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Cytokine production in equine peripheral blood mononuclear cells induced by SvSXP, a *Strongylus vulgaris* excretory/secretory protein

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Cytokine production in equine peripheral blood mononuclear cells induced by SvSXP, a *Strongylus vulgaris* excretory/secretory protein

Kan det exkretoriska/sekretoriska proteinet SvSXP från Strongylus vulgaris inducera cytokinproduktion i blodlymfocyter från häst?

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Abstract

The most pathogenic intestinal parasite in horses is *Strongylus vulgaris* (large bloodworm). When a horse becomes infected with *S. vulgaris*, the larva penetrates the intestinal wall and migrates through the horse's blood vessels. The parasite can cause serious injuries that can lead to severe colic and, in the worst case, death. Horses have been routinely dewormed since the 1970s, but in 2007, restrictions of deworming were introduced as increased resistance to some drugs (anthelmintics) was found in small bloodworms, cyathostomins. To be able to fight bloodworms and reduce the use of anthelmintics, a research project at the VH-faculty, SLU aims to develop a vaccine against *S. vulgaris*. When horses are infected with *S. vulgaris*, a type 2 immune response is activated in which the cytokines IL-4, IL-5, IL-9 and IL-13 are produced.

The purpose of the present study was to perform an *in vitro* experiment to investigate effects of parasite-derived excretory/secretory (ES) proteins on the cytokine gene expression. Equine blood leukocytes (PBMC) were used to analyze gene expression (up-regulation) for the following cytokines IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-α, TSLP, and IFN-γ to monitor the immune profile. First, attempts to extract ES-proteins to be used in the *in vitro* assays were made from different larval stages of cyathostomins because the access to *S. vulgaris* larvae was small compared to the number of cyathostomins. These attempts however failed, and therefore a recombinant *S. vulgaris* protein, SvSXP, was evaluated using equine PBMC. Different concentrations of SvSXP (0.1; 0.25; 0.5; 0.75 µg/ml) were used and qPCR was applied to examine whether the cytokines genes were upregulated.

Since no ES-proteins could be recovered from cyathostomin L3 larvae, only data from cytokine induction by the recombinant protein SvSXP is reported. Of the cytokines tested, upregulation of IL-9 and IFN-γ was observed, indicating a SvSXP-induced immune response in equine PBMCs. Thus, the SvSXP protein shows potential for further evaluation as an antigen in the development of a vaccine against *S. vulgaris*.

Sammanfattning

Den mest patogena tarmparasiten hos hästar är *Strongylus vulgaris* (stor blodmask). När en häst smittas med *S. vulgaris* tränger larven in i tarmväggen och migrerar genom hästens blodkärl. Parasiten kan orsaka allvarliga skador som kan leda till svår kolik och i värsta fall dödsfall. Hästar har rutinmässigt avmaskats sedan 1970-talet, men 2007 infördes begränsningar för avmaskning eftersom ökad resistens vid användning av avmaskningsmedel (anthelmintics) hittades hos liten blodmask (cyathostominer). För att kunna bekämpa blodmaskar och minska användningen av anthelmintika, pågår ett forskningsprojekt vid VH-fakulteten, SLU som syftar till att utveckla ett vaccin mot *S. vulgaris*. När hästar infekteras med *S. vulgaris* aktiveras ett immunsvar av typ 2 där cytokinerna IL-4, IL-5, IL-9 och IL-13 är närvarande.

Syftet med den här studien var att utföra ett *in vitro*-experiment för att undersöka effekterna av exkretoriska/sekretoriska (ES) parasitproteiner på cytokinproduktionen. Hästblodleukocyter (PBMC) användes för att analysera genuttryck (uppreglering) för cytokinerna IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-a, TSLP, och IFN-γ för att undersöka hur immunförsvaret reagerar. I de första försöken användes olika larvstadier av cyathostominer för att utvinna ES-proteiner som skulle användas i *in vitro*-analyserna. eftersom tillgången till *S. vulgaris* larver var liten jämfört med antalet cyathostominer. Försöken att isolera ES-proteiner misslyckades dock, varför förändringar av genuttrycket i PBMC från häst studerades med hjälp av ett rekombinant *S. vulgaris* protein (SvSXP). Olika koncentrationer av SvSXP (0,1; 0,25; 0,5; 0,75 μg/ml) användes och för att undersöka om cytokingenerna uppreglerades användes qPCR.

Av de testade cytokinerna kunde uppreglering av IL-9 och IFN-γ observeras vilket indikerade en immunreaktion i närvaro av SvSXP. SvSXP proteinet visar således potential för ytterligare utvärdering som ett antigen vid utveckling av ett vaccin mot *S. vulgaris*.

Content

Introduction

The large bloodworm (*Strongylus vulgaris*) is a parasite that infects horses and can pose major problems such as severe colic with serious consequences. The parasite can cause nonstrangulating intestinal infarction in the intestinal wall if thrombi in arteries or arterioles are formed due to the migrating bloodworm (Pihl *et al*., 2018). During the 70s, 80s and 90s in Sweden, it became common to use anthelmintics in oral pastas for deworming of horses. This easier handling increased the frequency of dewormings that most likely contributed to a development of resistance to some of the drug classes. In the related species, the small bloodworm, resistance against drugs containing benzimidazoles has developed but also other agents show reduced effect on the small bloodworm (Osterman & Tydén 2020).

To avoid building up anthelminthic resistance, restrictions were introduced in 2007 to ensure that only horses displaying clinical signs or high egg excretion are dewormed. The egg excretion is determined through testing feces from the horses. In 2018, a study by Tydén *et al*., (2009) was conducted to determine the prevalence of bloodworms in Sweden, ten years after the restrictions of using oral pastas for deworming were introduced. It turned out that 61% of the farms that participated in the study were infected by *S. vulgaris*, while only 14% of the farms in 1999 had *S. vulgaris* infections. (Tydén *et al*., 2019). Thus, alternative methods are needed to reduce the incidence of infections with *S. vulgaris*, *e.g.,* by development of vaccines against both the large and small bloodworms. At the section for veterinary immunology, SLU, a project financed by Stiftelsen Hästforskning aims to formulate a vaccine protecting against *S. vulgaris*.

The purpose of this Master´s thesis was to study if and how excretory/secretory (ES) proteins produced by equine bloodworms affect the cytokine production by equine peripheral blood mononuclear cells (PBMC). The studies were conducted *in vitro* analyzing the gene expression for a number of immune regulatory cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-α, TSLP, and IFN-γ) by qPCR.

Litterature review

Strongylus vulgaris

S. vulgaris is a parasite with a complex lifecycle from egg to its adult larval stage L5, as illustrated in Figure 1. Eggs produced by the adult females (egg-shedding adults) in the horse's colon and cecum are then later excreted via the horse's feces. In the feces, the eggs hatch and develop into L1 and then become L2 and then L3. When the bloodworm transforms into stage L3, it leaves the feces and live in the grass at the pasture, awaiting a host to infect (Nielsen & Reinemeyer 2018; Nielsen 2019). For development into L3, the worm needs a temperature between 8–39 ° C and a humid environment. At lower temperatures, it takes longer time for the bloodworm to develop into L3. At a temperature around 18 °C, 8-10 days are required, while at a temperature around 12 °C, 16-20 days are required for the development. The L3 stage is rather resistant to cold and dry environment and can survive a winter period on the pasture

before they end up within their host (Marchiondo *et al*., 2019). A study in Ukraine observed that a very small number of stage 3 larva could survive up to 12 months (Kuzmina *et al*., 2006). Only when the worm enters the horse's intestinal submucosa, it transforms from L3 to L4. The L4 stage penetrates the mucosa enters the blood vessels and migrates to the cranial mesenteric artery where it stays for 4-5 months and converts to its last stage, L5. After this transformation, the bloodworm reaches the central colon by returning to the intestinal wall through arterial lumina (Marchiondo *et al*., 2019). The size of the adult stage of the parasite differs between 20-40 mm (Equippo Lab).

