to our results. Studies in other European countries have also reported similar sample level prevalence over the last five years: Austria, 55.4% (Lichtmannsperger *et al.*, 2020); Belgium, 32.2% (Pinto *et al.*, 2021); Cyprus, 43.8% (Hoque *et al.*, 2022); France, 41.1–88.5% (Mammeri *et al.*, 2019b; Pinto *et al.*, 2021); Latvia, 33.8% (Deksne *et al.*, 2022); Netherlands, 32.5% (Pinto *et al.*, 2021); and Scotland, 48.7% (Wells *et al.*, 2019).

In previous studies, two- and three-week-old calves shed the highest number of *Cryptosporidium* spp. oocysts (Santín *et al.*, 2008; Coklin *et al.*, 2010). We obtained the same result in our models, which showed that calves aged 8-14 days were over 10 times more likely to be *Cryptosporidium* spp.-positive than calves up to seven days (**I**). In study **II**, calves that did not receive HL treatment shed the most oocysts at 10–12 days, while correctly treated calves had a shedding peak at 19–21 days of age. Due to these findings, study **III** focused only on the second week of life, during which *Cryptosporidium* spp. infection is more common.

The animal level prevalence in study **I** was low compared to study **II**, where 84.7% of the calves were shedding *Cryptosporidium* oocysts. It should be noted that in study **II**, there was an acute outbreak of cryptosporidiosis (with increased mortality) present at the time of sampling, which might have led to a higher overall shedding rate of oocysts in the faeces. Furthermore, study **II** was conducted in one large

dairy farm, while study **I** included samples from 53 cattle farms all over Estonia.

Three species of *Cryptosporidium* were identified: *C. parvum*, *C. bovis,* and *C. ryanae,* of which *C. parvum* was the most prevalent (**I**). This contrasted with reports from the neighbouring countries of Finland (Seppä-Lassila *et al.*, 2015) and Sweden (Silverlås *et al.*, 2010; Björkman *et al.*, 2015), where *C. parvum* was not the most prevalent species detected, but coincided with results from Latvia (Deksne *et al.*, 2022).

The most common subtype of *C. parvum* identified in our study was IIaA18G1R1 (**I** and **II**). Overall, ten different *C. parvum* subtypes were identified, with one of which (IIlA21R2) was novel (**I**). This indicates high genetic variability within *C. parvum* in Estonian calves. Although *C. parvum* is the most prevalent species of *Cryptosporidium* found in Estonia and Latvia, the most prevalent subtype was different in both countries. In Latvia, the most prevalent *C. parvum* subtype was IIaA15G2R1 (Deksne *et al.*, 2022), which was not detected in our study.

At least six of the ten subtypes identified in our study have also been found in humans (Soba and Logar, 2008; Chalmers *et al.*, 2011; Lassen *et al.*, 2014), highlighting the zoonotic potential of *C. parvum* shed by Estonian cattle. More awareness should be raised among risk groups, such as farm workers, that are under increased pathogenic pressure to contract cryptosporidiosis (Autio *et al.*, 2012), as well as their healthcare providers. The epidemiology of *C. parvum* also contains a sylvatic aspect, since several subtypes identified in study **I** have also been reported in wildlife (Krawczyk *et al.*, 2015) and fish (Certad *et al.*, 2015), suggesting that being in contact with these animals might be a risk factor for contracting cryptosporidiosis.

6.2. Acute phase proteins' profiles and treatment with halofuginone lactate

During *Cryptosporidium* spp. infections, the strength of the APR was dose-dependent (**II**). Previous studies on calves either found an association between *Cryptosporidium* spp. infection and increased SAA and Hp serum concentrations (Pourjafar *et al.*, 2011; Al-Zubaidi, 2015), or no association at all (Seppä-Lassila *et al.*, 2015). Interestingly, in the untreated group with high *Cryptosporidium* spp. oocyst shedding, the

mean SAA serum concentration was in the range of the reference values (established by Seppä-Lassila *et al.*, 2013), while the mean Hp serum concentration was 4.8 times higher than the reference value (**II**). Thus, in this case, Hp was a better biomarker of inflammation than SAA. The same phenomenon has been observed in previous studies of *Eimeria* spp. infections (Lassen *et al.*, 2015) and umbilical and respiratory infections (Seppä-Lassila *et al.*, 2015). According to study **II**, Hp also appears to be a more sensitive biomarker during parasitic infections than SAA, especially during the neonatal period. Hp seems to react already in the first week of calves' life, when the shedding of oocysts is low, suggesting that it responds to early *Cryptosporidium* spp. infections, whereas SAA reacts later. This could be because the serum concentration of SAA in newborns is influenced by the colostrum and normal physiological changes (Peetsalu *et al.*, 2022). Associations between APPs and *Cryptosporidium* spp. infections have also been studied for other species. Both SAA and Hp serum concentrations were higher in *C. parvum*-infected lambs than in healthy lambs (Dinler *et al.*, 2017), but no statistically significant associations were found between *Cryptosporidium* spp. infections and APPs (Niine *et al.*, 2018) in lambs up to three weeks of age.

As the incubation period of *Cryptosporidium* spp. infection is short (5–7 days), the adaptive immune response is unlikely to stop the development of clinical disease (Petry *et al.*, 2010; Abeywardena *et al.*, 2015). Thus, innate immunity, including APR, is more likely to play a role in the early stages of controlling *Cryptosporidium* spp. infections and disease development. HL treatment delayed oocyst shedding, induction of APR, and its magnitude during the first 2–3-weeks (**II**).

The connection between APR and *Cryptosporidium* spp. infections can be demonstrated by HL treatment (**II**). Early *Cryptosporidium* spp. infections cause a severe inflammatory response, whereas HL treatment delays the onset of cryptosporidiosis which in turn does not cause such a severe response.

While treatment with HL did not decrease the number of oocysts shed in faeces, it delayed the onset of shedding, reduced the severity of illness, and improved the survival of calves (**II**). Interestingly, this treatment also resulted in poorer ADWG at three months of age (**II**). The delay in shedding of oocysts has been demonstrated many times previously

(Jarvie *et al.*, 2005; Trotz-Williams *et al.*, 2011; Keidel and Daugschies, 2013). The impact of HL treatment on calf survival has not been previously demonstrated. We hypothesised that the survival chances of calves that did not have the best "starting position" (e.g., colostrum quality below optimal, lower birth weight) who were thus more susceptible to *Cryptosporidium* spp. infections, improved once the mass treatment with HL was initiated. The survival of calves improved even with the incorrect treatment regimen, showing that starting the treatment late is still better than no treatment at all, and mass treatment with HL can decrease calf mortality during an acute outbreak of cryptosporidiosis.

6.3. Faecal microbiota and *Cryptosporidium* spp.

The faecal microbiota of two-week-old female dairy calves was composed of the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria,* in order of decreasing relative abundance (**III**). This finding was comparable with those of other studies (Jami *et al.*, 2013; Myer *et al.*, 2017; Alipour *et al.*, 2018). Faecal microbiota diversity increases with age, as previously shown (Dill-McFarland *et al.*, 2017). In addition to age, HL treatment is also associated with increased diversity in the faecal microbiota.

Enteric pathogens can change the composition of the intestinal microbiota (Laurent and Lacroix-Lamandé, 2017). We showed that a higher number of *Cryptosporidium* spp. oocysts was associated with decreased microbial diversity in calves (**III**). The same phenomenon has also been reported in other studies (Rahman *et al.*, 2022). One study on mice (Mammeri *et al.*, 2019a) showed that infections with *Cryptosporidium* spp. were associated with an increase in the relative abundance of *Bacteroidetes* and a decrease in *Firmicutes* abundance. The same trend was observed in the study on dairy calves (**III**).

The *Firmicutes*-to-*Bacteroidetes* ratio is relevant to the microbial composition in the gastrointestinal tract (Myer *et al.*, 2017). In humans, a decreased ratio has been linked to weight loss and a decreased capacity to harvest energy from food (Ley *et al.*, 2006). An increase in *Bacteroidetes* abundance with a concurrent decrease in *Firmicutes* abundance has been suggested as an indicator of dysbiosis of the intestinal microbiota in humans (Sampson *et al.*, 2016) and dogs (Li *et al.*, 2017). We found that

an infection with *Cryptosporidium* spp. increases the *Firmicutes*-to-*Bacteroidetes* ratio, while HL treatment decreases it (**III**). This could explain the controversial results on future weight gain in calves with *Cryptosporidium* spp. infection that were not treated with HL who had better weight gain later in life compared to calves that received HL treatment. Of note, the *Firmicutes*-to-*Bacteroidetes* ratio has been shown to change with age in humans (Mariat *et al.*, 2009), and thus these changes can be considered as physiological and not always an indication of dysbiosis. Dividing changes into these categories is not yet clear for calves. Furthermore, the significance and possible implications of the *Firmicutes*-to-*Bacteroidetes* ratio have not been fully elucidated because there is great variability among individual calves (for instance, some calves have a very low faecal abundance of *Bacteroidetes* and thus an extremely high ratio). This could be explained by the rapid change in microbiota during the second week of life, or by the fact that calves are not monogastric animals like the populations of the studies mentioned above (humans and dogs).

Different segments of the gastrointestinal tract of calves have different microbiota compositions at both the phylum and genus levels (Myer *et al.*, 2017). These differences reflect the different functions and physiology of the segments, and this should be considered when comparing the results of different microbiota studies.

Four faecal bacterial genera, *Ruminiclostridium 9, Alistipes, Parasutterella*, and *Faecalibacterium*, were negatively associated with the number of *Cryptosporidium* spp. oocysts found in the faeces. These genera belong to three phyla: *Firmicutes* (*Ruminiclostridium 9* and *Faecalibacterium*), *Bacteroidetes* (*Alistipes*), and *Proteobacteria* (*Parasutterella*). We hypothesised that the presence of *Cryptosporidium* spp. may alter the environment of the intestinal microbiota less favourably for some bacterial species. Alternatively, *Cryptosporidium* spp. infection may be inhibited or reduced by a high abundance of certain bacterial species. As study **III** was cross-sectional, we could not prove causal relationships, and we could only speculate.

We showed that some bacterial genera in the faecal microbiota of two-week-old calves, such as *Fusobacterium*, have the potential to trigger an immune response in the host (**III**). Previous studies have suggested that *Fusobacterium* and *C. parvum* exert synergistic effects (Ichikawa-Seki *et al.*,

2019). Our study reported a higher relative abundance of faecal *Fusobacterium* than in previous studies (Oikonomou *et al*., 2013; Ichikawa-Seki *et al*., 2019). We did not find an association between *Cryptosporidium* spp. infection and *Fusobacterium* abundance, but there was a positive association between *Fusobacterium* abundance and SAA (**III**). This means that *Fusobacterium* can independently initiate APR in calves, showing the pathogenic potential of *Fusobacterium*. Abundance of faecal *Peptostreptococcus* was also positively associated with both the SAA and Hp serum concentrations.

Study **III** also found that the abundance of faecal *Flavonifractor* was negatively associated with Hp concentration in serum. Interestingly, serum Hp concentrations decrease at the beginning of an inflammatory process before increasing, as Hp binds available free haemoglobin in the blood to limit bacterial access (Eaton *et al*., 1982), resulting in a decrease in circulating Hp in the blood before the inflammatory response initiates Hp production in the liver. This means that Hp concentrations in serum below the reference range can also indicate an inflammation (Arthington *et al*., 2003; González-Barrio *et al*., 2021), depending on the sampling time. The inconsistent findings observed in this study regarding the association between Hp serum concentration and different bacterial genera may be related to this phenomenon. However, it should be noted that on a larger scale, the intestinal microbiota seems to influence the innate immunity response of the host only slightly. Therefore, the biological importance of these findings should be studied in greater detail.

Antimicrobial treatments and feed supplements can also alter the microbiota composition. A review by Amin and Seifert (2021) suggested that both direct and indirect exposure to antimicrobials during the neonatal period modifies the microbiota composition and their functional profile in calves. Penicillin treatment has been shown to cause a microbial variance of around 5% (Grønvold *et al*., 2011). Changes in microbiota are reversible, usually within a few weeks after treatment discontinuation (Holman *et al*., 2019). It would be interesting to know if any long-term effects on microbial composition could later influence the performance of these future dairy cows, especially since we found a similar negative effect of HL treatment on three- (**II**) and nine-month (Peetsalu *et al*., 2022) weight gain. However, studies in humans have shown that some bacterial species may never recover from antibiotic use

(Jakobsson *et al.*, 2010; Dethlefsen and Relman, 2011; Li *et al.*, 2019). We suggest that the effects of HL treatment on the microbiota might be long-lasting. Based on the pharmacodynamic properties of HL treatment (Intervet International BV, 2019), the mechanism towards the pathogen could be roughly the same as that of antimicrobials towards different bacteria, and may thus influence microbial composition. However, HL treatment did not have any association with bacterial genera within the microbiota in our study, whereas *Cryptosporidium* spp. did.

The long-term effects of *Cryptosporidium* spp. infections on young animals have not been extensively studied. We showed that *Cryptosporidium* spp. and HL can affect the survival of calves, and that HL treatment was associated with lower weight gain at three months of age, while *Cryptosporidium* spp. itself was associated with better weight gain in the same timeframe (**II**). Previous studies have also noted a marked decrease in nutrient absorption in calves with cryptosporidiosis (Olson *et al.*, 2004), but the mechanism behind this has not yet been addressed. We can speculate that *Cryptosporidium* spp. affects weight gain through its influence on microbial diversity. *Cryptosporidium* spp. decrease the Shannon index (index of microbial diversity) and, thus, the occurrence of some bacterial genera. If the remaining genera are efficient in obtaining nutrients from the calf feed, this change in microbiota composition can lead to weight gain. This effect has already been proven for antibiotics (Cox, 2016), so it would be interesting to investigate the mechanisms underlying the association between *Cryptosporidium* spp. infection and weight gain.

As HL treatment is widely used, it should always be considered a possible confounder when studying the effects of *Cryptosporidium* spp. and the microbiota of calves in field studies. Furthermore, we have shown that the effects of *Cryptosporidium* spp. and HL treatment cannot always be differentiated from one another; hence, this should be considered when conducting similar studies. Other enteric pathogens may affect HL treatment against *C. parvum*, and they should be considered when conducting efficacy studies (Delling and Daugschies, 2022). In study **II**, rotavirus, coronavirus, and *E. coli* were present in calves before the cryptosporidiosis outbreak, and the herd tested positive for the bovine viral diarrhoea virus at the time of the study. Although no other pathogens were found in our study, we cannot rule

out other pathogens that may have influenced *Cryptosporidium* spp. infections, HL treatment, or microbiota.

6.4. Perspectives of future research

In study **III**, we showed that *Cryptosporidium* spp. are associated with changes in microbiota composition. The question remains as to which comes first. Does the altered microbial composition in the intestines make the host more susceptible to infection with *Cryptosporidium* spp., or does the *Cryptosporidium* spp. infection affect the composition of the microbiota? This must be studied further, since this knowledge and the implementation of it could improve the health and welfare of calves by protecting them from cryptosporidiosis and its possible long-term (harmful) effects.

The potential of APPs as markers to predict future weight gain should not be disregarded, as the connection between stronger APR and lower weight gain in the future has been shown (**II**) (Peetsalu *et al.*, 2022). *Cryptosporidium* spp. infection, HL treatment, and APR should be studied as factors influencing future performance (e.g., fertility, production) and health. For now, the exact mechanisms and effects behind these factors are not known.

Even if the clinical cases and outbreak fit the picture of cryptosporidiosis, the co-existence of other pathogens can never be fully excluded in field studies. Thus, the co-existence and possible synergies of different pathogens as well as their host-interactions should be studied further. As *Cryptosporidium* spp. infections and HL treatment could not be differentiated from one another in this study, it would be interesting to investigate if and how they would affect the calves independently since HL is used as a prophylactic treatment of cryptosporidiosis regardless of the infection status of the calf. There is a possibility that HL itself might affect the intestinal microbial community of calves independently from *Cryptosporidium* spp. infection, as our study showed that not treated animals had higher weight gain than those treated with HL (**II**). Further studies of the independent effects of HL would require a controlled clinical trial instead of a field study.

As many species of *Cryptosporidium* are zoonotic, it should be investigated whether human and animal cases are related, that is, what species and

subtypes are involved. In Estonia, one case study has shown a zoonotic link (Lassen *et al.*, 2014). In the same study, the authors stated that many human cases of cryptosporidiosis (such as those among veterinary students) go undiagnosed, and thus the reported prevalence is low. To conduct a study comparing animal and human cases, there is a need to enhance the diagnostics of human cryptosporidiosis, and precise technologies (e.g., PCR) should be used. By comparing the species and subtypes involved in human, cattle, and potentially cases of cryptosporidiosis in other animal species, it would be possible to address and interfere with the chain of transmission and thus reduce the number of cases in both humans and animals.

7. CONCLUSIONS

In Estonian cattle farms, there is a high genetic diversity of *Cryptosporidium* spp. among calves. The animal level prevalence varies between farms, with an example of higher prevalence during an acute outbreak of death-causing cryptosporidiosis on a farm. As the parasite is potentially zoonotic, the need for awareness and prevention among risk groups and their health care providers is essential. There are treatment options for calves that can decrease mortality and delay the infection with *Cryptosporidium* spp. and the onset of oocyst shedding by one or two weeks, when the calves' immune system is more prepared to fight the invading pathogen.

During *Cryptosporidium* spp. infections, the more oocysts shed by the calves, the stronger their APR; Hp appears to be a more sensitive biomarker than SAA in two-week-old calves, as it reacts earlier and more strongly to *Cryptosporidium* spp. infections, and it is not influenced by the colostral intake of SAA. By delaying the infection, HL treatment also delays the induction of APR and decreases its magnitude. The host immune response can potentially also be triggered by some bacterial genera, such as *Fusobacterium* and *Peptostreptococcus*, and through this mechanism, a high abundance of certain bacterial genera during early life may have long-term effects on the calves. The long-term effects and interactions of APPs, *Cryptosporidium* spp. infection, HL treatment and microbiota are yet to be thoroughly studied.

The overall diversity of faecal microbiota is low in two-week-old calves shedding *Cryptosporidium* spp. oocysts when compared to calves with no *Cryptosporidium* spp. infection. The presence of *Cryptosporidium* spp. may alter the intestinal microbiota environment to less favourable direction for some bacterial species, or alternatively, a high abundance of certain bacterial species may inhibit or reduce the *Cryptosporidium* spp. infection. Still, the big question remains – which happens first, the changes in the microbiota or the *Cryptosporidium* spp. infection?

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SUMMARY IN ESTONIAN

Krüptosporiidide levimus, seos üldise põletikuvastuse, rooja mikrobioota ja halofuginoon laktaadi raviga vasikatel

Sissejuhatus ja kirjanduse ülevaade

Perekond *Cryptosporidium*

Ernest Edward Tyzzer avastas 1907. a parasiidi, alglooma, mida teatakse tänapäeval kui *Cryptosporidium*'i (Tyzzer, 1907). Hetkel sellesse perekonda kuulub 44 liiki, ning üle 120 genotüübi (Ryan jt, 2021). Krüptosporiidid on vasikate kõhulahtisuse põhjustajaks kõrvuti rotaviiruse, koroonaviiruse, *Escherichia coli* ja *Giardia´*ga. Veistel diagnoositi *Cryptosporidium* spp. esimest korda 1971. a (Panciera jt, 1971). Kõik *Cryptosporidium´*i perekonda kuuluvad parasiidid põhjustavad krüptosporidioosi, haigust, mis esineb imetajatel (sh inimesel), lindudel, reptiilidel, kahepaiksetel ja kaladel (Spickler, 2018). Globaalse levikuga *Cryptosporidium*'i levimus varieerub veistel vahemikus 3,4–96,6% (Thomson jt, 2017). Veiseid võib nakatada mitu *Cryptosporidium* liiki, kuid kõige levinum, eriti võõrutamata vasikate hulgas, on *C. parvum* (Chako jt, 2010). Eestis on 84% veisefarmidest *Cryptosporidium* spp. positiivsed, kusjuures levinud on kaks liiki: *C. parvum* ja *C. andersoni* (Lassen jt, 2009).

Cryptosporidium on minimaalselt invasiivne intratsellulaarne, kuid tsütoplasmaväline parasiit, mille elutsükkel lõpeb peremeesorganismi peensoole epiteelirakkude apikaalses osas (Petry jt, 2010; O'Hara ja Chen, 2011; Laurent ja Lacroix-Lamandé, 2017; Delling ja Daugschies, 2022). Parasiidil on üks nakkuslik sporosoidi staadium ootsüstis (4–6 μm), mis väljutatakse roojaga. Peiteoslaste ülekandetee on fekaal-oraalne (Ryan jt, 2016). Haigus väljendub vasikatel vesise, mõnikord ka verise kõhulahtisuse, loiduse, anoreksia, toitainete imendusmishäire ja kõhuvaluna (Naciri jt, 1999; Olson jt, 2004). Enamasti on tegu mööduva haigusega (Petry jt, 2010), kuid ägedal juhul võib nakkus lõppeda surmaga (Olson jt, 2004). Krüptosporidioosi inkubatsiooniperiood on 5–7 päeva (Abeywardena jt, 2015), ja ootsüste väljutatakse juba mõni päev pärast nakatumist (Innes jt, 2020). Ootsüste väljutatakse nakkuse puhul rohkelt, kuni 10^6–10^{10} ootsüsti grammi rooja kohta (*oocysts per gram of faeces;* opg) (Nydam jt, 2001; Armon jt, 2016), kusjuures nende

väljutamisega ei kaasne alati kõhulahtisust (Thomson jt, 2017). Ootsüstid on peremeesorganismist väljudes nakkuslikud (Kosek jt, 2001).

Euroopa Liidus on müügiloa saanud krüptosporidioosi ravim müüginimetusega Halocur, mille aktiivne toimeaine on halofuginoon laktaat (HL). Tootja andmetel võib ravimit kasutada nii haiguse profülaktikaks kui ka parasiidi põhjustatud kõhulahtisuse leevendamiseks vasikatel (Intervet International BV, 2019). *Cryptosporidium* spp. diagnoosimiseks kasutatakse erinevaid meetodeid, millest usaldusväärseimaks peetakse antikeha immunofluorestsentsi, kuna selle meetodi spetsiifilisus on kuni 97% (Ryan jt, 2016). Molekulaarsetest meetoditest on kõige levinumad polümeraasi ahelreaktsiooni (*polymerase chain reaction*, PCR) 18S ribosomaalse RNA (rRNA) määramine ja 60-kDa glükoproteiini (gp60) geeni määramine, mille abil saab eristada lisaks liikidele ka nende alamtüüpe (Xiao, 2010; Strong jt, 2000).

