Genomic variation and molecular mechanisms of the host response to gastrointestinal nematodes in small ruminants

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Abstract

Gastrointestinal nematode (GIN) infections are one of the major constrains for sheep and goat production worldwide. One of the promising control strategies is the genetic selection for resistant animals as there are no issues due to anthelmintic resistance and it aligns to demands for chemical-free food. Exploring possible phenotypic and genomic markers that could be used in breeding scheme besides understanding the mechanisms responsible for resistance were the main goals of this thesis.

Thesis consists of **General introduction**, a brief description of GIN biology and methods to control GIN with focus on phenotypic and genomic markers, **four papers** and **General discussion**. In **paper I**, a systematic review and meta-analysis were conducted to re-analyse and summarize the findings on immunoglobulins response to GIN in the literature and discuss the potential to use immunoglobulins as biomarkers of the host resistance. A conceptual model summarizing the role of immunoglobulins in resistance to GIN is proposed. In **paper II**, transcriptome profiling of the abomasal mucosa and lymph node tissues were compared between non-infected, resistant and susceptible Creole goats experimentally infected with *Haemonchus contortus*. Results indicated that the maintenance of the integrity of the mucosa has probably the priority for the host at late infection stage. In **paper III**, the dynamics of the response of the abomasal mucosa of resistant and susceptible Creole goats experimentally infected with *H. contortus* were compared. The immune response was activated through many relevant pathways including the Th1 immune response at different time post-infection. Interestingly, the results showed a simultaneous time series activation of Th2 related genes in resistant compared to susceptible kids. In **paper IV**, the genomic variants of Creole goats resistant and susceptible to *H. contortus* were discovered from RNA-sequencing data at four different times post-infection. Single nucleotide polymorphisms, insertions and deletions that distinguish the resistant and the susceptible groups were identified and characterized through functional analysis. The T cell receptor signalling pathway was one of the top significant pathways that distinguish the resistant from the susceptible group with genomic variants in 78% of genes in this pathway.

Keywords: Goats, *Haemonchus contortus*, genetic resistance, immune response, transcriptome

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French Abstract

Swedish Abstract

Dedication

To my loving, supportive, encouraging, and patient husband Mohamed Rashid, none of this would have been possible without your love and support.

To my family in Egypt for all their love and encouragement.

Thank you!!

Contents

List of publications

This thesis is based on the work contained in the following papers:

I Aboshady H. M., M.J. Stear, A. M. Johansson, E. Jonas, J.C. Bambou (2019). Immunoglobulins as Biomarkers for Gastrointestinal Nematodes Resistance in Small Ruminants. (submitted)

II Aboshady H. M., N. Mandonnet, M. J. Stear, R. Arquet, M. Bederina, J. Sarry, G. Tosser-Klopp, C. Klopp, A. M. Johansson, E. Jonas, J.C. Bambou* (2019). Transcriptome variation in response to gastrointestinal nematode infection in goats. *PLoS ONE* 14(6): e0218719. https://doi.org/10.1371/journal.pone.0218719

III Aboshady H. M.; Nathalie Mandonnet; Yoann Félicité; Julien Hira; Aurore Fourcot; Claude Barbier; Anna M. Johansson; Elisabeth Jonas; Jean-Cristophe Bambou (2019). Dynamic transcriptomic changes of goat abomasal mucosa in response to *Haemonchus contortus* infection. (submitted)

IV Aboshady H. M., N. Mandonnet, A. M. Johansson, E. Jonas, J.C. Bambou. Genomic variants from RNA-seq for goats resistant or susceptible to gastrointestinal nematode infection. (manuscript)

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The contribution of Aboshady H. M. to the papers included in this thesis was as follows:

I Performed the literature search, the statistical analysis and contributed to interpretation of the data and to writing of the manuscript.

II Formal analysis and writing – original draft.

III Performed bioinformatics and statistical analysis, interpretation of the data and writing – original draft.

IV Performed bioinformatics and statistical analysis, methodology, interpretation of the data and writing – original draft.

Abbreviations

DEG	Differentially expressed genes
dpi	Days post infection
FEC	Faecal egg count
GIN	Gastrointestinal nematode
GWAS	Genome-wide association study
MHC	Major Histocompatibility Complex
PCV	Packed cell volume
QTL	Quantitative trait loci
SNP	Single nucleotide polymorphism

1 General introduction

1.1 Global context

The world population is predicted to grow by over one third between 2009 and 2050 reaching expectably 9.8 billion in 2050 while it is expected to surpass 11.2 billion in 2100 according to official estimations from the united nation in 2017 (https://refugeesmigrants.un.org/es/node/100043622#collapseOne). The population is expected to increase rapidly in developing country in Africa and Asia, while the population in developed countries is expected to increase slightly.

This population growth leads to the challenge for agriculture to produce more food to feed a growing population and to adopt more efficient and sustainable production methods. Ruminant in general and small ruminants in particular are known for their ability to eat low valuable resources (low inputs) to produce high valuable products (increase outputs). Small ruminants have a very valuable contribution in production of goods for human needs throughout the world, ranging from food with precious animal proteins (meat and milk) to fibre and skins, draught power in the highlands, food security and important non-market services. Additionally, small ruminants make important contributions to human livelihoods in small farming systems and developing economies. Recent reports from the Food and Agriculture Organization (http://www.fao.org) showed that Asia counts for 37 and Africa for 22% of the 1.2 billion world sheep population together with 56 and 30% of the approximately 1 billion world goat population, respectively.

Goats, in particular, are known for their ability to survive in some of the most inhospitable regions of the world and are usually called the ‘poor man’s cow’ which underlines their importance in small farming systems. Recent

reports from the FAO showed that goat population is expanding and more than 95% of the population is found in developing countries.

1.2 Gastrointestinal nematode in small ruminants production

One of the main wedges for efficient livestock farming is management of animal health. Among the diseases that constrain the productivity of sheep and goats, gastrointestinal nematode (GIN) infection ranks highest on a global index. GIN parasite affects productive and reproductive performance and leads to economic losses (Mavrot, Hertzberg and Torgerson, 2015). Among all the species of GIN commonly found in small ruminants, *Haemonchus contortus*, *Trichostrongylus spp*. and *Teladorsagia circumcincta* are the most abundant and cause the greatest losses in production. *H. contortus* is known to be the most important nematode species of small ruminants in tropical and subtropical areas, meanwhile in temperate regions, the most economically important nematodes are *Trichostrongylus spp*. and *T. circumcincta* (Peter and Chandrawathani, 2005; O'Connor, Walkden-Brown and Kahn, 2006). However, it has been reported an increasingly common occurrence of *H. contortus* also in temperate areas such as in Sweden, France, Denmark and the Netherlands (Waller *et al.*, 2004). This phenomenon is expected to aggravate with accordance to expected increase in temperature worldwide and climate changes reported in the last IPCC (Intergovernmental Panel on Climate Change) report in 2019.

Generally, GIN have a simple direct life cycle presented in figure 1. The development of the nematode larvae has five stages within two phases: the free-living phase in the external environment and the parasitic phase. The free-living phase starts from eggs which are dispersed on the pasture by animal faeces. The eggs hatch thereafter into first stage larvae (L1) and develop to the second (L2) and third (L3) larval stage. L3 is a non-feeding stage which could last for weeks to months depending on the environmental condition. The parasitic phase starts after the host ingests forage containing infective larvae (L3). These L3 lose their sheath in the host to become parasitic L3 and migrate to their host organ (abomasum or small intestinal). The larvae enter the gastric glands where they have their third molt and develop into the fourth larval stage (L4), as which they move then back into the lumen of the gastrointestinal tract. After another molting, the L4 develop into immature adults (L5) for a short period of time before becoming mature adults (Soulsby, 1982).

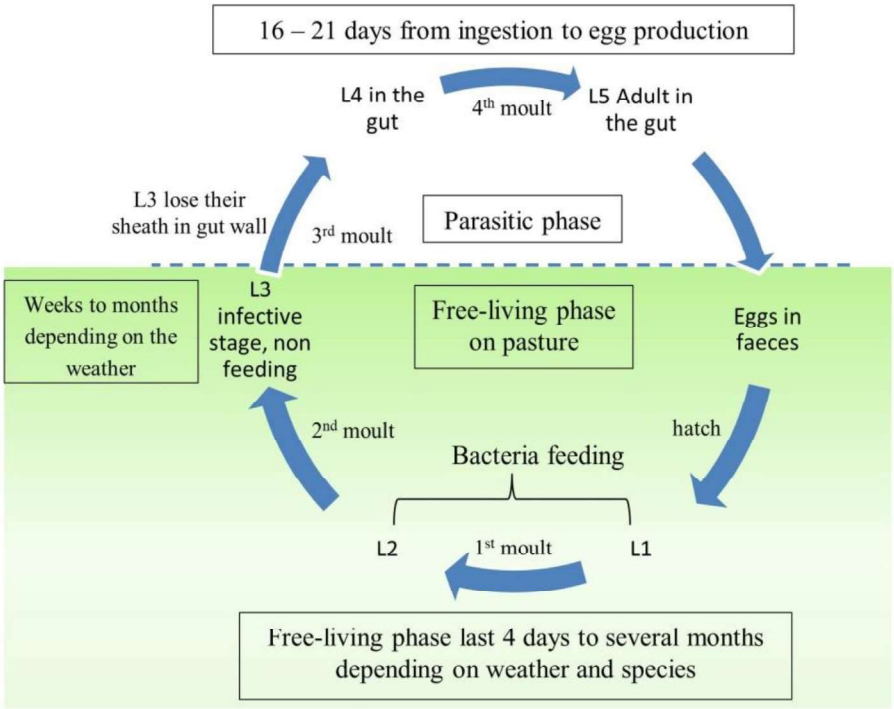

Figure 1. Gastrointestinal nematode life cycle.

1.3 Non-genetic methods to control GIN

There are different approaches to control GIN infection, each of them either target the parasite population in the host or on pasture, but all of them have the same goal which is minimize the impact of GIN on animal performance by minimizing host parasite contact (Jackson and Miller, 2006). The main methods that can be used to control GIN include chemical/anthelmintic methods, grazing management, nutrition, biological or vaccines.

Chemical control is the most widely used method for control GIN infection. The rapid and broad advance of anthelmintic drugs in the early 1960s offered an affordable and simple way to manage GINs. As a result, these drugs have been widely used as a cost-effective means for GIN control. The frequencies use of anthelmintic leads to pathogen resistance. Anthelmintics resistance have been reported all over the world (Kaplan, 2004; Jabbar *et al.*, 2006; Falzon *et al.*, 2014; Zvinorova *et al.*, 2016). *H. contortus* is prominent amongst the reports of anthelmintic resistance that has emerged (Peter and Chandrawathani, 2005). Growing anthelmintic resistance has created a compelling need to develop alternative options for the control of GIN infection.

Grazing management has been utilized for many years as a mean of parasite control to limit the host-parasite contact hence reducing pasture contamination. The different strategies can be considered as being either preventative, evasive, or diluting. The preventative strategy involves turning out parasite-free animals on clean pastures such as delayed turn-out, change of pastures between seasons, and the use of more aftermath. The evasive strategy involves moving animals from contaminated to clean pastures such as changing the pasture within the same season. The diluting strategy allows diluting pasture infectivity by mixed or alternate grazing with other host species (Cabaret, Bouilhol and Mage, 2002). However, these grazing methods are difficult to apply in extensive production systems and in those with common grazing, besides in many intensive systems there may not be sufficient land for grazing, or adequate numbers of non- susceptible animals, to provide a sufficient reduction in the numbers of GIN on pasture (Jackson and Miller, 2006).

Some plants that showed bioactive effects on internal parasite populations may help on controlling GIN by either acting directly upon the parasite population and/or indirectly by influencing host mediated regulatory mechanisms (Jackson and Miller, 2006). Consequently, optimized animal nutrition could play a role in controlling GIN infection. A first report for the possible use of tanniferous plants to control different worm species was reported in New Zealand (Niezen *et al.*, 1998). The authors showed that some condensed tannins plants (*Hedysarium coronarium*) were able to reduce parasite burdens while other condensed tannins plants (*Lotus pedunculatus*) maintain animal performance despite high worm burden. In this context, a highly rich source of condensed tannin (quebracho extracts) supplementation induced reduction in *H. contortus* fecundity and faecal egg counts (FEC) in goats (Paolini *et al.*, 2003). However, the same condensed tannin extracts have been found to reduce small intestine burdens (*Trichostrongylus colubriformis, Cooperia, Nematodirus* and *Bunostomum spp*) but not those from the abomasum (*H. contortus* and *T. circumcinta*) in sheep (Athanasiadou *et al.*, 2001). There is also extensive evidence in several breeds of sheep indicating the benefits of improving nutrition through supplementation of dietary protein as a mean of parasite control (Steel, 2003).

A biological control method through nematode-trapping fungi (*Duddingtonia flagrans*) has been used in small ruminants for parasite control (Larsen *et al.*, 1997). Resting spores of this fungus break the lifecycle of parasites bypass through the digestive tract, deposit in the faeces and develop along with the larvae then trapping and killing the larvae before they migrate to pasture (Terrill *et al.*, 2012). Work with nematode trapping fungi was discontinued because of lack of a commercial source of the spores.

Internal parasites can be controlled by the use of vaccines. The general approach for identifying candidate vaccine antigens is to screen for a protective fraction against target parasite through preliminary protection trials, then to purify the protective fraction, isolate and express the genes which encode this protein. Finally, a functional recombinant protein can be produced (Jackson and Miller, 2006). For example, some vaccines derived from the worm's intestinal gut cells. Consequently when the parasite feeds on the host, the parasite ingests antibodies that bind to functional proteins on its intestinal surface. As a result, digestive processes are compromised leading to starvation, loss of fecundity, weakness and at the end parasites lost from the infected site (Jackson and Miller, 2006; Terrill *et al.*, 2012). Recently, a recombinant form of *H. contortus* somatic antigen (rHc23) have been produced and used successfully for vaccination against GIN (González-Sánchez, Cuquerella and Alunda, 2018). The problem associated with the use of vaccines could be related to the cost, the need for regular re-vaccination and that continued exposure to larval antigens can stimulate a natural immunity (Jackson and Miller, 2006; Zvinorova *et al.*, 2016).

1.4 Genetic control of GIN

Animals can combat the adverse effects of parasites with two broad strategies: resistance and tolerance. Resistance is defined as the host ability to reduce the probability of infection, reduce the growth of the pathogen population within it, or recover from infection. Tolerance, by contrast, is defined, as the ability to limit the damage caused by a given parasite burden and maintaining health, performance and ultimately on fitness as infections levels increase (Kause, 2011). Resilience is related to tolerance, and describes an animal's ability to maintain performance in the face of a disease challenge (Råberg, Sim and Read, 2007; Bishop, 2012). Different between resistance and tolerance are shown in figure 2.

Figure 2 shows that by selecting for low FEC or parasite load we select for resistant animals that could differ in tolerance level (more tolerant has lower slope). Meanwhile, animals with high FEC or parasite load are susceptible but also some of them more tolerant (with low slope). Other tolerant animals are lost as they have medium infection load. This explains the complicity in defining resistant and tolerant individuals in disease respect. Another point is that in a breeding program we give more weight for production level.

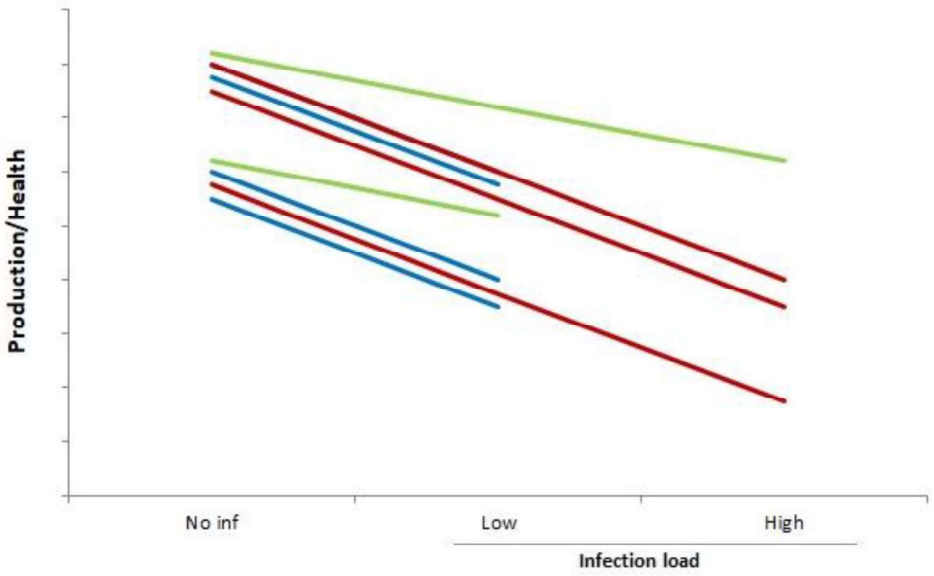

Figure 2. Schematic figure showing changes in productivity/health for different host genotypes (blue, red and green line) after being exposed to same infection dose. The blue genotype has lower parasite burdens (is resistant). The red genotype has higher parasite burdens (is susceptible), red and blue equal in tolerance (same slop). The green genotype has lower slope (is tolerant), thereby maintains production/health status.

The term disease resistance is often used loosely and generically to cover both resistance to infection as well as resistance to the disease consequences of infection, that is, disease tolerance. In general, the susceptibility to nematode infections seems to be related to genetic factors since evidence for genetic variation in resistance to nematode infection has been observed within and between breed (Sayers and Sweeney, 2005) which make opportunity to the use of genetic variation in resistance for the purpose of breeding animals for increased GIN resistance. Moreover, genetic variation in tolerance has been recorded as genetic variance in regression slopes of host performance along a gradient of increasing pathogen burden (Kause, 2011).

1.4.1 Classical selection approach, phenotypic markers to GIN

Appropriate phenotypes traits that could be considered as indicator for resistance to nematodes have been classified (Bishop, 2012; Coutinho *et al.*, 2015) as follows:

- Measures of resistance: FEC, worm burden, worm size and fecundity.
- Immune response: Eosinophilia, antibodies such as IgA, IgG and IgM.
- Measures of impact of infection: anemia, pepsinogen or fructose amine concentrations.

- Measures of resilience: growth rate, anemia and/or required treatment frequency in relation with FEC, worm burden, worm size or fecundity.

Of these traits, FEC and anemia are the most studied traits. In animals infected with *H. contortus*, anemia can be easily measured using either PCV or the Famacha score, which is an indicator of anemia in the eyelid (Bishop, 2012).

Genetic variation in resistance to GIN within and between breeds has been studied extensively in sheep and goats as reviewed by Zvinorova *et al.* (Zvinorova *et al.*, 2016). Successful selection for nematode resistance has been reported in sheep and goats (Vagenas *et al.*, 2002). Conventional breeding strategies are based on the use of indicator traits to select for resistance. FEC have been the main indicator for resistance to GIN. FEC has been found to be a low to high heritable trait in lambs within the heritability range from 0.01 to 0.65, which is sufficiently high in most breeds to make selective breeding feasible (Stear, Strain and Bishop, 1999; Bishop, 2012; Zvinorova *et al.*, 2016). Moderate heritability for FEC was found in kids ranging from 0.1 to 0.37 (Mandonnet *et al.*, 2001; Vagenas *et al.*, 2002), which makes it still possible to breed for improved resistance to nematodes in goats. In addition, the differences in the estimated FEC heritability may be related to the age of animals as it has been reported (Stear, Strain and Bishop, 1999) that the heritability of a single egg count in each month of lambs age (Scottish Blackface) was essentially zero at 1 and 2 months of age, then rose rapidly to 0.33 at 6 months of age. Moreover, genetic correlations between FEC and resistance to different species of nematodes tend to be related being close to 0.5 (Bishop *et al.*, 2004).