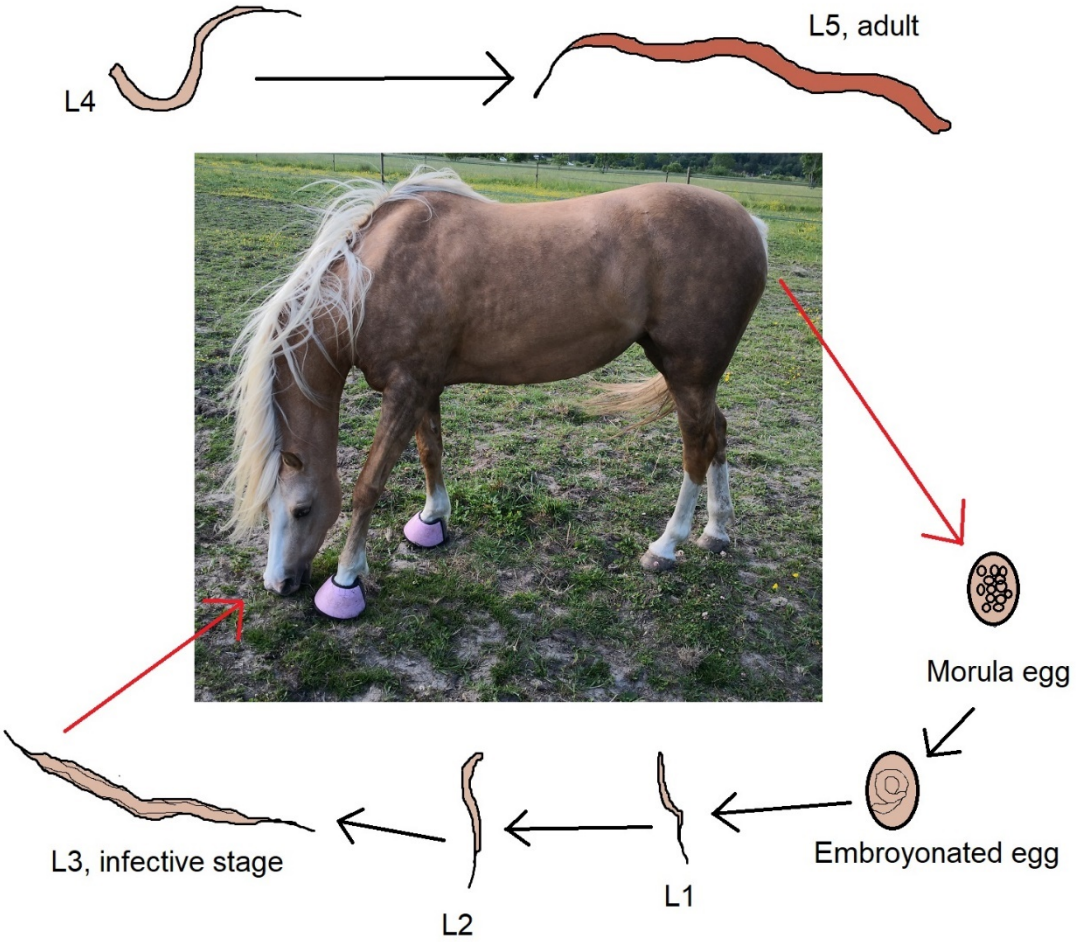


Figure 1*: Lifecycle of Strongylus vulgaris (Modified from Nielsen & Reinemeyer 2018)*

In Sweden, the prevalence of *S. vulgaris* in horses is increasing. At 40–60% of the herds, one or several horses are infected with this parasite (SVA, 2019). One of the reasons for this could be that the owners do not order specific tests discriminating between cyathostomins and *S. vulgaris* at examination of feces for presence of parasite eggs. An older study from US examining 43 ponies and 87 horses showed that 93% and 95.4% of the *S. vulgaris* infected animals, respectively, had arterial damage (Duncan, 1975). *S. vulgaris* develops into its fourth and fifth stages in the arteries, which means that blood flow to the large intestine can be blocked due to large thromboses caused by the larva (Nichol *et al*., 1987). When the blood supply to the intestine decreases, there may be a lack of oxygen transport into the tissue which

can cause painful colic and nerve damage and can even have a fatal outcome for the horse (Nichol *et al*., 1987; Caffrey & Ryan 1994: Nielsen *et al*., 2015; SVA, 2019)

Cyathostomins

Cyathostomins, also referred to as small bloodworms are the most common parasites in horses around the world (Corning 2009; Canever *et al*., 2013; Peachey *et al*., 2017). Unlike the large bloodworm, which reaches a size of 20–40 mm, the small bloodworm is only 0.5–20 mm (Equippo Lab). The lifecycle resembles that of the large bloodworm shown in Figure 1, but the time interval between the stages differs and the small bloodworm remains encysted in the intestinal mucosa. The parasite development from L1 to L3 is directly linked to temperature. At higher temperatures, the small bloodworm can develop from L1 to L3 in just three days. When the parasite is in the L3 stage they can infects the horse and develop into L5 in just 5–6 weeks after infection. The L5 stage migrates to the horse's cecum where they can lay the eggs (Corning 2009).

Large numbers of the small bloodworm can also cause problems in horses such as weight loss, lack of energy, diarrhea, weakness, severe colic and in worst case, death (Corning 2009; Canever *et al*., 2013; Peachey *et al*., 2017). In large numbers, these worms can cover the cell walls of the intestines, making it difficult for the body to absorb the nutrients (Corning 2009; Canever *et al*., 2013). In the final stage (L5) it poses an additional and serious problem for the horse. This condition called "larval cyathostominosis" can occur when large numbers of worms pass into the gut lumen, potentially causing great damage to the intestines and even creating severe colic and diarrhea. The mortality rate for this condition can be up to 50% (Corning 2009). Luckily, larval cyathostominosis is a rare condition (Nielsen, 2019).

The development of resistance to some anthelminthic drugs among the small bloodworm is an increasing problem worldwide (Corning 2009; Canever *et al*., 2013; Peachey *et al*., 2017; Tydén 2020). In 2007, the deworming routine was changed so a prescription, based on egg counting results, is needed to get deworming drugs (Tydén 2020). The resistance is primarily against Fenbendazole, Benzimidazole and Pyrantel (Corning 2009; Canever *et al*., 2013; Tydén 2020).

Excretory and secretory proteins

For the bloodworm to survive and avoid the hosts immune system, one of its strategies is to produce so-called ES-proteins (excretory/secretory proteins). These proteins are directly secreted in the extracellular matrix or excreted via vesicles (exosomes). ES-proteins have several important functions such as proteolysis, adhesion, and extracellular matrix organization. The ability to produce excretory and secretory proteins is regarded as a virulence factor. Through ES proteins, the parasite can control and regulate its host's immune reaction so that it poses no danger and thereby ES proteins play an important role for the parasite's survival in its host (Gomez *et al*., 2015).

Virulence factor refers to the Latin word virulentia, which means virus, that later was translated to poison. Vitulence refers to the intensity or degree of disease that an organism can cause in its host. By virulence factors, the organism has the special characteristic of being virulent (Willey *et al*., 2012). A virulent parasite utilizes the resources from the host without making significant damage, which gives the parasite time and opportunity to reproduce inside the host (Frank 1996).

By using ES proteins as a biomarker, the presence of a parasite in the host can be indicated. Because the host's immune system can identify most ES proteins as foreign, they can be immunogenic (Gomez *et al*., 2015). It is therefore important to study how the ES proteins are composed to better understand how they affect the host's immune system. It is also possible that ES proteins can be useful as vaccine antigens (Feng *et al*., 2009).