Põletikuvastus

Haigustekitaja invasioon või koekahjustus käivitavad organismis mittespetsiifilise põletikuvastuse, mille üheks osaks on ägeda faasi vastus (*acute phase response*; APR). Selles reaktsioonis on tähtsal kohal ägeda faasi valgud (*acute phase proteins*; APP), mille sünteesi eelkõige maksas algatavad proinflammatoorsed tsütokiinid (Baumann ja Gauldie, 1994). Krüptosporidioosi korral hakkavad nakatunud epiteelrakud tootma proinflammatoorseid tsütokiine, kemokiine ja antimikrobiaalseid peptiide (Thomson jt, 2017). Antud zoonoosi puhul on kõige tähtsamad proinflammatoorsed tsütokiinid interferoon gamma (IFN-γ), interleukiin 1 beeta (IL-1β), interleukiin 6 (IL-6) ja tuumori nekroosi faktor alfa (TNF-α) (Laurent ja Lacroix-Lamandé, 2017). Igal (looma)liigil on oma peamised APP-d, mida kasutatakse üldise põletikureaktsiooni markeritena. Veistel on nendeks seerumi amüloid A (*seerum amyloid A*; SAA) ja haptoglobiin (*haptoglobin*; Hp) (Eckersall ja Bell, 2010). Nende valkude sisaldus veres sõltub infektsioonist ning koekahjustusest, samuti kaasnevad muutused looma vanuse suurenemise tõttu (Orro jt, 2006, 2008; Tóthová jt, 2015).

Soolestiku mikrobioota

Esimestel elunädalatel toimuvad vasika rooja mikrobiootas olulised muutused (Alipour jt, 2018). Mikroobne mitmekesisus suureneb vanusega (Dill-Mcfarland jt, 2017); kõige suurem liigiline mitmekesisus

on tuvastatud võõrutamise ajal (Klein-Jöbstl jt, 2014). Kõige enam on soolestikus *Firmicutes´*e ja *Bacteroidetes´*e hõimkonda kuuluvaid baktereid, seda ka pärasooles (Jami jt, 2013; Myer jt, 2017). Vasika soolestiku mikroobne koostis ei sõltu ainult looma vanusest, vaid ka kasvust ja terviseseisundist (Oikonomou jt, 2013). Mikroobikooslust mõjutavad ka antibiootikumravi, antimikrobiaalsete jääkidega piima jootmine, kuumastress, võõrutamine ja kõhulahtisus (Grønvold jt, 2011; Oultram jt, 2015; Meale jt, 2016; van Vleck Pereira jt, 2016; Gomez jt, 2017 Chen jt, 2018).

Soolestiku mikrobioota kooslust võivad mõjutada ka siseparasiidid, nt *Cryptosporidium* spp. (Laurent ja Lacroix-Lamandé, 2017). Krüptosporidioosi puhul on täheldatud soolestiku suurenenud läbilaskvust, mis taandub paari nädala jooksul (Klein jt, 2008). Soolestiku läbilaskvust saab hinnata, mõõtes näiteks soolestiku rasvhappeid siduva proteiini (*intestinal fatty acid binding proteine*; I-FABP) hulka (Ok jt, 2020). On leitud, et tervetel ja krüptosporidioosiga hiirtel on erinev soolestiku mikroobikooslus (Ras jt, 2015). Hiirtel on nakkus seotud *Firmicutes´*e bakterite väiksema suhtelise arvukusega, ning *Bacteroidetes´*e bakterite suurema suhtelise arvukusega (Mammeri jt, 2019). Vasikatel on leitud, et *C. parvum* võib luua sünergistliku efekti *Fusobacterium´*iga (Ichikawa-Seki jt, 2019).

Töö eesmärgid

Väitekirja eesmärk oli selgitada soolestiku parasiidi *Cryptosporidium* spp. levimust vasikatel Eestis (**I**), nende seoseid üldise põletikuvastuse (**II** ja **III**), rooja mikrobioota (**III**) ja halofuginoon laktaadi raviga (**II** ja **III**). Väitekirja ülesanded olid:

1. Kirjeldada *Cryptosporidium* spp. levimust, roojaga väljutatavaid liike ning *C. parvum*'i alamtüüpe kuni kahe kuu vanustel vasikatel Eestis (**I**);

2. Selgitada välja *Cryptosporidium* spp. nakkuse seoseid üldise põletikuvastusega (APP-dega) kuni kuue nädala vanustel vasikatel (**II**);

3. Selgitada välja HL ravi efekte ja seoseid *Cryptosporidium* spp. nakkuse dünaamikaga kuni kuue nädala vanustel vasikatel (**II**);

4. Leida võimalikke seoseid rooja mikrobioota koostise ja mitmekesisuse ning *Cryptosporidium* spp. nakkuse ja üldise põletikuvastuse (APP-de) vahel kahe nädala vanustel vasikatel (**III**).

Materjal ja metoodika

Proovide kogumine

Väitekirja esimene uuring (**I**) hõlmas 486 vasikat vanuses kuni kaks kuud 53 veisefarmis üle Eesti. Roojaproovid (n = 486) koguti ajavahemikes aprill 2013–mai 2014 ning jaanuar–märts 2015.

Teine uuring (**II**) hõlmas 144 lehmikut, kes sündisid vahemikus 21. jaanuarist 16. märtsini 2015. a Kesk-Eesti piimafarmis (lehmade arv proovivõtu ajal umbes 1800). Vasikatelt koguti seerumi- (n = 901) ja roojaproovid (n = 767) iganädalaselt kuni kuue nädala vanuseni, lisaks võeti kordusproov kolme kuu vanuses. Vasikate kehakaalu mõõdeti vahetult peale sündi ning ühe ja kolme kuu vanuselt.

Kolmas uuring (**III**) hõlmas 112 kahe nädala vanust lehmikut. Samad loomad osalesid ka väitekirja teises (**II**) uuringus. Vasikatelt koguti seerumi- (n = 112) ja roojaproovid (n = 112) ning nende kehakaalu mõõdeti vahetult peale sündi ning üheksa kuu vanuselt.

II ja **III** uuringu proovivõtu ajal oli farmis akuutne krüptosporidioosi puhang, seetõttu raviti uuritavaid vasikaid HL-iga. Kõigil alla kahe nädala vanustel loomadel teostati massravi HL-iga, vanemaid loomi ei ravitud. Retrospektiivselt jagati uuringu vasikad kolme ravirühma: 1) korrektselt ravitud (ravi alustati 48 tunni jooksul sünnist, kestis nädal aega; **II** n = 65; **III** n = 52), 2) ebakorrektselt ravitud (ravi alustati hilja (rohkem kui 48 tundi peale sündi), kestis vähem kui nädal aega; **II** n = 45; **III** n = 39), ja 3) ravita (vasikad vanemad kui kaks nädalat; **II** n = 34; **III** n = 21).

Proovide analüüsimine

I uuringu jaoks analüüsiti roojaproove molekulaarseid meetodeid kasutades tuvastamaks ja kirjeldamaks *Cryptosporidium´*i liike ja alamtüüpe. Roojast eraldatud DNA-st määrati *Cryptosporidium* spp. olemasolu, kasutades PCR-i ning 18S rRNA geeni sekveneerimisel põhinevat meetodit.

Selle geeni suhtes positiivseid proove uuriti ka gp60 geeni olemasolu suhtes, et määrata roojas leiduvad *Cryptosporidium´*i liigid ja alamtüübid.

II ja **III** uuringus kasutati *Cryptosporidium* spp. ootsüstide leidmiseks roojast immunofluorestsentsmeetodit (tulemus: opg), ning vereseerumis määrati APP-de (SAA ja Hp) kontsentratsioon. **III** uuringu jaoks määrati proinflammatoorsete tsütokiinide (IL-1β, IL-6, ja TNF-α) ja I-FABP kontsentratsioon.

III uuringus mikrobioota koostise määramiseks eraldati roojast DNA ja sekveneeriti V3−V4 regioon, kasutades 16S rRNA-d.

Statistiline analüüs

I uuringus kasutati logistilise regressioonanalüüsi mudeleid dihhotoomsete muutujate (vasikas positiivne *Cryptosporidium* spp. ootsüstide suhtes, vasikas positiivne *C. parvum´*i ootsüstide suhtes, vasikas positiivne *C. parvum´*i alamtüübi IIa18G1R1 ootsüstide suhtes) jaoks. **II** uuringu jaoks kasutati lineaarse regressiooni mudeleid, et hinnata seoseid vasika vereseerumi SAA ja Hp tasemete ning *Cryptosporidium* spp. opg gruppide vahel erinevatel nädalatel, ja seoseid päevase massi-iibe ja HL-ravirühmade vahel. **III** uuringu jaoks kasutati lineaarseid regressiooni-mudeleid hindamaks seoseid mikrobiaalse mitmekesisuse (Shannoni indeksi), vanuse, kaasasündinud immuunsuse markerite (SAA, Hp, IL-1β, IL-6, TNF-α), *Cryptosporidium* spp. nakkuse ja HL-ravi vahel.

II ja **III** uuringus jagati vasikad rühmadesse SAA ja Hp kontsentratsioonide, ja *Cryptosporidium* spp. opg alusel. SAA ja Hp kontsentratsioonide (madal, keskmine, kõrge) alusel moodustati kolm rühma. *Cryptosporidium* spp. uurimiseks moodustati rühmad opg alusel: ootsüste pole, opg alla sama nädala positiivsete proovide opg mediaani, ja opg üle sama nädala positiivsete proovide opg mediaani.

III uuringus kasutati dispersiooni jagunemise analüüsi, et selgitada välja, kuidas näitavad üldise põletikuvastuse markerid, *Cryptosporidium* spp. nakkus, ja looma vanus rooja mikrobioota hajuvust perekonna tasandil. Juhumetsa analüüsi kasutati uurimaks, kui täpselt kirjeldavad rooja mikroobikooslust uurimistöös valitud muutujad (tsütokiinid, APP-d, *Cryptosporidium* spp. nakkus, HL-ravi ja I-FABP). Juhumetsa analüüsis leitud 30 kõige mõjukamat bakteriperekonda analüüsiti edasi negatiivsete

binomiaalmudelite abil leidmaks seoseid ja nende suundi bakteripere-kondade ja *Cryptosporidium* spp. nakkuse ja valitud muutujate vahel.

Uurimistulemused

Cryptosporidium'i liikide levimus

Esimeses uuringus (**I**) tuvastati *Cryptosporidium* spp. olemasolu 22,6% uuritud vasikatest. Leiti kolm liiki: *C. parvum* (95,5%), *C. bovis* (3,6%), ja *C. ryanae* (0,9%), ning kümme *C. parvum´*i alamtüüpi, millest üks oli uudne (IIlA21R2). Kõige levinum alamtüüp oli IIaA18G1R1 (35,8%), mis esines peaaegu pooltes *Cryptosporidium´*i suhtes positiivsetes farmides. Kokkuvõtvalt oli 66,0% (35/53) uuritud farmidest *Cryptosporidium´*i liikide suhtes positiivsed. Ootsüste väljutasid vasikad kõige rohkem teisel elunädalal.

Teises uuringus (**II**) leiti, et 84,7% uuritud farmi lehmikutest olid *Cryptosporidium* spp. positiivsed, ning tuvastati *C. parvum´*i alamtüüp IIaA18G1R1. Ootsüstide väljutamine algas vasikate teisel elunädalal; opg tase tõusis kolmanda elunädalani. Kolmandas uuringus (**III**) uuriti ainult kahe nädala vanuseid lehmikuid, kellest oli *Cryptosporidium* spp. positiivseid 51,8%.

Ravi halofuginoon laktaadiga

Teises uuringus (**II**) esines vasikate esimesel kolmel elukuul kokku 21 surmajuhtu, enamus kõhulahtisuse tagajärjel. Kõige rohkem surmajuhte oli HL-ravi mittesaanute rühmas, kus suri 14 looma (66,7% kõigist surnutest). Kahes ravi saanud rühmas oli suremus väiksem.

Ravi mittesaanud vasikatel oli teisel elunädalal vere Hp kontsentratsioon märkimisväärselt kõrgem võrreldes teiste HL-ravirühmade ja elunädalatega (**II**). Eri HL-i ravirühmade kolme kuu vanused vasikad olid erineva päevase massi-iibega. Korrektset HL-ravi saanud vasikad olid väiksema päevase massi-iibega kui vasikad, kes HL-ravi ei saanud (**II**).

*Cryptosporidium´*i liikide ja mikrobiootaga seotud põletikumarkerid

Vasikatel, kellel tuvastati teisel elunädalal roojas kõrgem *Cryptosporidium* spp. ootsüstide arv, oli ka kõrgem SAA ja Hp kontsentratsioon seerumis

(**II** ja **III**). Teisel elunädalal oli Hp kontsentratsioon seerumis kõrgem ka vasikatel, kes ei saanud HL-ravi.

Mõne hõimkonna bakterite esinemisele roojas leidus seos APP-dega kahenädalastel lehmikutel (**III**). *Fusobacteria* suhteline arvukus oli suurem, ja *Bacteroidetes* suhteline arvukus oli väiksem juhul, kui vere SAA ja Hp sisaldus oli kõrge. SAA ja Hp seletavad ainult 1,6% rooja mikroobikoosluse varieeruvusest.

SAA-ga kõige rohkem seotud rooja bakter oli *Fusobacterium*'i perekonnast, mille arvukus oli kõige väiksem madala SAA-ga grupis. Hp-ga oli positiivne seos *[Ruminococcus] torques* rühmal. Negatiivne seos aga leiti Hp seerumi kontsentratsiooni ja rooja *Flanifractor´*i arvukuse vahel.

Perekond *Peptostreptococcus* arvukus roojas oli positiivselt seotud nii kõrge SAA kui ka kõrge Hp kontsentratsiooniga seerumis (mõlemad $p < 0,001$) (**III**).

Mikrobioota koostis ja krüptosporidioos

Kolmandas uuringus (**III**) leiti, et rooja mikroobne mitmekesisus (mõõdetud Shannoni indeksiga) suurenes vasika vanusega ($p = 0,001$), ja vähenes, kui roojas oli suur arv *Cryptosporidium* spp. ootsüste ($p = 0,007$). HL-ravi on seotud väiksema mikrobiaalse mitmekesisusega. Mida kõrgem oli SAA ja Hp kontsentratsioon seerumis, seda suurem oli *Fusobacteria* ning väiksem *Bacteroidetes´*e suhteline arvukus roojas. *Firmicutes´*e-*Bacteroidetes´*e suhe oli kõrgem vasikatel, kes olid *Cryptosporidium* spp. positiivsed, ja madalam nendel, kes said HL-ravi ($p = 0,06$) (varem avaldamata tulemus). Uurimistöös valitud muutujad – vanus, tsütokiinid, ühendatud SAA ja Hp kontsentratsioonid, ning ühendatud *Cryptosporidium* spp. nakkus ja HL ravi – seletavad ainult 6% rooja mikrobiaalsest varieeruvusest; *Cryptosporidium* spp. nakkus üksi seletab ainult 0,68% varieeruvusest.

Neli rooja bakterite perekonda (*Ruminiclostridium 9*, *Alistipes*, *Parasutterella* ja *Faecalibacterium*) on statistiliselt oluliselt seotud *Cryptosporidium* spp. nakkusega, kõigi nende seos on negatiivne (**III**).

Järeldused

Eesti veisefarmide vasikatel on suur geneetiline *Cryptosporidium*'i liikide varieeruvus. Levimus looma tasandil on väga erinev, näiteks on kõrgem levimus farmis, kus äge krüptosporidioos põhjustab surmajuhtumeid. Kuna krüptosporiidid on potentsiaalselt zoonootilised, on riskirühmadesse kuuluvate inimeste ja nende tervishoiuteenuste osutajate teadlikkuse tõstmine ja seeläbi haiguse ennetamine hädavajalik. Vasikate jaoks on väljatöötatud ravimid, mis võivad vähendada suremust, lükata edasi krüptosporiididega nakatumist, ja ootsüstide eraldumise algust ühe või kahe nädala võrra, kui vasikate immuunsüsteem on paremini ettevalmistatud võitlema sissetungiva patogeeniga.

Mida rohkem ootsüste vasikad eritavad krüptosporidioosi nakkuse ajal, seda tugevam on nende APR; Hp näib olevat tundlikum biomarker kui SAA kahenädalastel vasikatel, kuna see reageerib varem ja tugevamalt krüptosporiidide nakkusele ja seda ei mõjuta SAA ülekandumine ternesest. Nakkuse edasilükkamise kaudu lükkab HL-ravi edasi APR-i esilekutsumist ja vähendab selle ulatust. Peremeesorganismi immuunvastuse võivad potentsiaalselt käivitada ka mõned bakteriperekonnad, nagu *Fusobacterium* ja *Peptostreptococcus*, ning selle mehhanismi kaudu võib teatud bakteriperekondade suur arvukus varases elus avaldada vasikatele pikaajalist mõju. APP-de, *Cryptosporidium*'i liikide, HL-ravi ja mikrobioota koostoimeid ja pikaajalisi mõjusid tuleb veel põhjalikult uurida.

Kahenädalastel vasikatel, kes eritavad *Cryptosporidium*'i ootsüste, on rooja mikrobioota üldine mitmekesisus madalam võrreldes vasikatega, kes ei ole krüptosporiididega nakatunud. Krüptosporiidide olemasolu võib muuta soolestiku mikrobioota keskkonna mõne bakteriliigi jaoks vähem soodsamaks või teise võimalusena võib teatud bakteriliikide suur arvukus pärssida või vähendada nakkust krüptosporiididega. Siiski jääb õhku suur küsimus – kumb juhtub enne, kas muutused mikrobiootas või *Cryptosporidium*'i nakkus?

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I

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Molecular epidemiology of *Cryptosporidium* spp. in calves in Estonia: high prevalence of *Cryptosporidium parvum* shedding and 10 subtypes identified

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Abstract

We investigated the molecular epidemiology of *Cryptosporidium* spp. in Estonia by testing fecal samples from 486 calves aged <2 months, raised on 53 cattle farms, for the presence of *Cryptosporidium* DNA. The parasites were identified and characterized by sequencing of the 18S rRNA gene and of the 60 kDa glycoprotein (*gp60*) gene. Moreover, using a questionnaire, we surveyed factors that could be relevant for animal-to-human and human-to-animal transmission of *Cryptosporidium* spp. on the farms. *Cryptosporidium* spp. were shed by 23% of the investigated calves and at least one shedding calf was found on 66% of the farms. *Cryptosporidium parvum* was the most common species shed, while *C. bovis* and *C. ryanae* were also detected. More than half of the calves aged 8–14 days shed *C. parvum*. Nine previously described *C. parvum* subtypes (IIaA14G1R1, IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1 and IIaA16G2R1) and an apparently novel subtype IIlA21R2 were found. Calves from farms that reported spreading manure on fields during spring had 10 times higher odds to shed *Cryptosporidium* spp. in their feces than calves from farms that did not. Calves aged 8–14 days had higher odds to shed IIa18G1R1 as well as IIaA16G1R1 than younger calves.

Research Article

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Introduction

Protozoan parasites of the genus *Cryptosporidium* can cause gastro-intestinal disease in several host species, including humans and cattle (Thompson *et al.*, 2016). *Cryptosporidium parvum* and *C. hominis* are considered responsible for most cases of human cryptosporidiosis (Cacciò and Chalmers, 2016). *Cryptosporidium hominis* is known as human-specific species, while *C. parvum* has a wider host spectrum that includes cattle. *Cryptosporidium parvum* has been observed as the dominant *Cryptosporidium* species shed by pre-weaned calves in many countries (Chako *et al.*, 2010), but not in countries nearby Estonia (Silverlås and Blanco-Penedo, 2013; Björkman *et al.*, 2015). Young calves infected with *C. parvum* can shed high numbers of oocysts in their feces (Xiao, 2010; Smith *et al.*, 2014). In addition to animal-to-human transmission of *C. parvum* by direct contact, feces of infected cattle may also contaminate, e.g. water supplies (McLauchlin *et al.*, 2000; Xiao, 2010; Wells *et al.*, 2015) or ready-to-eat vegetables (Åberg *et al.*, 2015).

Cryptosporidium parvum subtype families IIa and IId have been found in both humans and cattle (Xiao, 2010). In Sweden, one of the most common subtype of the *C. parvum* in cattle was IIaA16G1R1 (Silverlås *et al.*, 2010; Björkman *et al.*, 2015), which has been also found in humans (Silverlås *et al.*, 2010; Insulander *et al.*, 2013). In Estonia, the same subtype IIaA16G1R1 has been identified in both cattle and an immunocompetent human with clinical cryptosporidiosis (Lassen *et al.*, 2014). That case and results of a questionnaire study focusing on veterinary students (Dorbek-Kolin *et al.*, 2018) provide evidence for zoonotic transmission of *Cryptosporidium* in Estonia.

In Estonia, cryptosporidiosis in humans is a notifiable but under-reported disease (Lassen *et al.*, 2014; Plutzer *et al.*, 2018). Surveillance data do not provide a good overview of the epidemiology of *Cryptosporidium*, and the need to fill the knowledge gaps with a One Health approach is evident (Plutzer *et al.*, 2018). Previous studies have shown that almost all Estonian cattle farms had cattle that were shedding *Cryptosporidium* spp. (Lassen *et al.*, 2009). However, the zoonotic potential of the *Cryptosporidium* spp. shed and the circulating *C. parvum* subtypes have been unknown.

The main aim of this study was to characterize *Cryptosporidium* spp. shed by calves in Estonia, with special emphasis on the zoonotic potential. In addition, we surveyed factors

that could be relevant for transmission of *Cryptosporidium* spp. from animals-to-humans or from humans-to-animals on the farms.

Materials and methods

Study design

Sample size calculation was performed using OpenEpi (Dean *et al.*, 2015): 35 farms was the minimum sample size needed for this study. This calculation was based on a population size of 5572 cattle herds (Estonian Agricultural Register and Information Board, 2018*a*), absolute precision of 10% and an expected proportion of farms with calves shedding *C. parvum* of 10%. The aim was set to sample at least 50 farms. The sampling was proportionally stratified to the 15 Estonian counties according to the number of farms listed in the Estonian Animal Recording Centre (2013) in each county. Expecting that at a given moment, at least 30% of calves would be shedding *Cryptosporidium* spp. oocysts on a farm where *Cryptosporidium* spp. is present (Lassen *et al.*, 2009), it was evaluated that 10 calves per farm would be sufficient to find at least one calf shedding the parasite, if *Cryptosporidium* spp. was present on the farm.

Sampling

The samples were collected by veterinarians from April 2013 to May 2014 and from January to March 2015. Inclusion criteria for farms were: registration in the e-Business Register (Centre of Registers and Information Systems, 2018) and Estonian Agricultural Registers and Information Board (2018*b*), and herd size ⩾50 cattle to ensure a sufficient number of calves for the study. Farms were selected using a random number generator (Microsoft Excel, Microsoft Cooperation). Three farms were randomly chosen as potential replacements for each county and were included in case a farm that was originally selected opted out.

The veterinarians were instructed to collect individual fecal samples from the rectum of up to 10 calves ⩽2 months of age on each farm. Animal-level exclusion criterion was the calf being reported to be >2 months old. Samples were collected in disposable gloves and stored in a transportable cooler during transport to the laboratory. The samples were stored frozen at −18 °C until DNA extraction.