Other traits that could be used to breed for improved resistance to nematodes are packed cell volume (PCV) (Mandonnet *et al.*, 2001; Baker *et al.*, 2003; Coutinho *et al.*, 2015), blood eosinophils (EOS) (Dawkins, Windon and Eagleson, 1989; Stear *et al.*, 2002), worm size and number of eggs in utero in adult female worms which are strongly heritable traits (Stear *et al.*, 1995, 1997). Meanwhile, the numbers of larvae or adult worms present in the gut are weakly inherited (Stear *et al.*, 1997).

Another heritable trait that could be used in traditional breeding as indicator trait for resistance to different nematodes species is antibody responses and it has been found to be moderately to strongly heritable. For example, Smith et al. (1985) were the first to show strong correlation of 0.95 between increased lymphatic IgA concentrations and reduced mean worm length, in 4.5 and 10 month-old lambs. Despite that there is no review available to evaluate the possibility of using immunoglobulins as phenotypic biomarkers in breeding schemes.

Although selection for resistance is possible and effective for sheep and goats, there are other issues restricting it. The main problem with conventional breeding strategies is the indicator traits which are costly, time consuming to collect and the need to infect animals (Zvinorova *et al.*, 2016).

1.4.2 Molecular genetic markers associated with GIN resistance

Incorporation of genotype information, using genetic markers approach, focuses on identifying DNA markers, which may not necessarily be causative mutations for resistance themselves, but may be in linkage disequilibrium with the causative mutation (Sayers and Sweeney, 2005). In contrast to the classical selection, marker-assisted selection can be utilized to accelerate selection with more efficiency even in cases where the desirable alleles for the trait are found in low frequencies, beside avoiding the requirement for animals to be challenged with nematodes (Bishop, 2012; Zvinorova *et al.*, 2016). A summary for previous studies that examined different molecular genetic marker association with GIN resistance is presented in appendix 1.

Associations with candidate-genes or specific markers

Several studies examined the association of specific genes or markers with FEC. In searching for genes involved in resistance or susceptibility, the genetic markers that have been most frequently associated with nematode resistant are those from the major histocompatibility complex (MHC) region on *Ovis aries* chromosome 20 (Schwaiger *et al.*, 1995; Buitkamp *et al.*, 1996; Janßen *et al.*, 2002; Sayers, Good, Hanrahan, Ryan, Angles, *et al.*, 2005; Davies *et al.*, 2006; Valilou *et al.*, 2015). Genes of this complex play important roles in presenting antigens to host T lymphocytes, causing T cell activation (Zinkernagel and Doherty, 1979). MHC genes were reported to have high levels of polymorphism (Schwaiger *et al.*, 1995; Valilou *et al.*, 2015). In this context, Bolormaa *et al.* (2010) tested specific markers on goat chromosome 23 which is near to the MHC region and found it to be associated with goat resistance to nematodes. The second most frequently identified gene in studies for resistance to GIN infection is the interferon ɣ (*IFN-γ*) gene on *O. aries* chromosome 3 (Coltman *et al.*, 2001; Sayers, Good, Hanrahan, Ryan and Sweeney, 2005). *IFN-γ* is known to be one of the principal cytokines produced by Th1 cells as innate immune response resulting in a cell mediated immune response (Schallig, 2000). The role of MHC and *IFN-γ* genes in immune response and their association with resistance and/or susceptibility to GIN infection are discussed in detail later in discussion section.

A main obstacle with candidate-genes or specific markers studies is that it is relied on prior knowledge to predict the correct genes or markers, usually on the basis of biological hypotheses or the location of the gene or marker within a previously determined region (Hirschhorn and Daly, 2005). However, lots of genes have their functions yet to be defined.

Microsatellite-based QTL studies

Quantitative trait loci (QTL) mapping can help in understanding the complexity of parasite resistance by identifying candidate genomic regions. Studies using microsatellite markers have been conducted to identify genomic region associated with GIN resistance. Several microsatellite-based QTL on different chromosomes have been reported in the literature for sheep. Most reported genomic regions for nematode resistance in sheep are located on chromosome 1, 3, 6, 14 and 20 (Davies *et al.*, 2006; Gutiérrez-Gil *et al.*, 2009; Stear *et al.*, 2009; Dominik *et al.*, 2010; Matika *et al.*, 2011; Silva *et al.*, 2011). Genomic regions on chromosome 2 were also reported for nematode resistance in sheep in many studies (Crawford *et al.*, 2006; Davies *et al.*, 2006; Marshall *et al.*, 2012; Sallé *et al.*, 2012). In a few studies, some other potential genomic regions were identified on different ovine chromosomes. It should be also noticeable that some studies used microsatellites that only cover 8 or 9 chromosomes and not the whole genome (Crawford *et al.*, 2006; Davies *et al.*, 2006; Dominik *et al.*, 2010). Meanwhile in goats, the first genome scan was undertaken in goats of the Creole breed and identified 13 QTL for resistance, resilience and immune criteria (de la Chevrotière *et al.*, 2012). The main conclusion from microsatellite-based QTL studies is that most significant QTL effects tend to be scattered throughout the genome.

Results from microsatellite-based QTL studies are often difficult to utilize in breeding programs, primary because the QTL are generally detected within families, and the markers linkage with causative mutation is family specific (within-family linkage). This explains why previously identified QTL seem to disappear with new ones emerging between populations (Bishop, 2012; Zvinorova *et al.*, 2016)..

SNP studies

An alternative to microsatellite-based QTL is the single nucleotide polymorphism (SNP) associations, in which SNPs are associated with favorable phenotypes across an entire population. This technique uses SNPs that show population-wide linkage disequilibrium with the causative mutation, consequently the issue of family-specific linkage is avoided (Bishop, 2012).

The availability of SNP arrays such as the GoatSNP50k chip, the OvineSNP50k chip and OvineSNP600k chip made Genome-wide association study (GWAS) more prevalence. GWAS aim at understanding the genetic basis of complex traits, such as resistance to diseases and production traits by searching the whole genome for genetic variants associated with the studied trait, without prior assumptions (Hirschhorn and Daly, 2005).

Results from GWAS reported genomic regions for nematode resistance in sheep on chromosomes 6 (Riggio *et al.*, 2013, 2014; Benavides *et al.*, 2015) and 14 (Riggio *et al.*, 2013, 2014), both regions were previously reported in microsatellite-based QTL studies. Meanwhile, other genomic region identified in many QTL studies were not reported using GWAS. Regions on sheep chromosome 4 (Riggio *et al.*, 2014), 7 (Benavides *et al.*, 2015) and 19 (Riggio *et al.*, 2014) were identified in GWAS. The only GWAS for nematode resistance in goats was in Creole goat (Silva *et al.*, 2018). Results from this study identified a total of seven SNP (on the chromosomes 4, 6, 11, and 17) associated with nematode resistance and the identified genes near to these positions were related to the intestine damage, inflammation process, immune response, hemorrhage control, and muscle weakness.

Evidence from SNP association studies suggests that individual SNPs are likely to be associated with very small effects because of polygenic nature of the resistance trait (Kemper *et al.*, 2011). As a result, to achieve reasonable genetic progress many SNPs would need to be included in a breeding program (Bishop, 2012). Moreover, obtaining GWAS for parasite resistance requires genotyping and phenotyping large numbers of animals (McCarthy *et al.*, 2008).

1.4.3 Genome- wide expression studies

A detailed understanding of the genes and biological mechanisms involved in resistance and protective immunity will aid the development of direct genetic markers which consider sustainable nematode control methods (McRae *et al.*, 2015). Gene expression profiling or transcriptional profiling allows examining large numbers of transcripts simultaneously in order to identify those transcripts that contribute to an animal's susceptibility or resistance.

The first studies that described genome-wide gene expression differences in parasite-resistant and susceptible sheep used the cDNA microarray technology (Diez-Tascón *et al.*, 2005; Keane *et al.*, 2006, 2007; Rowe *et al.*, 2008; MacKinnon *et al.*, 2009; Andronicos, Hunt and Windon, 2010; Knight *et al.*, 2011). Microarray technology is a tool to address complex biological questions by measurement and analyses gene expression simultaneously from potentially thousands of genes (Diez-Tascón *et al.*, 2005). Studying differentially

expressed genes (DEG) via microarray has led to the identification of genes and biological processes involved in the development of a resistant phenotype. Out of the identified genes, biological processes and pathways; genes involved in the stress and/or immune response were the most common (Diez-Tascón *et al.*, 2005; Keane *et al.*, 2006, 2007; Rowe *et al.*, 2008; MacKinnon *et al.*, 2009; Andronicos, Hunt and Windon, 2010; Knight *et al.*, 2011). In microarrays, samples of RNA populations are hybridized with DNA spots to determine the extent of expression of each sequence. As a result microarray technology has inherent weaknesses in terms of repeatability and precision because it relies on hybridization (’t Hoen *et al.*, 2008).

Instead of testing the expression of thousands of genes through microarray, nowadays RNA sequencing (RNA-seq) provides a tool for analysing the entire transcriptome of an organism. Identifying DEG through whole transcriptome analysis via RNA-seq and functional analysis for these genes has been shown to provide a key role in the knowledge of mechanisms responsible for complex quantitative traits (Costa *et al.*, 2013). Whole transcriptome analyses via RNA-seq have been used recently to identify DEG in resistance and susceptible sheep to GIN infection (Gossner *et al.*, 2013; Ahmed *et al.*, 2015; Guo *et al.*, 2016; McRae *et al.*, 2016). Meanwhile, only one study in goats used RNA-seq technology to explore the genetic resistance to GIN infection (Bhuiyan *et al.*, 2017). Identified DEG via RNA-seq from sheep and goats studies were involved mainly in inflammatory and immune responses.

Through RNA-seq, besides allowing the detection of DEG, functional genes are sequenced at high coverage, allowing to full scale variants discovery in coding genes. This technique has been used as a method to detect SNPs in transcribed regions in an efficient and cost-effective way for different traits and species (Cánovas *et al.*, 2010; Sharma *et al.*, 2012; Wang *et al.*, 2015; Martínez-Montes *et al.*, 2017; Pareek *et al.*, 2017). Up to date, there is no study explored genomic variants via RNA-seq related to resistance to GIN in sheep or goats.

Generally, studies in genetic resistance to nematode strongly suggest that the genetic resistance to GIN in small ruminants is closely linked to the host immune response. However, it appears that the underlying mechanisms are different at least partly, from breed to breed (within sheep), between goats and sheep and depending on the parasite specie.

2 Objectives of the PhD project

The present thesis aims to unravel the genetic background of goat resistance to GIN by exploring the mechanisms involved in resistance and susceptibility. It additionally aims to study phenotypic and genomic markers that could be used as biomarker in breeding for resistance. More specifically the objectives are:

1. Evaluate the pertinence of the immunoglobulin responses (especially IgA and IgE) against GIN and their potential use as biomarkers in breeding schemes. (Paper I).

2. Identify the molecular pathways involved in the response of Creole goats to GIN infection by analyzing the transcriptome of abomasal mucosa and draining lymph nodes of infected versus non-infected and resistant versus susceptible kids (Paper II).

3. Identify the changes over time in the molecular pathways and immunity development in response of Creole goats to GIN infection by analyzing the transcriptome of abomasal mucosa of resistant and susceptible kids at different time point post infection (Paper III).

4. Discover the genomic variants in the abomasal mucosa transcriptomes of Creole goats resistant or susceptible to *Haemonchus contortus* and characterized the variants identified (Paper IV).

3 Results

Immunoglobulins as Biomarkers for Gastrointestinal Nematodes Resistance in Small Ruminants

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Abstract

The rise of anthelmintic resistance worldwide has led to the development of alternative control strategies for gastrointestinal nematodes (GIN) infections, which are one of the main constraints on the health of grazing small ruminants. Presently, breeding schemes rely mainly on fecal egg count (FEC) measurements on infected animals which are time-consuming and requires expertise in parasitology. Identifying and understanding the role of immunoglobulins in the mechanisms of resistance could provide a more efficient and sustainable method of identifying nematode-resistant animals for selection. In this study we review the findings on immunoglobulin response to GIN in the literature published to date (june 2019) and discuss the potential to use immunoglobulins as biomarkers. The literature review revealed 41 studies which measured at least one immunoglobulin: 35 focused on lamb immune response (18 used

non-naïve lambs) and 7 on yearlings. In this review we propose a conceptual model summarizing the role of immunoglobulins in resistance to GIN. We highlight the need for more carefully designed and documented studies to allow comparisons across different populations on the immunoglobulin response to GIN infection.

Introduction

Small ruminants are an important source of food and revenue[1,2]. The world's sheep and goat populations have increased steadily over the past decades, especially in developing countries[2]. One of the main constraints on small ruminant production is management of animal health. Infection with gastrointestinal nematode parasites has the greatest impact upon animal health and productivity[3]. The control of GIN in sheep and goats has been dependent on the use of anthelmintic treatment, however their extensive use has resulted in the anthelmintic resistance[4–6] which has been reported in multiple countries[7]. In addition, there is a growing demand from consumers to produce chemical-free food and increasing concern about animal welfare[8].

Therefore, two main axes of research have been identified to develop alternative control strategies for GIN management. The first option is the reduction of parasite burden on the pasture through grazing management. However, nematode-free pastures are not readily available under intensive grazing conditions. The second option to reduce GIN infections is the improvement of the host immune response through the genetic selection of lines or breeds of resistant animals, nutritional supplementation and/or vaccination.

A number of studies have already identified sheep breeds, such as the Florida Native[9,10], Santa Ines[11,12], Texel[13,14], St. Croix[9,15,16] and Red Massai sheep[17] that are resistant to various GIN species. There are also reports on differences between breeds in resistance to GIN infection in goats[18–20]. Moreover, variation among individuals within the same breed in

response to GIN infection has been observed in sheep[21] and goats[22,23], which could be used to breed resistant lines for several breeds. These variations were often applied to breed diverse lines in experimental studies for the identification of mechanisms or genetic regions for GIN resistance.

Several studies have indicated that genetic resistance to GIN is associated with a protective immune response which is mediated, at least partly, by the humoral response[24]. Understanding the differences in the humoral response between resistant and susceptible breeds, lines or individuals could help to design and implement appropriate control programs and sustainable breeding for GIN resistance. To our knowledge, there is no recent review on the association of immunoglobulin responses and the intensity of GIN infection (based on FEC and/or parasite burden counts). The role of different immunoglobulins in immunity to nematodes needs to be confirmed. The objective of this study was to evaluate the role of immunoglobulin responses (especially IgA and IgE) against GIN and their potential use as biomarkers in breeding schemes.

Results and Discussion

Immune response to GIN infection

Both cellular and humoral responses are actively involved in immune response against nematode infection. The main effectors of this immune response are T- and B-lymphocytes, plasma cells, mast cells, eosinophils, globule leukocytes, soluble cytokines and various immunoglobulin isotypes[25]. Incoming nematode larvae from GIN infection trigger local inflammation and mast cell degranulation, which damages the gastrointestinal mucosa[26]. Dendritic cells, macrophages and the other antigen-presenting cells, capture the nematode antigens within the intestinal mucosa and migrate to the regional lymph nodes to present these antigens to naïves T cells[24]. After T-cell differentiation, the secretion of type 1 T helper

(Th1) or type 2 T helper (Th2)-associated cytokines induces the migration to the site of infection of activated effector cells such as eosinophils and mast cells[24,27]. The type of helper CD4+ T lymphocytes that develop following an infection with nematodes is critical for the ability of the host animal to overcome an infection[28]. The Th1 cells produce interferon-gamma (IFNγ), interleukin (IL-2) and tumor necrosis factor-beta (TNFβ) for the activation of macrophages and the initiation of the cell-mediated immunity and the phagocyte-dependent protective responses[11,29]. The Th1 cells develop mainly following infections by intracellular parasites (viruses and some bacteria). When GIN antigens penetrate the gastrointestinal tissues of the host, macrophages and other cells which have receptors for nematode cell surface molecules are activated and induce a specific but mostly ineffective immune response[29].

The phagocyte-independent protective responses characterized by antibodies production, eosinophils activation and inhibition of several macrophage functions, are activated by the production of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13)[11,29]. The Th1 response inhibits the Th2 response through IL-10 [30], which makes Th1 and Th2 responses antagonistic to each other. Results from studies in sheep showed that CD4+ lymphocytes increase during the experimental infection of both susceptible and resistant sheep with GIN[31,32]. But compared with resistant sheep, susceptible produce more interferon gamma (IFNγ), fewer parasite-specific serum antibodies, blood and abomasal eosinophils[33]. The role of the two major types of T helper cells distinguishes resistant from susceptible sheep.

The acquired immune response after infection with *Haemonchus contortus* was compared in Barbados Blackbelly sheep, which are generally defined as a resistant breed, and Columbia sheep, a breed classified as susceptible[34]. Sheep of the resistant breed developed and sustained a Th2 response through increasing and maintaining IgG and blood eosinophil levels. Meanwhile sheep of the susceptible breed showed changes in the response starting

with an initial increase in IgG and blood eosinophils (Th2) but a later reduction in both, which suggests a switch to a Th1 response[34]. An earlier study suggested that in the relative absence of Th1 type secretions (i.e. cytokines), the Th2 cells secrete cytokines that promote mastocytosis, eosinophilia and the production of IgE and IgG1[35]. Gulf coast native (resistant) lambs showed a significantly higher expression of IL-4 mRNA (Th2) on day 10 post exposure to the nematode compared to Suffolk lambs (susceptible). On the other hand, the expression of IFN-γ mRNA and IL-10 (Th1 and regulatory T) on days 7, 10 and 14 post exposure was higher in Suffolk lambs (susceptible) compared with native lambs [25].

This confirms that if T helper cells of the Th2 type gain ascendancy after GIN infection, then a protective immune response ensues. In contrast, if an inappropriate Th1 type response predominates, effective resistance is unlikely to develop. The Th1 type response for GIN infection is most likely associated with susceptibility, while a Th2 type response is associated with resistant phenotypes in sheep[25,33,34]. As Th2-associated cytokines target plasma cells to produce nematode-specific antibodies and generate protective immune responses[24], we focus here on the immunoglobulin response against GIN.

Immunoglobulin response in sheep (IgA, IgE, IgG and IgM)

The association between different immunoglobulin isotypes, including IgA, IgE, IgG and IgM and GIN resistance has been widely studied in sheep (Table 1). Studies which measured at least one immunoglobulin parameter during GIN infection (Table 1) differed in sheep breed used, type of breed (resistant or susceptible), age of animals in the experiment, immunological status (naïve or non-naïve), infected parasite genus and infection type (natural, artificial with single dose or artificial with trickle doses) which makes the comparison between them rather complex.

When comparing publications which measured different immunoglobulins levels against different larva stages during GIN infection in sheep it was found that the majority of the studies examined the presence of L3 antigen-specific immunoglobulins (Fig 1). The third stage larva (L3) represents the stage with the first contact of the gastrointestinal nematodes with the host immune system. It can also be seen from the figure that IgA was the most commonly investigated immunoglobulin isoform in sheep.