To use ES proteins, you need to isolate them. Since the larva secretes these proteins during their infective stages, the ES proteins need to be collected *in vivo* or at *ex vivo* conditions simulating infection. In the study of Gadahi *et al*. (2016), two goats were infected with 10,000 worms of the *H. contortus* species in stage L3. At this stage, the larva is infective. When the infection was established and confirmed by egg counting, the animals were killed after 27 days for collection of adult worms in stage L5 and subsequent isolation of ES proteins. In the present Master´s thesis, laboratory experiments were preformed to extract ES proteins from cyathostomin larvae hatched from eggs in equine feces. The trials were made *in vitro* (outside biological context).

Cytokines

The main function of cytokines is to communicate between different cells and “direct” their interactions with each other. Cytokines are mainly produced by immune cells, myeloid and lymphoid cells but also other cells such as intestinal cells can produce a variety of cytokines. These proteins are produced when an immune response or an inflammatory response is necessary, such as at an infection or injury of the host. Then cells need to communicate with each other to activate the immune system. The same type of cytokine can be produced by different cell types. Different cytokines can have similar functions, also different functions can be performed from the same cytokine. Cytokines can both potentiate and down-regulate the production of each other, and the specific immune response tends to be dominated by T-helper cells of type 1 (Th1) or type 2 (Th2) (Sjaastad *et al*., 2010 & Arunabha *et al*., 2016).

When a parasite infects a host, the outer layer of cells in the gut, the epithelial cells, is the first tissue that comes in contact with the parasite. How these cells respond to the parasite damaging the tissue and breaking through the epithelial barrier that create an inflammation is still not fully discerned. When a parasite infects its host, it is generally inducing a T helper type 2 reaction. As the size of a helminth parasite is much larger in proportion to the cells of the immune system and other microbes, such as bacteria, viruses and fungi, the body must handle an infection from a parasite in another way. The body is creating an environment in the gut to disturb the parasite with help from the type 2 immune reaction. This means that the barrier that the worm needs to penetrate will be protected and the tissue to heal faster. In this early innate immune reaction,

the cytokine TSLP is activated which favors production of the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 (Sorobetea *et al*., 2018).

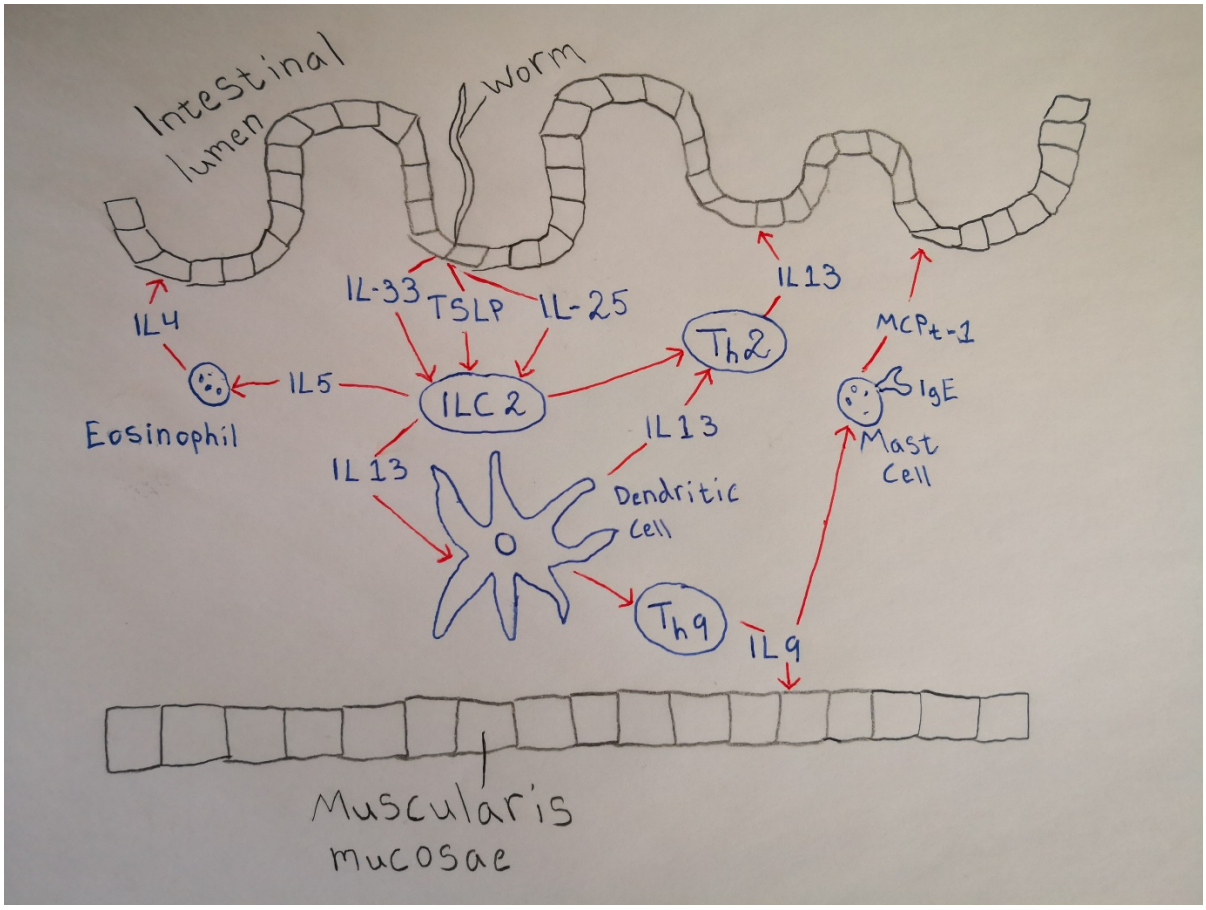


Figure 2*: A sketch showing cytokines involved in type 2 immune responses to a parasite infection*

SvSXP

There is no commercially available serological method today to diagnose that a horse has been infected by *S. vulgaris*. Only by examining the horses' feces for the presence of eggs and determining the number of eggs per gram feces (EPG value) can it be established that the horse has become infected by the small or the large bloodworm. The eggs of the large and small bloodworm cannot be separated by morphology, but culturing to make the eggs hatch and become L3 larvae generates a stage at which they can be differentiated from each other. In addition, *S. vulgaris* can reside in infected horses for more than four months without producing eggs and the infection will during that period not be detected by conventional egg counts. When the frequent deworming against bloodworms became restricted due to an increased resistance against some anthelminthic drugs noted in the small bloodworm, the prevalence of *S. vulgaris* increased. (Andersen *et al*., 2013).

In the presence of both *S. vulgaris* and cyathostomins, the horse's immune system produce antibodies IgG(T) (Andersen *et al*., 2013). IgG(T) is made from two different isotypes (IgG3 and IgG5), also called IgG3.5. The horse has up to 7 IgG isotypes, also called IGHG genes with different functions (Wagner *et al*., 2004). Regardless, the specificity of the antibody makes it possible to determine an immune response to a certain parasite. In case of infection with cyathostomins, the presence of IgG(T) can be detected by ELISA 5 weeks after infection. (Dowdall *et al*., 2002). However, it is more difficult to determine the time of infection with *S. vulgaris* by ELISA as the levels of IgG(T) specific for *S. vulgaris* may remain from previous infections (Nielsen *et al*., 2015).

In an experiment by Andersen *et al*. (2013), an ES-protein from *S. vulgaris* (SvSXP) was studied for its potential use in detection of an immune response to the parasite. The study involves 102 horses at different ages and showed a correlation between the number of *S. vulgaris* worms and the presence of SvSXP IgG(T) antibodies, where only horses >7 months of age produced antibodies to SvSXP. However, many younger healthy foals displayed no antibody production despite a high prevalence of *S. vulgaris,* suggesting that only migrating larval stages induce an immune resposne to SvSXP (Andersen *et al*., 2013).