Questionnaire

A questionnaire was designed to collect information on diarrhoea in calves on the farms as well as on factors with potential relevance for animal-to-human and human-to-animal transmission of *Cryptosporidium* spp. on farms (Supplementary Table S1). The questionnaire was filled in by the interviewing veterinarian either on the farm at the time of sampling or by phone interview following the farm visit. The questions were asked in Estonian language.

DNA extraction, polymerase chain reaction and sequencing

We used molecular methods to detect, identify and characterize *Cryptosporidium* spp. from the samples. Genomic DNA was extracted from 200 μg of thawed and homogenized feces using the PSP® Spin Stool DNA Kit (STRATEC Biomedical AG, Birkenfeld, Germany) according to the manufacturer's instructions.

Three microlitres of each DNA sample were submitted to polymerase chain reaction (PCR) amplification targeting the 18S rRNA gene (Xiao *et al.*, 1999). Nuclease-free water and *C. parvum* genomic DNA (kindly provided by the European Union Reference Laboratory for Parasites) were used as negative and positive controls. The nested reaction used 1 μL of the first-round PCR product. The thermal cycling conditions were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min and a final extension of 72 °C for 7 min in both the first and second rounds. The PCR products were run on 2% ethidium bromide-stained agarose gel and visualized under an ultraviolet transilluminator. Products of the expected size (approximately 825 bp) were submitted to sequencing for species identification.

The samples that tested positive were submitted to PCR amplification targeting the 60 kDa glycoprotein (*gp60*) gene for subtype identification (Peng *et al.*, 2001). Three microlitres of DNA sample were used in the first PCR reaction and 1 μL of PCR product in the nested PCR reaction. Nuclease-free water and *C. parvum* genomic DNA were used as negative and positive controls. Thermal conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min and a final extension of 72 °C for 7 min and 10 min in the first and second rounds, respectively. Electrophoresis was performed as described above. Products of approximately 490 bp were selected for subsequent sequencing.

The PCR products were cleaned up and sequenced with Applied Biosystems® 3130xl Genetic Analyzer by a two-directional procedure. Forward and reverse sequences were aligned with BioEdit v7.2.5 software (Hall, 1999) to generate single consensus sequences and correct mismatches. The resulting sequences were compared with nucleotide sequences (Accession numbers KJ941147, HQ005736, AM937006, AB242226) deposited in GenBank using BLASTn (nucleotide Basic Local Alignment Search Tool, Altschul *et al.*, 1990). *Gp60* subtypes were named in agreement with the system proposed by Sulaiman *et al.* (2005) based on the number of serine-coding trinucleotide repeats.

Statistics

Sample size and confidence intervals (CI) (Mid-P exact) were calculated using OpenEpi (Dean *et al.*, 2015). Further statistical analyses were performed using Stata IC 14.2 for Mac software (Stata Corporation, College Station, TX, USA). A calf was considered *Cryptosporidium* spp.-positive if its sample tested positive for *Cryptosporidium* spp. 18S rDNA. A farm was considered positive if at least one calf of the investigated calves tested positive. Multivariable logistic regression models were built for dichotomous animal-level outcomes: calf testing positive for *Cryptosporidium* spp., calf testing positive for *C. parvum*, calf testing positive for *C. parvum* subtype IIa18G1R1 and calf testing positive for *C. parvum* subtype IIaA16G1R1. The farm number was used as a random factor to account for clustering. Variables (Supplementary Table S1) with a P value ⩽0.20 in univariable analysis were first included in the model, followed by a stepwise backward elimination procedure. Biologically meaningful interactions and possible confounding effects were tested. P values <0.05 were considered significant.

Results

Sample

From each of the 53 farms included in the study, 3–14 (median 10) fecal samples were collected, resulting in a total of 522 individual fecal samples. A total of 36 fecal samples were excluded because the same farm had been sampled twice (the samples from second sampling were included), missing labels or insufficient amount of fecal sample available for the analysis. The final sample included in this study comprised individual fecal

Table 1. Animal-level prevalence of *Cryptosporidium* species and subtypes in fecal samples from calves ($n = 486$), including 454 calves ⩽2 months of age and 32 calves of unknown age, collected from 53 cattle farms in Estonia

Species	Subtype[a]	*n* positive	% positive	95% confidence interval
Cryptosporidium parvum		105	21.60	18.12–25.43
	IIaA18G1R1	34	7.00	4.97–9.53
	IIaA16G1R1	16	3.29	1.96–5.18
	IIaA20G1R1	9	1.85	0.90–3.37
	IIaA14G1R1	8	1.64	0.77–3.10
	IIaA16G2R1	5	1.03	0.38–2.27
	IIaA22G1R1	5	1.03	0.38–2.27
	IIaA21G1R1	5	1.03	0.38–2.27
	IIaA17G1R1	5	1.03	0.38–2.27
	IIlA21R2	5	1.03	0.38–2.27
	IIaA19G1R1	3	0.62	1.16–1.67
Cryptosporidium bovis		4	0.82	0.26–1.97
Cryptosporidium ryanae		1	0.21	0.01–1.01

[a]*Cryptosporidium parvum* subtype data for 95 samples (only species level for 10 *C. parvum*-positive samples).

samples from altogether 486 calves from the 53 farms, 3–14 (median 10) per farm. Information on age was available for 454 calves, and the age of the calves ranged from 1 to 59 days (median 15, mean 18.26). The prevalence estimates were based on the results from 486 calves, i.e. including also the 32 calves with no information of age.

Questionnaire

Of the 53 farms, 49 (92.5%) answered the questionnaire. Supplementary Table S1 shows the distribution of the answers. The majority of farms had more than 150 cattle (79.2%). One (2.1%) farm had bought cattle from abroad during the previous 5 years. Almost a quarter (24.4%) of the farms were located close to natural waterbodies. Altogether, 10 (18.9%) of the farms reported that *Cryptosporidium* spp. had been diagnosed in calves during the previous 5 years.

Prevalence of Cryptosporidium *spp.*

Cryptosporidium spp. DNA was amplified and sequenced from 110 (22.63%, 95% CI 19.08–26.51) of the 486 fecal samples. Of the 110 *Cryptosporidium* spp.-positive fecal samples, 105 (95.45%, 95% CI 90.22–98.32) were *C. parvum*-positive, four (3.64%, 95% CI 1.17–8.53) were *C. bovis*-positive and one (0.91%, 95% CI 0.05–4.40) was *C. ryanae*-positive. Of the 105 *C. parvum*-positive fecal samples, 95 (90.48%, 95% CI 83.68–95.06) were successfully sequenced and typed by *gp60* analysis.

At least one of the investigated calves was *Cryptosporidium* spp.-positive on 35 (66.0%, 95% CI 52.6–77.8) of the 53 farms (Table 2, Fig. 1). On 33 farms (62.3%, 95% CI 48.7–74.5), at least one of the investigated calves was *C. parvum*-positive. *Cryptosporidium bovis* was detected in fecal samples from two farms (3.8%, 95% CI 0.6–11.9), and on one of these farms, *C. parvum* was also detected. *Cryptosporidium ryanae* was detected as the only *Cryptosporidium* species on one farm.

Cryptosporidium parvum DNA was almost exclusively found in fecal samples of calves ⩽28 days old, with the exception of one calf that was 36 days old, and seven calves of unknown age (Fig. 2). A total of 64 (52.03%, 95% CI 43.21–60.76) of the 123 calves aged between 8 and 14 days shed *C. parvum*. *Cryptosporidium bovis* and *C. ryanae* were detected in feces of calves that were >14 days old.

Cryptosporidium parvum *subtypes*

A total of 10 different subtypes were identified (Table 1 and 2). The majority (9/10) of the subtypes were in the IIa subtype family, while one subtype was identified as novel IIlA21R2 (Accession numbers MH509210–MH509219). The most common subtype was IIaA18G1R1, which was found in 35.79% (34/95) of the *C. parvum*-positive samples and on 15 (45.5%) of the 33 farms where *C. parvum* was found. The second most common subtype was IIaA16G1R1, which was identified in 16 (16.84%) of the *C. parvum*-positive samples and on four (12.1%) of the *C. parvum*-positive farms. A single *C. parvum* subtype per farm was found on all except one farm. Three *C. parvum* subtypes (IIaA18G1R1, IIaA20G1R1 and IIaA21G1R1) were identified on a farm located in southern part of the country (Fig. 1).

Subtypes within the IIa family differed by the number of TCA repeats. The exception was the subtype IIaA16G2R1, which had an additional TCG triplet. The novel IIlA21R2 had no TCG triplets, an ACATCA sequence that repeated twice and several single nucleotide polymorphisms when compared with the other IIa subtypes.

Models

Based on the final multivariable model, calves from farms that spread manure on the fields during spring had 10.1 (CI 1.18–86.27) times higher odds to have *Cryptosporidium* spp. DNA in the feces than calves from farms that did not. Calves that were from farms that reported mortality during the first month of life in calves with severe diarrhoea, which had received veterinary treatment, had 6.2 times higher odds (CI 2.46–15.66) to shed *Cryptosporidium* spp. (Supplementary Table S2) and 7.4 times higher odds (2.60–21.10) to shed *C. parvum* (Supplementary Table S3). The odds of a calf aged 8–14 days being *Cryptosporidium* spp. or *C. parvum*-positive were 10.1 (CI 4.53–22.36) and 10.4 (CI 4.58–23.74) times higher than the odds of a calf aged up to 7 days, respectively (Supplementary Tables S2 and S3). Based on the final models, the odds of a calf being

Table 2. Farm-level prevalence of *Cryptosporidium* species and subtypes on cattle farms ($n = 53$) in Estonia

Species[a]	Subtype[b]	*n* positive	% positive	95% confidence interval
Cryptosporidium parvum		33	62.3	48.72–74.50
	IIaA18G1R1	15	28.3	17.44–41.48
	IIaA16G1R1	4	7.5	2.44–17.21
	IIaA20G1R1	4	7.5	2.44–17.21
	IIaA14G1R1	2	3.8	0.64–11.91
	IIaA22G1R1	2	3.8	0.64–11.91
	IIaA21G1R1	2	3.8	0.64–11.91
	IIaA16G2R1	1	1.9	0.09–8.95
	IIaA17G1R1	1	1.9	0.09–8.95
	IIIA21R2	1	1.9	0.09–8.95
	IIaA19G1R1	1	1.9	0.09–8.95
Cryptosporidium bovis		2	3.8	0.64–11.91
Cryptosporidium ryanae		1	1.9	0.09–8.95

A farm was considered positive if at least one of the 3–14 calves investigated from the farm tested positive.
[a]One farm had both *C. bovis* and *C. parvum*.
[b]*Cryptosporidium parvum* subtype data for 31 farms (only species level for two *C. parvum*-positive farms). A single *C. parvum* genotype per farm was found on all except one farm. Three *C. parvum* subtypes (IIaA18G1R1, IIaA20G1R1 and IIaA21G1R1) were found on that one farm.

Fig. 1. Map of Estonia showing 18 farms negative for *Cryptosporidium* spp. (grey circles), three farms positive for *Cryptosporidium* spp. other than *C. parvum* (grey diamonds), and 33 farms positive for *C. parvum* (black circles). We tested fecal samples from three to 14 (median 10) calves per farm, and a farm was considered positive if fecal sample of at least one of the sampled calves tested positive.

C. parvum-positive for subtypes IIa18G1R1 and IIaA16G1R1 were 4.00 ($P = 0.024$) and 25.47 ($P = 0.018$) times higher in a calf aged 8–14 days than in a calf aged up to 7 days.

Discussion

The results of this study showed that *Cryptosporidium* spp. were commonly shed by calves in Estonia. This result, which is based on molecular detection, is in line with the previous sample-level and farm-level microscopy-based prevalence estimates (Lassen *et al.*, 2009).

In this study, *C. parvum* was the predominant *Cryptosporidium* species detected. This is in contrast to reports from calves of comparable age groups from nearby countries Sweden (Silverlås *et al.*, 2010; Björkman *et al.*, 2015) and Finland (Seppä-Lassila *et al.*, 2015), where the dominant species found were *C. bovis*, and *C. bovis* and *C. ryanae*, respectively. The results of this study resemble the results from Belgium, Slovenia and the Czech Republic, where *C. parvum* accounted for most of the *Cryptosporidium* findings from calves (Geurden *et al.*, 2007; Soba and Logar, 2008; Kváč *et al.*, 2011).

Ten different *C. parvum* subtypes were identified in the fecal samples in this study, indicating a high genetic diversity within

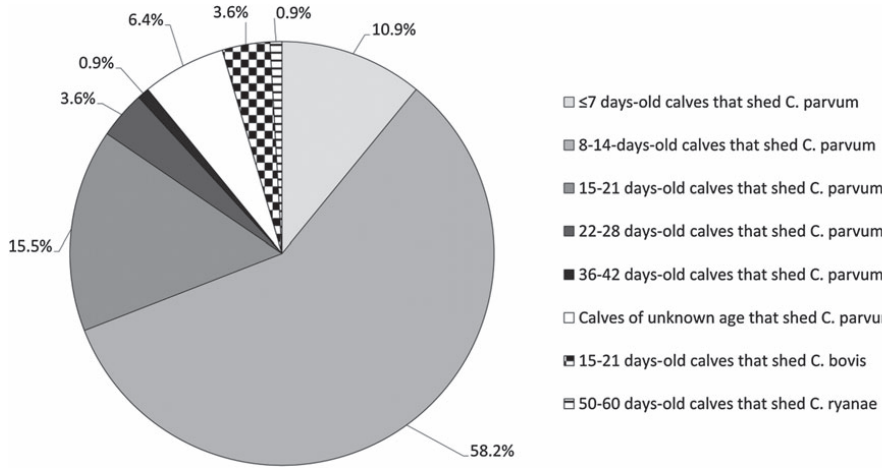

Fig. 2. Distribution of the 110 *Cryptosporidium* spp. shedding calves by age group and the *Cryptosporidium* species identified.

C. parvum in Estonia. The most frequently found subtype in this study, IIaA18G1R1, has been reported in cattle in Hungary (Plutzer and Karanis, 2007), the Czech Republic (Kváč *et al.*, 2011), the Netherlands (Wielinga *et al.*, 2008) and Serbia (Misic and Abe, 2007). IIaA16G1R1, the second most commonly found subtype in this study has been reported in the Czech Republic (Ondrácková *et al.*, 2009; Kváč *et al.*, 2011), Hungary (Plutzer and Karanis, 2007), Romania (Imre *et al.*, 2011; Vieira *et al.*, 2015), Slovenia (Soba and Logar, 2008) and Sweden (Björkman *et al.*, 2015). IIaA17G1R1 has been reported in Poland (Kaupke and Rzeżutka, 2015) and the UK (Smith *et al.*, 2014); IIaA16G2R1 in Belgium (Geurden *et al.*, 2007) and Spain (Quilez *et al.*, 2008); IIaA14G1R1 in Poland (Kaupke and Rzeżutka, 2015) and Spain (Ramo *et al.*, 2014); IIaA20G1R1 in Serbia (Misic and Abe, 2007); and IIaA21G1R1 and IIaA22G1R1 in Sweden (Silverlås *et al.*, 2010) and Germany (Broglia *et al.*, 2008). At least six of the subtypes identified in this study from calves, including the two most common ones, have also been found in humans (Soba and Logar, 2008; Chalmers *et al.*, 2011; Lassen *et al.*, 2014; Lobo *et al.*, 2014), highlighting the zoonotic potential of *C. parvum* shed by cattle. Subtype IIaA16G1R1, which has been connected to zoonotic transmission from a calf to a human in Estonia (Lassen *et al.*, 2014), was the second most common *C. parvum* subtype and was identified in feces of 3.3% of the calves and on 7.5% of the investigated farms.

The subtype allele family IIl (also indicated as IIj, Soba and Logar, 2008) has been reported from calves in Serbia (Misic and Abe, 2007), Poland (Kaupke and Rzeżutka, 2015) and Lithuania (Wielinga *et al.*, 2008), and in calves and humans from Slovenia (Soba and Logar, 2008). To our knowledge, the IIlA21R2 identified in this study is a new subtype.

Cryptosporidium parvum is commonly regarded as a zoonotic pathogen (Cacciò and Chalmers, 2016). However, not all *C. parvum* infections in humans result from zoonotic transmission. Molecular subtyping is a useful tool for determining whether human infections originate from animals. The results of studies like ours might be useful for back-tracing potential sources of *Cryptosporidium* infections and for evaluating the likelihood of the involvement of local cattle in outbreaks. Nevertheless, the definitive discrimination in such cases must resort to a multilocus approach (Chalmers *et al.*, 2016; Chalmers and Cacciò, 2016).

Either IIaA16G1R1 (Iqbal *et al.*, 2015) or IIaA16G2R1 (Ranjbar *et al.*, 2016), both reported to cause human cryptosporidiosis (Lassen *et al.*, 2014; Hijjawi *et al.*, 2017), was identified in 4.3% of the fecal samples. These subtypes have also been found in river water in Romania (Imre *et al.*, 2017) and sewage in Portugal (Lobo *et al.*, 2009). More studies are needed on the role of calves in shedding *C. parvum* in their feces and into the environment. In this study, a calf from a farm that reportedly spread cattle manure on the fields during spring had higher odds of shedding *Cryptosporidium* spp. as well as *C. parvum*. In the UK, a peak in human cryptosporidiosis cases caused by *C. parvum* during springtime was suspected to be linked with livestock (McLauchlin *et al.*, 2000). In the largest human cryptosporidiosis outbreak, which occurred in Milwaukee in 1993, run-off from cattle farms was suspected as the potential cause of the water contamination that resulted in human *C. parvum* infections (Mac Kenzie *et al.*, 1994). Several of the subtypes identified in the current study have been reported in wildlife (Krawczyk *et al.*, 2015) and fish (Certad *et al.*, 2015), which adds a sylvatic aspect to the epidemiology. *Cryptosporidium* spp. infection epidemiology is One Health epidemiology where humans, animals and the environment need to be considered.

High rates of *Cryptosporidium* spp. infection have been reported in calves of 1–3 weeks of age (Abeywardena *et al.*, 2015). In all our models, being 8–14 days old was a risk factor for the calf to shed *Cryptosporidium*. Young animals are usually more susceptible to *Cryptosporidium* spp., and may act as amplifiers and infection sources to other animals (Geurden *et al.*, 2010). In the current study, being 8–14 days old was a risk factor for shedding *C. parvum* subtype IIaA16G1R1 (OR 20.6), as well as for shedding IIaA18G1R1 (OR 4.0), indicating that this specific age group is a risk group for zoonotic subtypes. This information can be used to design measures that may improve animal health and reduce the occupational risks to humans: considering feces of this age group of calves as likely infective and handling them accordingly could be advisable.

Cryptosporidium parvum can cause high morbidity in calves, and the typical profuse diarrhoea can result in high mortality (Abeywardena *et al.*, 2015). Outbreaks with a high mortality in calves due to *C. parvum* have also been described in Estonia (Lassen and Talvik, 2009; Niine *et al.*, 2017). In the current study, calves from farms reporting mortality of calves with severe

diarrhoea that had received veterinary treatment had higher odds to shed *Cryptosporidium* spp. as well as *C. parvum* in feces.

The design of this study succeeded in obtaining a well-representative sample from cattle farms all over the country (Fig. 1). We chose not to concentrate the fecal samples before extracting the DNA. Consequently, calves shedding only a few oocysts may have been missed. The results are thus mainly representing calves shedding moderate-to-high numbers of oocysts, and the prevalence estimates should be considered conservative. It should be noted that PCR methods targeting the 18S rRNA and direct sequencing are likely to detect only the most abundant species and genotype in the specimen and underestimate the occurrence of mixed infections (Hadfield *et al.*, 2011; Mercado *et al.*, 2015).

The *gp60* sequence analysis we used is a common approach employed to characterize *C. parvum* (Xiao, 2010). The findings of this study indicate that subtypes of *C. parvum* that have also been found in humans were the rule, not the exception, in calves raised in Estonia. It would be important to characterize *Cryptosporidium* spp. from humans in the country as well, to evaluate the proportion attributable to zoonotic transmission.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018001348.

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Conflict of interest. None.

Ethical standards. The study was approved by the Ethical Committee of Ministry of Agriculture (currently, Ministry of Rural Affairs, permit nr. 7.2-11/46). The participating farms were recruited during farm visits for unrelated reasons, or contacted by phone by a veterinarian. Participation was voluntary and oral informed consent was given.

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Cryptosporidium outbreak in calves on a large dairy farm: Effect of treatment and the association with the inflammatory response and short-term weight gain

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ABSTRACT

Cryptosporidium spp. infections in neonatal dairy calves can cause diarrhoea and, in rare cases, death. The infection is usually self-limiting, but halofuginone lactate (HL) can be used prophylactically. Calves (n = 144) in the study were born during a 2-month period on one farm. A total of 901 serum and 767 faecal samples were collected. Based on HL treatment, the calves were divided into 3 groups: I) not treated, II) treated incorrectly (treatment started > 48 h after birth, or lasted < 7 days), and III) treated correctly (started < 48 h after birth, and lasted ≥ 7 days). Over the 3-month observation period, 14.6% (n = 21) of the calves died, of which most (67%) had not been treated with HL. Correctly performed treatment of cryptosporidiosis significantly delayed the onset of oocysts shedding (P < 0.001) and reduced haptoglobin (HP) and serum amyloid A (SAA) concentrations in the second week of life. HP concentration and HL treatment were negatively associated with weight gain at 3 months of age. *Cryptosporidium* positive faecal samples were significantly (P < 0.001) more likely to be diarrhoeic but *Giardia* or *Eimeria* positive samples were not. Correct prophylactic treatment with HL delayed the shedding of *Cryptosporidium* oocysts and improved survival, but was negatively associated with weight gain. Incorrect treatment had a low impact on mortality and resembled no treatment regarding the proportion of calves shedding oocysts. Acute phase response (APR) in the second week of life seemed to be positively associated with shedding high amounts of *Cryptosporidium* oocysts.

1. Introduction

Cryptosporidium can be found in cattle herds worldwide (O'Handley and Olson, 2006) and has also been found in Estonian dairy farms (Lassen et al., 2009). *Cryptosporidium* infection in dairy calves can lead to villous atrophy in the small intestine mucosa and increase intestinal permeability (Wyatt et al., 2010). Consequently, these pathologies can lead to diarrhoea and increased risk of mortality (Delafosse et al., 2015). Neonatal calves have a higher risk of being negatively affected and shed *Cryptosporidium* oocysts more frequently than adult livestock (Maddox-Hyttel et al., 2006; Featherstone et al., 2010). The incubation period of cryptosporidiosis varies on average from 5 to 7 days, but symptoms can start as early as 2 days post-infection (Abeywardena et al., 2015). Infected calves typically excrete oocysts with faeces for about 2 weeks (Fayer et al., 1998; O'Handley et al., 1999). Under experimental conditions, the *Cryptosporidium* oocysts count in faeces rises a day before the onset of diarrhoea, peaks, and drops 2 days before the diarrhoea becomes less severe (Operario et al., 2015). In previous longitudinal studies, the highest number of *Cryptosporidium* oocysts were found during the second or third weeks of the calves' lives (Santín et al., 2008; Coklin et al., 2010).