Total antibody response

Results from Douch et al.[36] and Gauly et al.[37] suggested that resistant sheep have higher total antibody levels and that the antibody level could be used in selection for resistance. Romney rams, selected based on their serum antibody levels at an age of 6 months to reduce FEC, underwent a natural parasite challenge, and it was predicted that the genetic gain was 51 to 67 % of the genetic gain achieved when FEC was directly used as a selection trait [36]. The total antibody level in both 4 and 5 months old Rhön (resistant) sheep were significantly higher compared to Merinoland (susceptible) lambs following experimental infection with *H. contortus* [37].

The correlation between *Trichostrongylus colubriformis*-L3 or -adult total antibody and FEC (-0.62 and -0.55) or nematode burden (-0.56 and -0.63) was high in Romney progeny selected for low and high FEC following a natural GIN challenge[38].

IgA response

It has been suggested that local immune effectors expressed in the abomasal mucosa, particularly IgA, play an important role in immunity acquired both naturally and experimentally[39]. In sheep, secretory and plasma IgA derive predominantly from the gastrointestinal tract[24]. The correlation between gastric mucus IgA and peripheral IgA is positive and highly significant, ranging from 0.618 to 0.779[40,41]. Several studies reported an

146 increase of the IgA response after GIN infection, higher levels were recorded for resistant
147 breeds[16,39,42].
148 The level of IgA against the CarLA antigen (a carbohydrate larval surface antigen expressed
149 on the L3 of all trichostrongylid nematode species) has been suggested to be a suitable means
150 to measure the level of resistance to GIN[43,44]. Different studies found that CarLA is a target
151 antigen for host IgA which binds to the larval surface antigen and prevents larvae from
152 establishing at their preferred sites in the intestinal epithelial folds[43–45]. In this context, a
153 different L3-specific surface antigen (CarLA) was detected from *Trichostrongylus*
154 *colubriformis, Haemonchus contortus* and *Ostertagia circumcincta* with similar molecular
155 weight (35-kDa), and from *Cooperia curticei* and *Nematodirus spathiger* with a different
156 molecular weight (22-kDa on blots of L3 extracts) [43]. IgA in saliva had a negative genetic
157 correlation with FEC (r = −0.5) and animals with high levels of anti-CarLA IgA have
158 typically 20–30% lower FEC than animals with low or undetectable titers[45]. A simple way to
159 use these results for the selection of animals resistant to parasite infection could be to
160 measure anti-CarLA IgA in saliva.
161 The faecal egg output in St. Croix hair-type sheep (resistant breed) can rapidly reduce in
162 response to *H. contortus* artificial infection following a 45-day breeding season, which was
163 accompanied with higher levels of circulating antigen-specific antibody IgA compared to a
164 susceptible composite line of wool-type sheep (50% Dorset, 25% Finnsheep and 25%
165 Rambouillet breeding)[16]. The increase in anti-*T. circumcincta* IgA antibody and eosinophil
166 concentrations were associated with an increase in the frequency of early L4[42]. Also Ellis et
167 al.[39] found a correlation (r = 0.534, P = 0.007) between L3 antigen-specific IgA levels in
168 efferent gastric lymph and the percentage of inhibited L4s. In addition, a negative correlation
169 (r = -0.565, P = 0.005) between total *T. circumcincta* burden measured at necropsy and L3
170 antigen-specific IgA levels in efferent gastric lymph was reported. .

171 A negative association was reported between IgA activity against L4 and both egg counts
172 and worm length when studying resistance to *T. circumcincta* in Scottish Blackface and
173 Churra sheep[40,46]. Gastric mucus IgA against L4 somatic antigen was highly and negatively
174 correlated (r = -0.71, $P < 0.01$) with the number of eggs per female in utero and also with the
175 length of adult females (r = -0.552, $P < 0.01$). Results for IgA against somatic antigen from
176 the adult stage were similar to those with activity against L4, but correlations were somewhat
177 weaker[40]. Negative genetic correlations were found between IgA and FEC, worm length,
178 worm fecundity and worm burden (r = -0.78, -0.53 and -0.62, -0.36, respectively) in Scottish
179 Blackface lambs exposed to natural mixed infection[47]. It was suggested that parasite
180 development such as worm growth and fecundity in sheep can be regulated via an IgA
181 response, possibly in conjunction with eosinophils[11,41].
182 In this paper, we have collected data from previous studies that measured correlations
183 between FEC and blood IgA levels against different larval stages. We also calculated
184 correlations using raw data for FEC and blood IgA level from these studies before re-
185 analyzing all the data. Figure 2 shows the correlations between FEC and blood IgA levels
186 against different larval stages from different studies. The overall correlation between IgA in
187 blood and FEC was negative (r = -0.36, 95% CI = -0.46, -0.26). Correlations between FEC
188 and blood IgA activity against L3 or adult were -0.39 (95% CI = -0.51, -0.28) and -0.47 (95%
189 CI = -0.85, -0.09), respectively. Only one study measured the correlation between FEC and
190 blood IgA activity against L4, L5 or ESP. Although IgA is produced locally at the mucosal
191 surfaces and serum IgA is derived from the gastrointestinal tract[24], few studies measured
192 mucosal IgA, possibly because of the difficulties in sampling mucus. Serum IgA is easier to
193 measure and highly correlated with mucosal IgA[40,41]. We were not able to re-analyze mucosal
194 IgA. Although CarLA saliva IgA antibody test is currently being marketed (CARLA®
195 SALIVA TEST) as a powerful new tool for measuring parasite immunity in sheep

(https://www.agresearch.co.nz/doing-business/products-and-services/carla-saliva-test/), no correlation was found between saliva IgA and serum or mucosal IgA[40]. However, antibodies in saliva may be binding directly to ingested L3.

IgE response

The degranulation of mucosal mast cells is induced by the cross-linking of IgE on their surface. Following reinfection, a negative correlation between the concentrations of globule leucocytes (intraepithelial mast cells) and the *T. circumcincta* worm burden have been shown in sheep[48]. Indeed, the IgE antibody response mainly directed against L3 antigens, would be more prominent in previously infected sheep [49].

In Romney sheep selected for almost two decades for high or low FEC following *T. colubriformis* infection, sheep from the low FEC line had higher total IgE (97 to 103%) and *T. colubriformis*-specific IgE (59 to 98%) compared to sheep from the high FEC line[50,51]. Similarly, after 7 weeks of grazing on a contaminated pasture, low FEC (based on the accumulated weekly measure) lambs (Greyface × Suffolk) had significantly higher systemic levels of IgE anti-HMW*Tc* (a major high molecular weight complex allergen from *T. circumcincta* L3) than high FEC lambs[52]. In addition, after 13 and 18 weeks on pasture, serum IgE anti-HMW*Tc* assays demonstrated an even greater difference between low and high FEC lambs. We conclude from these studies that high levels of IgE are associated with low levels of FEC in sheep. Pettit et al.[53] showed that during the first and second year on pasture a higher concentration of blood circulating IgE-bearing cells was associated with a lower FEC of Scottish Blackface lambs.

Other studies comparing resistant and susceptible sheep breeds confirm the role of IgE as an important effector of the immune response to nematode infection in sheep[13,33]. Sayers et al.[13] reported that the most notable difference of mucosal antibody isotype when comparing Texel

220 (GIN resistant breed) and Suffolk (GIN susceptible breed) is IgE, after natural GIN infection.
221 Mucosal IgE in Texel was significantly negative correlated with FEC (r = -0.48) and
222 abomasum worm burden (r = -0.47). Also, the total IgE content in 5-6 month old Gulf Coast
223 Native lambs increased significantly and was highest 7-14 days after artificial infections with
224 *H. contortus* compared to the level in Suffolk lambs, which was confirmed in a natural
225 infection experiment with the highest level of IgE at 14-42 days post infection[33].

226 **IgG response**

227 A review of studies investigating IgG and FEC after parasite infection in different sheep
228 breeds[12,34,38,54] suggested that IgG (especially IgG1) increases following infection and is
229 associated with increased resistance to some GIN infections in sheep. Romney lambs had
230 elevated levels of antibodies to *T. colubriformis* L3 excretory/secretory antigens which
231 consisted predominantly of IgG1, reaching peak levels between days 42 and 77 post
232 infection[54]. Bisset et al. (1996) found that the *T. colubriformis*-specific IgG1 response against
233 both L3 and adult secretory/excretory antigens, was significantly higher in Romney lambs
234 bred for low FEC compared to those selected for high FEC. IgG1 was negatively correlated
235 with FEC (r = -0.60 and -0.48) and strongyle burden (r = -0.53 and -0.57) for both L3 and
236 adult worm secretory/excretory antigens[38].

237 Barbados Blackbelly lambs, which are resistant to *H. contortus*, had higher amounts of IgG
238 anti-L3 between weeks 9 and 15 after infection. On the other hand, in the 9th week, sheep
239 from the less resistant Columbia breed had lower IgG levels compared to the uninfected
240 group[34]. An increase in IgG anti-L3 and IgG anti-adult *H. contortus* was also observed in
241 Santa Ines ewes (a resistant breed) and its cross with Dorper, Ile de France, Suffolk and
242 Texel, as a consequence of exposure to GIN larvae on pasture[55]. Santa Ines lambs had, after
243 artificial infection with *T. colubriformis*, significantly higher specific serum levels of IgG

anti-L3 and IgG anti-adult than the uninfected control group from week 4 ($P < 0.05$) to 13 ($P < 0.01$) post-infection[56]. A significant negative correlation was reported between *H. contortus* burden and IgG against L3 ($r = -0.72$), IgA against L5 ($r = -0.61$) and mast cells ($r = -0.73$) in Santa Ines male lambs, but not in Ile de France, which may be the reason that Santa Ines sheep had a lower FEC and worm burden than Ile de France sheep[12]. In a serial infection trial with Santa Ines crossbred lambs, *H. placei* infection induced high levels of IgG anti-L3 and IgG anti-adult compared with a control group, while animals serially infected with *H. contortus* induced high levels of IgG anti-adult but not IgG anti-L3 compared with the uninfected control group[57].

IgM response

IgM has an important role in the immune response as it represents the first class of antibodies produced following the initial exposure to a foreign antigen[58]. However, it is not normally present in the gastrointestinal mucus[59]. Only a few studies have investigated the IgM response in sheep during GIN infection and the findings from two studies did not suggest a major effect of IgM in sheep[33,38]. In selected Romney ram progeny, bred for genetic divergence in FEC, the correlations between IgM and both FEC or GIN burden were weak following an extended period of exposure to naturally contaminated pasture[38]. IgM did not differ between Gulf Coast Native (a resistant breed) and Suffolk lambs (a susceptible breed) in both artificial infections with *H. contortus* and natural infection experiments[25].

In contrast, GIN challenge in Romney lambs gave rise to elevated levels of IgM anti-L3 after artificial infection with *T. colubriformis* L3 [54]. A moderate increase of IgM serum antibody levels against both larval and adult antigens was found in Texel lambs in both primary infections and challenge infections with *H. contortus*[60]. Jejunal IgM anti-L3, after *T. colubriformis* infection, was the highest in resistant Merino animals with a tertiary infection,

whereas susceptible animals with one infection had the lowest titres. This difference was not observed for abomasal IgM anti-L3 after *H. contortus* infection[61].

Immunoglobulin response in goats

The strategies developed by goats appear to be different from those observed in sheep, to regulate GIN infections[62] and to establish immunity[63], but only few studies have investigated the immune response against GIN in goats. Bambou et al.[64] found that serum antibodies IgA anti-L3, IgE anti-L3 and IgA anti-ESP, IgE anti-ESP increased significantly after L3 *H. contortus* infection in both susceptible and resistant 11 months old Creole kids. At the same time IgG anti-L3 and IgG anti-ESP levels were weak in both groups. Similarly, McBean et al.[23] reported no consistent differences in IgA, IgE or IgG levels between Scottish Cashmere goats selected for low FEC (generations F2 to F9) and control lines (unselected) after artificial infection with *T. circumcincta* and during the grazing season. On the other hand, IgA anti-ESP, IgA anti-L3 and IgE anti-L3 were genetically correlated with FEC (0.84 ± 0.13, 0.72 ± 0.18 and -0.32 ± 0.08, respectively), while they did not find any phenotypic correlation between them[65]. A phenotypic correlation is the correlation between records of two traits on the same animal. Genetic correlation, traditionally calculated from pedigree data, is a measure of the genetic relationship between two traits[66]. A high genetic correlation between two traits is generally supported by genes that are usually co-inherited. The phenotypic correlation estimate the correlation between two traits and is depend both on additive genetic and of environmental effects[66]. Recently, it has been suggested that the lack of functionality of the immune response mediated by IgA and eosinophil against natural nematode infection in Boer goats would be due to a dysfunctional transmembrane domain of the high affinity IgA receptor[67]. Altogether these results indicate that the humoral response

against GIN infection is less effective in goats than sheep and does not probably play a major role in resistance to nematode infections in goats.

Genomic studies on GIN resistance

Identifying genetic markers of resistance and/or susceptibility could improve the efficiency of breeding programs. The first approach used to search for genetic markers associated with host resistance was a study of associations between variants of genes related to the host immune response and phenotypic resistant traits[68]. The genetic markers that have been most frequently associated with nematode resistant are those from the major histocompatibility complex (MHC) region on *Ovis aries* chromosome 20[69–74]. MHC genes are highly polymorphic[69,74] and play important roles in presenting processed antigens to host T lymphocytes, causing T cell activation. The second most frequently region identified in studies for resistance to GIN infection is the interferon ɣ (IFNG) gene on *O. aries* chromosome 3[75,76]. The majority of studies of quantitative trait loci (QTL) focused on the association/ linkage between genomic regions and FEC. Only few studies examined the association between genomic regions and immunoglobulins level during GIN infection. Table 2 shows the genomic regions that were reported to be linked or associated with immunoglobulin-mediated resistance to GIN infection. Similarly to phenotypic studies, the majority of genomic studies focussed on the IgA response during GIN infection[73,75,77–79], two studies considered the IgG response[80,81] and only one study examined the IgE response[80].

Factors that impact animal response to GIN infection

A number of factors impact the immune response to parasite infection. We focus here on the factors of the animal itself, such as genetic differences, age of the animal and immunological experience, infection period, or type of infection (i.e. natural or experimental).

Age of the animal and immunological experience

Our review on immune responses to parasite infection in sheep suggests that the age of the animal influences the immune response to GIN infection. Even though the traits investigated were different, three studies have identified age-related differences in the immune response[82–84]. High eosinophil counts were significantly correlated with low FEC in naturally infected with *T. circumcincta* Scottish Blackface lambs which were at least 3 months of age, where the correlations were -0.33, -0.14 and -0.24 after 3, 4 and 5 months of age, respectively[83]. Also the correlations between IgE activity and FEC in Texel lambs following 4 weeks of natural mixed nematode infection were only significant at 5 and 6 months, while it was not significant at 7 months of age when natural exposure would be declining due to the onset of late autumn and winter[82]. We found that many studies discussed their results without taking the age structure of the chosen experimental cohort as an important factor into account. However, from the studies using age different cohorts, we conclude that differences in immune reactivity might be partly explained by age.

Immunological experience has a huge impact on the response to parasite infection and is partly confounded with animal age. Immune response was reported to be low and delayed in primary-infected lambs while it is higher and rapid in previously-infected animals[60,85,86]. The majority of the studies (85%, n=35) focus on lamb immune response as model for nematode infection and half of these studies (n=18) used non-naïve lambs. It has been shown that genetic variation in resistance to GIN is associated with the development of an acquired immune response, which explains why the pathophysiological impact of these parasitic infections is more important in growing lambs compared to mature sheep[87]. Also, in lambs genetic variation in FEC is not correlated with genetic variation in the total number of worms but rather to female worms length and consequently their fecundity [88]. In contrast to lambs,

mature sheep may have the ability to limit both fecundity and worm numbers[87]. Hence the immune mechanisms could differ.

Infection period

Days post infection (d.p.i) represents also a very important factor that may affect the results obtained. The majority of the research measured host immune response within 0 to 6 weeks post infection, while other studies indicate that they did not find significant immune response until about 9 weeks post infection[34,37,42].

IgG level at 60 d.p.i was significantly correlated with worm burden (r = 0.235 to 0.247) and total antibody level was significantly correlated with worm length (r = 0.316) and FEC (r = -0.148), whereas the correlations were not significant at 30 d.p.i in Merinoland sheep infected with *H. contortus*[37]. A similar difference was also observed in Rhön sheep, the total antibody value was significantly correlated with worm burden (r = 0.372 to 0.378) at 60 d.p.i but not at 30 d.p.i [37]. Similarly, in Blackface lambs exposed continuously to infection of *T. circumcincta*, anti-*T. circumcincta* IgA levels were inversely correlated with FEC and increased with time from r = -0.17 (NS) at 14 d.p.i to r = -0.44 at 84 d.p.i ($P < 0.001$)[42]. Moreover, the difference of the anti-*T. circumcincta* IgA level between resistant and susceptible sheep diverged with time and was significant from 56 d.p.i. onwards[42].

Comparing immune response to GIN in resistant and susceptible sheep breeds, sheep of both Barbados Blackbelly (resistant) and Columbia (susceptible) breeds showed a significant increase in blood eosinophils from 1 to 9 weeks post infection, these levels decreased suddenly thereafter in Columbia lambs[34]. A similar pattern was observed in the same study for IgG anti-L3 levels, Barbados Blackbelly lambs had a higher amount of IgG anti-L3 between weeks 9 and 15 after infection with a positive significant regression (0.79), while the regression in Columbian lambs was negative and not significant (-0.59) at week 9 . These

results suggest that at the beginning of the infection the immune response may not differ a lot between resistant and susceptible animals but differences become more apparent 9 weeks after infection.

An interlaced issue is the effect of age and infection period, significantly greater nematode-specific serum antibody activities were reported in Texel compared to Suffolk sheep for all isotypes (IgG1, IgG2, IgA and IgE) at 14 and 17 weeks of age with increasing divergence between the breeds as age increased [13]. Meanwhile these differences could be explained as the effect of infection period as in this experiment the lambs were exposed to natural infection after staying on pasture within 1 to 3 days of birth, so these differences in response could be due to age or infection period or a combination of both.

Infection type (natural / artificial)

The majority of the studies measuring at least one immunoglobulin as a response to GIN infection used single infections (n = 25, 59.5%) with high number of GIN larvae or natural infections (n = 10, 24%). A natural infection occurs gradually and results from large single infections may not reflect the pattern of a natural infection[34]. However, in a natural infection, we cannot control infection dose or determine the exact time of infection; consequently the results may not accurately reflect the difference between susceptible and resistant animals. A suggested solution is to use artificial infection of different doses with a low number of larvae weekly (trickle infection) with which infection dose, the time of infection and infection specificity are controlled[34]. We only found 12% (n = 5) of the studies used trickle infection for their experimental design when measuring immunoglobulin response to GIN infection. The only study that measured the immune response to GIN infection under single and trickle infection found that mean optical densities for serum IgG in naïve lambs (resistant) was significantly higher than in Suffolk lambs (susceptible) in single infection groups at 14 and

21 d.p.i with no significant difference between these breeds in trickle infection groups at same time points[33].

Summary for immunoglobulins role against GIN infection

Results from published studies, which have reported significant correlations between different immunoglobulins and parasite parameters (FEC, worm burden and worm length) are summarized in Table 3. These studies suggest a role for L3 antigen-specific IgA, IgE and IgG responses in resistance to GIN in sheep. Also the role of L4 antigen-specific IgA for the resistance to GIN in sheep appears more important than the role of IgA against L3.