The use of the ES-protein SvSXP as an antigen candidate for the diagnose of *S. vulgaris* infection looks promising. The presence of IgG(T) antibodies in horses infected with *S. vulgaris* is high (Andersen *et al*., 2014). Further experiments with SvSXP were performed by Nielsen *et al*. (2014) on foals naturally infected with *S. vulgaris* to study whether they develop antibodies to SvSXP using an ELISA technique. In accordance with Nielsen *et al*. (2014), foals exposed to high numbers of worms did not form antibodies to *S. vulgaris* earlier than 12 weeks after infection, and no response was detected earlier than five months. These findings are in agreement with the suggestion that SvSXP is produced in the later larvae stages (Nielsen *et al*., 2015).

Helminths and the immune system of horse's

When horses become infected with helminths, the immune system will react to the foreign object. However, innate immunity is not enough to protect the horse from the bloodworms. This means that the parasites are not eliminated by the horse's early defenses. The horse's immune system reacts more strongly when there are higher numbers of parasites that develop into L3 larvae, which can protect older horses from the serious consequences that can lead to acute arteritis and colic. On the other hand, foals and younger horses that have not developed any immunity against the parasites can become very ill with a severe infection (Klei, 1986). Parasites in the gut cause a local inflammation mainly mediated by lymphocytes, mast cells and eosinophils (Dennis *et al*., 1992; Pittaway *et al*., 2014). In addition, presence of parasite in the lumen can affect the bacterial environment in the horses' intestines. A disturbance in the bacterial flora caused by helminths interacting with the gut bacterial microbiome can be a triggering factor in horses' suffering from colic (Walshe *et al*., 2019).

When parasites infect their host, Th2 cytokines are commonly produced in response to the ES proteins that the parasite produces. This allows the immune system to produce cytokines such as IL-4, IL-5, IL-10, and IL-13 (Mulchay *et al*., 2004; Mulchay *et al*., 2005; Coakley *et al*., 2016). Although the body responds to the invasion, the parasite has developed several ways to protect itself and evade the host's response. In humans, helminths are known to block responses from Th1-Th17 cells, which are an important part of the immune system's response to parasite infestations (McSorley *et al*., 2013)

Material and method

Collection of cyathostomins

Fecal egg counts in 4 horses were performed using the McMaster technique (Zajac *et al*., 2014). Three grams of feces were mixed with 42 ml of water and filtered through a 150 µm cell-strainer removing fecal debris. To obtain the eggs the filtrate was centrifuged in a 50 ml tube for 3 min at 248 x g and the supernatant discarded. Eggs were resuspended in a saturated saline solution and, floating eggs collected and counted in a light microscope using the McMaster counting chamber. With the dilution steps one counted egg represents 50 eggs in one gram of feces and eggs per gram of feces (EPG) was calculated for each horse. Bloodworm eggs were found in the feces of the four horses, with an EPG ranging from 300 to 1600 (Table 1).

Table 1. Eggs per gram feces (EPG)

Horse	EPG
1	650
2	1600
3	800
4	300

To obtain bloodworm larvae, feces from horse 1-4 were mixed with 50% vermiculite and moisturized with water. The fecal cultures were incubated in a jar placed in a humid environment at room temperature. After 14 days the jars were turned upside down on a dish with high edges. Water was added in the dish to allow the worms to swim out. The next day, the water and worms were collected and washed once in PBS. Morphological analysis of the samples revealed the presence of cyathostomins but not *S. vulgaris*.

In vitro culture of cyathostomin for isolation of ES-proteins

Cyathostomins were harvested and used for the *in vitro* experiments. In the first experiment intended to isolate proteins excreted/secreted during culture (ES proteins), 50, 100 and 150 worms were transferred to three separate 50 mL tubes containing 10 ml PBS. The worms were washed in PBS and the larvae were pelleted by centrifugation at 650 rpm for 5 minutes. The supernatants were removed, and the tube was filled up with RPMI containing 2 % PEST and 1µg/mL Fungizone, followed by centrifugation at 650 rpm for 5 minutes. The larvae were then transferred to three separate cell culture flasks, containing 40 ml culture medium, *i.e.* phenol-free RPMI 1640 supplemented with 2% PEST, 1 µg/ml Fungizon and a protease inhibitor

cocktail (Sigma), and incubated at 37 °C for three days. Once a day, the worms were examined by light microscopy, and dead parasites were removed using a 10 µl pipette. After three days, the media and larvae were transferred to 50 mL tubes and centrifuged at 650 rpm for 5 min. Supernatants were collected and used for protein quantification analysis. The amount of excretory and secretory larval proteins was estimated according to the Microplate Assay Protocol from DC protein assay Instruction (Bio Rad). A protein standard was made using bovine serum albumin (BSA) starting at 2 mg/ml protein, and then diluted stepwise 1:2. Up to eight dilutions were made and the last concentration contained 0.015 mg/ml. The larval ES-samples were diluted in series 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 1: 64 and 1:128 and 5 µl of each dilution were first placed in each wells of a dry microplate. A total of 50 wells were used for the test where two negative controls were included with water. Then 25 µl of Reagent A supplemented with 20 µl/ml of Reagent S was added in each sample. Finally, 200 µl of reagent B was placed in each well and incubated for 15 min. The absorbance was read at 650 nm (Multiscan).

Due to undetectable levels of ES-protein, the protocol was modified. Approximately 1000 cyathostomin L3 larvae were picked under the microscope using a 10 µl pipette and placed in a 50 ml tube and then washed one time in PBS and two times in RPMI containing 2 % PEST and 1 µg/ml Fungizone. Then, the larvae were cultured in 40 ml culture medium with at 37 ° C for 3 days. The worms were examined every 24 hours to study their activity. At day three, the medium containing larvae was placed in a 50 ml tube and centrifuged for 5 min at 650 rpm the supernatant was collected and concentrated using Cetriprep® 3K centrifugal filters (Merck Millipore). This filter concentration was performed by placing 15 ml of liquid in the tube and centrifuging three times. First spin for 90 minutes, second 35 minutes and third 10 minutes at 3000 x g. By this, 15 ml of the supernatants were concentrated to 0.6 ml before assayed undiluted and then 1:10 followed by dilutions of 1:2. The amount of protein was measured by the same method as before with the Microplate Assay Protocol but using a Tecan reader to determine the absorbance at 750 nm.

Further attempts were made with 1000 worms in the same way as in the previous trial but, using ex-sheated worms. For ex-sheatment, the worms were collected and placed in 10 ml tube with PBS, centrifuged 5 min at 400 rpm. The supernatant was removed, leaving approximately 1 ml with the worms. Five ml of pre-warmed (37°C) 0.1% Milton solution was added an incubated for 3-4 minutes in room temperature for the ex-sheating to occur. To stop the process, 5 ml of PBS were added, and the tube was centrifuged at 400 rpm for 5 minutes. The supernatant was removed, and the worms was examined in the microscope to see if they had ex-sheated. The worms were then washed with RPMI containing 2% PEST and 1 µg/ml amphotericin three times. After three days of incubation, the Microplate Assay Protocol was used to determine the protein content in the growth medium.