Giardia's role as a pathogen in production animals is debated (Geurden et al., 2010). *Giardia* infections can be chronic and last for months (Grit et al., 2014). *Giardia* and *Eimeria* have multifactorial pathogenesis that leads to microvilli alteration, diarrhoea, and weight loss in production animals (Olson et al., 1995; Geurden et al., 2010; Lassen et al., 2015). In a recent study, *Giardia* infection was also associated with haemorrhagic diarrhoea in calves (Lee et al., 2016). In cases of experimental inoculation with the parasite, dairy calves usually survive the infection with only minor repercussions (Grit et al., 2014). Calves who are infected after birth start shedding oocysts around the third week of life (O'Handley et al., 1999). The *Giardia* infection rate is related to the age and has been found to peak 6 weeks after birth (Winkworth et al., 2008; Coklin et al., 2010).

If *Cryptosporidium* and *Giardia* infections are concurrent, it could cause morphological damage to the jejunum to a lesser extent; this

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could be because of the antagonistic nature of the coinfection (Ruest et al., 1997). Very low doses of both parasites (around 10 oocysts/cysts) are required to mount an successful infection (Rendtorff, 1954; Okhuysen et al., 1999).

For the prophylactic treatment of *Cryptosporidium*, halofuginone lactate (HL) can be used; it has cryptosporidiostatic effect that in most cases has been proven to be effective in reducing the excretion of oocysts (Joachim et al., 2003; Silverlås et al., 2009).

The acute phase response (APR) is a series of complex physiological events occurring after tissue injuries or infections (Cray et al., 2009). In response to APR, the concentrations of specific acute phase proteins (APP), serum amyloid A (SAA) and haptoglobin (HP), can increase in ruminant serum > 1000-fold (Ceciliani et al., 2012; Eklund et al., 2012). In cases of viral (foot-and-mouth disease and bovine respiratory syncytial virus) or parasitic (*Eimeria* and *Cryptosporidium*) infections, APP concentrations can increase in domestic ruminants' blood serum (Orro et al., 2011; Pourjafar et al., 2011; Stenfeldt et al., 2011; Lassen et al., 2015). In neonatal calves, APPs go through significant changes during first 2 to 3 weeks of life (Orro et al., 2008; Tóthová et al., 2015), suggesting that APPs have a role in the adaptation of neonate calves to the new environment. In reindeer calves, lambs, and beef calves, high concentrations of SAA measured in the second week of life have been associated with lower weight gain recorded many months later (Orro et al., 2006; Peetsalu et al., 2013; Seppä-Lassila et al., 2015, 2017).

Cryptosporidium infection has been shown to increase the APP concentration in dairy calves (Pourjafar et al., 2011). However, the effect of *Cryptosporidium* infection combined with prophylactic treatment on the immune system and growth remains unknown. In this study, we examined the effects of untreated, incorrect treatment, and correct treatment with HL in an outbreak of cryptosporidiosis in neonatal calves.

2. Materials and methods

2.1. Ethics statement

This study was conducted based on ethical permission issued by the Ethical Committee of Animal Experiments in the Estonian Ministry of Agriculture (no. 7.2-11/2).

2.2. The farm

This study took place on a large dairy farm in Järvamaa County, Central-Estonia. The average milk production per cow in 2015 was 10,000 kg (Estonian Livestock Performance Recording Ltd., 2015). During the study, there were about 1800 dairy cows in the farm.

2.3. Animals

Inclusion criteria: all of the female calves born from January 21 to March 16, 2015, were included in the study (n = 145). Exclusion criteria: twins (1 pair of twins born) and male calves. One animal was dropped from the study because she died before any samples were collected.

The calves were separated from their mothers immediately after birth. In the first 4 weeks, the calves were kept in individual pens with wooden floors and straw bedding. After that, they were moved to group pens with concrete flooring and straw and sawdust bedding. Group pens were composed of 8–10 calves. Both individual and group pens were housed in the same building until the animals were 2 months old. Immediately after birth, the calves were weighed with a digital scale (MS4 PW, Excell Precision Co., Ltd, Vilnius, Lithuania). Additional weight measurements were taken around 1 and 3 months of age with a digital scale (KERN EOS 150K100NXL, Kern & Sohn GmbH, Balingen, Germany) and measuring tape (ANImeter, Albert Kerbl GmbH, Buchbach, Germany), respectively.

2.4. Feeding

The calves were fed 3 l of unpasteurised colostrum in the first 2 h of life. The colostrum given to the calves was collected from the dam and the quality examined visually and with a hydrometer (Kruuse colostrum densimeter, Jørgen Kruuse A/S, Langeskov, Denmark). If the colostrum was of unsatisfactory quality (n = 2), deep frozen colostrum from another dam was provided. The calves were fed 2–3 kg of warmed unpasteurised raw milk twice per day with free access to hay and starter feed (Prestarter, Agrovarustus OÜ, Tartu, Estonia) up to 15–17 days of age. Then their feed was switched to milk powder (Josera GoldenSpezial, Josera GmbH & Co. KG, Kleinheubach, Germany) solution (1 l of warm water + 140 g of milk powder) of 2 × 3 l/day for 1 week with free access to starter feed (Prestarter, Agrovarustus OÜ) and hay. At 1 month of age, the milk powder product was changed (Josera IgluStart, Josera GmbH & Co. KG) and decreased each week with 0.5 l per feeding. Around weaning time (70–80 days of age), the calves received 2 × 2 l/day. After weaning, the calves had free access to starter feed (Starter, Agrovarustus OÜ), hay, and silage. No significant changes were made to the feeding regiments or feed itself during the study period.

2.5. Treatments

All of the calves were vaccinated on the second day after birth against parainfluenza virus type 3 (PI3V) and bovine respiratory syncytial virus (BRSV) (Rispoval, Zoetis Belgium SA, Louvain-la-Neuve, Belgium). At 3 months of age, all of the calves were vaccinated against bovine herpesvirus-1 (BoHV-1) (Hipravobis, Laboratorios HIPRA, S.A., Girona, Spain). Prophylactic treatment against *Eimeria* infection was done once by administrating toltrazuril (Cevazuril, Ceva Santé Animale, Libourne, France) to every calf between 29 and 65 days of age. Prophylactic treatment of the *Cryptosporidium* infection was done using HL (Halocur, Intervet International B.V., Boxmeer, Netherlands).

The study was designed as observational cohort study. Based on the HL treatment regime, the calves were divided retrospectively into 3 groups: I) not treated (n = 34), II) treated incorrectly (treatment started > 48 h after birth, or lasted < 7 days) (n = 45), and III) treated according to manufacturer's instructions (started < 48 h after birth, and lasted ≥ 7 days) (n = 65).

All animals in the study requiring medical treatment received it from the farm's veterinarian. Diarrhoea was treated by administering electrolyte solutions, and if needed, antibiotics were also given. Antibiotics were also used to treat respiratory infections.

No necropsies were performed on the study animals that died. The farm's veterinarian noted the most likely cause of death based on symptoms, such as diarrhoea, respiratory distress, or lameness.

2.6. Sample collection

Once per week, up to 6 weeks of age, serum and faecal samples were collected from each calf. Follow-up sample collection was done at around 3 months of age.

Faecal samples were collected with a clean disposable latex glove directly from the rectum and placed into clean sealable plastic cups and marked with the last 5 numbers of the animal's ear tag. If the rectum was found empty and the calf could not be stimulated to defecate using finger, the sample collection was abandoned (n = 158). In addition, 83 faecal samples were not collected due to the unexpected death of 21 calves. Faecal samples were stored in an insulated container with cooling elements for 2 h and then kept at 4 °C for a maximum of 48 h until analysis. In total, 767 faecal samples were collected from 144 calves.

Serum samples (n = 901) were collected from the jugular vein in sterile evacuated test tubes using an 18-G sterile needle. Blood samples were transported to the laboratory and centrifuged (3000 RCF for

10 min). All of the serum samples were then stored at − 20 °C until further analysis.

In order to avoid dehorning affecting the APP serum concentrations, all of the blood samples were collected immediately before the procedure. For technical reasons, 8 calves were not sampled before dehorning and were marked as compromised.

2.7. Parasites

Faecal samples were prepared for *Cryptosporidium* and *Giardia* detection in a similar method described for *Eimeria* detection by Lassen et al., 2009, but with slight modifications. In detail, sample preparation followed the same steps: weighing, mixing, diluting, and centrifuging, but after the supernatant was removed and before saturated sugar and solution ($\rho = 1.26$ g/cm^3) was added, a 20 µl subsample of the 1 ml suspended pellet was fixed on glass slides well (14 mm diameter latex wells). Staining was done using fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Crypto/Giardia Cel, Cellabs Pty Ltd., Sydney, Australia). The slides were examined using an epifluorescence Nikon Eclipse 80i microscope using 200–400 × magnification. *Cryptosporidium* and *Giardia* oocysts were differentiated visually based on morphology, and considered positive if at least 1 oocyst or cyst was found. All of the oocysts and cysts on the slide were counted and the approximate number of oocysts per gram of faeces (OPG) was calculated, corrected to the total area of the well and to the dilution of the sample (De Waele et al., 2010). In case there were too many oocysts to count, 3 random visual fields on the slide were picked and all of the oocysts were counted in the field of view (Lassen and Lepik, 2014). The counts of each visual field were averaged and multiplied with the fraction of the visual field surface area divided by the total slide surface area to calculate the total number of oocysts on a slide.

DNA was extracted from 12 FITC *Cryptosporidium*-positive faecal samples collected at 3th March from calves borned between 21st January-20th February 2015 (mean age 21 days) using the PSP® Spin Stool DNA Kit (STRATEC Biomedical AG, Birkenfeld, Germany). The DNA was submitted to PCR amplification targeting the 18S rRNA gene of *Cryptosporidum* spp. as described by Zintl et al. (2007), and the 60 kDa glycoprotein (gp60) gene as described by Peng et al. (2001). The PCR products were run on a 2% ethidium bromide stained agarose gel and visualized under an UV transilluminator. Products of approximately 825 bp from the 18S rRNA and approximately 490 bp of the gp60 amplifications were cleaned and submitted to sequencing in two directions using Applied Biosystems® 3130xl Genetic Analyzer. Forward and reverse sequences were aligned using the BioEdit v7.2.5 software (Hall, 1999) to generate consensus sequences and correct potential mismatches. The GenBank BLASTn (Altschul et al., 1990) tool was used to find similarities between the sequences of our PCR products and with deposited nucleotide sequences in the library. Sequences of the 18S rRNA products were used to determine the species of *Cryptosporidium*, and gp60 was used to determine the subtype.

The faecal samples were classified as diarrhoeic or non-diarrhoeic based on visual examination. The remaining 1 ml of concentrated faecal sample from above was examined with a light microscope using the flotation method (Roepstorff and Nansen, 1998) for possible parasites (*Eimeria* spp. and intestinal nematodes). *Eimeria* spp. were differentiated visually based on morphology (Levine, 1985).

2.8. Acute phase proteins and gamma-glutamyltransferase

The concentration of SAA was measured by commercial ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland). The HP concentration was assessed via the method defined by Makimura and Suzuki, 1982), with an alteration using tetramethylbenzidine (0.06 mg/ml) as a substrate and using microtitration plates (Alsemgeest et al., 1994). Bovine acute phase serum (pooled and lyophilised) were used to generate standard curves. Standard provided by the European Commission Concerted Action Project (number QLK5-CT-1999-0153) was used to standardise the assay of bovine plasma sample with a known HP concentration. The range of the standard curve was 75–1160 mg/l.

The intra-assay and inter-assay coefficients of variations for SAA were <11% and <13% and for HP were <13% and <10%, respectively.

Analysis of GGT activity was measured using the kinetic method with L-γ-glutamyl-3-carboxy-4-nitroanilide (Persijn and van der Slik, 1976) in a clinical chemistry analyzer (Accent-200 GGT, PZ Cormay S.A., Łomianki, Poland).

2.9. Statistical analysis

Linear regression models were used to check if HL treatments were associated with changes in the HP or SAA concentrations in the first 6 weeks of life. HP or SAA were the dependent variables and both were logarithmically transformed in order to meet the presumption of normal distribution. The explanatory variables were the age (days) at sample collection and HL treatment as a categorical variable.

A random-effects logistic regression model was constructed to investigate if *Cryptosporidium*- or *Giardia*-positive faecal samples in the first 6 weeks of life were more likely to be diarrhoeic. *Eimeria* was excluded from these models, as all the faecal samples from the first 6 weeks of life were negative. The sample being diarrhoeic was added as a binary dependent variable. Explanatory variables were *Cryptosporidium*-positive faecal samples, *Giardia*-positive samples, and age (days). Parasite-positive samples were categorised as follows: 0 = no oocysts or cysts found; 1 = the oocyst or cyst count in the sample below the median count; and 2 = the oocyst or cyst count in the sample above the median count. The calves were added to the model as random intercepts.

A logistic regression model was used to examine if *Eimeria*-positive calves were diarrhoeic at 3 months of age. The dependent variable was diarrhoea (binary) and the explanatory variables were the total number of *Eimeria* oocysts in 1 g of sample (OPG) and the age (days) at sample collection.

For assessing the odds of death within the first 6 weeks of age, a retrospective case-control logistic regression model was constructed. The case group ($n = 14$) consisted of animals who died before 43 days of age. The control group ($n = 49$) consisted of animals born ± 3 days to a matching case group of animals that did not die before 43 days of age and whose dams were also either primiparous or multiparous. The dependent variable was death; the explanatory continuous variables were *Cryptosporidium* oocyst count in faecal samples, birth weight, GGT, and APPs (SAA and HP) at the first week of life; and the independent variable was the dam being primiparous or multiparous. Backward step-wise elimination procedure was used for final model.

Linear regression models were used to evaluate if HP and SAA concentrations differed on weekly bases over the first 6 weeks of life based on the *Cryptosporidium* oocyst count found in the faecal samples. The dependent variables were HP or SAA, and both were logarithmically transformed in order to meet the presumption of normal distribution. The explanatory variables were age at sample collection (days) and *Cryptosporidium* infection intensity as a categorical variable (0 = no oocysts found in faecal sample; 1 = low; sample containing less than the median number of oocysts (OPG) when compared to other same weeks' positive results; and 2 = high; sample containing more than the median number of oocysts when compared to the other same weeks' positive results). Bonferroni's multiple comparison correction procedure was used to control Type I errors.

The average area under the curve (AUC) was calculated using the trapezoidal method for different APPs and the parasite oocyst count over 6-week periods as:

$$AUC = \sum [(t_i - t_{i-1})f_{i-1}] + [0.5(t_i - t_{i-1})(f_i - f_{i-1})]$$

where t_i = the time of observation, t_{i-1} = the previous time of observation, f_i = APP concentration at the time, and f_{i-1} = APP concentration at the previous time. AUCs were used as summary measures for concentrations of APPs and the oocyst counts over time. The AUC value was divided with the calves' age in order to be comparable between different animals. $AUC_{average}$ = AUC/age at sample collection. The AUC calculation was performed if the calf had 4 observations or more and was not compromised (had serum sample collected prior to dehorning).

Multiple linear regression models were used to determine the association between APPs-AUC results and *Cryptosporidium* and *Giardia* infection. The SAA- and HP-AUC results were used as dependent variables. The independent variables of AUCs for both parasites were: the oocyst or cyst count in the faecal samples, the age and GGT concentration at the first sample collection, and HL treatment as categorical variable. The dependent variables SAA- and HP-AUC results were logarithmically transformed to meet the presumption of normal distribution.

Multiple linear regression models were used to describe the APPs and the *Cryptosporidium* and *Giardia* infections possible association with average daily weight gain (ADWG). The dependent variable was ADWG at the age of 1 month or at the age of 3 months. The independent variables were SAA, HP average-AUCs, *Cryptosporidium* and *Giardia* oocysts-AUCs, age (days) at the first collection of the first sample, age (days) at weight measurement, proportion of diarrhoeic faecal samples, HL treatment categories, and primiparous or multiparous dam's offspring as a categorical variable.

In the linear and logistic regression models, independent variables were selected according to their P values using backward stepwise elimination. Independent variables were eliminated from a model if $P > 0.05$. Variables that changed the coefficient of the remaining variables with > 10% were kept as confounders.

Statistical data analysis was done using STATA 14.1 (StataCorp LP, College Station, TX, USA). Basic data management was done using Excel 2013 (Microsoft, Redmond, WA, USA) and Python 3.5.1 (Anaconda 4.0.0 by Continuum Analytics, Austin, TX, USA). The level of a significant result was $P \leq 0.05$.

3. Results

3.1. Parasite infection, diarrhoea, and halofuginone lactate treatment

The treatment initiated to control the outbreak of diarrhoea in the calves with HL was started on February 17 and ended on March 22, 2015. In total, 110 calves were treated an average of 6 times (range 1 to 9). On average, the earliest treatment started on day 3 of life (range 0 to 14) (Fig. 1).

In first six weeks of life, a total of 655 faecal samples (by HL treatment groups: I) no treatment: $n = 131$, II) incorrect treatment: $n = 228$, III) correct treatment: $n = 296$) and 774 serum samples (by HL treatment groups: I) no treatment: $n = 156$, II) incorrect treatment: $n = 272$, III) correct treatment: $n = 346$) were collected. Additionally, 112 faecal and 130 serum samples were collected at 3 months of age.

Cryptosporidium oocysts were found in 33.3% (218/655) of faecal samples and 84.7% (122/144) of calves. *Giardia* cysts were found in 30.8% (202/655) of faecal samples and 76.4% (110/144) calves. Protozoan infections detection and average age of first detection according to different HL treatment groups are presented in Table 2. Mixed protozoan infections were found in 5.8% (38/655) of faecal samples in 22.2% (32/144) of calves.

The median OPG in a positive *Cryptosporidium* and *Giardia* faecal sample in the first six weeks of life, by HL treatment groups was: I) no treatment: 242,844 and 14,035 with range of 70–2,755,554 and 69–469,367, II) incorrect treatment: 333,027 and 79,112 with range of 70–3,646,621 and 70–1,401,208, III) correct treatment: 363,436 and 29,386 with range of 69–10,145,426 and 71–2,652,344.

Out of the 12 FITC *Cryptosporidium*-positive faecal samples the 18S rRNA gene was successfully amplified in six samples and sequence analysis identified these as *C. parvum*. Seven (7/12) samples successfully amplified the gp60 gene; five of which were positive and two that were negative in the amplification of the 18S rRNA gene. Sequence analysis of the gp60-positive samples identified them as *C. parvum* subtype IIaA18G1R1.

Diarrhoea was diagnosed in 53% (344/655) of samples and in 92% (132/144) of calves during the first 6 weeks of age and in 29% (32/112) of samples and in 29% (32/112) of calves at 3 months of age. For the full observation period, 92.4% (133/144) of calves had at least 1 sample that was considered diarrhoeic. The model indicated that in the first 6 weeks of the calves' lives, *Cryptosporidium*-positive samples were associated with diarrhoea but not *Giardia* (Table 1). The age of the calf was negatively associated with diarrhoea (OR = 0.98; $P = 0.007$). Calves shedding *Eimeria* oocysts did not have increased odds of being diarrhoeic ($P = 0.2$).

In general, the highest proportion of *Cryptosporidium*-positive samples was found in calves that were 16–18 days old. When looking at the proportion of animals shedding oocysts by treatment category, calves that got no treatment or were treated incorrectly peaked around 10–12 days of age, while correctly treated animals peaked around 19–21 days of age (Fig. 2).

Eimeria oocysts were detected in 9.5% (73/767) of all the faecal samples collected. *Eimeria* was only detected in faecal samples collected at approximately three months of age (median age: 99 days). Four species of *Eimeria* were detected in a total of 73 faecal samples: *E. bovis* (71.2%, 52/73), *E. zuernii* (45.2%, 33/73), *E. ellipsoidalis* (37.0%, 27/73) and *E. auburnensis* (16.4%, 12/73). Additional results of *Eimeria* infection, grouped by different HL treatments can be found in Table 1S. No helminth eggs or nematode larvae were found.

3.2. Survival

In first 3 months of life, 21 calves (14.6%) died or were euthanised (Fig. 1 and Fig. 1S). The average age of death was 29 ± 24 days (median 16, range 8 to 83). The reasons listed by the farm veterinarian for mortalities were diarrhoea ($n = 12$), respiratory infection ($n = 6$), euthanised because of massive inflammation of the carpal joint or septic umbiliculitis ($n = 2$), and unknown cause ($n = 1$).

In total, 66.7% (14/21) of the calves in the group that got no HL treatment died. In the groups of calves that were treated incorrectly or correctly, 2 and 5 died, respectively (Fig. 1S). Based on the veterinarian's diagnosis, 71% of the deaths in the no treatment group were caused by diarrhoea, and in the other groups, 1 animal succumbed to diarrhoea.

In the retrospective case-control logistic regression model, the odds of a calf dying within the first 6 weeks of life increased with higher SAA concentrations (OR = 1.01; $P = 0.041$). Factors that decreased the odds of a calf dying were: higher GGT activity (OR = 0.99; $P = 0.004$), larger birth weight (OR = 0.76; $P = 0.019$), and having a primiparous mother (OR = 0.11; $P = 0.022$) (Table 3).

3.3. Acute phase proteins

In the linear regression models, no associations were found between HP, SAA, or HL treatment in the parasitic infection category (average AUC) ($P > 0.05$) during the first 6 weeks (average AUC).

The average SAA concentration increased during the first 2 weeks of life and then decreased. The average HP concentration peaked in the second week of life. In the first 2 weeks of life, calves with a high number of *Cryptosporidium* oocysts in their faecal samples also had elevated serum concentrations of HP compared to calves in the groups with fewer oocysts in their faeces (Fig. 3). Similarly, in the second week of life, calves with a high number of *Cryptosporidium* oocysts in their faeces also had higher SAA serum concentrations in their serum

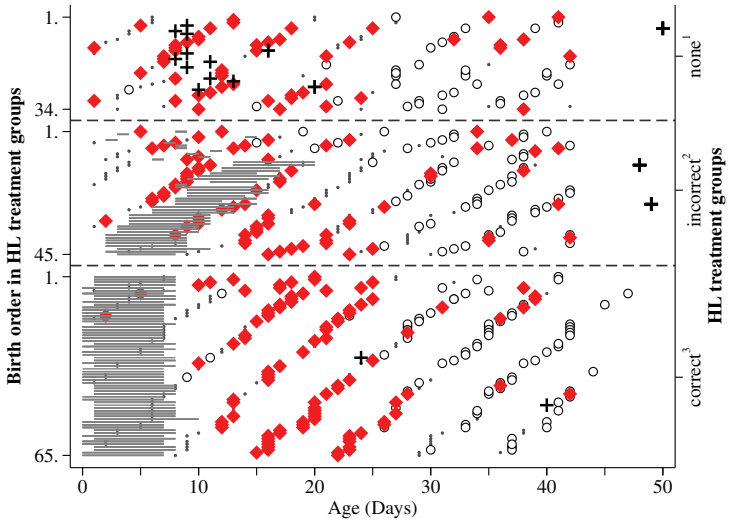

Fig. 1. *Cryptosporidium* and *Giardia* infection patterns differentiated by halofuginone lactate (HL) treatments. Calves were assigned into groups retrospectively based on HL treatment regiments (separated by dashed lines on figure): [1]) not treated (n = 34), [2]) treated incorrectly (treatment started > 48 h after birth, or lasted < 7 days) (n = 45), and [3]) treated according to manufacturer's instructions (started < 48 h after birth, and lasted ≥ 7 days) (n = 65). (◆) *Cryptosporidium* positive; (○) *Giardia* positive; (·) *Giardia* and *Cryptosporidium* negative; (+) death (n = 17); horizontal lines represent HL treatment and the length represents the treatment in days. The y-axis represents the birth order of calves in different HL treatment groups, starting with the oldest and ending with the youngest; x-axis represent the age of the calf.