Three major mechanisms of immunity to nematodes have been described in sheep, regulation of the establishment rate of infective larvae, suppressed nematode growth and thus fecundity, and the expulsion of adult worms; a combination of these mechanisms is possible[24]. Figure 3 shows the gastrointestinal nematode life cycle and summarizes the suggested role of different immunoglobulins in the three major mechanisms of immunity to nematodes. Reduced parasite establishment and survival is associated with IgE activity mainly against incoming third stage larvae (L3) in concert with mast cells as cross-linking of IgE on the mast cell surface leading to mast cell degranulation[82,89] with more prominent response in previously infected animals[49]. Reduced parasite growth and fecundity is correlated with increased local IgA activity against fourth stage larvae[59,89,90]. Increased number of inhibited larvae is correlated with IgG1 activity against the third stage larvae[54,60] beside IgA activity against the third and fourth stage larvae[89,90].

Conclusions

The selection of animals with a high immune response to GIN infection is a promising method of reducing the negative impact of these infections on grazing small ruminants. We

have highlighted factors that differ across studies and affect the immune response to GIN infection. Indeed, it is essential that future studies take into account and mention the age of the animals, the infection experience and the type of infection (i.e. single vs trickle). One another important point is the need to normalize the measurements of immunoglobulin concentrations. The use of different units and/or optical density to measure immunoglobulin levels at different time post-infection produces results that are incomparable between studies. One way to overcome this issue is to use the change ratio between day 0 or an uninfected group and different time post-infection or to develop methods for the quantification of the immunoglobulins with standard curves. This effort for standardization should potentially allow to take advantage of the research results produced by implementing breeding programs for higher resistance to GIN infection.

Material and Methods

The study methodology followed the guideline of "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement"[91]. The literature search had been conducted using electronic databases. Web of Science and PubMed were used as databases to cover the literature on small ruminants and parasites. Besides, the reference lists from five literature reviews, all published after 2008, were additionally searched[24,28,29,89,92]. Studies were closely evaluated and selected for inclusion if they measured at least one immunoglobulin type and the data were extractable.

Information extracted from each study included nematode genus, host (sheep or goat), infected breed, host classification (resistant or susceptible), host sex, host age (months), age at weaning (weeks), infection period (days post infection), sample tissue, infection type (artificial or natural), number of animals per group, immunological state (naïve or non-naïve), total number of infected larvae, larval details and infection method for artificial infection

(single or trickle). Measured traits of interest included both parasitological and immunological parameters. If data were provided in graphical form, traits means were extracted using WebPlotDigitizer (version 3.8)[93]. These summary measures beside the study information previously mentioned were entered into an electronic spreadsheet in Microsoft Excel and a dataset was built. The Metafor package in R (version 3.5.1) was used to analyze the correlation coefficient between FEC and immunoglobulins. A REML model was used in which the effect size was calculated according to the number of animals used in each study with 95% confidence interval.

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Contributions

HMA performed the litterature search, the statistical analysis and contributed to interpretation of the data and to writing of the manuscript. MJS contributed to the drafting and critical revision of the manuscript. AJ and EJ had full access to all the study data and contributed to the study quality assessment and writing the manuscript. JCB developed the hypothesis, performed the litterature search, screening of the papers and contributed to interpretation of the data and to writing of the manuscript.

Competing Interests

The authors declare no competing interest

Availability of data and material

All data supporting the results of this study are included within this article.

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784 **Table 1.** Studies in sheep with at least one immunoglobulin against gastrointestinal nematode
785 measured

Breed	Gen[1]	n[2]	Age Class	Immune status	GIN sp.[3]	Inf.[4]	Reference
Texel Suffolk	R S	6 5	lamb	non-naïve	*T. circumcincta*	AIs	94
Santa Ines Ile de France Suffolk	R S S	16 9 11	yearling	non-naïve	*H. contortus*	NI	11
Santa Ines Dorper x Santa Ines Ile de France x Santa Ines Suffolk x Santa Ines Texel x Santa Ines	R	15 15 15 15 15	yearling	naïve	*H. contortus*	NI	55
Merino	R&S	14	lamb	naïve & non-naïve	*H. contortus & T. colubriformis*	AIs	61
Blackface	R&S	57	lamb	naïve	*T. circumcincta*	AIt	42
Romney	R&S	65	lamb	non-naïve	natural mixed	NI	38
St. Croix hair Wool[5]	R S	10 10	lamb	non-naïve	*H. contortus*	AIs	16
St. Croix hair Wool[5]	R S	6 6	lamb	non-naïve	*H. contortus*	AIs	95
Santa Ines		20	lamb	non-naïve	*T. colubriformis*	AIt	56
Manchego	-	9	lamb	naïve & non-naïve	*H. contortus*	AIs	96
Scottish Blackface	-	1000	lamb	non-naïve	natural mixed	NI	47
Romney	-	10	lamb	naïve	*T. colubriformis*	AIt	54
Romney	R&S lines	1547	lamb	non-naïve	*T. colubriformis*	NI	36
Scotch Mule[6]	-	23	lamb	non-naïve	*T. circumcincta*	AIs	39
Rhön Merinoland	S R	133 244	lamb	naïve	*H. contortus*	AIs	37
Marino Manchego	R	14 12	lamb	naïve & non-naïve	*H. contortus*	AIs	97
Castellana		13	lamb	naïve & non-naïve	*H. contortus*	AIs	85
Churra	-	14	lamb	naïve & non-naïve	*H. contortus*	AIs	98
Blackbelly	R	16	lamb	non-naïve	*T. colubriformis*	NI & AIs	99
Scottish Blackface-cross	-	46	yearling	naïve & non-naïve	*T. circumcincta*	AIs	100
Scottish Blackface		30	lamb	non-naïve	*T. circumcincta*	AIs	41
Canaria Hair Canaria	R S	18 19	yearling	non-naïve	*H. contortus*	AIs	101
Greyface cross Suffolk	-	28	lamb	naïve & non-naïve	*T. circumcincta*	AIs	49
Greyface cross Suffolk	-	28	lamb	naïve & non-naïve	*T. circumcincta*	AIs	102
Texel	-	256	lamb	naïve & non-naïve	*H. contortus*	AIs	103
INRA 401	-	81	lamb	naïve & non-naïve	*H. contortus*	AIs	86
St. Croix hair Wool[5]	R S	26 26	lamb	non-naïve	*H. contortus*	AIs	15
Scottish Blackface	R&S	20	lamb	non-naïve	*T. circumcincta*	AIs	21

Churra	-	22	yearling	non-naïve	*T. circumcincta*	AIs	[40]
Blackbelly Columbia	R S	27 29	lamb	naïve	*H. contortus*	AIs	[34]
Texel	R	-	lamb	non-naïve	*T. circumcincta*	NI	[82]
Santa Ines crossbred	-	54	lamb	non-naïve	*H. contortus & H. placei*	AIt	[57]
Suffolk Texel	S R	57 85	lamb	naïve	*T. circumcincta*	NI	[13]
Texel	-	256	lamb	naïve & non-naïve	*H. contortus*	AIs	[60]
Gulf Coast Native Suffolk	R S	30 30	lamb	non-naïve	*H. contortus*	AIst	[33]
Romney	R&S lines	816	lamb	non-naïve	*T. colubriformis*	NI	[50]
Romney	R&S lines	21	yearling	naïve & non-naïve	T. colubriformis	AIs	[51]
Romney x Texel x Finnish Landrace	-	614	lamb	non-naïve	*H. contortus*	NI	[45]
Santa Ines Ile de France	R S	10 12	lamb	non-naïve	*H. contortus & T. colubriformis*	NI	[12]
INRA 401 Barbados Black Belly	S R	28 25	lamb	naïve & non-naïve	*H. contortus*	AIs	[104]
Marino	R line	20	yearling	non-naïve	*T. colubriformis and/or T. circumcincta*	AIt	[105]
St. Croix Florida Native Dorset/Rambouillet	R R S	20 12 16	lamb	naïve & non-naïve	*H. contortus*	AIs	[9]

[1]Gen.: Genotypes Resistant (R) and Susceptible (S)

[2]n: Number of animals/genotype

[3]GIN sp.: Gastrointestinal nematode species

[4]Inf.: Infection protocol, single artificial infection (AIs), trickle artificial infection (AIt), natural infection (NI).

[5]50% Dorset 25% Finnsheep 25% Rambouillet,

[6]Blackface ewe × Blue-faced Leicester ram,

795 **Table 2.** Genomic regions associated with immunoglobulin-resistance to GIN infection.

Chr[1]	Breed	Infection, parasite species	Association	Marker intervals (MI)/SNP/candidate gene (CG)/allelic effects	Reference
0	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: s27388.1	[78]
1	Churra	Strongylidae	IgA	OAR1 markers at position 37cM (ILSTS044)	[106]
1	Spanish Churra	Natural, mainly T. circumcincta	IgA (L4)	MI: BMS835 - ILSTS044	[77]
3	Soay	Natural, mainly T. circumcincta	IgA (L4)	CG: (o(IFN)-γ)*126 allele	[107]
3	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	MI: KD103 - LYZ	[73]
3	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: OAR3_227580261.1	[78]
3	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: OAR3_215619424.1	[78]
4	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: OAR4_90051359.1	[78]
4	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: OAR4_87762617.1	[78]
4	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: s57016.1	[78]
5	Romane × Martinique Black Belly backcross	Artificial, H. contortus	IgG (ESP)	MI: OAR5_67605574.1–OAR5_67883800_X.1	[81]
5	Romane × Martinique Black Belly backcross	Artificial, H. contortus	IgG (ESP)	MI: OAR5_100699982.1–DU183841_402.1	[81]
6	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: OAR6_107079726.1	[78]
8	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: OAR8_53084022.1	[79]
8	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: s42819.1	[79]
9	Churra	Strongylidae	IgA	OAR9 markers at 50cM position	[106]
9	Romane × Martinique Black Belly backcross	Artificial, H. contortus	IgG (ESP)	MI: OAR9_85325486.1–s48117.1	[81]
10	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: s56461.1	[79]
10	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: OAR10_23921485.1	[79]
10	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: s61799.1	[79]
11	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: DU232778_232.1	[79]
12	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: s68938.1	[79]
14	Romane × Martinique Black Belly backcross	Artificial, H. contortus	IgG (ESP)	MI: OAR14_48832510.1–s30682.1	[81]
14	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: OAR14_21336208.1	[79]

15	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: s75729.1	[79]
20	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	MI: BM1815 - DRB1	[73]
20	Rhönschaf	Artificial, H. contortus	IgL	DYMS1 (DYA) allele C	[71]
20	Scottish Blackface	Natural, mainly T. circumcincta	IgA (L3)	SNP: OAR20_40924783_X.1	[78]
21	Romane × Martinique Black Belly backcross	Artificial, H. contortus	IgG (ESP)	MI: s27845.1–OAR21_14592163.1	[81]
23	Romney × Coopworth	Natural, mainly T.colubriformis	IgE (total)	MI: Centomere - BM226	[80]
23	Romney × Coopworth	Natural, mainly T.colubriformis	IgG (L3)	MI: McMA1 - ADCYCAP1	[80]
25	Churra	Natural, mainly T. circumcincta	IgA (L4)	SNP: s21640.1	[79]
26	Romane × Martinique Black Belly backcross	Artificial, H. contortus	IgG (ESP)	MI: OAR26_21857857.1–OAR26_22456940.1	[81]

[1]Chromosome

798 **Table 3.** Immunoglobulin classes reported to be significantly correlated with resistance traits
799

Ig class	Larval stage	Parasitological parameter	Breed	State	Inf.[1], GIN sp.[2]	Reference
AB	L3, adult	WormBurden	Romney	non-native	NI, *H. contortus*	38
		FEC	Merinoland	native	AI, *H. contortus*	37
		WormBurden	Rhön	native	AI, *H. contortus*	37
		WormLength	Merinoland	native	AI, *H. contortus*	37
IgA	L3	FEC	Blackface	non-native	AI, *T. circumcincta*	42
			Texel, Suffolk	native	NI, *T. circumcincta*	13
			Romney*Texel* FinnishLandrace	non-native	NI, *H. contortus*	45
			Pelibuey	native	NI, *H. contortus*	108
		WormBurden	Blackface	non-native	AI, *T. circumcincta*	42
			Texel, Suffolk	native	NI, *T. circumcincta*	13
		WormBurden, inhibited L4	Scotch Mule (Blackface ewe * Blue-faced Leicester ram)	native&non	AI, *T. circumcincta*	39
	L4	FEC	Churra	non-native	AI, *T. circumcincta*	40
		WormLength	Scottish Blackface	non-native	AI, *T. circumcincta*	41
			Churra	non-native	AI, *T. circumcincta*	40
	L5	FEC	Churra	non-native	AI, *T. circumcincta*	40
		WormBurden	Santa Ines	non-native	NI, *H. contortus*	12
IgE	L3	FEC	Texel	non-native	NI, *T. circumcincta*	82
			Texel	native	NI, *T. circumcincta*	13
		WormBurden	Texel, Suffolk	native	NI, *T. circumcincta*	13
IgG	L3	FEC	Blackbelly and Columbia	native	AI, *H. contortus*	34
		WormBurden	Merinoland	native	AI, *H. contortus*	37
			Santa Ines	non-native	NI, *H. contortus*	12
	L3, adult	WormBurden, L4Burden, AdultBurden	Merino	non-native	AI, *H. contortus*	97
IgG1	L3	FEC	Texel, Suffolk	native	NI, *T. circumcincta*	13
			Romney*Texel* FinnishLandrace	non-native	NI, *H. contortus*	45
		WormBurden	Romney	non-native	NI, *H. contortus*	38
IgG2	L3	FEC, WormBurden	Texel, Suffolk	native	NI, *T. circumcincta*	13

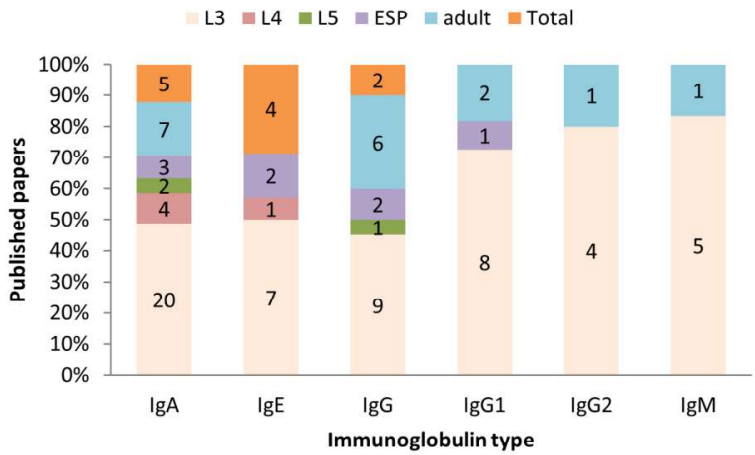
800

801 [1]Inf.: Infection protocol, artificial infection (AI), natural infection (NI).

802 [2]GIN sp.: Gastrointestinal nematode species

803

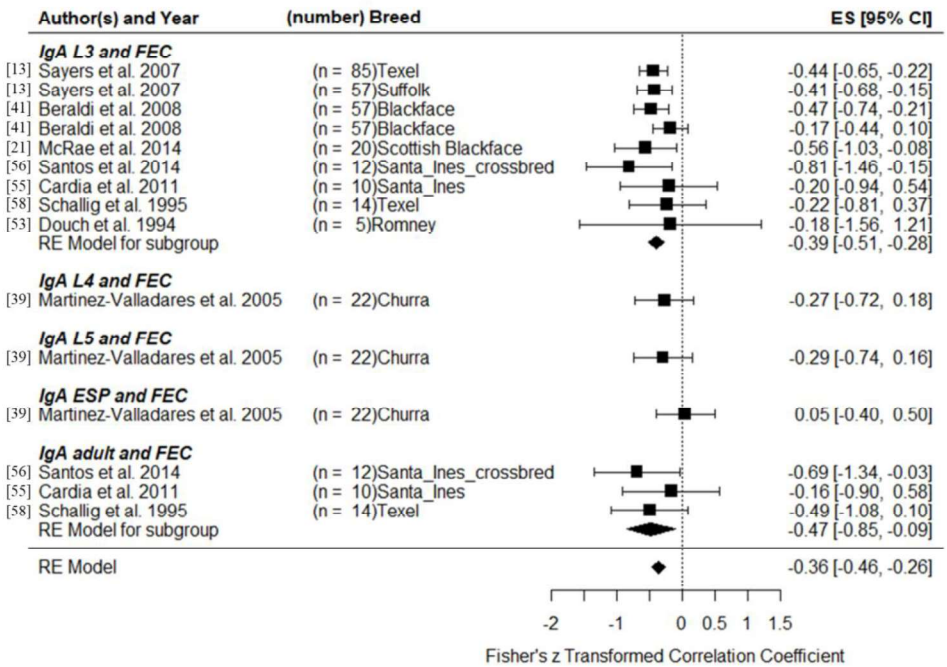
804 **Figures**



805

806 **Figure 1.** Percentages and numbers of published articles measuring different

807 immunoglobulins levels against different larva stage during GIN infection.



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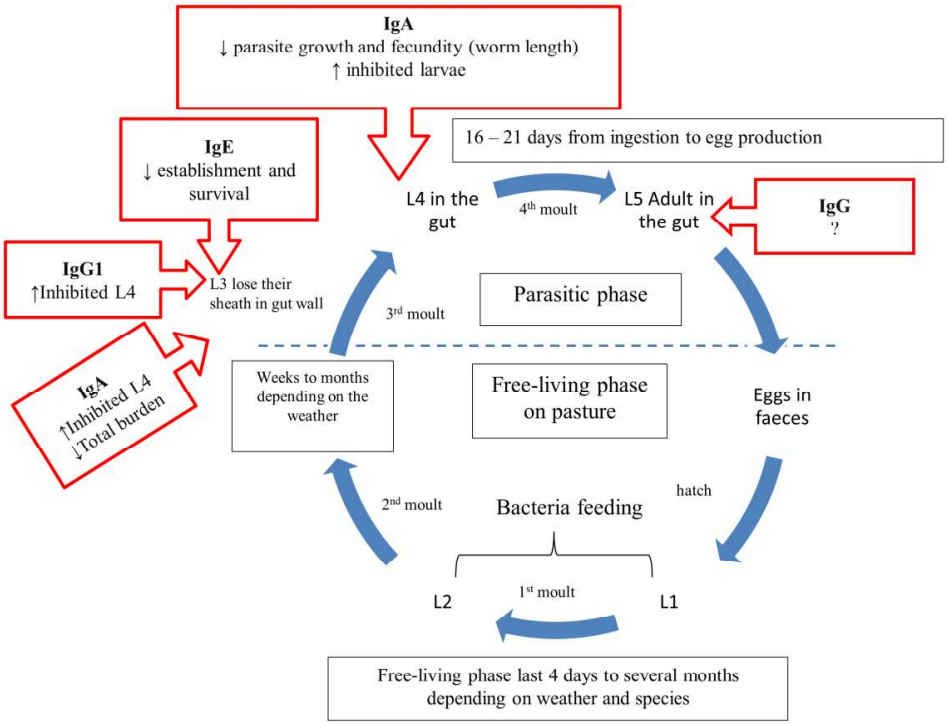
809 **Figure 2.** Forest plot for the correlation between IgA and fecal egg count (FEC). ES: effect

810 size, CI: confidence interval.



811

812 **Figure 3.** Immunoglobulins suggested role in resistant to gastrointestinal nematode during its

813 life cycle.