Isolation of PBMC, cultivation and *in vitro* stimulation with recombinant SvSXP protein

Blood from 4 horses was collected in 3 heparinized tubes for each horse. The blood was pooled individually into larger tubes and let to sink for 15-20 minutes. Five ml of the leukocyte enriched plasma was placed on 2.5 ml Ficoll Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) in 15 ml tubes and centrifuged at 1700 rpm for 25 min with no brake. After the density centrifugation the PBMC were enriched as a band on top of the Ficoll solution. A pipet was used to collect this layer and transferred into a new tube. The cells were washed in PBS and centrifuged at 1000 rpm for 8 min without break. The pelleted cells were resuspended in new PBS and washed 3 times more. After the last wash, 4 ml of the growth medium was added, and the cells were counted. The cell concentration was adjusted to 5-6 x 10^6 cells/ml. For each horse, a 6-well plate was used. One ml of the cell suspension was placed into 5 wells and incubated at 37°C with 6.5% CO_2 in air for 1-2 hours. Meanwhile, 5 different concentrations of SvSXP were diluted: 0, 0.1, 0.25, 0.5 and 0.75 µg/ml. One ml of the medium with and without SvSXP was added to each well with equine PBMC.

For *in vitro* culture of equine PBMC a growth medium was used consisting of RPMI 1640 medium (BioWhittaker, Cambrex Bioscience, Verviers, Belgium) supplemented with HEPES (20 mM), l-glutamine (2 mM), penicillin (200 IU/mL), streptomycin (100 µg/mL), 2-mercaptoethanol (50 µM), and 5% fetal calf serum (Invitrogen, Life Technologies, Carlsbad, CA, USA).

Harvesting of cells

After 18 hours of incubation, the cells were harvested. The cells were transferred to 2 ml tubes and centrifuged at 500 g for 5 min and the supernatant was discarded. Meanwhile, 1 ml Trizol was added to each empty well to lyse remaining cells that had adhered to the bottom of the well. The trizol containing lysed cells was transferred to its corresponding tube for lysis of the cells before storage at -80°C.

RNA preparation from Trizol samples

Extraction of RNA was performed by combining Trizol with the EZNA Total RNA Kit (Omega Bio-Tek, Norctoss, GA). The trizol containing lysed cells were mixed with a pipet 5-10 times and incubated at room temperature (RT) for 5 minutes. 200 µl of chloroform was added to each tube, vortexed for 15 seconds and incubated 2-3 min at RT. The tubes were then centrifuged at 12 000 g at 4°C for 15 min. The upper phase in the tubes was transferred to a 1.5 ml Eppendorf tube and an equal volume of 70% Ethanol was added. 600 µl of this mixture was applied on an RNeasy spin column (EZNA total RNA kit) placed in a 2 ml collection tube and centrifuged for 60 seconds at 10 000 g. The void volume in the bottom of the tube was discarded before 500 µl of RNA wash buffer I was added to the column and the centrifugation was repeated. This procedure was repeated two times with RNA wash buffer II. After the last wash, the spin column was placed into a new collection tube and centrifuged for 2 min at 14 000 g. Thereafter, the column was placed into a new Eppendorf tube 1.5 ml, and 35 µl of DEPC-water was added and incubated for 2 min before a new run into the centrifuge

at 14 000 g for 2 min at RT. The RNA concentration was then measured by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Montchanin, DE).

cDNA synthesis

cDNA was synthesized using the GoScript Reverse transcription system (Promega) as outlined below.

Preparation of reagents

Eight tubes were marked "+rt" and in each tube the following substances where added: 1.2 µl 10xRxn buffer, DEPC-water (the amount depending on the RNA concentration) and 1.2 µl DNAse. Two new 1.5 ml tube were used, one with 29 µl oligo dT and the other one with 11 µl DNase STOP. Two new 1.5 ml tubes were used to make the master mix for +rt and -rt. In each the following substance were added: Goscript 5x reaction buffer, PCR nucleotide mix, RNAsin and Nuclease free water (different amount in (+rt and -rt). In +rt, Goscript reverse transcriptase was added. The tubes without reverse transcriptase (-rt) were included as a control for the DNAse reaction and later checked for any contaminating genomic DNA.

DNAse reaction

Into the eight tubes marked +RT, 1.2 µg RNA from the RNA preparation was added in each tube with different volume and incubated for 30 min at 37°C. Thereafter, 1.2 µl DNase-STOP was added in each tube and incubated at 65°C for 10 min.

cDNA reaction

In each of the eight tubes of +rt, 3.3 µl oligo-DT was added and incubated at 70°C for 5 min and then on ice for 5 min. 2.75 µl from each +rt tube was transferred to eight new tubes marked -rt. Into each of the +rt tubes, 41.25 µl of the +rt master mix was added and 8.25 µl -rt master mix into the eight tubes -rt. After 5 min the eight tubes with both the +rt and -rt were incubated at 42°C for 1 hour before being incubated at 70°C in for 15 min.

qPCR

In this step, gene expression for the different cytokines was estimated using RPL32 as a reference gene. First the cDNA was diluted 1:5. Then, the primers were mixed with the PCR-mix (Quantitect SYBR Green PCR mix, Qiagen), F (forward primer), R (reverse primer) and water in Eppendorf tubes using different volumes for different cytokines. 23 µl of the qPCR mix was added to each well and then 2 µl of the cDNA in each well. A sealing tape was placed over the wells and the plate was centrifuged a few seconds before running in the qPCR machine using the following cycles:95°C for 15 min to open the DNA strings followed by a lower temperature in 30 seconds, optimized for every primer pair. Thereafter the temperature was increased again to 72°C to make the replicates. These steps were repeated 39 times to make more replicates and later, detect a Cq value (Hellman *et al*. 2018; Hellman *et al*., 2021).

Table 2: Primer data

Cytokine	Water (µl)	F and R (µl)	PCR-mix (µl)	Primer concentration (µM)	Anneal temperature °C (Optimized)
IL-4	357	42	525	0.4	55
IL-5	336	52,5	525	0.5	58
IL-6	357	42	525	0.4	55
IL-9	336	52.5	525	0.5	59
IL-10	336	52.5	525	0.5	58
IL-13	357	42	525	0.4	56
TNF-α	336	52.5	525	0.5	56
TSLP	357	42	525	0.4	55
IFN-γ	357	42	525	0.4	55

Calculation

Differences in gene expression vas calculated according to Vandesompele *et al.* (2002).

By collecting the Cq value from the reference gene, RPL32, and the Cq values for the genes of interest the difference in Cq values (ΔCq) was calculated:

$Cq_{(RPL32)} - Cq_{(x)} = \Delta Cq$

Then we want ΔΔCq. For each horse we run samples with no SvSXP (medium control) and 4 different concentrations of SvSXP. We will use the $\Delta Cq_{(med)}$ and the ΔCq from the different concentrations of SvSXP (x).

$\Delta Cq_{(x)} - \Delta Cq_{(med)} = \Delta\Delta Cq$

With the ΔΔCq, we can calculate the fold change by:

$2^{-\Delta\Delta Cq}$

A fold change value above 2 indicates an up-regulation compared to the medium control. A fold change value under 0.5 indicates down-regulation and between 0.5–2 is not differentially expressed.