Table 1
Logistic regression model examining the association between diarrhoea in 144 calves during the first 6 weeks of life and the concentration of *Cryptosporidium*, the concentration of *Giardia* oocysts in faecal samples, and age at sample collection. Calves were added as random intercepts. Final model is presented.

Variable (n = no. of samples)	OR	Confidence interval 95%	P-value
Cryptosporidium negative (n = 437)	1.0	–	–
Cryptosporidium low (n = 109)	1.93	1.23; 3.03	0.004
Cryptosporidium high (n = 109)	2.22	1.39; 3.56	0.001
Giardia negative (n = 453)	1.0	–	–
Giardia low (n = 101)	1.45	0.83; 2.55	0.192
Giardia high (n = 101)	1.58	0.91; 2.74	0.102
Age at sample collection (days)	0.98	0.96; 0.99	0.007

n (observations) = 655, average n (observations) per calf = 4.5.

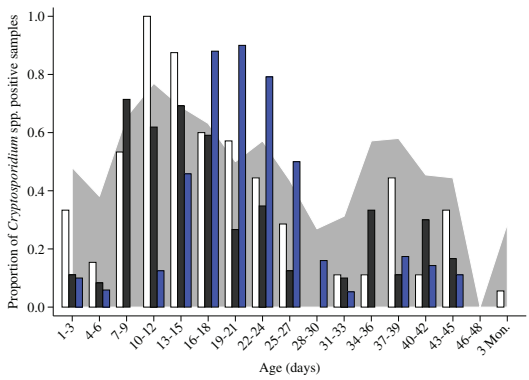

Fig. 2. Proportion of positive *Cryptosporidium* spp. faecal samples with different halofuginone lactate (HL) treatment category: (white) not treated (n = 34); (black) treatment start was delayed or duration was < 7 days (n = 45); (blue) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment) (n = 65). Total proportion of diarrhoeic faecal samples presented as grey area in the background.

compared to the other groups (Fig. 3).

The HP concentration during the second week of life was higher in the HL untreated group compared to the incorrectly (P = 0.001) or correctly treated groups (P = 0.001) (Fig. 4). HL treatment in the second week of life was not associated with SAA (P > 0.05).

3.4. Weight gain

The average birth weight was 41.19 ± 5.1 kg (range 27 to 52 kg). Linear regression predicted a negative association (P = 0.004) between HP-AUC and ADWG at 3 months of age. Correct treatment had a negative effect (P = 0.003) on ADWG after a 3-month period when compared to the group that did not receive treatment (Table 4). Information on weight and ADWG by different HL treatment groups is presented in Table 5.

4. Discussion

Several pathogens potentially fit the differential diagnosis of diarrhoea in calves, including coronavirus, rotavirus, *E. coli*, and *Salmonella*. Before the start of the study, veterinarians on the farm had performed rapid pen-side tests with positive results for coronavirus, rotavirus, and *Cryptosporidium*. In addition, the herd had tested positive for bovine viral diarrhoea virus at the time of the study (personal communication from farm veterinarian). The significant increase in SAA and HP concentrations and presence of diarrhoea in calves has been observed in calves during rotavirus, coronavirus and *E. coli* infections (Balikci and Al, 2014). In case of naturally occurring rotavirus or coronavirus co-infection with *Cryptosporidium* versus mono-infection, significantly higher SAA and HP concentration increase has been previously reported (Pourjafar et al., 2011). Nevertheless, best to our knowledge there are no experimental studies where potential co-pathogenic synergism between rotavirus and *Cryptosporidium* infection has been demonstrated in calves. Our results indicated that *Cryptosporidium* infections effect to the production of APP was dose-dependent and associated clinical signs could be attributed to the *Cryptosporidium* infection (Fig. 4). This study reflects the conditions of a farm, and though the outbreak fits the picture of cryptosporidiosis, it is not possible to exclude the co-existence of other pathogens.

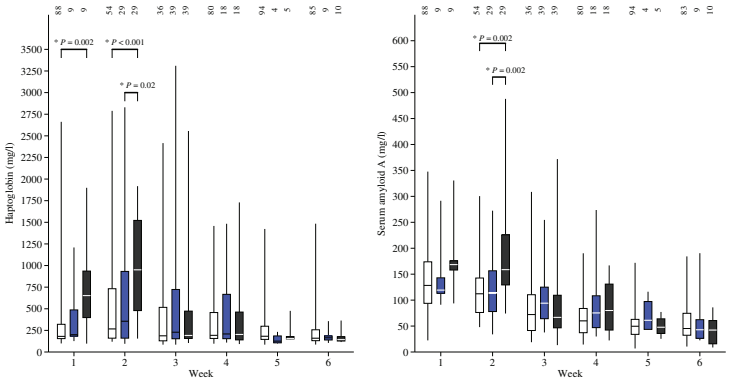

Fig. 3. Haptoglobin (HP) and serum amyloid A (SAA) concentrations in serum and different categories of *Cryptosporidium* oocyst counts in faecal samples. White = negative (no oocysts found), blue = low (below median oocysts per gram (OPG)), black = high (more than median OPG found in a faecal sample). The number of calves in each group is marked above each bar. Results from the 3 months of age were not presented because only one calf had a *Cryptosporidium* positive faecal sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

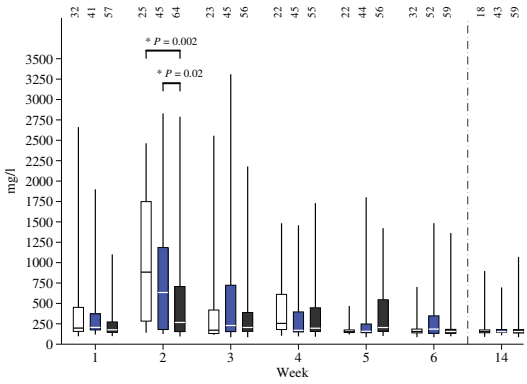

Fig. 4. Haptoglobin (HP) concentrations in serum and different halofuginone lactate (HL) treatment groups. Statistically significant differences demonstrated with a horizontal bar on top of second week results. The number of animals in a group shown at the top of a bar. HL treatment groups: white = no treatment; blue = incorrect treatment (treatment start was delayed or was < 7 days long); black = correct treatment (started in the first 48 h of life and had ≥ 7 days of treatment). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. Parasite infection, diarrhoea, and halofuginone lactate treatment

This study investigated the dynamics and treatment of what the farm veterinarians considered an outbreak of cryptosporidiosis. Only *C. parvum* isotype IIaA18G1R1 was found and which has been previously detected in calves faeces (Misic and Abe, 2007; Plutzer and Karanis, 2007; Brook et al., 2009). We suspect that this subtype was the main cause of cryptosporidiosis in current study, but due to relatively small PCR sample size, which was collected in single time point during the study, it was difficult to say whether there were other subtypes present. Almost all of the calves (84%) were shedding *Cryptosporidium* spp. oocysts or *Giardia* spp. cysts in their faeces, but both parasites were found in only 6% of the faecal samples, and thus did not indicate an antagonistic effect. This may be explained by the differences in the parasites infection patterns (Xiao and Herd, 1994; Santín et al., 2008, 2009). However, studies of morphological changes of the jejunum have suggested the possibility of an antagonistic effect of the two parasites (Ruest et al., 1997). Most of *Cryptosporidium* infections happened before 1 month of age, similar to what has been reported previously in longitudinal studies (Harp and Goff, 1998; O'Handley et al., 1999; Geurden et al., 2007). Calves that were shedding high amounts of *Cryptosporidium* oocysts had higher odds of being diarrhoeic than calves shedding *Giardia* or *Eimeria*, supporting *Cryptosporidium* as the causative agent of the symptoms. Some authors have suggested that *Giardia* infection itself does not cause diarrhoea (Maddox-Hyttel et al., 2006; O'Handley and Olson, 2006). A previous study in dairy cattle on several Estonian farms found a negative correlation between diarrhoea and the presence of *Eimeria* spp. in faeces, but a positive correlation between diarrhoea and higher amounts of *Cryptosporidium* spp. oocysts (Lassen et al., 2009). In Finnish calves, the opposite was observed; *Eimeria* spp. was associated with diarrhoea, while *Cryptosporidium* and *Giardia* were not (Seppä-Lassila et al., 2015). This illustrates that intestinal parasites, including *Cryptosporidium,* are important agents of disease in calves in the area, but the general clinical picture varies.

The initiation of the *Cryptosporidium* infection's prophylactic treatment with HL exemplified the connection between APR and the parasitic infection under natural conditions. The HL treatment did not seem

Table 2
Calves tested for presence of *Cryptosporidium* oocysts and *Giardia* cysts in faeces and average age of first positive sample grouped by halofuginone lactate (HL) treatment regiments[a] in the first six weeks of life.

(*n* = no. of calves)	*Cryptosporidium*		*Giardia*	
	No. of animals tested positive	Average (± SD) age of first positive sample (days)	No. of animals tested positive	Average (± SD) age of first positive sample (days)
Not treated (*n* = 34)	26 (77%)	11 ± 7	23 (68%)	28 ± 10
Incorrectly treated (*n* = 45)	41 (91%)	12 ± 7	41 (91%)	31 ± 7
Correctly treated (*n* = 65)	55 (84%)	16 ± 5	46 (71%)	31 ± 8
Total (*n* = 144)	122 (85%)	14 ± 6	110 (76%)	30 ± 8

[a] I) not treated; II) treatment start was delayed or duration was < 7 days; III) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment).

Table 3
Retrospective case control logistic regression modelling of factors associated with mortality of calves up to 43 days of age. Final model is presented.

Variable (n = no. of calves)	OR	Confidence interval 95%	P-value
SAA (mg/l)[a]	1.013	1.001; 1.026	0.041
GGT (IU/l)[a]	0.993	0.988; 0.998	0.004
Birth weight (kg)	0.762	0.607; 0.957	0.019
Multiparous (n = 23)	1.0	–	–
Primiparous (n = 40)	0.111	0.017; 0.731	0.022

n (observations) = 63 (the case group (n = 14) and the control group (n = 49)), SAA = Serum amyloid A, GGT = gamma glutamyltransferase.
[a] Sample collected first week of life.

Table 4
Association of average daily weight gain (g/days) of 109 calves at 3 months of age, haptoglobin (HP) average area under the curve (AUC), halofuginone lactate (HL) treatment[a] and age at weight measurement. Final model is presented.

Variable (n = no. of calves)	Estimate	Confidence interval 95%	P-value
HP average AUC (mg/g/day)	− 0.16	− 0.27; − 0.05	0.004
Not HL treated (n = 18)	0	–	–
Incorrect HL treatment (n = 42)	− 55.53	− 126.63; 15.58	0.125
Correct HL treatment (n = 49)	− 107.22	− 176.37; − 38.06	0.003
Age at weight measurement (days)	2.15	− 0.75; 5.05	0.145
Intercept	708.69	390.65; 1026.72	0.000
n (observations) = 109			

[a] I) not treated; II) treatment start was delayed or duration was < 7 days; III) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment).

to decrease the number of *Cryptosporidium* oocysts shed in faeces, similar to what has been reported in one study (Weber et al., 2016), but contrary to another (Keidel and Daugschies, 2013). Nevertheless, the correctly performed prophylactic treatment had a delaying effect on the onset of shedding (Fig. 2 and Table 2), seemed to improve survival (Fig. 1S), but resulted in a poorer ADWG (Table 5). Previous investigations have also reported that HL can cause a delay in oocyst shedding (Jarvie et al., 2005; Trotz-Williams et al., 2011; Keidel and Daugschies, 2013), but not an impact on the survival of calves. Calves have higher risk of succumbing to dehydration and acidosis due to diarrhoea in their first week of life (Foster and Smith, 2009). Prophylactic HL treatment may delay the development of cryptosporidiosis and help calves cope with very strong infection pressure (Abeywardena et al., 2015). It has been suggested that HL may have a positive therapeutic effect in calves aged 8–14 days (Klein, 2008). Other authors (Silverlås et al., 2009; Almawly et al., 2013) have reported that the therapeutic treatment effect of HL on calves' health seems to be limited, similar to the findings in this study.

4.2. Survival

Most of the calves' deaths in the current study were concentrated in a relatively short period, and were found to be related to infections of the digestive system in the group that did not receive HL treatment. Shortly after the mass treatment with HL started, the death rate dropped (Fig. 1). This suggests that the mortalities were related. *Cryptosporidium* infections and the treatment may have reduced the severity of the illness and raised the chance of survival (Fig. 1S). Higher GGT activity had a positive effect on survival, which suggests colostrum quality and adsorption of antibodies had an important role in the animal's ability to survive the infection. It has been shown that high levels of immunoglobulin G and long fatty acids in colostrum have some protective effect against diarrhoea caused by *Cryptosporidium*, but not against the infection itself (Lopez et al., 1988; Schmidt and Kuhlenschmidt, 2008; Weber et al., 2016). This could mean that the animals who were at a weaker starting position due to poorer quality colostrum and lower birth weights more easily succumbed to *Cryptosporidium* infection. Only after starting the mass treatment with HL did the survival chances of these calves improve. Even incorrect treatment with HL seemed to have a positive effect on survival; thus, we could conclude that no treatment would be the worst option during a massive increase in cryptosporidiosis cases, especially when most of the deaths are diarrhoea-related.

The incubation period of *Cryptosporidium* infection is 5–7 days, which is so short that the adaptive immune response is unlikely to stop the development of clinical disease (Petry et al., 2010; Abeywardena et al., 2015). APR as an innate immune response is faster and more likely to play a role in controlling the infection and the development of disease in the early stages. Interestingly, higher SAA concentrations had a negative impact on the survival of calves. This suggests that APR was triggered more profoundly in severely affected animals (Fig. 3). Although we cannot rule out other common digestive system pathogens, the evidence suggests that *Cryptosporidium* played a major role in diarrhoeic calves, and that the correct HL treatment was able to delay the APR induction and decrease its magnitude (Fig. 4).

4.3. APPs

The HL prophylactic treatment delayed *Cryptosporidium* infection and seemed to affect the APR in the second week of life. HP, but not SAA, serum concentrations were significantly lower in the animal groups that were correctly treated compared to the untreated and incorrectly treated calves (Fig. 4). This increase in concentrations of APP coincided with an increased proportion of the calves shedding *Cryptosporidium* spp. oocysts (Fig. 2) and mortality (Fig. 1S) in the second week of life. The HP median concentration in heavily shedding animals was 4.8 times higher (950 mg/l) than the reference value (< 196 mg/l) of calves that age while the SAA median value (158 mg/l) did not exceed the reference value (< 178 mg/l) (Seppä-Lassila et al., 2013). We

Table 5
Results of weight measurement and average daily weight gain (ADWG) at 1 and 3 months (± SD) by different halofuginone lactate (HL) treatment groups.[a]

HL treatment group	n (observations)	Age (days)	Weight (kg)	ADWG (g/day)
1 month of age				
Not treated	22	29.2 ± 4.4	54.1 ± 5.7	433.6 ± 160.2
Incorrectly treated	44	27.8 ± 3.8	53.4 ± 6.3	449.5 ± 168.2
Correctly treated	56	31.2 ± 4.6	53.9 ± 5.7	388.4 ± 122.8
Total	122	29.6 ± 4.5	53.8 ± 5.9	418.6 ± 148.9
3 months of age				
Not treated	19	108.6 ± 12.2	134.5 ± 17.3	861.6 ± 121.0
Incorrectly treated	43	99.8 ± 12.9	120.4 ± 19.3	794.4 ± 129.4
Correctly treated	58	100.4 ± 6.6	117.7 ± 14.9	751.3 ± 123.7
Total	120	101.5 ± 10.6	121.3 ± 17.9	784.2 ± 130.3

[a] I) not treated; II) treatment start was delayed or duration was < 7 days; III) treatment was done correctly (started in the first 48 h of life and lasted ≥ 7 days of treatment).

speculate that this drastic difference was caused by the nature of the *Cryptosporidium* infection and likely because localised damage to the small intestine was more prone to trigger an immunological response that increased HP rather than SAA concentrations. Previously, relatively small studies (1 to 6 animals) reported an increase in HP and SAA in dairy calves as a response to *Cryptosporidium* infections, especially before the onset of diarrhoea (Enemark et al., 2003a,b; Pourjafar et al., 2011). Although this study shed more light on the subject, there is a lack of research on the role of APR in *Cryptosporidium* and *Giardia* infections in cattle.

4.4. Weight gain

Although about 67% of the calves died in the group that did not receive HL treatment, it was surprising to find that the treatment had significant negative effects on the daily weight gain when the calves reached 3 months of age. In general, the calves' weight gain met the expectations of Holstein breed calves at 3 months of age (Retamal and Risco, 2011), averaging around 121.3 kg. We expected that *Cryptosporidium* infection would have a lasting negative effect on the growth of the surviving calves. The effect of the infection should have been most obvious in calves that were infected but not treated. However, the largest effect was observed in animals that had the correct treatment. Other authors have not found a significant positive or negative effect of HL treatment on the growth rate (Jarvie et al., 2005; Trotz-Williams et al., 2011). It is important to remember that HL treatment does not stop calves from being infected and shedding large numbers of *Cryptosporidium* oocysts, damaging the host's cells and consuming resources for replicating (Silverlås et al., 2009). There were no significant changes in the feeding regimens in first 3 months of the calves' lives, ruling out differences in nutrition as an explanation. A possible explanation for the observed effect is the delay and possible expansion of the parasites' life cycle in the host due to the effect of HL. The categorisation of different HL treatment groups was very strongly influenced by birth order. As a result, we were not able to exclude time as a confounding factor in average daily weight regression models.

Elevated concentrations of SAA in the second week of life have been negatively associated with the growth rate in reindeer calves (Orro et al., 2006), lambs (Peetsalu et al., 2013) and beef calves (Seppä-Lassila et al., 2017). In the current study, *Cryptosporidium* shedding was associated with higher serum concentrations of SAA and HP in the second week of the calves' life, indicating that this period of adaptation was critical. However, only the HP overall response had a negative association with short-term weight gain at 3 months of age. The elevated HP concentrations may have triggered a stronger APR as a response to the infection and consequently affected the growth of the calf.

5. Conclusions

In the outbreak, there was a strong association between *Cryptosporidium* infection and diarrhoea, but not with *Giardia* or *Eimeria* infections. Correctly performed prophylactic HL treatment against cryptosporidiosis delayed the onset of oocyst shedding and improved the chances of survival. However, the growth rate was negatively affected by correct treatment and a strong APR. Correct treatment was associated with lower HP concentrations in the second week of life. The study demonstrates a possible connection between *Cryptosporidium* infection and APR in dairy calves.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2017.12.015.

Conflict of interest

None.

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Faecal microbiota in two-week-old female dairy calves during acute cryptosporidiosis outbreak – Association with systemic inflammatory response

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ABSTRACT

In the present study, relationships between the intestinal microbiota and innate immunity response, acute cryptosporidiosis, and weight gain in female dairy calves were investigated. A total of 112 calves born during a natural outbreak of cryptosporidiosis on one dairy farm was included in the study. Microbiota composition was analysed by means of 16S ribosomal RNA gene amplicon sequencing from faecal samples collected during the second week of life, while the status of *Cryptosporidium* spp. infection was determined using immunofluorescence. Serum samples from the second week of life were colourimetrically analysed for the following markers of acute inflammation: acute-phase proteins (serum amyloid A and haptoglobin) and pro-inflammatory cytokines (interleukin-1 beta, interleukin-6, and tumour necrosis factor-alpha). Statistical analyses were performed using random forest analysis, variance-partitioning, and negative binomial regression. The faecal microbiota of the two-week old calves was composed of the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* (in order of decreasing abundance). Microbial diversity, measured in terms of the Shannon index, increased with the age of the calves and decreased if a high count of *Cryptosporidium* spp. oocysts was found in the faeces. *Fusobacterium* was positively associated with *Cryptosporidium* spp. oocyst count and serum amyloid A concentration. *Peptostreptococcus* was positively associated with haptoglobin and serum amyloid A concentrations, and negatively associated with average daily weight gain at 9 months of age. The markers of innate immunity, in combination with age, explained 6% of the microbial variation. These results suggest that some components of the intestinal microbiota may have a long-lasting negative effect on animal growth through the stimulation of the systemic innate immune response.

1. Introduction

Every dairy farmer's dream is to have a farm full of productive and healthy animals (Hansson and Lagerkvist, 2016). The neonatal period is critical because it is during this time that the immune system interacts with the outside world for the first time, and these early responses of the immune system may have long-term effects on future weight gain, production, and health (Seppä-Lassila et al., 2018). The development of the neonate's immune system depends on microbial stimulation, as well as parasitic and viral stimulation (Chase et al., 2008; Ianiro et al., 2022; Kelly and Coutts, 2000). The gastrointestinal and respiratory tracts are predominant sites of microbial contact (Renz et al., 2012). To the authors' knowledge, the development of intestinal microbiota in neonatal calves has not been thoroughly studied. In a recent study carried out in Finland, small amounts of microbial DNA were observed in the rectums of newborn calves, and the rectal microbiota changed rapidly during the first week of life (Alipour et al., 2018). It has been found that in beef cattle, taxonomic groups belonging to the phyla *Firmicutes* and *Bacteroidetes* are dominant in the gastrointestinal tract in general, as well as in the colon (Jami et al., 2013; Myer et al., 2017). The highest species

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richness and diversity have been detected at weaning (Klein-Jöbstl et al., 2014). One genus, *Faecalibacterium* spp., has been found to be positively associated with weight gain and negatively associated with diarrhoea in dairy calves during the first week of life (Oikonomou et al., 2013). This indicates that *Faecalibacterium* may be beneficial for neonatal calves.

Cryptosporidiosis, caused by the zoonotic protozoan parasite *Cryptosporidium* spp., is a common clinical problem in calves. It has been demonstrated that calves in their second week of life have the highest number of *Cryptosporidium* spp. oocysts (Santín et al., 2008). *Cryptosporidium parvum* (*C. parvum*) is associated with clinical diseases, although other *Cryptosporidium* species are also found in cattle (Thomson et al., 2017). The worldwide prevalence of *C. parvum* varies from 3.4% to 96.6% (Thomson et al., 2017), whereas in Estonia, 66% of the investigated farms had at least one calf shedding *Cryptosporidium* spp. oocysts (Santoro et al., 2018). It has been suggested that the ability of *Cryptosporidium* spp. to infect the gut may be affected by the age-related changes in the gut microbiota or changes in the diet of calves (Thomson et al., 2017). As *Cryptosporidium* spp. infects intestinal epithelial cells (McDonald et al., 2000), I-FABP (intestinal fatty acid binding protein) can be a good biomarker for evaluating the intestinal epithelial damage and permeability in calves (Ok et al., 2020).