PLOS ONE

RESEARCH ARTICLE

Transcriptome variation in response to gastrointestinal nematode infection in goats

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Abstract

Gastrointestinal nematodes (GIN) are a major constraint for small ruminant production. Due to the rise of anthelmintic resistance throughout the world, alternative control strategies are needed. The development of GIN resistance breeding programs is a promising strategy. However, a better understanding of the mechanisms underlying genetic resistance might lead to more effective breeding programmes. In this study, we compare transcriptome profiling of abomasal mucosa and lymph node tissues from non-infected, resistant and susceptible infected Creole goats using RNA-sequencing. A total of 24 kids, 12 susceptible and 12 GIN resistant based on the estimated breeding value, were infected twice with 10,000 L3 *Haemonchus contortus*. Physiological and parasitological parameters were monitored during infection. Seven weeks after the second infection, extreme kids (n = 6 resistant and 6 susceptible), chosen on the basis of the fecal egg counts (FEC), and 3 uninfected control animals were slaughtered. Susceptible kids had significantly higher FEC compared with resistant kids during the second infection with no differences in worm burden, male and female worm count or establishment rate. A higher number of differentially expressed genes (DEG) were identified in infected compared with non-infected animals in both abomasal mucosa (792 DEG) and lymph nodes (1726 DEG). There were fewer DEG in resistant versus susceptible groups (342 and 450 DEG, in abomasal mucosa and lymph nodes respectively). 'Cell cycle' and 'cell death and survival' were the main identified networks in mucosal tissue when comparing infected versus non-infected kids. Antigen processing and presentation of peptide antigen via major histocompatibility complex class I were in the top biological functions for the DEG identified in lymph nodes. The *TGFβ1* gene was one of the top 5 upstream DEG in mucosal tissue. Our results are one of the fist investigating differences in the expression profile induced by GIN infection in goats.

(EGS-ABG, Erasmus Mundus) and the division of animal genetics of INRA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Gastrointestinal nematode (GIN) infection is one of the most important economic constraints in small ruminant production. These parasites have a negative impact on animal health and welfare, but their main effect is reduced productivity and thus economic return [1,2]. For the last 50 years control strategies have been based mainly on the use of anthelmintics but unfortunately, the selection pressure created by their repeated use has led to the rapid development of anthelmintic resistance in GIN populations worldwide [3–5]. The constant increase in the prevalence of anthelmintic-resistant GIN strains together with increasing demand for chemical-free animal products and potential environmental consequences of anthelmintics increase the need for novel alternative control strategies. The improvement of host response against GIN through genetic selection of resistant lines or breeds is among the most promising strategies.

The feasibility of different selection programs has been studied, in both temperate and tropical conditions, mainly in sheep and to a lesser extent in goats [1,6,7]. Selection is in most studies mainly based on fecal egg count (FEC), however, some selection schemes include other relevant traits such as production and measures of anemia and blood eosinophilia under conditions of either natural or experimental infection with GIN [8–11]. Fecal egg count is a moderately heritable trait ($h^2 \sim 0.3$) for which response to selection has been shown in sheep and also in goats [1]. Despite numerous studies comparing intra- or inter-breed resistance variation in small ruminants [12–15], the detailed mechanisms involved in genetic resistance remain unclear. One challenge for the coming years is the identification of new biological resistance and/or susceptibility markers to improve the efficiency and timeliness of breeding programs. Comparative transcriptomic analysis is a pertinent method for understanding the molecular genetic basis of complex traits such as host resistance. Several previous studies have been undertaken to identify genes and biological processes associated with the host response to GIN in the duodenum [16–18] and the abomasal mucosa [19,20] and the draining lymph nodes [21–25]. Although goats are more susceptible to GIN infections than sheep, most of the research programs have aimed to investigate host-GIN interactions in sheep. The need for more studies on the goat model has become increasingly important since it was reported that goats develop a different set of strategies than sheep to regulate parasitic infections [26], and they may differ in the sequence for establishing immunity [27].

The aim of the present study was to identify the molecular pathways involved in the response of Creole goats to GIN infection by analyzing the transcriptomes of abomasal mucosa and draining lymph nodes of infected versus non-infected and resistant versus susceptible kids.

Materials and methods

Ethics statement

All animal care handling techniques and procedures as well as the license for experimental infection, blood sampling and slaughtering were approved by INRA, according to the certificate number A-971-18-02 of authorization to experiment on living animals issued by the French Ministry of Agriculture, before the initiation of the experiment.

Animals and experimental design

Twenty four 10-month old Creole kids with limited natural GIN infection (FEC < 100) were reared indoors at the experimental facility of INRA in Guadeloupe (PTEA, Plateforme Tropicale pour l'expérimentation Animale). Animals had been bred for resistant and susceptible to

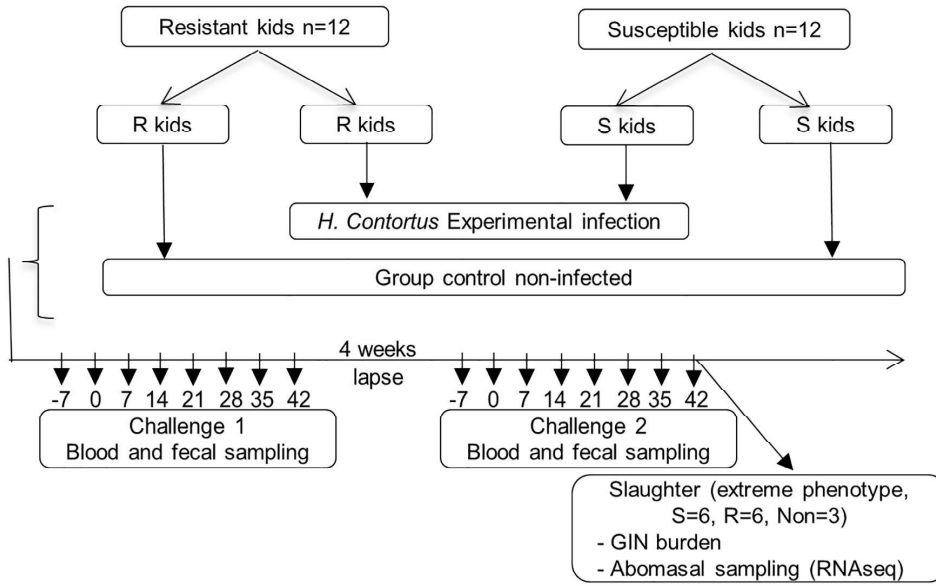

Fig 1. Experimental design. S, susceptible; R, resistant; Non, non-infected.

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GIN according to estimated breeding values of FEC. The average predicted breeding values on FEC of the resistant and susceptible kids differed by 1.04 genetic standard deviation (n = 12 resistant and n = 12 susceptible). Two consecutive challenges of 7 weeks with a 4 week interval between the end of challenge 1 and the start of challenge 2 were carried out (Fig 1). For each challenge, kids were orally infected with a single dose of 10,000 *Haemonchus contortus* third-stage larvae (L3) at Day 0. A group of 6 kids (n = 3 resistant and n = 3 susceptible) from the 24 animals was used as non-infected controls. Fecal and blood samples from each animal were recovered once a week during the course of the experimental infection. At the end of the second infection (42 days post infection), animals with extreme FEC measured during the infection (6 resistant and 6 susceptible) and 3 non-infected control animals were slaughtered with a penetrating captive bolt immediately followed by exsanguination for sampling of abomasal mucosa and lymph nodes tissue and GIN burdens quantification (Fig 1).

Parasitological techniques and blood samples

FEC was determined using a modified McMaster technique for rapid determination as described by Aumont [28]. Blood samples (2 x 3 ml) were individually collected once a week from each animal by using disposable syringes and 20-Ga needles in two plastic tubes (one coated with EDTA, ethylenediamine tetraacetic acid K3, Becton Dickinson, Plymouth, UK) by jugular venipuncture. Blood samples previously placed in EDTA coated tubes were used to measure the number of circulating eosinophils according to the method of Dawkins [9] with a Malassez cell counter. The packed cell volume (PCV) was measured using the capillary micro-haematocrit method. Blood samples collected in plastic tubes without EDTA (serum tubes; Becton Dickinson) were centrifuged for 5 min. at 5000 rpm then serum were frozen at -20˚C until analysis. Serum pepsinogen levels were determined using a micromethod for routine determination according to Dorny and Vercruysse [29]. At slaughter the abomasum contents

were stored in 5% formalin for total male, female and worm burden counts. Samples of the fundic abomasum and the draining lymph nodes tissues (approximatively 1cm X 1cm) were collected with a sterile scalpel and snap frozen in liquid nitrogen then stored at -80˚C until total RNA extraction.

RNA extraction and quality analysis

Total RNA was extracted from frozen tissues samples using the NucleoSpin RNA isolation kit (Macherey-Nagel, Hoerdt, France) in accordance with the manufacturer's instructions, except DNase incubation which was performed with twice the indicated amount of enzyme. The total RNA concentration was measured with NanoDrop 2000 (ThermoScientific TM, France) and the quality was quantified using a Agilent 2100 Bioanalyzer (Agilent Technologies, France). The extracted total RNA was stored at -80˚C until use.

Library preparation and sequencing

High-quality RNA (RIN $>$ 7.5) from all tissues samples (abomasal mucosa and lymph nodes) was used for the preparation of cDNA libraries according to Illumina's protocols (Illumina TruSeq RNA sample prep kit for mRNA analysis). Briefly, poly-A mRNA was purified from 4μg of total RNA, fragmented and randomly primed for reverse transcription to generate double stranded cDNA. The cDNA fragments were then subjected to an end repair process, consisting of the addition of a single 'A' base, and the ligation of indexed Illumina adapters at both ends of cDNA. These products were then purified and enriched by PCR to create the final barcoded cDNA library. After quality control and quantification, cDNA libraries were pooled in groups of 6 and sequenced on 5 lanes on the HiSeqTM 2000 (Illumina NEB, USA) to obtain approximatively 30 million reads (100 bp paired-end) for each sample with insert sizes ranging from 200 to 400 base pairs.

Quality control and read mapping to the reference genome

The quality control check on raw reads in FASTQ format were processed using FASTQC and the Q20, Q30 and GC contents of the clean data were calculated. The sequences obtained by RNA-Seq were splice-aligned, for each library, using STAR (version 2.3.0e with standard parameters) [30]. The reads were mapped to the *Capra hircus* genome (assembly ARS1). The resulting alignment files were merged to produce a global splice alignment reference with samtools sort, merge and index (version 0.1.19-44428cd using standard parameters). This reference was processed using the Cufflinks program [31] (version v2.1.1 with standard parameters and Ensembl ref_CHIR_1.0_top_level.gtf as reference) to identify expressed transcripts and genes. The reference transcript and gene model files were used to quantify the expression in each library with featureCounts (version 1.4.5-p1 using standard parameters) [32]. The same reference was used for all samples. The resulting count files were merged using Unix cut and paste commands to produce a global count file on which the statistical analyses were performed. The three outputs of this first part of the process gathered the count file, the reference transcriptome file and the unaligned read sets.

Expression profiling

Differentially expressed genes (DEG) of read counts were identified using the Bioconductor package DESeq2 [33] run within the R software (v3.4.1). Four comparisons were performed: 1- mucosa tissue from infected versus non-infected animals; 2- lymph node tissue from infected versus non-infected animals; 3- mucosa tissue from infected resistant versus infected

susceptible animals and 4- lymph node tissue from infected resistant versus infected susceptible animals. Low expression tags were filtered, keeping only genes that achieved at least 5 counts in each condition. To account for multiple testing, genes were filtered using a Benjamini and Hochberg false discovery rate (FDR) of < 0.001.

Gene ontology (GO) and pathway enrichment analysis

GO analysis was used as an international standardized gene functional classification system to describe properties of genes and their products. DEG are functionally grouped into the three GO domains (biological processes, cellular components, and molecular processes) looking for significantly enriched functions compared to the genomic background. GO enrichment analysis and GO annotations plotting were performed using the clusterProfiler R-package. Due to the lack of goat (*Capra hircus*) GO data, GO were analyzed based on human annotation. All enriched GO terms that possessed a p-value < 0.01 were displayed.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is the major public pathway database of biological systems that integrates genomic, chemical and systemic functional information [34]. KEGG pathway enrichment analyses were performed using DAVID [35,36]. Pathways with a Q-value < 0.05 were considered significant. Moreover, analysis of canonical pathways and regulator effects as well as network analysis were performed using Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA) for DEG in each comparison.

Quantitative real-time PCR (qRT-PCR) validation

Gene expression of 7 genes (n = 5 for the abomasal mucosa and n = 5 for the lymph nodes; 3 genes were common to both tissues) was measured by q-PCR in order to validate the results obtained in the RNAseq analysis. Goat ACTB (actin beta) gene whose expression remained stable among the samples was used as the endogenous control for all reactions. A total of 2 μg of high quality total RNA (RIN > 7.5) was used to synthetize the cDNA using M-MLV Reverse Transcriptase (Promega, Charbonières, France) according to the manufacturer's instructions. All qPCR reactions were carried out in 48-well plates in a Prime Pro 48 Real-Time PCR System and analyzed with the ProStudy Software v5.2.10 (Techne, Staffordshire, UK). Taqman predesigned gene expression assay (Table 1) and the universal PCR master mix were purchased from Applied Biosystems and the analyses were performed according to the manufacturer's instructions (ThermoFisher Scientific, Applied Biosystems, Courtaboeuf, France). Samples were analyzed in duplicate in a total volume of 20 μl containing: 4 μl of cDNA, 10 μl of 2X TaqMan Fast Advanced Master Mix, 1 μl of TaqMan Gene Expression Assays 20X (ThermoFisher Scientific, Applied Biosystems, Courtaboeuf, France) and 5 μl of distilled RNAse DNAse-free

Table 1. List of target genes for qPCR validation and assay IDs according to the manufacturer.

Gene symbol	Gene description	Assay IDs
ACTB	*actin beta*	Ch04810274_s1
CCL20	*C-C motif chemokine ligand 20*	Ch04791475_m1
CLEC4E	*C-type lectin domain family 4, member e*	Ch04688119_m1
Galectin-9	*LOC102189615*	Ch04788979_m1
IFI6	*interferon alpha inducible protein 6*	Ch04807049_g1
IL13	*interleukin 13*	Ch04684348_m1
PGLYRP1	*peptidoglycan recognition protein 1*	Ch04786957_m1
TLR4	*toll-like receptor 4*	Ch04654181_m1

https://doi.org/10.1371/journal.pone.0218719.t001

water. Relative gene expression values were determined using relative quantification ($2^{-\Delta\Delta Ct}$ method, [37]).

Statistical analysis on the phenotypic data

The FEC variable was log transformed in order to normalize its variance. The statistical analyses of the phenotypic (FEC, eosinophilia, pepsinogen and PCV) differences between animals in the infected, non-infected, resistant and susceptible groups were tested by using PROC MIXED in SAS (v. 9.4, SAS Inst. Inc., Cary, NC, USA, 2012) and differences were considered significant when $P < 0.05$. The results are presented after back-transformation. The association between parasitological (number of males, number of females, worm burden and establishment rate) and physiological (FEC, eosinophilia, pepsinogen and PCV) variables at slaughter were determined using Pearson's correlation coefficient (v. 9.4, SAS Inst. Inc., Cary, NC, USA, 2012).

Results and discussion

Phenotypic and parasitological measurements

The packed cell volume (PCV) values significantly decreased during the first challenge in both resistant and susceptible kids until 28 days post-infection (d.p.i.) with no significant difference between the groups (Fig 2a). A small decrease was observed during the second challenge in both groups, with values significantly different than control kids from day 28 to 42 post-infection (Fig 2b). During the second infection, the PCV was negatively correlated with establishment rate (r = -0.66, $P < 0.01$) and other physiological variables, including fecal egg counts (FEC) (r = -0.47, $P < 0.01$), eosinophilia (r = -0.30, $P < 0.01$) and pepsinogen (r = -0.40, $P < 0.01$ Table 2). Similar correlations between PCV and FEC were previously reported in sheep infected with *H. contortus* in Florida Native, Rambouillet sheep and their crossbreed (r = -0.45) [38] and in Pelibuey sheep (r = -0.35, $P < 0.01$) [39]. A stronger negative correlation (r = -0.78) was reported for Caribbean hair sheep and conventional wool sheep [40].

The FEC remained at zero until 14 d.p.i. in both resistant and susceptible groups during both the first (Fig 2a) and second infection (Fig 2b). Susceptible kids had significantly higher FEC than resistant kids during the second infection (Fig 2b). No significant differences between resistant and susceptible kids in worm burden (1669±673 and 2556±673, respectively), male worm count (1042±392 and 1371±392, respectively), female worm count (627±285 and 1186±285, respectively) or establishment rate (12.35±6.75 and 19.70±6.75, respectively) were observed in samples from kids slaughtered at the end of the second challenge. The correlations of worm burden with FEC (r = 0.63, P < 0.05) and PCV (r = -0.61, P < 0.05) were similar to those previously reported in sheep [38,40].

These results suggested that the adaptive immune response influences genetic resistance which is expressed during the second experimental infection but not during the first one. In keeping with a previous study the lower FEC observed in resistant compared with susceptible kids was not correlated with differences in worm burden and establishment rate [11]. The control of fecundity in goats is probably driven by other mechanisms than IgA activity [41] in contrast with sheep in which IgA was suggested to be the major mechanism controlling GIN fecundity [42,43].

In sheep, numerous studies have shown that eosinophil response is associated with local IgA response to control GIN fecundity [42,44,45]. In this experiment, there was no significant correlation of eosinophilia and pepsinogen with FEC. However, eosinophilia and pepsinogen showed significant positive correlations with male worm count (r = 0.62 and r = 0.57, $P < 0.05$, respectively), female worm count (r = 0.64 and r = 0.58, $P < 0.05$, respectively),

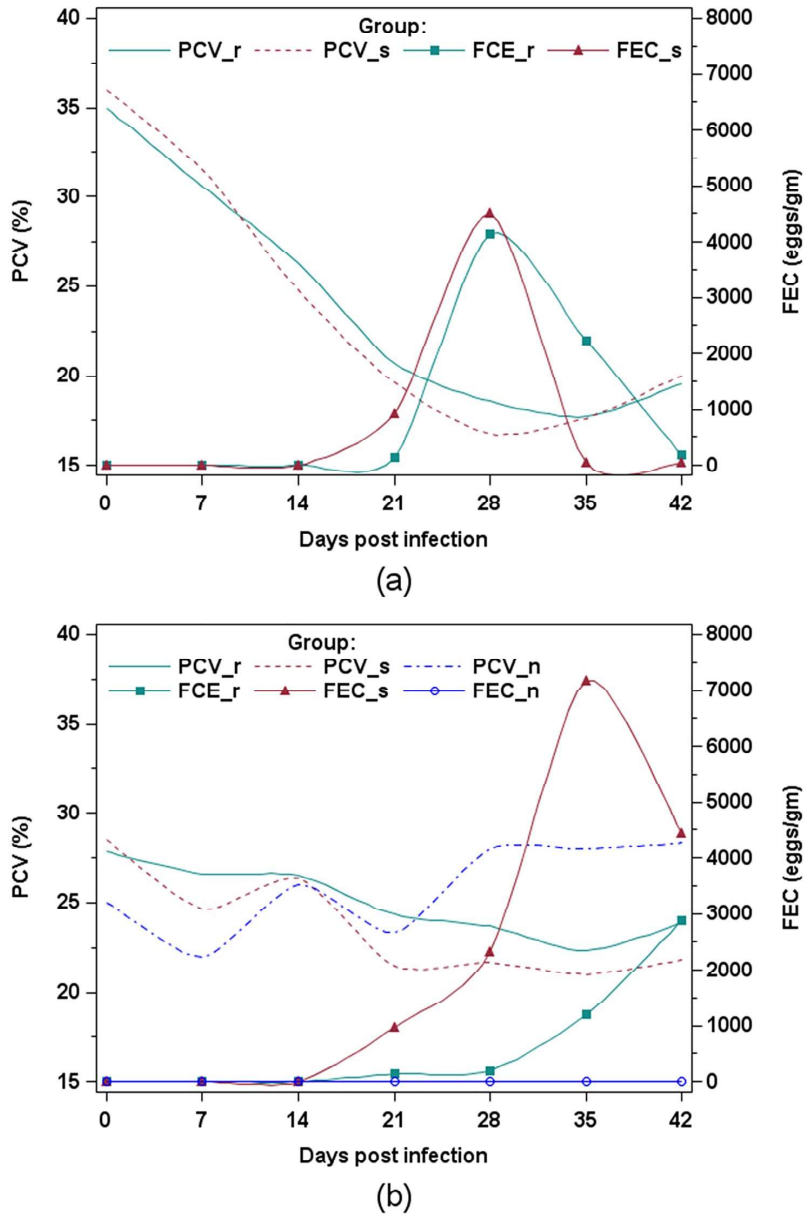

Fig 2. Least square means of fecal egg counts (FEC) and packed cell volume (PCV) in resistant (r) and susceptible (s) Creole kids infected with 10,000 *H. contortus* larvae (L3) and non-infected (n) animals. (a) first infection, (b) second infection.