Results

Attempts to isolate ES proteins from cyathostomins

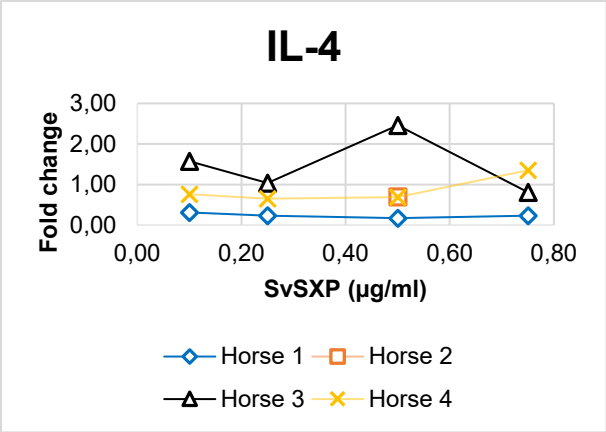
In the first experiment with 50, 100 and 150 worms, the amount of protein was under the detection limit for the method applied. Therefore, more worms (1000 worms) were used in the following experiment and filters were used to concentrate the amount of protein from the culture supernatant. Also, culture supernatants from the first experiment were concentrated by filtering showing that the amount of protein was in the same low range in both experiments (Table 3). Another attempt was performed to isolate ES proteins using 1000 ex-sheated worms. This experiment also showed similar concentrations as the last attempt (Table 3). After the same concentration process was done by using CFU-filter in both experiment with 100 worms (concentrated 15 ml to 0,6 ml). The protein concentration was the same. Thus, it turned out that the amount of protein determined in both experiments with 1000 worms most likely reflected the amount of protein already included in the RPMI medium (glutamine). Therefore, the protein concentration was determined in the plain RPMI medium supplemented but without worms. The RPMI was also concentrated with CFU-filters (15 ml to 0,6 ml). Also, this amount of protein was close to the previous recorded concentrations (Table 3). This means that no ES-proteins could be isolated at the present experimental condition.

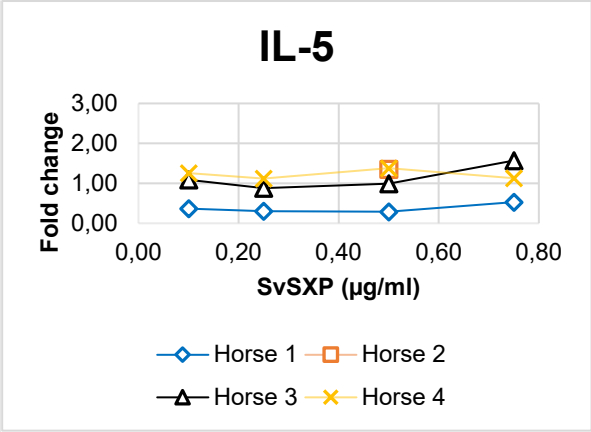
Table 3: Protein concentration

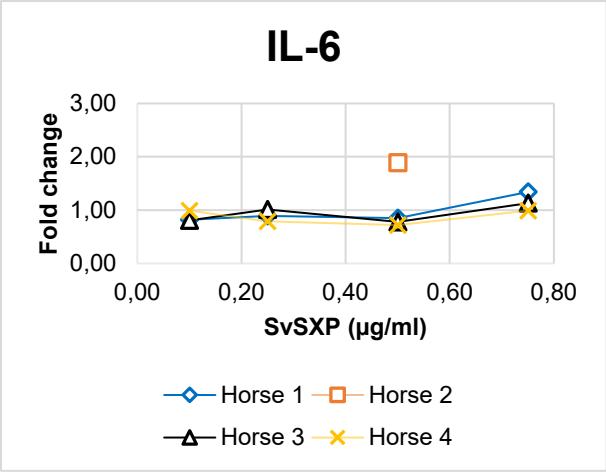
Samples	Protein concentration (μg/ml)
Plain RPMI	470.6
L3 larva	452.9
L3 Exsheated larva	478.8

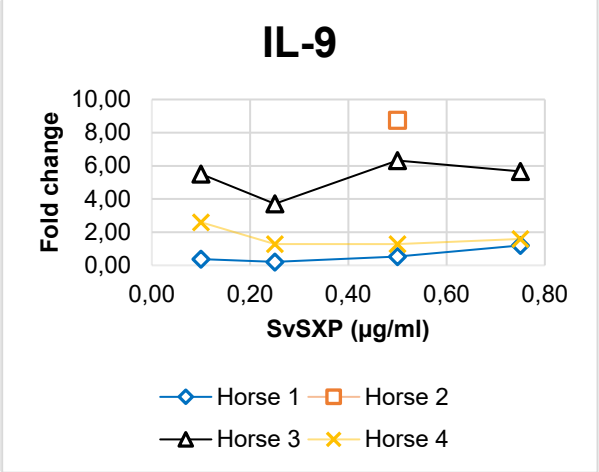
Using SvSXP

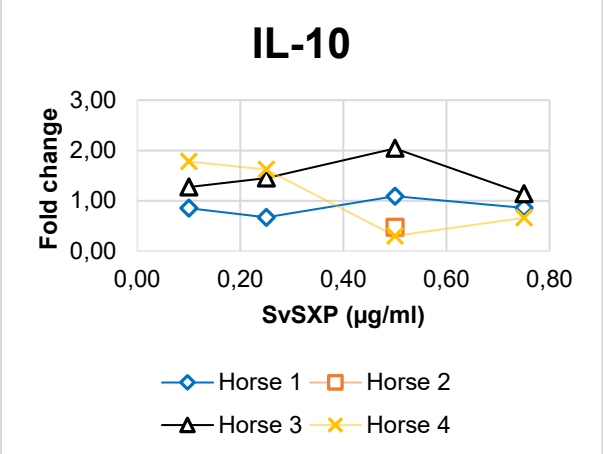
Instead of collecting our own ES-proteins from cyathostomins or *S. vulgaris* we therefore used a recombinant protein from *S. vulgaris*, SvSXP, in our further experiments with equine PBMC. During the procedure, some of the blood from horse 2 was lost and only the medium control and 0.5 μg/ml SvSXP could be analyzed. The relative gene expression for IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-α, TSLP and IFN-γ in PBMC collected from four horses as determined by qPCR is summarized in Figures 3 and 4.

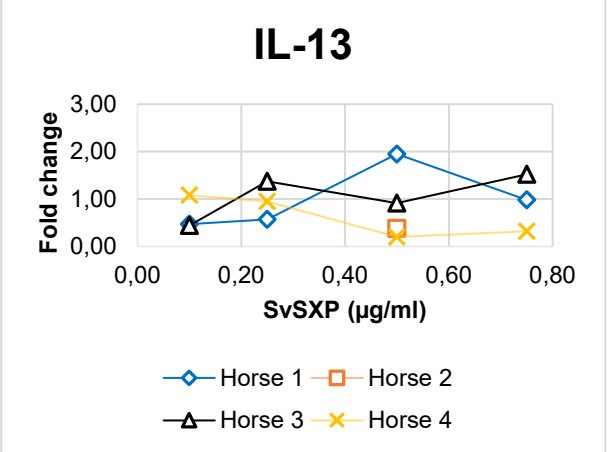

IL-4
Fold change
3,00
2,00
1,00
0,00
0,00
0,20
0,40
0,60
0,80
SvSXP (µg/ml)
Horse 1
Horse 2
Horse 3
Horse 4


IL-5
Fold change
3,00
2,00
1,00
0,00
0,00
0,20
0,40
0,60
0,80
SvSXP (µg/ml)
Horse 1
Horse 2
Horse 3
Horse 4


IL-6
Fold change
3,00
2,00
1,00
0,00
0,00
0,20
0,40
0,60
0,80
SvSXP (µg/ml)
Horse 1
Horse 2
Horse 3
Horse 4


IL-9
Fold change
10,00
8,00
6,00
4,00
2,00
0,00
0,00
0,20
0,40
0,60
0,80
SvSXP (µg/ml)
Horse 1
Horse 2
Horse 3
Horse 4


IL-10
Fold change
3,00
2,00
1,00
0,00
0,00
0,20
0,40
0,60
0,80
SvSXP (µg/ml)
Horse 1
Horse 2
Horse 3
Horse 4


IL-13
Fold change
3,00
2,00
1,00
0,00
0,00
0,20
0,40
0,60
0,80
SvSXP (µg/ml)
Horse 1
Horse 2
Horse 3
Horse 4