In Europe, halofuginone lactate (HL) has been approved as a prophylactic and therapeutic treatment for calf cryptosporidiosis. HL is used to prevent and reduce diarrhoea caused by *C. parvum,* and in both indications, the oocyst excretion has been demonstrated to be reduced (European Medicines Agency, 2019).

Tissue damage (*e. g.* trauma or infection) initiates a host inflammatory response called the acute phase response (APR) (Baumann and Gauldie, 1994). This response is a vital part of innate immunity and is an important protective mechanism in neonatal infections. Acute phase proteins (APPs) are produced by the liver, induced by proinflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) (Baumann and Gauldie, 1994). In cattle, the two major APPs are haptoglobin (Hp) and serum amyloid A (SAA) (Eckersall and Bell, 2010). The concentration and kinetics of systemic APPs during the inflammatory response are related to the severity of tissue damage and the time-course of the inflammation process (Baumann and Gauldie, 1994; Kent, 1992; Petersen et al., 2004). Thus, measuring the circulating levels of APPs provides valuable information about the ongoing APR and can be used as non-specific disease markers (Thompson et al., 1992; Van Leeuwen and Van Rijswijk, 1994; Petersen et al., 2004). In general, changes in APPs after birth reflect the physiological adaptation of the newborn to extrauterine life (Orro et al., 2008).

In humans, it has been shown that the immunity effects of early-life microbial exposure are durable and persist into later life (von Mutius, 2007; Ege et al., 2011). Immunological maturation may have a long-term impact on an animal's success as a production animal (Chester-Jones et al., 2017). High SAA concentrations in studies with 2–3-week-old beef calves (Seppä-Lassila et al., 2017), rearing calves (Seppä-Lassila et al., 2018), reindeer calves (Orro et al., 2006), and lambs (Peetsalu et al., 2019) predict lower long-term weight gain up to the age of slaughter. These observations suggest that early factors activating an APR can have a long-term negative impact on an animal's life, including lower weight gain. To the authors' knowledge, the relationships of neonatal infections with APR and the development of the immune system have not yet been thoroughly addressed.

The main aim of this study was to investigate the relationship between faecal microbiota and systemic innate immunity response in 2-week-old calves, as measured in terms of the APP and pro-inflammatory cytokine concentrations in the serum. Genera associated with the systemic innate immunity response were further analysed to explore the potential relationship between microbiota and growth rate during the first 9 months of life. In addition, we investigated the relationship between acute cryptosporidiosis and the intestinal microbiota and measured I-FABP concentration in blood as it is a marker of intestinal permeability.

2. Materials and methods

The present study is a part of a large-scale study, the previous parts of which have been published by Niine et al. (2018a) – APR of calves up to 6 weeks of age during an acute cryptosporidiosis outbreak; and Peetsalu et al. (2022) – association of colostrum cytokine and APP concentrations with APR of calves during the first 3 weeks of life.

2.1. Animals

The calves included in this study were all from the same large dairy farm located in Järva County in Central Estonia. At the time of the study, there were approximately 1800 Estonian Holstein dairy cows on the farm. All the included calves ($n = 112$) were females born between 21 January and 16 March 2015. Twins and male calves were excluded from the study. For this study, we analysed faecal and serum samples taken during the second week of the calves' lives.

The calves were separated from their mothers immediately after birth and weighed on a digital scale. They received three litres of colostrum from their dams once within 2 h after birth. Colostrum quality was determined by means of visual examination and a Kruuse colostrum densimeter (Jørgen Kruuse A/S, Langeskov, Denmark). If the specific gravity of the colostrum was <1035 or total protein was <50 g/l, deep frozen colostrum from another dam was provided to the calf. At 9 months of age, the calves were weighed again. A more detailed description of the management of farm and veterinary services provided to calves is presented in Niine et al. (2018a). Briefly, after the onset of an acute cryptosporidiosis outbreak, treatment with HL (Halocur, Intervet International B.V., Boxmeer, Netherlands) was initiated. The calves were retrospectively allocated into three groups, based on the HL treatment regimen they received: 1) not treated, 2) treated incorrectly (treatment started >48 h after birth and lasted <7 d), and 3) treated correctly (treated according to manufacturer's instructions; started <48 h after birth and lasted ≥7 d).

2.2. Feeding

Twice a day, the calves were fed 2–3 kg of warmed unpasteurised milk, in addition to free access to hay and prestarter feed (Prestarter, Agrovarustus OÜ, Estonia). This feeding regimen was maintained until the age of 15–17 d, after which the calves received milk powder (Josera GoldenSpezial, Josera GmbH & Co. KG, Germany) solution (140 g of milk powder diluted in 1 l of warm water) twice a day (3 l/day) and continued free access to prestarter feed.

2.3. Sample collection

Serum and faecal samples were ethically collected from calves during their second week of life. Serum samples ($n = 112$) were taken from the jugular vein using an 18-G needle into sterile evacuated tubes (VACUETTE® TUBE 9 ml CAT Serum Clot Activator). The samples were then transported to the laboratory and centrifuged (1800 ×*g* for 10 min). Serum samples were stored at −20 °C until analysis. Faecal samples (n = 112) were collected directly from the rectum using disposable gloves. The collected faecal samples were stored at 4 °C for up to 48 h until further analysis (detection of *Cryptosporidium* spp. oocysts and DNA extraction).

2.4. Laboratory analysis

The SAA concentration was measured using a commercial ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland). The concentration of Hp was measured using a method defined by Makimura and Suzuki (1982), with alterations of using tetramethylbenzine (0.06

mg/ml) as a substrate and using microtitration plates (Alsemgeest et al., 1994). The detection limits were 0.3 mg/l for SAA and 60 mg/l for Hp. The concentrations of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) were measured using commercial ELISA kits from Cusabio Biotech (Wuhan, Hubei, China). The detection limits for IL-1β, IL-6, and TNF-α were 15.6 ng/l, 2.5 ng/l, and 50.0 ng/l, respectively. The concentration of intestinal fatty acid binding protein (I-FABP), a marker of intestinal permeability, was measured using a commercial ELISA kit (bovine I-FABP ELISA kit (MBS035016); MyBiosource Inc., San Diego, CA, USA). The results were read using a spectrophotometer (Magellan Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The inter- and intra-assay coefficients of variability for all protein detection methods were < 15%.

Faecal samples were prepared for the detection of *Cryptosporidium* spp. using an immunofluorescence staining method, as described by Niine et al. (2018a). The results were measured as the approximate number of oocysts per gram of faecal matter (opg). Faecal DNA was extracted from approximately 200 μg of faeces using the PSP® Spin Stool DNA Kit (Stratec Biomedical AG, Birkenfeld, Germany) and the bead beating process, according to the manufacturer's instructions.

2.5. Microbiota analysis

The hypervariable regions V3-V4 of the 16S ribosomal RNA (rRNA) genes were sequenced using the Illumina MiSeq platform in the DNA core facility of the University of Helsinki, as described previously (Alipour et al., 2018; Husso et al., 2020). Samples and controls were first amplified using 1× Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific), 2.5% DMSO (Thermo Scientific), 500 nM of 16S rRNA V3 and V4 gene primers (341F and 785R; Metabion), and 1.25 μl of DNA extract, in 25 μl total volume. The thermal cycling conditions included an initial denaturation step at 98 °C for 30 s, and 12 cycles of denaturing at 98 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 20 s. The final extension step was at 72 °C for 5 min. T100™ Thermal Cycler (Bio-Rad Laboratories) was used.

The 2nd round PCR amplifications were performed using an Illumina forward and reverse primer set, Phusion Hot-Start II polymerase (Finnzymes/Thermo Scientific), High Fidelity buffer and 2.5% DMSO. The following thermal cycling conditions were applied with an Arktik thermal cycler (Finnzymes/Thermo Scientific): initial denaturation at 98 °C for 30 s, 17 cycles at 98 °C for 10s, 65 °C for 30s, 72 °C for 10s, and a final extension at 72 °C for 5 min. The final 16S rRNA gene amplicons were sequenced on an Illumina MiSeq sequencer using the v2 600 cycle kit paired-end (325 bp + 285 bp).

In addition to calf faecal samples, we sequenced no template controls, a ZymoBIOMICS Microbial Community Standard (Zymo Research, USA), and an in-house adult cow faecal standard. The observed composition of the commercial standard matched the expected composition provided by the manufacturer (data not shown).

The detailed bioinformatics pipeline is described in the Supplementary Materials. Briefly, the read quality was first inspected using FastQC and MultiQC (Andrews et al., 2015; Ewels et al., 2016). Leftover primes and spacers were then trimmed using Cutadapt version 1.10 (Martin, 2011). A mapping file was created for QIIME2 and validated using Keemei (Rideout et al., 2016). The FASTQ- files were imported into QIIME2 version 2018.4, where the DADA2 plugin was used to denoise and filter the reads, call amplicon sequence variants (ASVs), and generate a feature table (Callahan et al., 2016; Bolyen et al., 2019). A naïve Bayes classifier was trained in QIIME2 against the SILVA v132 97% database, extracted to only include the V3–V4 reference region, and used to assign taxonomy to ASVs (Quast et al., 2013; Bokulich et al., 2018). Singleton sequences and sequences derived from chloroplasts or mitochondria were removed.

2.6. Statistical analysis

We used variance partitioning analysis to investigate how the systemic innate immunity response markers, *Cryptosporidium* spp. infection, and age explain faecal microbiota variability on the genus level. This analysis partitions the variation in faecal microbiota data into components with respect to different tables of exploratory variables and their combinations using adjusted R-squares in redundancy analysis ordination (Borcard et al., 1992). Continuous variables were used for variance partitioning (except HL treatment).

Random forest analysis was used to investigate how well faecal microbiota explain our chosen parameters – cytokines, APPs, *Cryptosporidium* spp. infection, HL treatment, and I-FABP. For random forest analysis, continuous variables (except IL-1β) were divided into low, moderate, and high concentration groups of similar size, based on the concentration levels from lowest to highest in serum. Based on the concentration of IL-1β, the calves were divided into two groups: low (below the detection level of the assay) and high (above the detection level of the assay). Calves were also divided into groups based on the amount of *Cryptosporidium* spp. oocysts shed in their faeces (opg); these groups were: no oocysts found, low oocyst count, and high oocyst count. The division into low- and high oocyst groups was based on the median value of oocysts found. For treatment of HL, the calves were divided into three groups: not treated, treated incorrectly, and treated correctly. All the categorical variables and their values are presented in Table 1. Genus-level microbiota data were used for the statistical analysis. All genera with a prevalence of ≥10% in 112 calves were included (n = 102).

Table 1
Categorical variables used in the statistical analysis.

Variable	Group	Value	n
SAA (mg/l)	Low	34.0–101.2	38
	Moderate	102.1–153.2	37
	High	154.3–487.9	37
Hp (mg/l)	Low	95–218	38
	Moderate	221–884	37
	High	891–2830	37
IL-1β (ng/l)	Low	<15.6*	99
	High	16.9–207.4	13
IL-6 (ng/l)	Low	<2.5*–4.2	38
	Moderate	4.3–8.6	37
	High	9.3–130.7	37
TNF-α (ng/l)	Low	<50*–221	38
	Moderate	230–396	37
	High	429–4205	37
I-FABP (ng/l)	Low	63–234	38
	Moderate	236–303	37
	High	308–1797	37
ADWG (g/d)	Low	406–646	34
	Moderate	648–741	33
	High	750–982	33
Cryptosporidium spp. (opg)	No oocysts found	0	54
	Low oocyst count	208–399,910	29
	High oocyst count	475,293–5,764,653	29
HL treatment group	Not treated	n/a	21
	Treated incorrectly	n/a	39
	Treated correctly	n/a	52

Continuous variables from calves (n = 112) were categorised into three similar sized groups based on the concentrations, from lowest to highest (low, moderate, and high). Average daily weight gain data from 100 calves were available. The median value of oocysts found in *Cryptosporidium*-positive samples was used to categorise them into low and high oocyst groups. Calves treated with halofuginone lactate (HL) were divided into groups treated incorrectly (treatment started >48 h after birth and lasted <7 d), and correctly (treatment started <48 h after birth and lasted ≥7 d).

SAA – serum amyloid A, Hp – haptoglobin, IL-1β – interleukin 1-beta, IL-6 – interleukin 6, TNF-α - tumour necrosis factor alpha, I-FABP – intestinal fatty acid binding protein, HL – halofuginone lactate, n/a – not applicable, ADWG – average daily weight gain. * Under the detection limit of the ELISA kit.

The 30 most influential genera in the random forest analysis, as measured by mean decrease in model accuracy, were further analysed using negative binomial regression, to investigate the associations of microbial genera with *Cryptosporidium* spp. and marker variables. Genus was always included as a response variable. Age at sampling and *Cryptosporidium* spp. oocyst count were included as categorical explanatory variables in all models. The abundance of all the bacteria in the sample was included as an exposure variable. Wald test *p*-values were calculated for categorical variables, and Holm-Šidák adjusted p-values were calculated to account for multiple comparisons (out of 30 Wald test *p*-values). For pairwise comparisons between 3-level categorical variables after a significant Holm-Šidák corrected Wald test, Bonferroni correction was used. Graphs of the observed proportions of negative binomial probabilities along with the Poisson model were used to evaluate how negative binomial distribution suited to the models used. Association with average daily weight gain (ADWG) was investigated for all genera ($n = 5$) that were significantly associated with I-FABP or any systemic innate immunity response marker. ADWG at 9-months of age (low, moderate, and high; $n = 34$, 32, and 34, respectively) was used as an explanatory categorical variable. To control for possible confounding factors, age at sampling and *Cryptosporidium* spp. oocyst counts in faeces were included as categorical explanatory variables, and calf birth weight was included as a continuous explanatory variable. Bonferroni correction was used for pairwise comparison of p-values for categorical variables.

Linear regression models were used to evaluate the associations between microbial diversity (Shannon index) and age, innate immunity response marker variables, *Cryptosporidium* spp. infection, and HL treatment. The Shannon index was a response variable; age (continuous variable), protein groups, *Cryptosporidium* spp. infection groups, and HL treatment groups were explanatory variables. Stepwise backward elimination procedure was used to select the final models. The linearity assumption between the response and continuous explanatory variables was evaluated using Lowess smoothing curve graphs. Interactions and confounders (change in coefficient of over 10% after variable elimination) were controlled for. Residual plots were used to evaluate the overall model assumptions.

For variance partitioning analysis package 'vegan' (Oksanen et al., 2019) and for random forest analysis package 'randomForest' (Liaw and Wiener, 2002) with R version 4.0.1 (http://www.R-project.org/) were used. For negative binomial regression models and linear regression model, STATA/IC 14.2 for Windows (StataCorp LP, College Station, TX, USA) was used. Excel 2016 (Microsoft, Redmond, WA, USA) was used for the initial data management.

3. Results

3.1. Microbiota composition on phylum level

The most prevalent phylum in all 2-week-old calves in our study ($n = 112$) was *Firmicutes*, with a mean relative abundance (±SD) 50.9 ± 26.8%. This was followed by *Bacteroidetes* (28.6 ± 25.8%), *Proteobacteria* (8.4 ± 12.4%), *Fusobacteria* (7.9 ± 15.9%), and *Actinobacteria* (3.9 ± 9.0%) (Fig. 1A). These five phyla contributed to 99.7% of all bacterial taxa found in the faecal samples. Age-related changes were observed in the composition of the microbiota, as shown in Fig. 1A. The overall trend of *Bacteroidetes* increased, whereas the relative abundances of *Firmicutes*, *Actinobacteria*, and *Fusobacteria* declined. Bacterial diversity, as measured using the Shannon index, increased with age ($p = 0.001$) (Fig. 2A).

As seen in Fig. 1B, the mean relative abundance of *Firmicutes* was the highest in the group with high *Cryptosporidium* spp. oocyst counts. The relative abundances of *Bacteroidetes* and *Actinobacteria* were, on average, lower in the high oocyst count group. A high count of *Cryptosporidium* spp. oocysts was negatively associated with Shannon's diversity index ($p = 0.007$) (Fig. 2B).

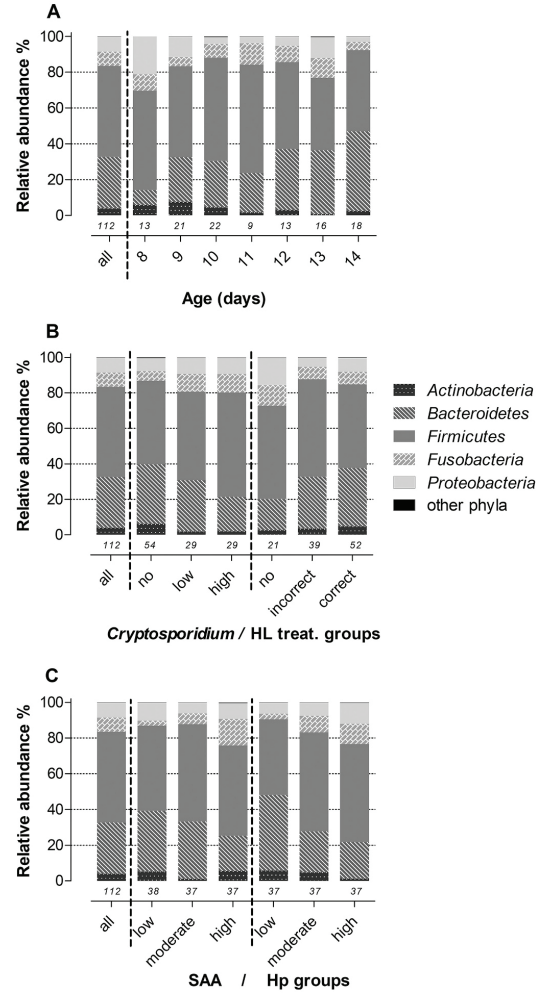

Fig. 1. Microbiota composition at phylum level in calves based on days of age (A), *Cryptosporidium* spp. infection status and halofuginone lactate (HL) treatment groups (B), and serum amyloid A (SAA) and haptoglobin (Hp) concentrations groups (C). The small numbers given in italics under the columns indicate the number of calves in that group. Column 'all' shows the composition of microbiota in all calves included in this study. The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – low oocyst count (below the median of the same week), and high – high oocyst count (above the median of the same week). HL treatment groups were: no – no treatment received, incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d.

The mean relative abundances of *Actinobacteria* were the highest in the HL treatment groups that did receive treatment (incorrect or correct), and *Fusobacterium* was the highest in the not treated group (Fig. 1B). *Cryptosporidium* spp. infection and HL treatment groups correlated with each other: correct HL treatment and *Cryptosporidium* spp. negative results; incorrect HL treatment and low *Cryptosporidium* spp. oocyst counts; and no HL treatment and high *Cryptosporidium* spp. oocyst counts mirrored each other.

The mean relative abundance of the phyla was similar in all three

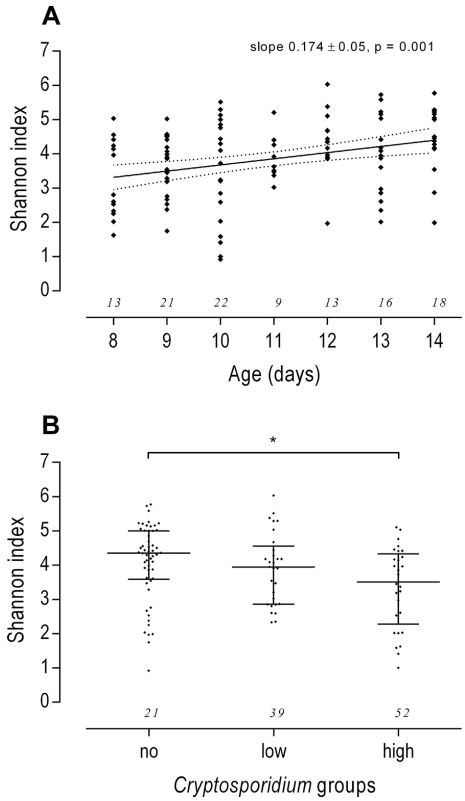

Fig. 2. Faecal microbiota diversity by age (days) (A) and *Cryptosporidium* spp. infection group (B), as evaluated using linear regression model. The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – low oocyst count (below the median of the same week), and high – high oocyst count (above the median of the same week). The small numbers given in italics under the columns indicate the number of calves in that group.
* Significant difference, Bonferroni-corrected $p < 0.05$.

groups of SAA and Hp (Fig. 1C). The higher the SAA and Hp concentrations, the greater the relative abundance of *Fusobacteria* and lower the relative abundance of *Bacteroidetes*.

3.2. Variance-partitioning analysis

The variation in intestinal microbiota at the genus level explained by the study variables is shown in Fig. 3. As seen in this figure, most of the variation was explained by factors other than APPs, *Cryptosporidium* spp. infection, HL treatment, cytokines, or age; a total of 6.0% of microbial variation was explained by these variables. However, it should be noted that APPs (SAA and Hp) independently explain half (3.0%) of the explainable microbial variation, and cytokines (IL-1β, IL-6, TNF-α) were completely overlapped by the APP explanation. In other words, cytokines did not explain the variability in microbiota more than the APPs. The combined APP group and the combined *Cryptosporidium* spp. infection and HL treatment group overlapped with each other for 1.2% of the explained microbiota genus variation. Age was more independent and explained approximately 1.4% of the 6.0% microbial variation.

When *Cryptosporidium* spp. infection and HL treatment groups were added separately to the variance partitioning analysis, all microbial variation explained by HL treatment overlapped with the *Cryptosporidium* spp. infection group. Therefore, *Cryptosporidium* spp. infection and HL treatment groups were added as a combined explanatory variable. When SAA and Hp were included separately in the model, 1.0% (together 1.16%; Fig. 3) of the explained microbiota variations overlapped with each other.

3.3. Random forest analysis

Random forest analysis classified the calves' faecal samples (n = 112) into SAA, Hp, *Cryptosporidium* spp. infection, and HL treatment groups. The relative importance of the 30 most influential genera (out of 102) is shown in Fig. 4. The proportion of groups that were classified correctly by this random forest analysis based on bacterial genera was 42.9% for SAA, 43.7% for Hp, 45.5% for *Cryptosporidium* spp. infection, and 46.2% for HL treatment. The 30 most influential genera by group were further analysed using negative binomial models, to investigate associations with variable groups (Holm-Šidák corrected Wald test *p*-values). In these models, we also accounted for the possible confounding factors age and *Cryptosporidium* spp. infection in the APPs.

Fusobacterium was the most influential genus for SAA, and the fourth most influential genus for Hp. The most influential genus for Hp was [*Ruminococcus*] torques group, but it was not found to be associated with SAA. *Ruminiclostridium 9* was the most influential genus for *Cryptosporidium* spp. infection, and it was ranked 16th for HL treatment. *Erysipelotrichaceae* UCG-009 was the most influential genus for HL treatment, but it was not found to be associated with *Cryptosporidium* spp. infection.