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Table 2. Correlation coefficients between parasitological and physiological variables at slaughter.

	FEC	Eosinophilia	Pepsinogen	PCV
Male	0.61*	0.62*	0.57*	-0.61*
Female	0.66*	0.64*	0.58*	-0.61*
Worm burden	0.63*	0.63*	0.58*	-0.61*
Establishment rate	0.41	0.64*	0.73*	-0.66*
FEC		0.12	0.03	-0.47*
Eosinophilia			0.30*	-0.30*
Pepsinogen				-0.40*

* $P < 0.05$. FEC: fecal egg counts. PCV: packed cell volume

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worm burden ($r = 0.63$ and $r = 0.58$, $P < 0.05$, respectively) and establishment rate ($r = 0.64$ and $r = 0.73$, $P < 0.05$, respectively, Table 2). The positive correlations in goats compared to the negative correlations in sheep suggest that different mechanisms are involved in the genetic resistance in goats and sheep.

Transcriptome analysis

1. RNA sequencing. Sequencing of the RNA samples resulted in an average of 8.8±2.0 million reads per sample aligned to a unique region of the goat genome. These reads corresponded to 21399 genes of the *C. hircus* genome (assembly ARS1). A total of 15007 to 15585 genes had at least 5 counts in goat abomasal and lymph node tissues. These genes were subsequently used in the comparative analysis.

2. Differential gene expression. The number of DEG in each comparison is shown in Table 3. The number of DEG was higher in lymph node tissue compared with mucosa tissue in both comparisons (infected versus non-infected (1726 and 792) and resistant versus susceptible (450 and 342, respectively). Meanwhile, the fold change range was on average higher in the mucosal tissue compared with lymph node tissue. Human orthologues were mapped for 82–89% of the DEG and these orthologues were used for the GO and IPA analysis (Table 3).

The number of DEG identified in our study was relatively high compared to RNA sequencing studies of lymph node tissues comparing resistant and susceptible sheep [24,25] and lower than the number of DEG in the blood transcriptome of resistant and susceptible goats [46]. There may be variations among species and tissues. However, Bhuiyan [46] used fold change values ≥ 2.5 or ≤ -2.0 as the basis to identify DEG, while we used all significant DE genes on the basis of a FDR < 0.001.

Functional classification analysis

1. Gene ontology (GO). An enriched GO term analysis including 'biological processes', 'molecular functions', and 'cellular components' was performed using the DEG from each

Table 3. Number of genes differentially expressed for each comparison.

Comparison	FDR < 0.001	Log2 fold change range	Human orthologs
Inf. vs non-inf. mucosa	792	-22.77, 4.55	648 (82%)
Inf. vs non-inf. lymph	1726	-9.41, 4.84	1519 (88%)
Res. vs sus. mucosa	342	-4.38, 8.19	303 (89%)
Res. vs sus. lymph	450	-2.67, 2.39	384 (85%)

FDR, false discovery rate.

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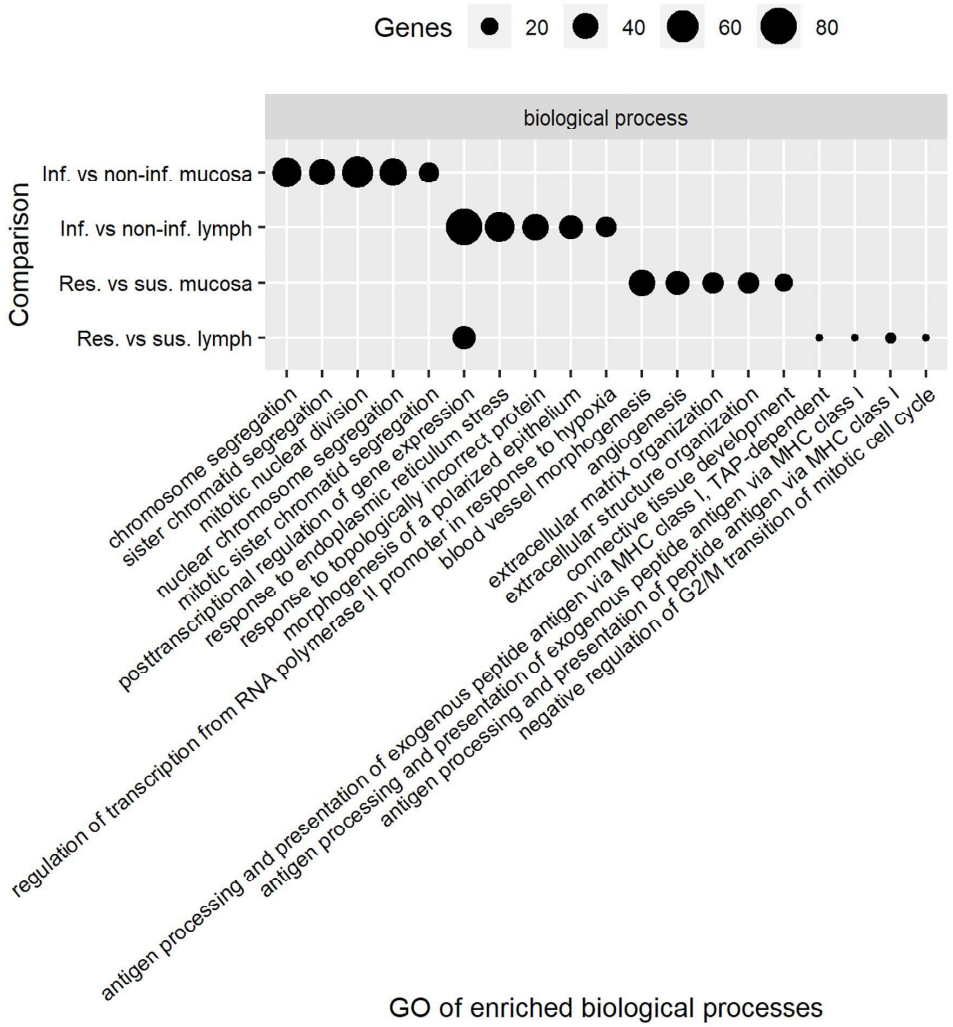

Fig 3. Top 5 biological processes that changed among groups. Infected versus non-infected (Inf. vs non-inf.), resistant versus susceptible (Res. vs sus.).

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comparison. The top 5 significant functional groups in each term are presented in Figs 3 and 4. The top significant biological processes for the DEG identified using the comparison of mucosa tissue from non-infected and infected animals were chromosome segregation and mitotic nuclear division, while it was blood vessel morphologenesis and angiogenesis for the comparison between resistant and susceptible animals using the same tissue. Post-transcriptional regulation of gene expression was the top significant biological process for DEG identified in abomasal lymph node tissue of infected versus non-infected kids and resistant versus susceptible kids.

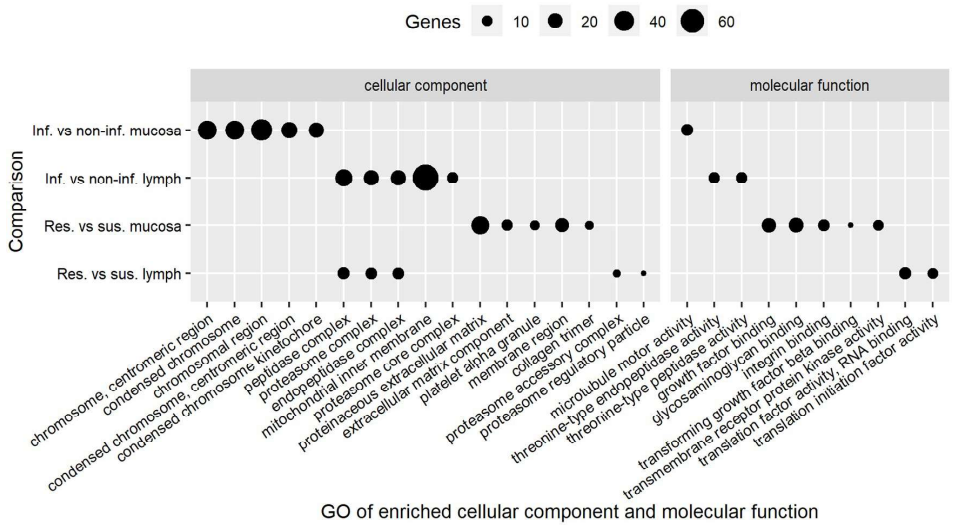

Fig 4. Top 5 cellular components and molecular functions that changed among groups. Infected versus non-infected (Inf. vs non-inf.), resistant versus susceptible (Res. vs sus.).

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The second, third and fourth top biological functions for the DEG identified from the comparison of lymph node tissue from resistant and susceptible goats were related to antigen processing and presentation of peptide antigen via major histocompatibility complex (MHC) class I (Fig 3). The 'antigen processing and presentation of peptide antigen via MHC class I' was also reported as one of the major functional annotation cluster of genes differentially expressed in abomasal lymph nodes in sheep breeds known to differ in GIN resistance [24]. The implication of the MHC Class I molecules in the mechanisms underlying genetic resistance to *H. contortus* was reported through an association between reduction in FEC and a homozygotes allele for the MHC class I (*OMHC1-188*) in sheep [39]. MHC class I present intracellular peptides at the cell surface to CD8+ T cells when intracellular pathogens such as viruses induce cellular expression of viral proteins. Some of these viral proteins are tagged for degradation, with the resulting peptide fragments entering the endoplasmic reticulum and binding to MHC class I molecules [47]. Meanwhile, MHC class II present peptides from extra-cellular pathogens at the cell surface of CD4+ T cells which help to trigger an appropriate immune response including localized inflammation or lead to a full-force antibody immune response due to activation of B cells [47]. Our results in goats and other results from previous studies in sheep [24,39] suggest that MHC class I plays a role in resistance to GIN infection. Moreover, other studies in goats indicated that humoral response is not correlated with GIN resistance in goats [41,48,49].

The complexes peptidase, proteasome and endopeptidase were the top three significant cellular components for the DEG in abomasal lymph node tissue of both infected versus non-infected kids and resistant versus susceptible kids, which reflects the role of MHC class I. The top significant molecular function terms for the comparison of mucosa tissue from susceptible and resistant kids were growth factor binding and glycosaminoglycan binding while for lymph node tissue it was translation factor activity, RNA binding and translation initiation factor activity (Fig 4). Growth factor binding was found to be one of the most enriched molecular

Table 4. Top 5 significant canonical pathways identified by Ingenuity pathway analysis using DE genes.

Comparison	Ingenuity Canonical Pathways	P value	Overlap ratio[a]
Infected versus non-infected mucosa	Mitotic Roles of Polo-Like Kinase	5.50E-06	0.18
	Eicosanoid Signaling	4.60E-05	0.15
	Role of CHK Proteins in Cell Cycle Checkpoint Control	6.69E-05	0.16
	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.73E-04	0.16
	Hereditary Breast Cancer Signaling	2.20E-04	0.10
Infected versus non-infected lymph	Protein Ubiquitination Pathway	1.55E-09	0.19
	Factors Promoting Cardiogenesis in Vertebrates	2.39E-06	0.24
	Molecular Mechanisms of Cancer	5.98E-06	0.14
	STAT3 Pathway	3.10E-05	0.23
	Adipogenesis pathway	5.34E-05	0.18
Resistant versus susceptible mucosa	Osteoarthritis Pathway	3.93E-06	0.07
	Human Embryonic Stem Cell Pluripotency	6.54E-05	0.07
	Factors Promoting Cardiogenesis in Vertebrates	7.07E-05	0.09
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.23E-04	0.06
	Regulation of the Epithelial-Mesenchymal Transition	1.63E-04	0.06
Resistant versus susceptible lymph	Protein Ubiquitination Pathway	5.89E-05	0.06
	EIF2 Signaling	2.56E-04	0.06
	Regulation of eIF4 and p70S6K Signaling	8.35E-04	0.07
	Granzyme B Signaling	3.31E-03	0.19
	Cell Cycle Regulation by BTG Family Proteins	4.46E-03	0.11

[a] The ratio is calculated by taking the number of DE genes that participate in a Canonical Pathway, and dividing it by the total number of genes in that Canonical Pathway.

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functions while comparing blood from resistant and susceptible goats [46]. The translation initiation factor activity pathway was previously found to be associated with genes more highly expressed in samples from the duodenum of susceptible compared with resistant sheep [18].

2. Pathway enrichment analysis. The Ingenuity pathway analysis identified 96 significant canonical pathways for the DEG for mucosa and 94 for lymph node samples from infected versus non-infected kids, and 37 pathways for mucosa and 35 for lymph node samples in the comparison of resistant versus susceptible kids. The KEGG pathway enrichment analysis on the other hand identified 25 (mucosa) and 22 (lymph node) pathways in infected versus non-infected kids, and 11 (mucosa) and 8 (lymph node) significant pathways for resistant versus susceptible kids. The top 5 canonical pathways for each comparison group are presented in Table 4 and the top 5 KEGG pathways in Table 5. The overlap ratio (the number of DEG involved in a particular pathway divided by the total number of genes in that pathway) was in general higher for KEGG than that for the canonical pathways.

The cell cycle pathway was one of the most significant pathways identified by canonical or KEGG pathways for the DEG identified in mucosa tissue from infected versus non-infected kids (Tables 4 and 5). The protein ubiquitination pathway was the most significant canonical pathway for DEG in lymph node tissue in both the comparison of infected versus non-infected and of resistant versus susceptible kids (Table 4). Meanwhile, proteasome and biosynthesis of antibiotics were in the top KEGG pathways for the same comparisons (Table 5).

We found that the 'Cell Cycle Regulation by BTG Family Proteins' pathway was one of the most significant canonical pathways identified for DEG in abomasum lymph node tissue from susceptible versus resistant kids. The same pathway was identified as significantly impacted

Table 5. Top 5 significant KEGG pathways identified by DAVID using DE genes.

Comparison	DAVID KEEG Pathways	P value	Overlap ratio[a]
Infected versus non-infected mucosa	Cell cycle	3.20E-06	0.14
	Fructose and mannose metabolism	1.20E-04	0.25
	Amino sugar and nucleotide sugar metabolism	4.10E-04	0.18
	Fat digestion and absorption	1.10E-03	0.18
	Protein export	1.80E-03	0.24
Infected versus non-infected lymph	Protein export	3.10E-09	0.60
	Protein processing in endoplasmic reticulum	1.10E-08	0.24
	Proteasome	5.70E-08	0.40
	Biosynthesis of antibiotics	8.00E-04	0.16
	Central carbon metabolism in cancer	3.20E-03	0.22
Resistant versus susceptible mucosa	Regulation of lipolysis in adipocytes	2.50E-04	0.13
	PI3K-Akt signaling pathway	2.40E-03	0.04
	TGF-beta signaling pathway	3.50E-03	0.08
	PPAR signaling pathway	6.90E-03	0.08
	Malaria	1.10E-02	0.10
Resistant versus susceptible lymph	Proteasome	1.20E-06	0.21
	Carbon metabolism	2.90E-04	0.10
	Biosynthesis of antibiotics	3.30E-04	0.07
	Ribosome biogenesis in eukaryotes	2.30E-03	0.10
	RNA transport	4.70E-03	0.07

[a] The ratio is calculated by taking the number of DE genes that participate in a KEGG Pathway, and dividing it by the total number of genes in that KEGG Pathway.

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during parasitic infection in the abomasum of resistant cattle [50]. Transforming growth factor beta (TGF-β) is a multifunctional cytokine belonging to a super family including three different isoforms (*TGFβ1*, *TGFβ2*, *TGFβ3*), which are related to the TGF-β signaling pathway besides many other signaling proteins produced by all white blood cells lineages and it is best known for its regulatory activity and induction of peripheral tolerance [51]. Recently, it was shown that the cytokine profile modulated by *rHCcyst-3* increases the secretion of *IL-10* and *TGF-ß1* in goat monocytes. This contributes to induce an anti-inflammatory environment which is favorable for worm survival [52]. The 'TGF-β signaling pathway' was reported to be a significant pathway regulated by DEG identified in blood of resistant versus susceptible goats [46]. We found that the 'TGF- β signaling pathway' was in the top 5 significant KEGG pathways and in the top 10 canonical pathways for the DEG in the comparison of mucosa samples between resistant and susceptible kids. Moreover, Transforming growth factor beta-1 (*TGFβ1*) was the first upstream regulator gene that was differently expressed in mucosa tissue of resistant versus susceptible and the fifth in infected versus non-infected kids, with a prediction to be inhibited in resistant kids (Table 6). TGF-β receptor 1 was reported as highly expressed in lymph node samples of wool (susceptible) sheep compared with hair (resistant) sheep at 27 day post infection with *H. contortus* [21].

3. Genetic associated diseases and networks. The top 5 diseases and biological functions for DEG identified for each comparison are presented in Fig 5. For all comparisons, 'cancer' and 'organismal injury and abnormalities' were in the top 5 diseases identified by IPA. Comparing infected with non-infected kids we found 'Cell death and survival' in the top molecular and cellular functions for mucosal tissue and lymph node samples. Moreover, molecular and cellular function was also one of the top functions when comparing lymph node tissue from

Table 6. Top 5 upstream regulators identified by Ingenuity pathway analysis using DE genes.