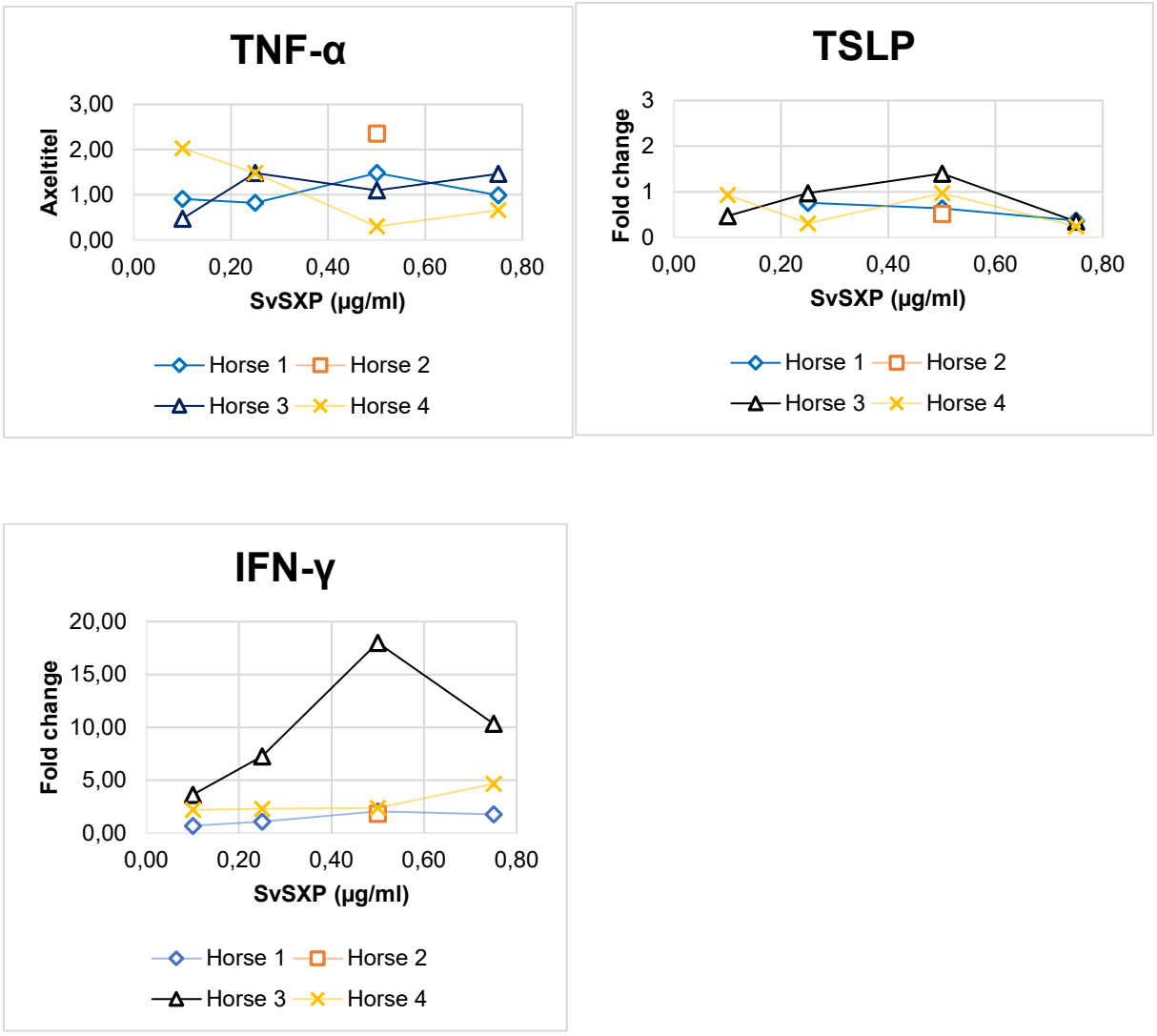


Figure 3: *Relative gene expression (fold change, FC value) for the cytokines IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-α, TSLP, and IFN-γ in PBMC collected from four horses and cultured in 18 hours. RPL32 was used as reference gene.*

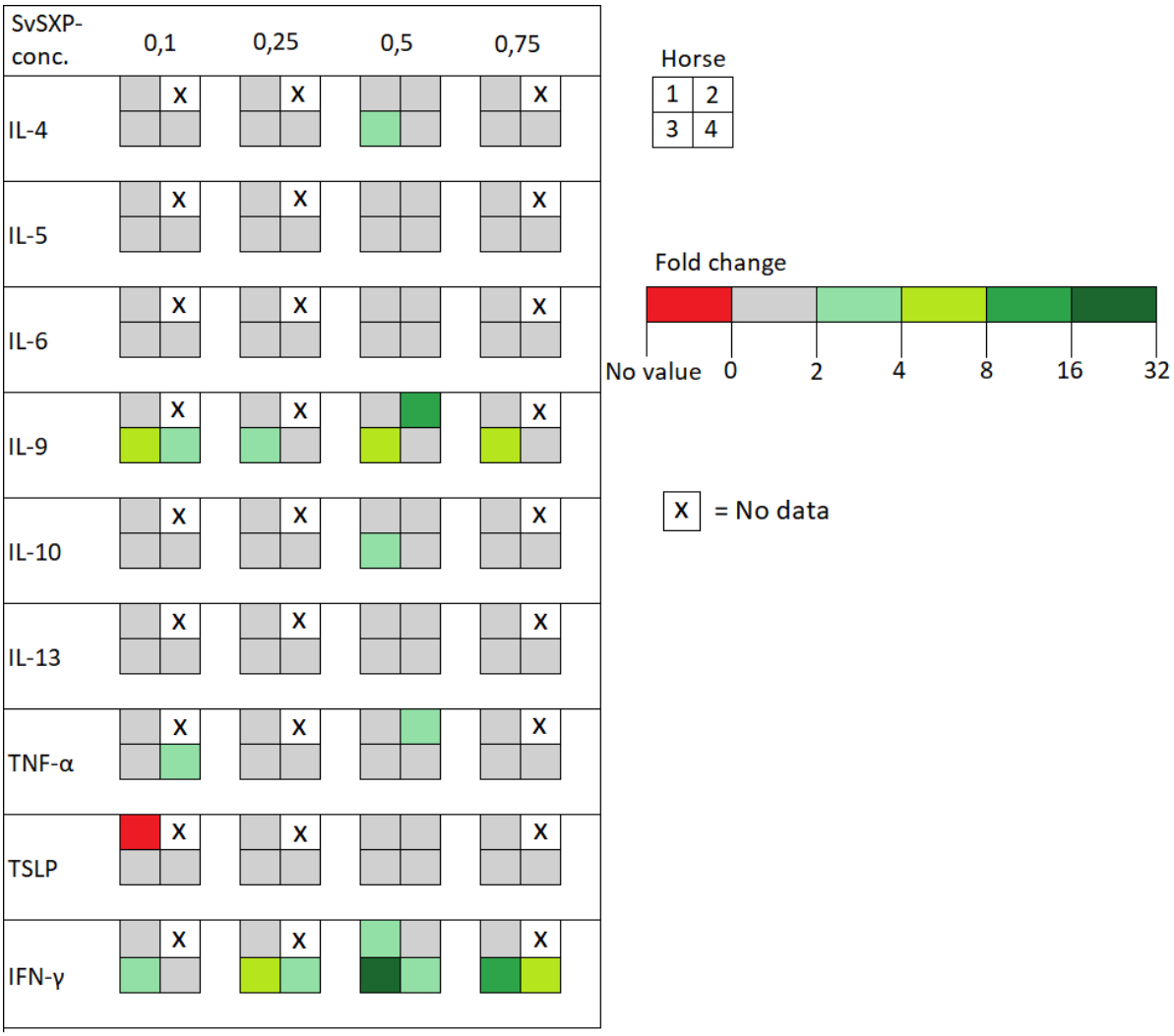


Figure 4: *Total view over the fold change value from cytokines IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-α, TSLP and IFN-γ. PBMC collected from four horses and cultured for 18 hours with SvSXP concentration 0.1; 0.25; 0.5 and 0.75 µg/ml. Red color indicates that no value could be calculated and grey indicates no gene expression. The four shades of green indicate the grade of gene up-regulation.*