3.4. Negative binomial models

The association between *Cryptosporidium* spp. infection and the presence of *Ruminiclostridium 9* was negative (Fig. 5A). The abundance of *Ruminiclostridium 9* was higher in the no *Cryptosporidium* spp. oocysts group than in the low and high oocyst count groups (Bonferroni corrected $p = 0.039$ and $p < 0.001$, respectively). This bacterial genus was found in 59% of the calves studied ($n = 112$). We also found other bacterial genera (*Alistipes*, *Parasutterella*, and *Faecalibacterium*) with a similar association, except that both the no and low *Cryptosporidium* spp. oocyst count groups had significantly higher abundances than the high oocyst count group (Fig. 5B–D). The 30 most influential genera did not show any statistically significant associations with the HL treatment groups in the negative binomial models.

The *Fusobacterium* genus was positively associated with SAA (Fig. 6A), and was found in 83% of the calves (n = 112). The moderate and high SAA groups had significantly higher *Fusobacterium* abundance than the low SAA group (Bonferroni corrected $p = 0.027$ and $p < 0.001$, respectively). The *Collinsella* genus was found to be associated with SAA and found in 99% of the faecal samples. The association was found to be non-linear, since in the moderate SAA group, abundance of *Collinsella* was lower than in the low or high SAA groups. However, the distribution of *Collinsella* abundance did not follow a negative binomial distribution; thus, the models (for SAA and *Cryptosporidium* spp. infection groups) were not validated (data not shown).

The high Hp concentration group had a positive association with genus [*Ruminococcus*] torques group abundance (Fig. 6B) and a negative association with *Flavonifractor* abundance (Fig. 6C).

Peptostreptococcus was found in 44% ($n = 49$) of the calves, and its abundance was positively associated with high concentrations of both SAA and Hp (Fig. 7A–B). All genera that were significantly associated with inflammatory markers were further analysed for possible associations with nine-month weight gain ($n = 100$), using similar negative binomial models. These five genera were *Fusobacterium*, *Peptostreptococcus*, *Collinsella*, [*Ruminococcus*] torques group, and *Flavonifractor*. The association between weight gain at the age of 9 months and *Peptostreptococcus* was negative (Fig. 7C). The high ADWG group had a lower abundance of *Peptostreptococcus* than the low and moderate

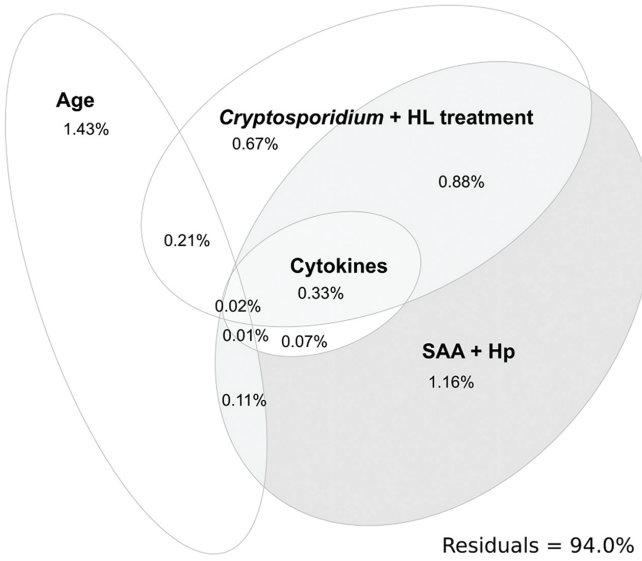

Fig. 3. Variance partitioning analysis of the calves' ($n = 112$) intestinal microbiota at the genus level ($n = 102$). Results are represented as percentiles of the 6.0% of microbial variance explained by these variables. The continuous variables included in the analysis were age at sampling in days ('Age'), cytokines in ng/l (including interleukin-1β, interleukin-6, tumour necrosis factor-α) ('Cytokines'), serum amyloid A (SAA) and haptoglobin (Hp) together in mg/l ('SAA + Hp'), and *Cryptosporidium* spp. infection (opg) and halofuginone lactate (HL) treatment groups together ('*Cryptosporidium* + HL treatment').

ADWG groups (Bonferroni corrected $p < 0.01$ and $p = 0.030$). No statistically significant associations were found between cytokines, I-FABP, HL treatment groups, and genera (data not shown).

4. Discussion

Infection with enteric pathogens can alter the composition of microbiota, and alterations in the composition of microbiota can confer or promote infection by pathogens (Laurent and Lacroix-Lamandé, 2017). The present study shows that there are some genera that have the potential to trigger the inflammatory response, but on a larger scale, microbiota seems to influence the innate immunity response only slightly. For example, *Peptostreptococcus* was positively associated with both APPs investigated (SAA and Hp) and negatively associated with long-term ADWG. Higher oocyst shedding of *Cryptosporidium* spp. is associated with decreased microbial diversity of faecal microbiota of neonatal calves, as was also reported by Rahman et al. (2022); age has the opposite effect.

The rectal microbial 16S rRNA gene profile of 2-week-old calves was dominated by *Firmicutes*, followed by *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria*, all of which constituted 99.7% of the microbiota. We found that microbial diversity increased with age, as demonstrated in a previous study (Dill-Mcfarland et al., 2017), and that a high *Cryptosporidium* spp. oocyst count was negatively associated with diversity (Fig. 2). In negative binomial models of *Cryptosporidium,* the four genera associated with *Cryptosporidium* spp. infection (*Ruminiclostridium 9*, *Alistipes*, *Parasutterella*, and *Faecalibacterium*) decreased in abundance, the more oocysts were found. Carey et al. (2021) found that a low abundance of *Megasphaera* was associated with diarrhoeal symptoms during cryptosporidiosis in human infants. They also found that the microbiome of these children was predictive of diarrhoea prior to and at the time of *Cryptosporidium* spp. infection. In mice, *Cryptosporidium* spp. infection has been associated with an increase in the relative abundance of *Bacteroidetes* and a decrease in that of *Firmicutes* (Mammeri et al., 2019). These findings indicate that the presence of *Cryptosporidium* spp. may steer the intestinal bacterial environment to a less favourable direction for some bacterial species. Alternatively, a high abundance of some bacterial species may inhibit or reduce *Cryptosporidium* spp. infection. As this was a cross-sectional study, we can only speculate about the causal relationships.

However, there have been studies suggesting that *Fusobacterium* and *C. parvum* have a synergistic effect (Ichikawa-Seki et al., 2019). Previous studies have hinted to the fact that some specific microbial phyla (indole producing bacteria) may modulate the immune response against *Cryptosporidium* spp. infections in humans (Laurent and Lacroix-Lamandé, 2017). The fact that *Cryptosporidium* spp. influences the microbial composition of calf faeces, or vice versa, needs to be considered when conducting field studies, so that the results of these studies can accurately represent the current situation in farms. *Cryptosporidium* spp. are widespread worldwide (O'Handley and Olson, 2006), and their influence on the host should not be ignored. This study is a field cohort study that reflects this situation.

HL is used as the standard care, as there are no alternatives in the European market. HL is used as a prophylactic treatment for *Cryptosporidium* spp. infection, which functions by reducing the excretion of oocysts (Silverlås et al., 2009), delaying shedding, and improving the survival of calves (Niine et al., 2018a). This treatment needs to be considered as a possible confounder of the objectives of this study. Associations between microbiota and innate immunity response in the present study were obtained from statistical analyses, where the influence of HL treatment and *Cryptosporidium* spp. infection have been accounted for, and should thus not be biased by them. In some countries, HL is so widely used that having a non-treatment control group in field studies is considered unethical (Seale et al., 2019); thus, HL treatment should always be considered when studying the effects of *Cryptosporidium* spp. and microbiota of calves in field studies.

We found that HL treatment was associated with increased faecal microbial diversity. It should be noted that the effects of *Cryptosporidium* spp. infection and HL treatment on faecal microbiota cannot always be differentiated, as they are 100% overlapping in variance partitioning analysis. However, in the negative binomial models, four genera were negatively associated with *Cryptosporidium* spp. infection, and no genera were associated with the HL treatment groups. This indicates that *Cryptosporidium* spp. infection may modulate the faecal microbiota more than HL treatment.

The *Fusobacterium* genus includes several species of gram-negative

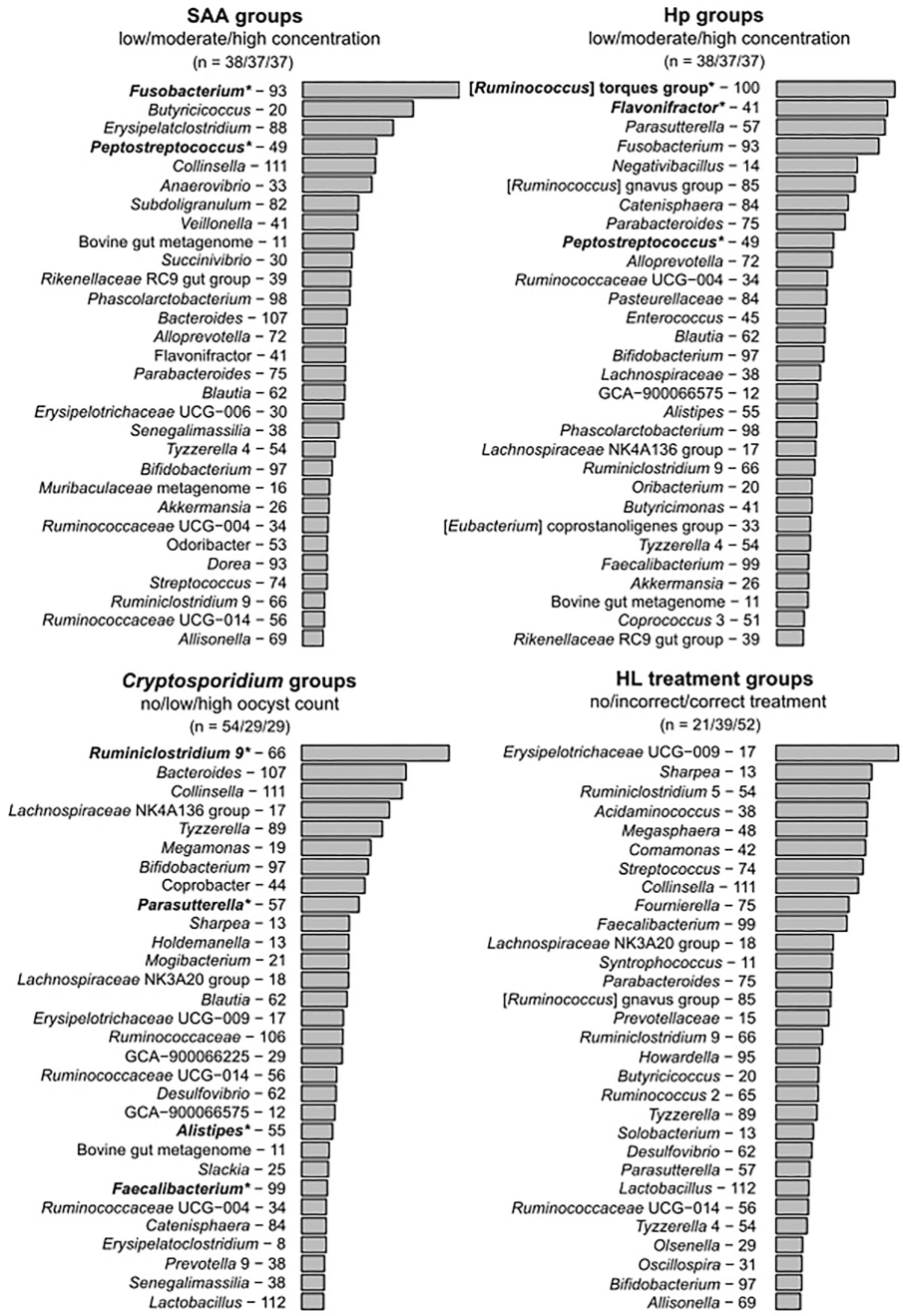

Fig. 4. Random forest analysis results of the 30 most influential genera (out of 102) by groups of calves ($n = 112$). Lengths of the bars (mean decrease in accuracy) show the relative importance of genera. Serum amyloid A (SAA) and haptoglobin (Hp) groups were defined according to concentrations, from lowest to highest. *Cryptosporidium* spp. infection groups were defined according to faecal oocyst count (no oocyst and below or above the median of the same week), while halofuginone lactate (HL) treatment groups were defined on the basis of the received treatment (incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d). The number after the genus name represents the number of calves in which this genus was found.
* Genera with significant association with variable group (Holm-Šidák corrected Wald test), as analysed using negative binomial models.

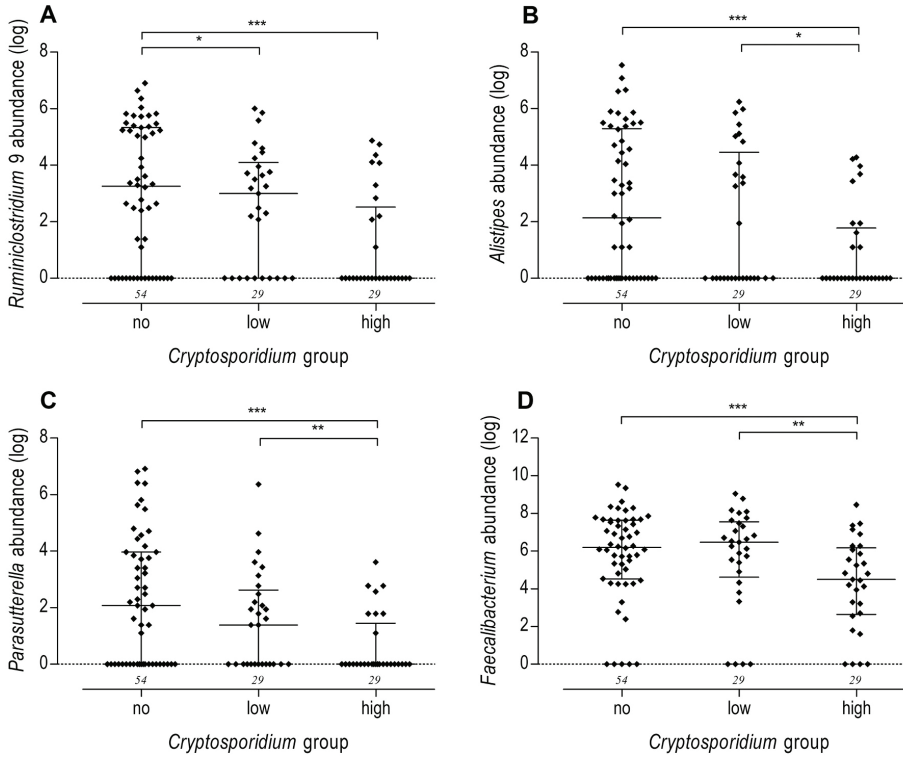

Fig. 5. Association of *Cryptosporidium* spp. groups with four genera, as assessed using negative binomial model. *Cryptosporidium* spp. infection groups (no oocysts found, low oocyst count, and high oocyst count) and *Ruminiclostridium* 9 (A), *Alistipes* (B), *Parasutterella* (C), and *Faecalibacterium* (D) abundances in samples are shown. Log-transformed abundance of these genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.
* Significant difference, Bonferroni-corrected $p < 0.05$.
** Significant difference, Bonferroni-corrected $p < 0.01$.
*** Significant difference, Bonferroni-corrected $p < 0.001$.

rods that are obligately anaerobic, non-spore forming, and can be motile or non-motile (Hofstad, 2006). *Fusobacterium* is an opportunistic bacterium with pro-inflammatory effects, among other properties (Wong and Yu, 2019). There are both pathogenic and non-pathogenic species of *Fusobacterium*, and all known species affect both animals and humans, with the two most common pathogenic species being opportunistic *F. necrophorum* and *F. nucleatum* (Nagaraja et al., 2005). In our study, the relative abundance of *Fusobacterium* (7.9%) was higher than reported previously. Previous studies have found abundances of 0.76%–5.67% in calves of up to 7 weeks of age (Oikonomou et al., 2013), and 2.0% from birth to 15 d of age in pathogen-free calves (Ichikawa-Seki et al., 2019). *Fusobacterium* abundance was positively associated with SAA concentration (Fig. 6). High *Cryptosporidium* spp. oocyst counts were also positively associated with SAA concentration in the second week of life in these calves (Niine et al., 2018a), but as we controlled the *Cryptosporidium* spp. infection groups in the *Fusobacterium* and SAA model, this means that *Fusobacterium* independently initiated the calves' APR. A study by Ichikawa-Seki et al. (2019) showed an increase in *Fusobacterium* in calf faecal matter to 14.1%, coinciding with infection with *C. parvum*. They also found more severe diarrhoea in calves with concurrent *Cryptosporidium* spp. and *Fusobacterium* infections. The authors of that study suggested that *Fusobacterium* can have an important aggravating effect on *Cryptosporidium* spp. infections. Our study did not show a direct association between *Cryptosporidium* spp. infection and *Fusobacterium,* but as SAA is directly related to the severity of clinical disease, an additional positive association between SAA and *Fusobacterium* supports the synergistic effect of these two infections with clinical disease in calves.

The gut microbiota is influenced by genetics (Fan et al., 2020), both paternal and maternal (Amin and Seifert, 2021). The bacterial composition varies within the gastrointestinal tract of calves, and is influenced by the colostrum and microbiota on the dam's skin, at both the udder and vagina (Yeoman et al., 2018). However, the differences in the overall microbial composition of calves in this study are probably influenced by the acute outbreak of cryptosporidiosis and HL treatment, or other factors leading to *Cryptosporidium* spp. infection, including *Fusobacterium* overgrowth. Since this was a cross-sectional study, causal relationships could not be proven.

Variance partitioning analysis (Fig. 3) showed that most of the microbial variation (residuals 94.0%) was not explained by the variables investigated in this study. The cytokine group completely overlapped with the larger APP group. We can, therefore, state that APPs reflect the activation of cytokines (which have a shorter half-life than APPs) as markers of inflammation. Cytokines are known to increase the levels of APPs. It should be noted that since no associations were found in the negative binomial model, the associations of cytokines with microbiota

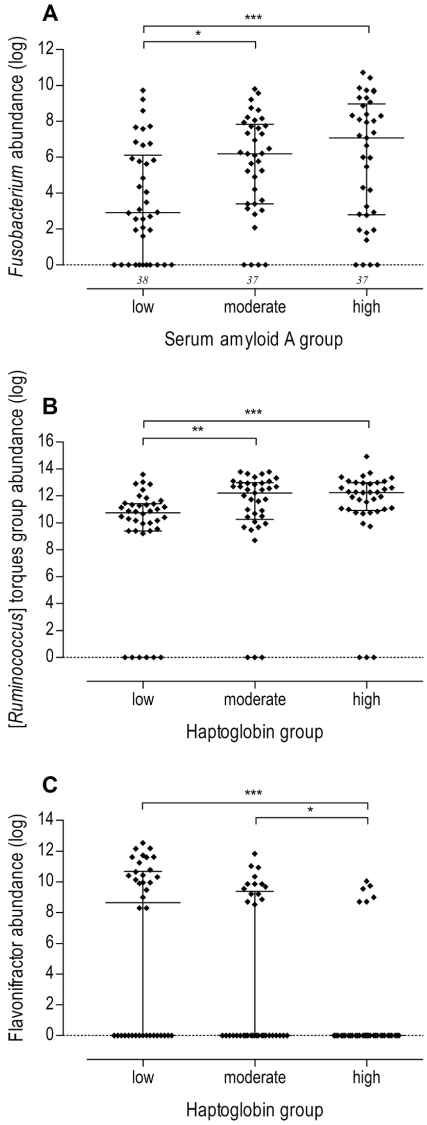

Fig. 6. Association of APPs with three genera, as assessed using negative binomial model. The results of negative binomial models of the associations of serum amyloid A (low, moderate, and high) and *Fusobacterium* abundance (A), as well as those of haptoglobin group (low, moderate, and high) and [*Ruminococcus*] torques group (B), and *Flavonifractor* abundance per sample (C). Log-transformed abundance of bacteria genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.
* Significant difference, Bonferroni-corrected $p < 0.05$.
** Significant difference, Bonferroni-corrected $p < 0.01$.
*** Significant difference, Bonferroni-corrected $p < 0.001$.

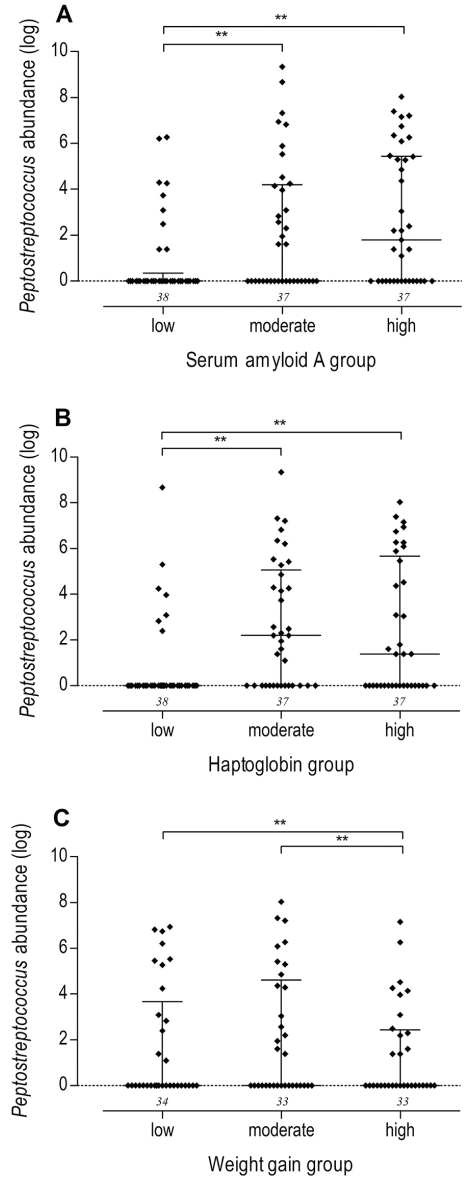

Fig. 7. Associations of APPs and weight gain with *Peptostreptococcus* using negative binomial model. The results of negative binomial models of the associations of serum amyloid A (A), haptoglobin (B) ($n = 112$) and weight gain (C) (n = 100) groups, and *Peptostreptococcus* abundance per sample. Log-transformed abundance of bacteria genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.
** Significant difference, Bonferroni-corrected $p < 0.01$.
*** Significant difference, Bonferroni-corrected $p < 0.001$.

seem to be relatively negligible compared to those of APPs.