Comparison	Upstream regulator	P value	Predicted Activation
Infected versus non-infected mucosa	*ERBB2*	9.06E-14	Inhibited
	RABL6	1.08E-13	Inhibited
	NUPR1	1.21E-11	Activated
	FOXM1	2.66E-11	Inhibited
	TGFβ1	1.40E-10	
Infected versus non-infected lymph	*CST5*	2.38E-05	
	SYVN1	5.41E-05	
	FSH	6.42E-05	
	XBP1	2.65E-04	Inhibited
	ESR1	3.20E-04	
Resistant versus susceptible mucosa	*TGFβ1*	4.71E-14	Inhibited
	beta-estradiol	3.05E-11	Inhibited
	FGF2	6.60E-11	
	estrogen	7.49E-10	
	TAZ	1.22E-09	
Resistant versus susceptible lymph	*5-fluorouracil*	1.47E-06	Inhibited
	HNF4A	3.11E-05	
	E2F1	1.30E-04	
	CD 437	1.67E-04	Inhibited
	valproic acid	1.82E-04	Inhibited

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resistant versus susceptible kids despite being exposed to the same GIN population. It was therefore the shared significant biological function for multiple comparisons which mainly controls infection consequences. Figs 6 and 7 show the DEG that control cell survival as identified in the mucosal tissue from infected versus non-infected (Fig 6) and resistant versus

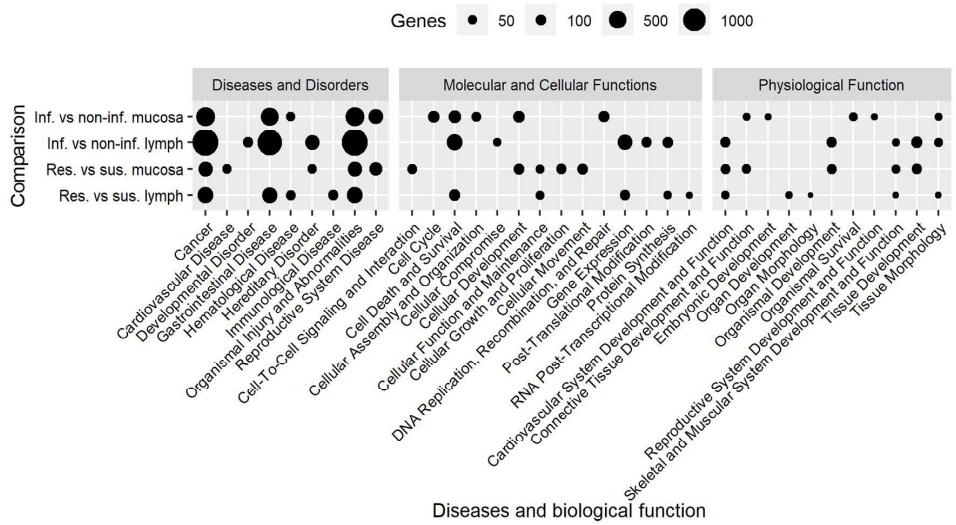

Fig 5. Top 5 diseases and biological function identified by Ingenuity pathway analysis using DE genes.

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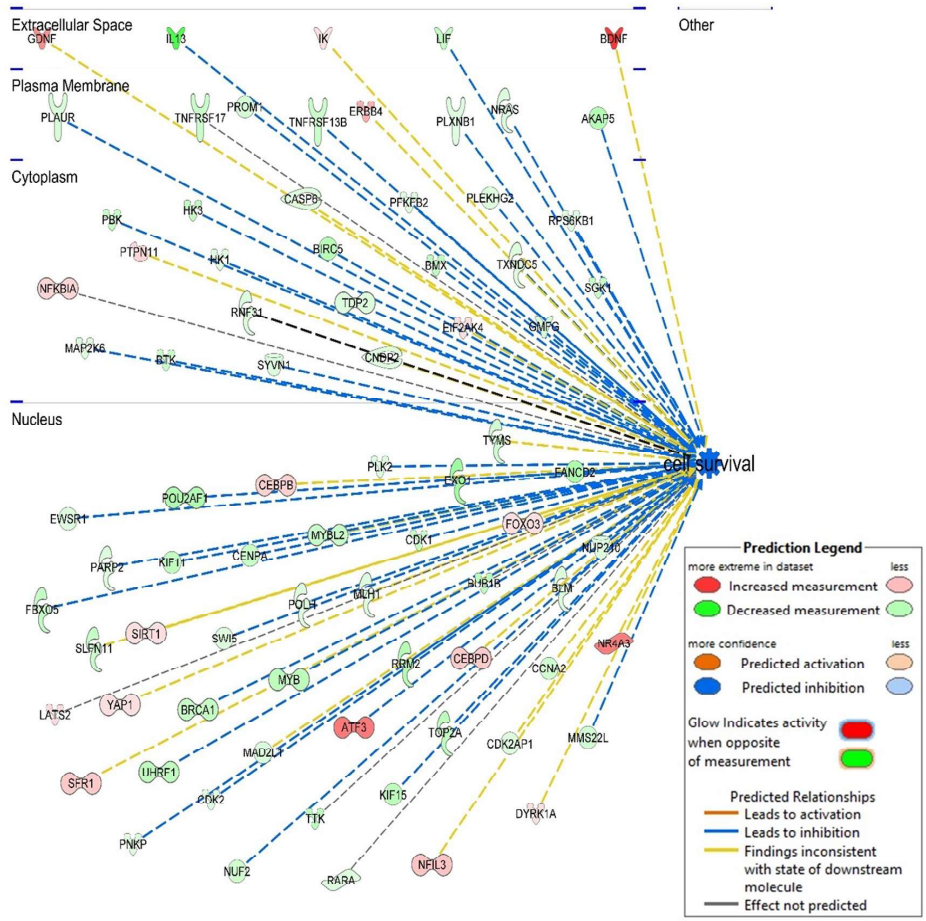

Fig 6. Genes controlling cell survival that were differently expressed in infected versus non-infected mucosal tissue.

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susceptible kids (Fig 7). The DEG in the abomasal mucosa, the ecological niche of *H. contortus*, were part of pathways for reproductive system diseases for both comparisons of infected versus non-infected and resistant versus susceptible kids. Besides, Beta-estradiol and estrogen were also in the top five upstream regulators genes differently expressed in mucosal tissue of resistant versus susceptible kids (Table 6).

We compared our results for diseases and biological functions with other studies. Four of the top five significant diseases that we identified in the comparison of abomasal mucosa in infected versus non-infected kids were also reported as being significantly different expressed in the abomasal mucosa of 'immune' sheep at day 0 (non-infected) compared with 2 days after infection with *Teladorsagia circumcincta*, another important nematode parasite of sheep and goats [53]. Moreover, the same study [53] reported the same top 5 molecular and cellular functions that we found for the same comparison (mucosa samples from infected versus non-

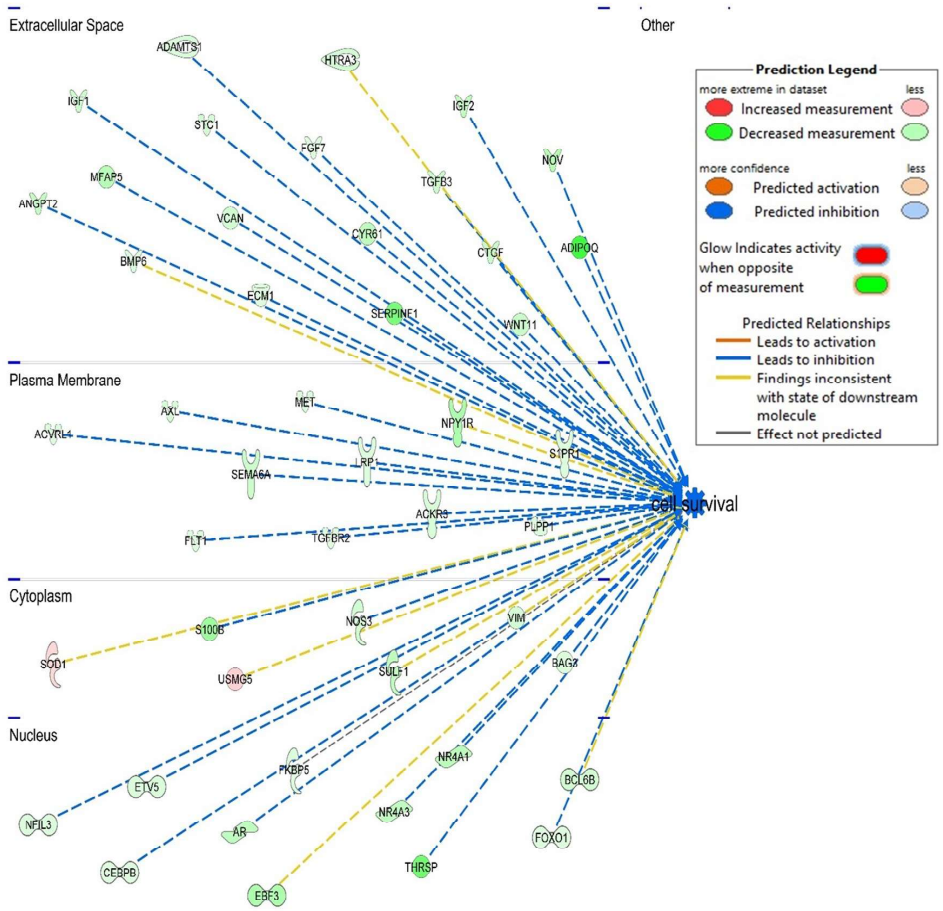

Fig 7. Genes controlling cell survival that were differently expressed in resistant versus susceptible mucosal tissue.

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infected animals). When comparing the lymph node transcriptome using resistant and susceptible sheep infected with *T*. *circumcincta*, the gastrointestinal and hematological diseases were identified to be significant [23]. We identified both these functions for the DEG in the same tissue when comparing resistant and susceptible kids. These results indicate that *H*. *contortus* and *T*. *circumcincta* infection in goats and sheep activate the same functions and that the biological process involved are the similar across different hosts and parasites.

The top 5 networks identified by IPA for each comparison are shown in Table 7. The results showed that the abomasal mucosa of infected kids activate or inhibit genes that target 'cell cycle' and 'cell death and survival' networks. It confirms results from canonical and KEGG pathways which identified cell cycle pathways as the most significant pathways. This suggests that the maintenance of the integrity of the mucosa, which is the barrier between the lumen and the organism, is probably the priority for the host. We also found that DEG in lymph

Table 7. Top 5 networks for each comparison identified by Ingenuity pathway analysis using DE genes.

Comparison	Network	IPA Score[a]	Genes
Infected versus non-infected mucosa	Cell Cycle, Reproductive System Development and Function, Cellular Movement	38	30
	Cell Cycle, DNA Replication, Recombination, and Repair, Cancer	36	29
	Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair	36	29
	Cell Death and Survival, Hematological System Development and Function, Cell Signaling	30	26
	Cell Signaling, Lipid Metabolism, Small Molecule Biochemistry	30	26
Infected versus non-infected lymph	Cancer, Organismal Injury and Abnormalities, Metabolic Disease	35	34
	Cancer, Organismal Injury and Abnormalities, Respiratory Disease	32	33
	Post-Translational Modification, Cell Cycle, Neurological Disease	32	33
	Post-Translational Modification, Amino Acid Metabolism, Small Molecule Biochemistry	30	32
	Dermatological Diseases and Conditions, Organismal Injury and Abnormalities, Antimicrobial Response	28	31
Resistant versus susceptible mucosa	Cellular Movement, Cellular Development, Embryonic Development	43	26
	Cardiovascular System Development and Function, Organismal Development, Cell-To-Cell Signaling and Interaction	36	23
	Cancer, Organismal Injury and Abnormalities, Cell Signaling	34	22
	Cellular Development, Connective Tissue Development and Function, Tissue Development	32	21
	Organ Morphology, Skeletal and Muscular System Development and Function, Tissue Morphology	27	19
Resistant versus susceptible lymph	Protein Synthesis, Molecular Transport, RNA Post-Transcriptional Modification	61	34
	Protein Synthesis, Gene Expression, RNA Post-Transcriptional Modification	40	26
	Antimicrobial Response, Inflammatory Response, Developmental Disorder	40	26
	Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry	37	25
	Cancer, Endocrine System Disorders, Organismal Injury and Abnormalities	33	23

[a] IPA network score is expressed as the -log (Fisher's exact test p-value).

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node tissues control post-translational modification in infected compared with non-infected kids. Further, DEG control RNA post-transcriptional modification and protein synthesis in resistant compared with susceptible kids (Table 7).

Validation of expression by qRT-PCR

RNA sequencing results were validated by performing RT-qPCR for seven genes, five from the mucosal tissue (CCL20, IFI6, IL13, galectin 9, TLR4) and five from lymph node tissue (CLEC4E, IFI6, IL13, PGLYRP4, TLR4) for both comparisons of infected versus non-infected and resistant versus susceptible kids. As shown in Fig 8, the $\log_2$ fold change levels of selected genes measured by RT-qPCR were in good agreement with the values from the sequencing data. The gene expression patterns from qPCR in mucosa and lymph node tissues were strongly correlated with the sequencing results (correlation coefficient 0.73 and 0.86 respectively).

Conclusion

Our results suggested that in our biological model, the mechanisms underlying genetic resistance were not expressed during the first challenge. Meanwhile, results from the second challenge suggested that resistance in Creole goats would be primarily mediated through reduced worm fecundity with a probable role for MHC class I. The consequences of infection were mainly controlled through 'Cell death and survival' as the top cell function at this stage of infection (42 d.p.i), which suggests that the maintenance of the integrity of the mucosal barrier is one of the priorities of the host response.

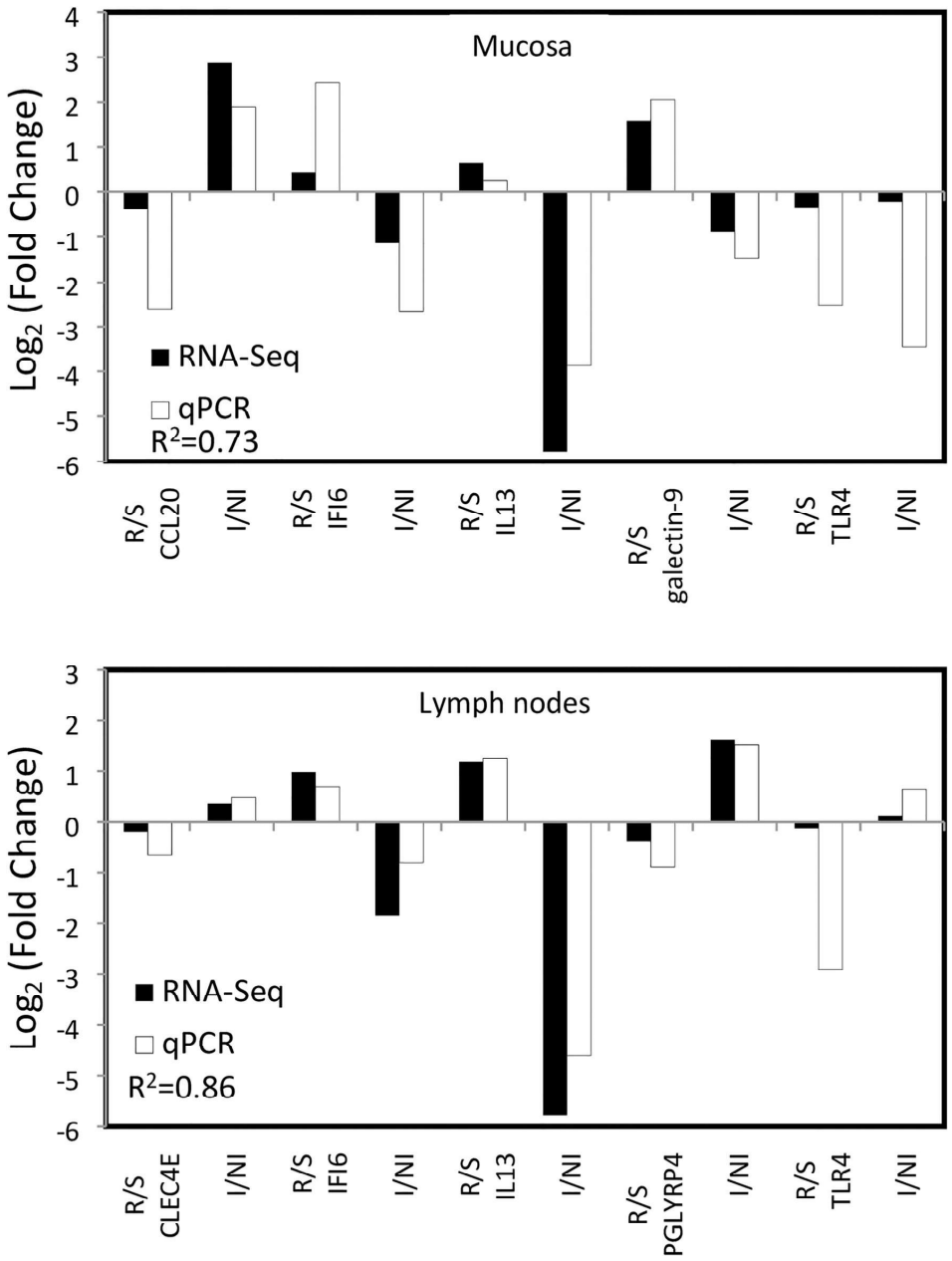

Fig 8. Comparison of fold changes of deferentially expressed genes measured by RNA-Seq (black) and qPCR analyses (white) according to the groups (resistant vs. susceptible, R/S and infected vs. non-infected, I/NI) and the tissues (mucosa and lymph nodes).

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Dynamic transcriptomic changes of goat abomasal mucosa in response to *Haemonchus contortus* infection

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Abstract

Gastrointestinal nematode (GIN) infections are one of the major constraints for grazing sheep and goat production worldwide. Genetic selection for resistant animals is a promising control strategy. Whole-transcriptome analysis via RNA-sequencing (RNA-seq) provides knowledge of the mechanisms responsible for complex traits such as resistance to GIN infections. In this study, we used RNA-seq to monitor the dynamic of the response of the abomasal mucosa of Creole goat kids infected with *Haemonchus contortus* by comparing resistant and susceptible genotypes. A total of 8 cannulated kids, 4 susceptible and 4 resistant to GIN, were infected twice with 10,000 L3 *Haemonchus contortus*. During the second infection, abomasal mucosal biopsies were collected at 0, 8, 15 and 35 days post infection (dpi) from all kids for RNA-seq analysis. The resistant animals showed early activation of biological processes related to the immune response. The top 20 canonical pathways of differentially expressed genes for different comparison showed activation of the immune response through many relevant pathways including the Th1 response. Interestingly, our results showed a simultaneous time series activation of Th2 related genes in resistant compared to susceptible kids.

Keywords

Goats, *Haemonchus contortus*, genetic resistance, immune response, RNA-seq

Introduction

Gastrointestinal nematodes (GIN) are an important constraint on grazing ruminants worldwide. These parasites can cause mortality especially in small ruminants but their main effect is reduced productivity [1, 2]. Anthelmintic treatments are the mainstay of current treatment but are threatened by the evolution of drug resistance in parasite populations [3]. Besides, the environmental side-effect of anthelmintic residues is no longer desirable for sustainable production and the increased demand for chemical-free animal products. Therefore, there is a need for additional control strategies. The introduction of resistance to GIN traits in small ruminants breeding schemes, would be a promising sustainable method to control GIN infection [1, 4, 5].

Resistance against most of the common diseases are complex traits involving many genes, the detection of causative variations is therefore a complex task. Currently selection against GIN relies on indirect measures such as fecal egg count (FEC), packed cell volume (PCV) and blood eosinophilia [6–9]. A major disadvantage of these method is that animals must be infected either naturally or experimentally for these measures. An alternative is the identification and the selection of genes that are responsible for resistance to GIN infection. Several studies investigated the molecular and cellular processes associated with GIN resistance in different tissues such as duodenal [10–12] and abomasal mucosa [13, 14] and draining lymph nodes [15–19] mainly in sheep. However, only a few studies have investigated the biological processes associated with GIN resistance in goats.