After 18 hours incubation of the PBMC with different concentrations of SvSXP, an up-regulation of IL-9 and IFN-γ was seen in three of the four horses (Fig 4), especially at a concentration of 0.5 SvSXP. Also, IL-4 and IL-10 were occasionally slightly up-regulated. For TNF-α, horse one and three showed a modest up-regulation at concentration 0.5. The medium control was not detected in the TSLP assay. Therefore, the reactions to the various SvSXP concentrations were normalized to the 0.1 µg/mL SvSXP sample instead of the medium control. The calculation with medium control is $\Delta Cq_{(x)} - \Delta Cq_{(med)} = \Delta\Delta Cq$. Instead the calculation for ΔΔCq in TSLP is: $\Delta Cq_{(x)} - \Delta Cq_{(0.1)} = \Delta\Delta Cq$

Discussion

Isolation of ES-proteins

Several attempts were made to isolate excretory/secretory proteins (ES-proteins) from cyathostomins using L3 larvae. The access to *S. vulgaris* was low and therefore cyathostomins were used in the attempts to set up a procedure for isolation of ES-proteins. Unfortunately, these attempts failed as no ES-proteins could be extracted. In contrast, Gadahi *et al*. (2016) using goats infected with *Haemonchus contortu* that were killed when the larvae had reached the L5 stage, succeeded to extract ES-proteins. According to Rathorea *et al*. (2006) it may be that L3 larvae do not produce any ES-proteins at *in vitro* culture. Thus, the *in vitro* conditions could contribute to our failure in isolation of ES-protein also from the ex-sheated cyathostomins L3 larvae. When the parasite is in contact with the intestinal mucosa, they develop into L4 and L5 that later are exposed to the host's immune system and might then start to produce ES-proteins. Thus, by using larvae in stage L4 or L5, ES-proteins could maybe be extracted. In a study by Paz-Silva *et al*. (2011) similar attempt to extract ES-production from cyathostomin were successful, using a higher concentration of worms. In the present study, only 1000 larva were used in 40 ml RPMI and in Paz-Silva *et al*. (2011) study, 1000 larva / 1.5 ml RPMI were used. The concentration of larva / ml RPMI was thus higher and could be a major factor explaining the failure to isolate ES-proteins in this study.

Cytokine reaction

When a parasite invades its host, it is in most cases the type 2 helper cells that reacts (Henry *et al*., 2017). To study which cytokines that are likely to be induced at infection with *S. vulgaris*, a recombinant ES protein, SvSXP, was used as an antigen. The results show that of the nine cytokines tested only IL-9 and IFN-γ were up-regulated. IL-9 is a cytokine that is produced by type 2 innate lymphoid cells (ILC2) and by Th2 cells. The cytokine IL-9 can activate mast cells that together with ILC2 form a strong link to promote Th2 differentiation (Goswami & Kaplan, 2012; Henry *et al*., 2017). In humans, the presence of IFN-γ could indicate an anti-parasitic response (Bando *et al*. 2018). Mostly, the immune Th2 response is protective. But parasites can mislead the immune system to their advantage and to protect themselves, the parasite can re-direct the Th2 response to a Th1- type with production of the cytokine IFN-γ. In a study by Hellman *et al*. (2019), up-regulation in IFN-γ was observed in cell culture with equine PBMC and UV-irradiated *S. vulgaris* larvae. The reason for that reaction could be to prevent immunopathology with self-regulating mechanism in Th1. In humans, immune cells produces IFN-γ in response to the ES production from hookworm in vitro (Hsieh *et al*., 2004) but still the outcome could be very different *in vivo* (Seladi-Schulman 2020).

The noted up-regulation of IL-9 and IFN-γ in the present study, gives an indication that the horses' immune cells react to SvSXP. However, it is difficult to determine in this experiment whether it is Th2 that is activated as other cytokines such as IL-4, IL-5 and IL-13 that also belong to Th2 profile were not up-regulated.

The use of FCS (fetal calf serum) could affect the result. For the cells to grow, the use of serum is required. The serum contains proteins that can affect the cells and the outcome of the results (Hoekstra & Scherpho 1997). FCS proteins can stick to other surface and pose a risk to neutralize the recombinant protein (Personal communication with Hellman 2021) Also, ES-proteins could be a decoy antigen (Bungiro & Cappello 2005). By decoy antigen, it means that the proteins can deceive horse's immune system.

Horses

An individual variation in the cytokine response was evident as shown in Figure 4. Horse's numbers 2 and 3 reacted more strongly to SvSXP compared to horses 1 and 4. The immune system reacts when a foreign substance enters the body, and this reaction may be more active to a foreign substance that the individual has been exposed to previously (Sjaastad *et al*., 2012). When examining the presence of bloodworms in the horses that were part of this project, it turned out that all the horses had eggs of bloodworms in their feces. Horses 2 and 3 displayed higher egg counts (1600 and 800 EPG, respectively) than horses 1 and 4 (650 and 300 EPG, respectively). The different numbers of bloodworms in the horses could affect the immune response to SvSXP. In the study with Andersen *et al.* (2013), horses with no previous infection and low numbers of *S. vulgaris* also had a low immune reaction.

Using recombinant SvSXP instead of native excretory/secretory proteins

Since no ES proteins could be recovered from our *in vitro* cultures of cyathostomins, the recombinant protein SvSXP was used as antigen from *S. vulgaris*. There is currently only one study (Andersen *et al*., 2013) describing that SvSXP could be used as a potential antigen in the diagnosis of infections with *S. vulgaris*. To replace native ES proteins with a recombinant product, equine PBMC ould reacts different from when exposed to native ES proteins.. Altogether, ES-proteins contain a large variety of different types of proteins that can react with equine PBMC while the recombinant SvSXP represent one of these proteins The SvSXP protein is a serine-x-proline rich ES-protein cloned from an immunogenetic screen of *S. vulgaris* infected horses. SvSXP with close orthologs in other roundworms (Andersen *et al*., 2013) is produced and secreted by the L4 and L5 larvae stages of *S. vulgaris* (Nielsen *et al*., 2014).

Further studies

As the resistance of cyathostomins to anthelminthic drugs has increased, it is important to find some way to prevent the horses from becoming infected. The consequences of large amounts of bloodworms in horses can be devastating in form of severe colic and in the worst case, death. By finding a suitable antigen, a vaccine can be developed. Although SvSXP is a good candidate to use as an antigen, it would be interesting in future attempts to extract real ES-proteins from both cyathostomins and *S. vulgaris* to make a comparison with the recombinant form of SvSXP that was used in this project.

In this study, only four horses were used, of which horse #2 only received a concentration of 0.5 microgram SvSXP after an accident in the handling of the blood. In future studies, it

would be interesting to use more horses and use horses that have no presence of bloodworms. Because the horses in this experiment with a larger number of eggs in the feces gave a stronger reaction of SvSXP, it could be interesting to determine the impact of previous exposure to the bloodworm.

Conclusion

An upregulation of the cytokines IL-9 and IFN-γ was recorded after exposure of equine PBMC to the SvSXP protein. Production of IFN-γ can be observed in presence of parasites to strengthen the anti-parasitic response. Also, IL-9 belong to the Th-2 type of immune reaction that is commonly activated at parasitic infections. Together, the results show that, SvSXP is an interesting candidate as an antigen in the development of future vaccines against *Strongylus vulgaris*.

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