Fusobacterium was the most influential genus explaining SAA, and the fourth most influential genus explaining Hp in the random forest analysis. The positive association between *Fusobacterium* and SAA was confirmed by a negative binomial model. However, *Fusobacterium* was not associated with the calves' ADWG at 9 months. The genus that was positively associated with SAA and Hp and negatively associated with ADWG was *Peptostreptococcus* (Fig. 6). *Peptostreptococcus* spp. are anaerobic, non-spore-forming, gram-positive cocci (Wanger et al., 2017). In humans, an increase in *P. anaerobius* abundance in the faecal and mucosal microbiota is associated with colorectal cancer (Cheng et al., 2020). However, little is known about the effects of this genus in ruminants or other animal species. SAA has been shown to increase in response to microbial colonisation (Murdoch et al., 2019). This process can trigger APR without manifesting as a clinical disease, but as a subclinical infection, and microbiota composition itself might have a long-lasting effect on an animal's weight gain and future immune response. SAA is not simply a marker for inflammation and infection; it can also be seen as a marker for the induction of an adaptive immune response, as it attracts Th2 T-cells (Xu et al., 1995), and thus provides an indication of the production of memory T-cells. The neonatal immune system is biased toward a Th2-dominant response with minimal Th1 activity (Zaghouani et al., 2009), and SAA and IL-6 have been shown to be related to this bias (Furuhashi et al., 2012; Schulte et al., 2008). Furthermore, SAA has been shown to be a soluble pattern recognition receptor related to Th2 immunity (Smole et al., 2020), and thus, SAA can also be a marker for the Th2-bias. These findings suggest that some factors initiating systemic APR affect overall health, and thus the future weight gain of the animal, probably by inducing biased priming of the immune system. We speculate that this is caused by subclinical rather than clinical disease, as our previous studies have shown a negative association between serum SAA concentrations during the second week of life and further gain in clinically healthy ruminants (Orro et al., 2006; Seppä-Lassila et al., 2017; Seppä-Lassila et al., 2018; Peetsalu et al., 2019). In the same calves as in the present study, SAA, Hp, and IL-6 serum concentrations in the second week of life were also negatively associated with ADWG at 9 months (Peetsalu et al., 2022 – submitted for publication). Multiple studies in different species, namely beef calves (Seppä-Lassila et al., 2017), dairy calves (Seppä-Lassila et al., 2018), reindeer calves (Orro et al., 2006), and lambs (Peetsalu et al., 2019) living under different conditions, have found this negative association, which cannot be explained by management factors such as climate, feeding, and colostrum intake. *Peptostreptococcus* may be one of the factors behind this phenomenon of the neonatal period's long-term influence on weight gain.

Serum Hp concentrations were positively associated with [*Ruminococcus*] torques group and negatively associated with *Flavonifractor* abundance (Fig. 5). Although controversial, these results support the possible stimulatory effect of microbiota on the calves' inflammatory responses. Studies in rearing calves (Seppä-Lassila et al., 2018) and lambs (Niine et al., 2018b) also found a positive association between Hp concentration in the second week and future weight gain. Mild inflammation can also be associated with lower systemic Hp concentrations in calves (Arthington et al., 2003; Seppä-Lassila et al., 2015). This can be explained by the binding of blood Hp to free haemoglobin (Eaton et al., 1982), resulting in a decrease in circulating Hp in the blood before the inflammatory response initiates Hp production in the liver. The associations found in these previous studies support our hypothesis that there are some early APR-activating factors that can have a negative long-term impact on an animal's life, including lower weight gain.

5. Conclusions

The results of our study show that there are associations between microbial composition and the systemic innate immune response, mainly with respect to SAA and Hp, but not with pro-inflammatory cytokines. This indicates that the bacterial composition has a systemic effect on neonatal calves. However, most of the variability in the intestinal microbiome does not affect the systemic innate immune response, as only a fraction of all faecal microbial variability can be accounted for by APR marker variation. The positive association of the genus *Peptostreptococcus* and APPs, and the negative association of this same genus and ADWG indicate that a high abundance of certain bacterial genera during early life may have long-term effects on the calf. *Cryptosporidium* spp. infection is negatively associated with microbial diversity. There is a need to further investigate the development and composition of intestinal microbiota to better understand how altering the microbial composition may benefit or harm the calf during the neonatal period and in its future life.

Ethics statement

Sample collection was conducted with permission issued by the Ethical Committee of Animal Experiments of the Estonian Ministry of Agriculture (no. 7.2–11/2).

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.07.008.

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Institution and occupation

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2015–2018	Finnish Food Safety Authority (Evira), Länsi-Kalkkuna Oy; official veterinarian in meat inspection
19.05.–15.08.2014	Univet Oy, Univet Kouvola Eläinsairaala; small animal veterinarian

Education

2016–2022	Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, doctoral studies
2009–2015	Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, degree in veterinary medicine (DVM)
2006–2009	University of Tartu, Faculty of Science and Technology, Geography, Bachelor of Science in natural sciences (B.Sc)
2003–2006	Langinkosken lukio [high school], Kotka, Finland
2000–2003	Kotkan Keskuskoulu [secondary school], Kotka, Finland
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R&D related managerial and administrative work

Special roles or positions:

2019–…	Academic ethics committee of Estonian University of Life Sciences; substitute member
2015–…	Veterinary Medicine Curriculum Committee in Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences; board member
2009–2015	Course president during veterinary studies (in Estonian: *kursusevanem*)

Memberships in professional networks and societies:

2019–...	Finnish Veterinary Hygienist's society (EHY Ry)
2018 − ...	Finnish Epidemiology Society (SES)
2018 − ...	Society for Veterinary Epidemiology and Preventive Medicine (SVEPM)
2016 − ...	Estonian Veterinary Association (ELÜ)
2015 − ...	Scandinavian-Baltic Society of Parasitology (SBSP)
2011 − ...	Finnish Veterinary Association (SELL)

Additional career information

Teaching:
VL.1266 Basic virology (3 ECTS)
VL.1267 Special virology (3 ECTS)
VL.1280 Animal infectious diseases I (3 ECTS)
VL.1281 Animal infectious diseases II (3 ECTS)
VL.1268 Introduction to veterinary studies (2 ECTS)
VL.1218 Basics of evidence-based veterinary medicine (sporadic lectures)
VL.0121 Parasitology and parasitic diseases (sporadic lectures)
VL.0373 Herd health and veterinary prophylaxis (sporadic lectures)

Awards and nominations:

- Finalist in the competition of 'Young Epidemiologist of the Year, 2018', Finnish Epidemiology Society
- 'Science in three minutes' laureate, 2017, Estonian Academy of Sciences
- Teacher of the year 2017 elected by SUOLET ry [*Suomalaiset Eläinlääketieteen Opiskelijat Tartossa*] 3rd year students

Field of research
Veterinary epidemiology, immunology, and parasitology

Participation in scientific projects

- ERASMUS+ 2021-1-FI01-KA220-HED-000022988 "Outbreak investigation game OIG" (14.02.2022−13.02.2025); University of Helsinki, Norwegian University of Life Sciences, Estonian University of Life Sciences, MedEdu Oy and Swedish University of Agricultural Sciences.
- PSG268 "Cow culling and mortality in modern high-yielding dairy herds – a multidisciplinary approach" (1.01.2019−31.12.2022); Kerli Mõtus; Estonian University of Life Sciences.
- COVSG9 "Polymer nanocomposites applied to anti-viral additives and antiviral coatings for surfaces protection" (1.11.2020−31.12.2021); Erwan Yann Rauwel; Estonian University of Life Sciences.
- V190115VLTR "Risk-based meat inspection and integrated meat safety assurance (4.03.2019−3.03.2023)"; Mati Roasto; Estonian University of Life Sciences.
- IUT8-1 "Fertility and health in dairy cattle (1.01.2013−31.12.2018)"; Andres Valdmann; Estonian University of Life Sciences.
- 8P160014VLVP "*Eestis vähe uuritud algloomtõbede molekulaarepidemioloogia ning putuksiirutajatega levivate nakkuste metagenoomika ja siirutajate ökoloogia* (1.02.2016−31.12.2017)"; Arvo Viltrop; Estonian University of Life Sciences.

Important professional training

- Competence course in laboratory animal science 'Laboratory animal science', Estonian University of Life Sciences, 2021; Estonia
- Workshop on 'Cox proportional hazard models', Swedish University of Agricultural Sciences (SLU), 2018; Sweden
- NOVA PhD course 'Questionnaire design and management', Swedish University of Agricultural Sciences (SLU), 2018; Lithuania
- SEARMET Winter School 'Molecular biology in Animal Sciences', Estonian University of Life Sciences, 2017; Estonia

- NOVA PhD course 'Molecular Epidemiology of Infectious Diseases, University of Helsinki, 2017; Finland
- NOVA course 'Animal Welfare – from Fork to Farm', University of Helsinki, 2016; Finland

Academic degrees

- Elisabeth Dorbek-Kolin, Master's Diploma (Veterinary Medicine); 2015; (sup) Brian Lassen, Maria Vang Johansen; 'Prevalence of *Taenia saginata* cysticercosis in Estonian cattle'; Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Department of Basic Veterinary Sciences and Population Medicine.
- Elisabeth Dorbek-Kolin, Bachelor of Science in Natural Sciences (Geography); 2009; (sup) Anneli Kährik; '*Maakondade arenguerinevuste mõju suitsiidsusele Eestis'* [Developmental differences in Estonian counties and their influence on suicide rates in Estonia]; Univerity of Tartu, Faculty of Science and Technology, Institute of Ecology and Earth Sciences, Department of Geography.

ELULOOKIRJELDUS

Eesnimi Elisabeth
Perekonanimi Dorbek-Kolin
Sünniaeg 16.10.1987
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Töökohad ja ametid

01.09.2015–...	Eesti Maaülikooli veterinaarmeditsiini ja loomakasvatuse instituudi veterinaarse biomeditsiini ja toiduhügieeni õppetooli loomade infektsioonhaiguste assistent
01.02.2020–31.01.2021	European Food Safety Authority (EFSA), risk assessment & scientific assistance (RASA) department, animal and plant health (ALPHA) unit, animal health and welfare (AHAW) team; praktikant
2015–2018	Elintarviketurvallisuusvirasto (Evira), Länsi-Kalkkuna Oy; lihainspektsiooni järelevalve veterinaar
19.05.–15.08.2014	Univet Oy, Univet Kouvola Eläinsairaala; loomaarst

Haridustee

2016–2022	Eesti Maaülikooli veterinaarmeditsiini ja loomakasvatuse instituudi doktorant
2009–2015	Eesti Maaülikooli veterinaarmeditsiini ja loomakasvatuse instituudi veterinaaria eriala
2006–2009	Tartu Ülikooli loodus- ja tehnoloogia teaduskonna loodusteaduste (geograafia) bakalaureus
2003–2006	Langinkosken lukio [keskkool], Kotka, Soome
2000–2003	Kotkan keskuskoulu [põhikool], Kotka, Soome
1994–2000	Aittakorven ala-aste [algkool], Kotka, Soome

Teadusorganisatsiooniline ja -administratiivne tegevus

Positsioonid:

2019–…	EMÜ akadeemilise eetika komisjoni asendusliige
2015–...	EMÜ VLI õppemetoodika komisjoni veterinaarmeditsiini erialakomisjoni liige
2009–2015	Kursusevanem (veterinaaria õpigute ajal)

Teadusorganisatsioonide liikmelisused:

2019–...	Soome Hügieeniloomaarstide Ühing (EHY Ry)
2018 − ...	Soome Epidemioloogia Selts (SES)
2018 − ...	Veterinaarepidemioloogia ja Ennetava Meditsiini Ühing (SVEPM)
2016 − ...	Eesti Loomaarstide Ühing (ELÜ)
2015 − ...	Skandinaavia-Baltimaade Parasitoloogia Ühing (SBSP)
2011 − ...	Soome Loomaarstide Ühing (SELL)

Teenistuskäigu lisainfo

Õppetöö:

VL.1266 Üldviroloogia (3 ECTS)
VL.1267 Eriviroloogia (3 ECTS)
VL.1280 Loomade infektsioonhaigused I (3 ECTS)
VL.1281 Loomade infektsioonhaigused II (3 ECTS)
VL.1268 Sissejuhatus veterinaaria õpingutesse (2 ECTS)
VL.1218 Tõenduspõhise veterinaarmeditsiini alused – üksikud loengud
VL.0121 Parasitoloogia ja invasioonihaigused – üksikud loengud
VL.0373 Karja tervis ja veterinaarprofülaktika – üksikud loengud

Auhinnad ja nominatsioonid:

- 'Aasta noor epidemioloog, 2018' finalist, Soome Epidemioloogia Selts
- 'Teadus kolme minutiga' laureaat, 2017, Eesti Teaduste Akadeemia
- 'Aasta õppejõud 2017' auhind, valitud SUOLET ry [*Suomalaiset Eläinlääketieteen Opiskelijat Tartossa*] 3.aasta tudengite poolt

Teadustöö põhisuunad

Veterinaarepidemioloogia, immunoloogia ja parasitoloogia

Osalemine uurimisprojektides

- ERASMUS+ 2021-1-FI01-KA220-HED-000022988 "*Outbreak investigation game OIG*" (14.02.2022−13.02.2025); University of Helsinki, Norwegian University of Life Sciences, Eesti Maaülikool, MedEdu Oy and Swedish University of Agricultural Sciences.
- PSG268 "Piimalehmade praakimine ja hukkumine kaasaegsetes kõrgetoodangulistes piimakarjades - multidistsiplinaarne uuring" (1.01.2019−31.12.2022); Kerli Mõtus; Eesti Maaülikool.
- COVSG9 "*Polymer nanocomposites applied to anti-viral additives and antiviral coatings for surfaces protection*" [Polümeersete nanokomposiitide kasutamine anti-viraalsetes lisandites ja pinnakatetes] (1.11.2020−31.12.2021); Erwan Yann Rauwel, Eesti Maaülikool.
- V190115VLTR "*Risk-based meat inspection and integrated meat safety assurance* (4.03.2019−3.03.2023)"; Mati Roasto, Eesti Maaülikool.
- IUT8-1 "Piimalehmade sigimine ja tervis (1.01.2013−31.12.2018)", Andres Valdmann, Eesti Maaülikool.
- 8P160014VLVP "Eestis vähe uuritud algloomtõbede molekulaarepidemioloogia ning putuksiirutajatega levivate nakkuste metagenoomika ja siirutajate ökoloogia (1.02.2016−31.12.2017)", Arvo Viltrop, Eesti Maaülikool.

Olulisemad erialased enesetäiendamised

- Kompetentsi kursus *'Laboratory animal science'*, Eesti Maaülikool, 2021; Eesti
- Töötuba '*Cox proportional hazard models*', Swedish University of Agricultural Sciences (SLU), 2018; Rootsi
- NOVA PhD kursus '*Questionnaire design and management'*, Swedish University of Agricultural Sciences (SLU), 2018; Leedu
- SEARMET talvekool '*Molecular biology in Animal Sciences'*, Eesti Maaülikool, 2017; Eesti
- NOVA PhD kursus '*Molecular Epidemiology of Infectious Diseases*', University of Helsinki, 2017; Soome
- NOVA kursus '*Animal Welfare – from Fork to Farm'*, University of Helsinki, 2016; Soome

Teaduskraadid

- Elisabeth Dorbek-Kolin, magistrikraad (veterinaarmeditsiin); 2015; (juh) Brian Lassen; Maria Vang Johansen; *Prevalence of Taenia saginata cysticercosis in Estonian cattle* (Taenia saginata tsüstitserkoosi levimus Eesti veistel); Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, Veterinaaria alusteaduste ja populatsioonimeditsiini osakond.
- Elisabeth Dorbek-Kolin, bakalaureusekraad (geograafia); 2009; (juh) Anneli Kährik; Maakondade arenguerinevuste mõju suitsiidsusele Eestis; Tartu Ülikool, Loodus- ja tehnoloogia teaduskond, Ökoloogia ja maateaduste instituut, Geograafia osakond.

LIST OF PUBLICATIONS

1.1 Articles in journals. Scholarly articles indexed by Web of Science Science Citation Index Expanded, Social Sciences Citation Index, Arts & Humanities Citation Index and/or indexed by Scopus (excluding chapters in books)

Dorbek-Kolin, E., Husso, A., Niku, M., Loch, M., Pessa-Morikawa, T., Niine, T., Kaart, T., Iivanainen, A., Orro, T. (2022) Faecal microbiota in two-week-old female dairy calves during acute cryptosporidiosis outbreak – Association with systemic inflammatory response. Research in Veterinary Science, 151, pp. 116-127. DOI: 10.1016/j.rvsc.2022.07.008

Peetsalu, K., Niine, T., Loch, M., **Dorbek-Kolin, E.,** Tummeleht, L., Orro, T. (2022) Effect of colostrum on the acute-phase response in neonatal dairy calves. Journal of Dairy Science, 105 (7), pp. 6207-6219. DOI: 10.3168/jds.2021-21562

Santoro, A., **Dorbek-Kolin, E.,** Jeremejeva, J., Tummeleht, L., Orro, T., Jokelainen, P., Lassen, B. (2019) Molecular epidemiology of Cryptosporidium spp. in calves in Estonia: High prevalence of Cryptosporidium parvum shedding and 10 subtypes identified. Parasitology, 146 (2), pp. 261-267. DOI: 10.1017/S0031182018001348

Seppä-Lassila, L., Oksanen, J., Herva, T., **Dorbek-Kolin, E.,** Kosunen, H., Parviainen, L., Soveri, T., Orro, T. (2018) Associations between group sizes, serum protein levels, calf morbidity and growth in dairy-beef calves in a Finnish calf rearing unit. Preventive Veterinary Medicine, 161, pp. 100-108. DOI: 10.1016/j.prevetmed.2018.10.020

Niine, T., **Dorbek-Kolin, E.,** Lassen, B., Orro, T. (2018) Cryptosporidium outbreak in calves on a large dairy farm: Effect of treatment and the association with the inflammatory response and short-term weight gain. Research in Veterinary Science, 117, pp. 200-208. DOI: 10.1016/j.rvsc.2017.12.015

Dorbek-Kolin, E., Åhlberg, T., Tummeleht, L., Tappe, D., Johansen, M.V., Lassen, B. (2018) Prevalence of cysticercosis in Estonian pigs and cattle. Parasitology Research, 117 (2), pp. 591-595. DOI: 10.1007/s00436-017-5710-9

Plutzer, J., Lassen, B., Jokelainen, P., Djurković-Djaković, O., Kucsera, I., **Dorbek-Kolin, E.,** Šoba, B., Sréter, T., Imre, K., Omeragić, J., Nikolić, A., Bobić, B., Živičnjak, T., Lučinger, S., Stefanović, L.L.,

Kučinar, J., Sroka, J., Deksne, G., Keidāne, D., Kváč, M., Hůzová, Z., Karanis, P. (2018) Review of cryptosporidium and giardia in the eastern part of Europe, 2016. Eurosurveillance, 23 (4), art. no. 16-00825. DOI: 10.2807/1560-7917.ES.2018.23.4.16-00825

3.4. Articles/presentations published in conference proceedings

Åhlberg, T., **Dorbek-Kolin, E**., Tummeleht, L., Johansen, M.V., Lassen, B. (2015) Prevalence study of cysticercosis in Estonian pigs and cattle. CSBSP6 (6th Conference of the Scandinavian Baltic Society for Parasitology), Uppsala, Sweden.

Kaura, R., **Dorbek-Kolin, E.,** Loch, M., Viidu, D.-A., Orro, T., Mõtus, K. (2022). Association between acute phase proteins and clinical signs of respiratory disease in dairy calves. 31st World Buiatrics Congress 2022 Abstract Book: 31st World Buiatrics Congress, Madrid, Spain.

3.5. Articles/presentations published in local conference proceedings

Åhlberg, T., **Dorbek-Kolin, E**., Johansen, M.V., Lassen, B. (2014). Prevalence study of cysticercoses in Estonian pigs and cattle. COST Action TD1302 CYSTINET. 1st Working group meeting & 2nd Management Committee meeting CYSTINET. Evora, Portugal.

5.2. Conference abstracts

Kaura, R., **Dorbek-Kolin, E.,** Loch, M., Viidu, D.-A., Orro, T., Mõtus, K. (2022) Systemic acute phase response (APR) to lower respiratory tract bacterial pathogens. SVEPM annual conference, Belfast, UK.

6.2. Textbooks and other study materials (excluding university textbooks)

Dorbek-Kolin, E., Karus, A., Praakle, K., Saar, T., Must, K., Randoja, H., Viltrop, A., Kõrgesaar, K. (2018). Biosafety and biosecurity manual. Estonian University of Life Sciences. http://hdl.handle.net/10492/5453

Dorbek-Kolin, E., Karus, A., Praakle, K., Saar, T., Must, K., Randoja, H., Viltrop, A., Kõrgesaar, K. (2018). Bioohutuse ja bioturvalisuse juhend. Estonian University of Life Sciences. http://hdl.handle.net/10492/5452

6.3. Popular science articles

Dorbek-Kolin, E. (2019). Krüptosporidioos – tundmatu vaenlane? Pilt, Ebe; Olvet, Triin (Toim.). Teadus kolme minutiga 2017-2019. Tallinn: Argo kirjastus. (Elav teadus; 21).

Timonen, A., Mõtus, K., **Dorbek-Kolin, E.,** Reimus, K., Aland, A., Viltrop, A. (2018) Veterinaarepidemioloogide ühingu aastakonverents 2018 Tallinnas. Eesti Loomaarstlik Ringvaade, 2, 23-25.

Giudici, A., **Dorbek-Kolin, E.,** Joonas, E., Roots, F.-S., Urvik, J., Uuemaa, M., Laas, O., Laansalu, T. (2017) Kuidas kolme minutiga maailma muuta? Sirp, 31, 31.

Dorbek-Kolin, E., Pirkkalainen, H. (2016) Konverents Eläinlääkäripäivät 2015 ülevaade. Eesti Loomaarstlik Ringvaade, 1, 19-20.

6.4. Popular science books

Dorbek-Kolin, E. (2018) Meenutusi elust enesest. Enn Ernits, Toivo Järvis (Ed.). Meenutusi: 170 aastat loomaarstiõpet Tartus. Eesti Maaülikool, pp. 302-307.

VIIS VIIMAST KAITSMIST

THAISA FERNANDES BERGAMO
KLIIMAMUUTUSE MÕJU HINDAMINE RANNANIIDU TAIMEKOOSLUSELE MESOKOSMI KATSE JA MEHITAMATA ÕHUSÕIDUKIGA KOGUTUD ANDMETE PÕHJAL
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28. november 2022

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ECOPHYSIOLOGICAL MECHANISMS CHARACTERIZING THE FRESH- AND BRACKISH MICROBIOTA
Juhendajad: professor **Daniel Philipp Ralf Herlemann**, doktor **Kairi Käiro**, teadur **Helen Tammert**
2. detsember 2022

MIHKEL MÄESAAR
LISTERIA MONOCYTOGENES'E JA CAMPYLOBACTER SPP. LEVIMUS JA ARVUKUS TOIDUS NING TÜVEDE MOLEKULAARNE ISELOOMUSTUS EESTIS
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MARILI VESTER
INVASIIVSE PAGOGEENI LECANOSTICTA ACICOLA GENEETILINE MITMEKESISUS, PÄRITOLU JA UUED PEREMEESTAIMED PÕHJA-EUROOPAS
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10. jaanuar 2023

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