It has been shown that whole-transcriptome analysis via RNA-seq is a key tool to investigate the molecular mechanisms responsible for complex quantitative traits such as resistance to GIN infection [20]. A detailed understanding of the genes and biological mechanisms involved in

resistance and protective immunity would provide new phenotypic and genetic markers for effective breeding schemes [21].

Previously, we investigated the transcriptome variation in response to GIN infection in goats at 42 days post infection (dpi) [22]. The results indicated that the maintenance of the integrity of the mucosa was probably the priority for the host at this late infection stage (42 dpi). The present study aimed to identify the changes over time in the molecular pathways and immunity development in response of Creole goats to GIN infection by analyzing the transcriptome of abomasal mucosa of resistant and susceptible kids at different time point post infection.

Materials and Methods

Ethics statement

All animal care handling techniques and procedures as well as the procedures for experimental infection, tissue sampling and slaughtering were approved by the French Ethic Committee n°069 (Comité d'Ethique en Matière d'Expérimentation Animale des Antilles et de la Guyane, CEMEAAG) authorized by the French Ministry of Higher Education, Research and Innovation. The experiment was performed at the INRA Experimental Facilities PTEA (Plateforme Tropicale d'Expérimentation sur l'Animal), in Guadeloupe (French West Indies) (16° 20' latitude North, 61° 30' longitude West), according to the certificate number A 971-18-02 of authorization to experiment on living animals issued by the French Ministry of Agriculture.

Animals and experimental design

Eight 9-month old Creole kids were chosen from the experimental flock of PTEA (Plateforme Tropicale d'Expérimentation sur l'Animal) in which the estimated breeding value (EBV) was calculated regularly since 1995 for each animal for FEC at 11 months of age following natural mixed infection on pasture taking into account the FEC of its ascendants and pedigree. Before

88 the experiment the kids were reared at pasture with a limited level of GIN contamination (FEC <
89 500). The FEC of the 8 kids (n=4 resistant and n=4 susceptible), chosen on the basis of their
90 extreme EBV in their cohort, were not statistically different. The EBV was estimated by taking
91 into account the FEC of their ascendants and their pedigree. The averages EBV of the 2 groups
92 were distant by 1.04 genetic standard deviation. The animals were drenched with moxidectine
93 (Cydectine®, Fort Dodge Veterinaria S.A., Tours, France, 300 µg/kg) and housed indoors under
94 worm-free conditions in a single pen, one month before the start of the experiment. Kids were
95 orally infected with a single dose of 10,000 *Haemonchus contortus* third-stage larvae (L3) in two
96 consecutive challenges. Each challenge lasted for 5 weeks with 8 weeks interval between the end
97 of challenge 1 and the start of challenge 2. At the end of the challenge 1, the kids were drenched
98 with moxidectine (Cydectine®, Fort Dodge Veterinaria S.A., Tours, France, 300 µg/kg) and
99 four weeks later a fistula was surgically implanted in the abomasum of each animal to allow
100 abomasal mucosa sampling at 0, 8, 15 and 35 days post infection (dpi). After another period of
101 four weeks, the animals were orally infected with a single dose of 10,000 *H. contortus* L3
102 (challenge 2). For FEC measurements during the experimental infection, approximately 10 g of
103 faeces were collected in plastic tubes directly from the rectum of each animal, and transported
104 from the experimental facility to the laboratory in refrigerated vials. The samples were
105 individually analysed using a modified McMaster method for rapid determination and FEC was
106 expressed as the number of eggs/g faeces [9].
107 **Surgical procedure**
108 The custom designed abomasal cannula consisted of a flexible plastic tube with a length of 7 cm
109 and a diameter of 2cm with a rounded base of 4 cm in diameter. This flexible plastic was chosen
110 to limit the possibility of mechanical abrasion of the mucosal surface of the abomasum. The

111 animals were fasted 16 h before cannula insertion surgery. The animals were premedicated with
112 ketamine (2mg/kg IV, Le Vet Pharma, Wilgenweg, Netherlands), xylazine (0.2mg mg/kg IM, Le
113 Vet Pharma, Wilgenweg, Neitherlands) and oxytetracycline (20 mg/kg IM, Eurovet Animal
114 Health, Handelsweg, Neitherlands). The animals were positioned in left lateral recumbency. Skin
115 over the surgical site was shaved and prepped with povidone iodine (Vétédine, Laboratoire
116 Vetoquinol S.A., Lure, France). A ventral midline incision was made to locate and externalise
117 the abomasum. A 3 cm purse-string suture (Silk 2-0) was placed midway between the lesser and
118 greater curvature and a stab incision was made in the center to insert the cannula. Then, the
119 purse-string suture was tightened and tied off. To maintain the abomasum in an anatomically
120 correct position, another stab incision was made in the abdominal wall at 10cm from the
121 laparotomy incision on the right paramedian area to enable the cannula to be passed freely
122 through. An external flange was placed over the external part of cannula and fixed with adhesive
123 fabric plaster strip. A sterile compress was inserted into the cannula as stopper. After the surgical
124 procedure, all the animals were housed individually with free access to fresh water and hay.
125 **Biopsy sampling procedure**
126 Biopsy specimens were taken from the abomasal mucosa using a flexible endoscope (FG-24V,
127 Pentax, France). The biopsies samples of 2×2×2mm taken with the endoscopic forceps with
128 window (model KW1815S) were quickly snap frozen into liquid nitrogen and stored at -80°C
129 until RNA extraction. The animals were restrained in a harness made with a surgical drape
130 allowing animal legs to protrude and which exposed the cannula. No sedation was used since no
131 signs of discomfort or pain were observed during or after the procedure. The sterile compress
132 inserted into the cannula was removed and the abomasal contents collected. The endoscope was
133 introduced into the abomasal lumen and 3 biopsies per animal and per time points were taken

from the abomasal folds of the fundic mucosa. At each time point the whole fundic mucosa was observed and no sign of mucosal injury due to the previous sampling was observed.

RNA extraction and sequencing

Total RNA was extracted using the NucleoSpin® RNA isolation kit (Macherey-Nagel, Hoerdt, France) following the manufacturer's instructions, except that DNase digestion was performed with twice the indicated amount of enzyme. The total RNA concentration was measured with NanoDrop 2000 (ThermoScientific TM, France). The RNA integrity was verified using an Agilent Bioanalyzer 2100 (Agilent Technologies, France) with a RNA Integrity Number of > 7.5. The extracted total RNA was stored at -80°C until sequencing.

High-quality RNA from all samples was processed for the preparation of cDNA libraries using an Illumina TruSeq RNA sample prep kit for mRNA analysis following the Illumina's protocols. After quality control and quantification, cDNA libraries were pooled in groups of 6 and sequenced on 5 lanes on the HiSeqTM 2000 (Illumina® NEB, USA) to obtain approximatively 30 million reads (100 bp paired-end) for each sample with insert sizes ranging from 200 to 400 base pairs.

Bioinformatics and data analysis

The quality control check on raw reads in FASTQ format were processed using FASTQC and the Q20, Q30 and GC contents of the clean data were calculated. The Salmon software (version 0.9.1) was used for transcript quantification [23]. NCBI RefSeq reference transcript of the *Capra hircus* genome (assembly ARS1) was used to build the index within Salmon. The reads from each sample were mapped to the same index and quantified. Unix commands were used to obtain corresponding gene and transcript identifiers from the NCBI RefSeq annotation of the *Capra hircus* (ARS1). Using these identifiers, the tximport (version 1.8.0) package was used to import

157 data into the R software (v3.5.1) and summarize the TPM estimates obtained from the Salmon
158 tool of all samples at the gene level [24]. This process produced a global count file on which the
159 statistical analyses were performed. A threshold of greater than or equal to 5 counts across
160 samples was applied in order to remove genes showing low expression.
161 Partial least squares discriminant analysis (PLSDA) had been conducted using the mixomics
162 package within R [25]. In this analysis, x was the matrix of gene expression values (count table)
163 and the classes of y were given as resistant and susceptible. Each row of the x matrix represented
164 the gene expression values for a sample, and each column corresponded to a gene.
165 Differentially expressed genes (DEG) of read counts were identified using the Bioconductor
166 package DESeq2 within R [26]. Ten comparisons were performed; three comparing day 0 with
167 day 8, 15 or 35 post infection in the susceptible group, another three comparing the same days in
168 the resistant group and four comparing samples from resistant versus susceptible animals at day
169 0, 8, 15 and 35 post infection. To account for multiple testing, genes were filtered using a
170 Benjamini and Hochberg false discovery rate (FDR) of < 0.001. Final DEG were determined on
171 the basis of their fold change values to be $\log2 \geq 1.0$ for up-regulated genes and ≤ -1.0 for down-
172 regulated genes. Gene ontology (GO) analysis for the biological processes was performed to
173 identify the biological function classification of the genes, which describes properties of genes
174 and their products. DEG are functionally grouped into the biological processes looking for
175 significantly enriched functions compared to the human genomic background due to the lack of
176 goat (*C. hircus*) GO data. GO enrichment analysis and GO annotations plotting were performed
177 using the clusterProfiler R package [27]. All enriched GO terms that possessed a p-value < 0.01
178 were displayed and the top 5 biological processes for each comparison were plotted. Analysis of

canonical pathways and regulator effects were performed using Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA) for DEG in each comparison.

Faecal egg counts (FEC) were measured twice a week after infection from 21 to 36 days post infection. The FEC variance was normalized using log transformation. PROC MIXED procedure (v. 9.4, SAS Inst. Inc., Cary, NC, USA, 2012) was used to test statistical differences. The differences were considered significant when $P < 0.05$. The results are presented after back transformed.

Quantitative real-time PCR (qRT-PCR) validation

To validate the results of the RNAseq analysis, the gene expression for a total of 9 genes (n=6 for each comparison: resistant *vs* susceptible at 0, 15 and 35 dpi, and resistant and susceptible for 0 *vs* 8 dpi, 0 *vs* 15 dpi and 0 *vs* 35 dpi) was determined by qRT-PCR. The endogenous control for all reactions was goat *ACTB* (*actin beta*) gene whose expression remained stable among the samples. The cDNA was synthetize with a total of 2 µg of high quality total RNA (RIN > 7.5) by using M-MLV Reverse Transcriptase (Promega, Charbonières, France) according to the manufacturer's instructions. All qRT-PCR reactions were carried out in 48-well plates in a Prime Pro 48 Real-Time PCR System and analyzed with the ProStudy Software v5.2.10 (Techne, Staffordshire, UK). Taqman® predesigned gene expression assay (Table 1) and the universal PCR master mix were purchased from Applied Biosystems and the analysis were performed according to the manufacturer's instructions (ThermoFisher Scientific, Applied Biosystems, Courtaboeuf, France). Samples were analyzed in duplicate in a total volume of 20 µl containing: 4 µl of cDNA, 10 µl of 2X TaqMan® Fast Advanced Master Mix, 1 µl of TaqMan® Gene Expression Assays 20X (ThermoFisher Scientific, Applied Biosystems, Courtaboeuf, France)

and 5 µl of distilled RNAse DNAse-free water. Relative gene expression values were determined using relative quantification ($2^{-\Delta\Delta Ct}$ method, [28]).

Results

Parasitological Measures

A significant effect of the group (i.e. resistant vs susceptible), the dpi and their interaction ($P < 0.001$) was observed for FEC (Figure 1). At 21 dpi no difference was observed between groups. Thereafter the FEC was significantly lower in resistant compared to susceptible animals whatever the dpi.

RNA sequencing and variance analysis

Alignment of RNA sequencing to the reference *Capra hircus* genome (assembly ARS1) resulted in an average of 4.5±0.1 million reads per sample. These reads correspond to 23258 genes of the goat genome. A total of 15188 out of the 23258 annotated genes (65%), showed at least 5 read counts per row and were used in the subsequently analysis. The multilevel PLSDA for gene expression of infected resistant and susceptible kids explained more than 20% of the variance in its two dimension components (Figure 2). Component 1 represented 11% of the whole variability and component 2 represented also 11% of the variation.

Differential gene expression

The numbers of DEG for each comparison are shown in Table 2. The numbers of DEG were low for the comparison between groups (R vs S) whatever the time point. For the comparison within infected resistant or infected susceptible the numbers of DEG were lower for 0 versus 15 dpi (678 and 1748, respectively) compared with 0 versus 8 or 0 versus 35 dpi. Meanwhile the highest number for DEG was recorded for the comparison of 0 versus 35 dpi of infected susceptible (3316) and infected resistant (2263). The fold change was on average higher when comparing

224 different time points within each group (from -11.15 to 24.17 and from -11.83 to 9.30 for R or S
225 respectively) than between groups at different days. Human orthologues were mapped for 72-85
226 % of the DEG (Table 2).

227 **Validation of expression by qRT-PCR**

228 qRT-PCR for nine genes was performed to validate RNA sequencing results. For the comparison
229 of resistant versus susceptible animals at 0, 8, 15 and 35 dpi, the genes selected randomly among
230 the DEG were: *DUOXA2*, *IFI6*, *CYP4F2*, *OLFM4* and *TFF3*. For the comparison of 0 versus 8,
231 15 and 35 dpi within the resistant and the susceptible animals the genes were respectively: IFI6,
232 CYP4F2, OLFM4, TFF3, TLR4 and NKX6-3, CCL20, OLFM4, LST1, TFF3. The log2 fold
233 change levels of the selected genes measured by qRT-PCR were in good agreement with the
234 values from the sequencing data (Figure 3). The gene expression patterns from qRT-PCR were
235 highly correlated with the sequencing results: the correlation coefficients were respectively 0.91,
236 0.96 and 0.81 for the comparison of resistant versus susceptible animals at different time points
237 and the comparison of 0 versus other time points within the resistant and the susceptible animals.
238

239 **Functional classification analysis**

240 ***Gene ontology (GO)***

241 An enriched GO term analysis for biological processes was performed using the DEG from each
242 comparison. The top 5 significant biological processes in each term are presented in Figure 4.
243 Comparing 0 versus 35 dpi, four out of the top 5 biological processes were the same for the
244 resistant and the susceptible kids; meanwhile leukocyte differentiation was in the top biological
245 process only for the resistant kids. The comparison of infected resistant at 0 versus 8 dpi showed
246 biological processes related to the immune response within the top 5 significant processes (*e.g.* T

247 cell activation, leukocyte cell-cell adhesion and lymphocyte differentiation). Positive regulation
248 of the innate immune response was in the top 5 biological processes when comparing susceptible
249 with resistant at 35 dpi.

250 ***Pathway enrichment analysis***

251 The Ingenuity Pathway Analysis was used to compare results from different comparison over
252 time. The top canonical pathways (Figure 5) and the top upstream regulators (Figure 6) were
253 compared. When comparing day 0 versus 35 post infection, the top 20 canonical pathways
254 showed a high activation of the immune response through dendritic cell maturation, *IL-8*
255 signaling, Leukocyte extravasation signaling, *NFAT* in regulation of the immune response, *P13K*
256 signaling in B lymphocytes, Th1 pathway and B cell receptor signaling pathways. In resistant
257 compared with susceptible kids the B cell receptor signaling pathway was activated at 8 dpi
258 while dendritic cell maturation and Th1 pathways were activated at 35 dpi.

259 The top 10 upstream regulators of the DEG for different comparisons showed that some genes
260 like *TGF-β1*, *TNF-α*, *IFN-γ*, *IL1-β* and *IL-6* were in the group of the top significant upstream
261 regulators in both infected resistant and susceptible kids specially when comparing 0 versus 35
262 dpi. These genes were still significantly differently expressed between resistant and susceptible
263 kids at 35 dpi (Figure 6). The *TGF-β1* gene was the top significant upstream regulator that was
264 differently expressed in resistant compared with susceptible kids in the abomasal mucosa.

265 **Differential of CD4+ T cell**

266 Genes related to the CD4+ T cell activation and the fold change comparing resistant versus
267 susceptible kids at 0, 8, 15 and 35 dpi are presented in Figure 7. The CD4+ T cell differentiation
268 pathway showed a significant difference and a positive fold change for the majority of genes
269 controlling the Th1 pathway when comparing resistant versus susceptible kids at 35 dpi. The

expression of genes controlling the Th2 pathway showed time series activation in resistant compared with susceptible kids at different dpi: *IL2RG* activated at 8 dpi, *IL4R* and *STAT6* at 15 dpi, *GATA3* and *CCR4* at 35 dpi. Meanwhile the expression of *IL4R* and *STAT6* at 35 dpi is higher in susceptible kids. The expression levels for genes controlling the Th17 pathway showed a positive fold change for *STAT3* and *RORC* in resistant kids at 15 dpi, then for *IL17F* at 35 dpi while for *STAT3* the expression was higher in susceptible kids at 35 dpi. Comparing resistant versus susceptible at 0 dpi (before the experimental infection), the expression of *IL17F* was three times higher in resistant kids. No difference of *FOXP-3* expression was observed between resistant and susceptible whatever the dpi, while the expression of *TGF-β1* was significantly higher in resistant kids at 8 dpi and lower at 35 dpi.

Discussion

This study aimed to investigate the kinetic changes in mucosal molecular pathways and immunity development of resistant and susceptible Creole kid goats in response to *H. contortus*. The classification of the animals as resistant or susceptible was explained at 22% by the gene expression profile. *H. contortus* infection induced a high number of DEG in the mucosa of both resistant and susceptible animals whatever the time points while the numbers of DEG were much lower when comparing resistant versus susceptible animals at the different time points of infection. This result indicates that the majority of genes involve in the host response against *H. contortus* infection were similar in susceptible and resistant animals.

GO of enriched biological processes showed an earlier activation of immune biological processes in resistant kids. Indeed, the top biological processes at 8 dpi were T cell activation, leukocyte cell-cell adhesion and lymphocyte differentiation. One of the top significant pathways was B cell receptor signaling. In keeping with this results, McRae *et al.* reported an early immune response

294 to *Teladorsagia circumcincta* in resistant sheep at 7 dpi [19]. The same top four biological
295 processes were observed in resistant and susceptible animals when comparing 0 and 35 dpi.
296 However, none of these processes appeared in these top biological processes when comparing
297 susceptible with resistant animals at 35 dpi, suggesting that at 35 dpi the host priority at the
298 abomasal mucosa interface would be similar for resistant and susceptible kids.
299 The Th1 pathway was one of the top pathways identified in most of the comparison performed in
300 this study. Upstream regulators of the genes involved in the Th1 processes include *TNF-α* and
301 *IFN-γ*, which were also identified as DEG. In accordance with this result, a transient increase of
302 the expression of *TNF-α* and *IFN-γ* was observed earlier after *H. contortus* infection in sheep
303 both in the abomasal mucosa and the draining lymph nodes [29–31]. However, a non-protective
304 Th1 response associated with an increased expression of cytokines, as TNF-α and *IFN-γ*, was
305 observed respectively in susceptible and primary infected sheep infected with *H. contortus* [32,
306 33]. Indeed, studies on murine models demonstrated for a long time that the protective response
307 against GIN parasites is better associated with the Th2 polarization of the immune response [34],
308 while host susceptibility is associated with a Th1 response [35, 36]. In ruminants, the Th1/Th2
309 dichotomy remains controversial despite studies showing a correlation between host resistance
310 and a polarized Th2 immune response [37–39]. A simultaneous increased expression of Th1- and
311 Th2-type cytokines was shown in cattle infected with *Ostertagia ostertagi* [40–42]. Similarly,
312 looking at differential of CD4+ T cell, we found signals for Th1 and Th2 activation at 35 dpi in
313 resistant animals when comparing them with susceptible animals. Caucheteux *et al.* [43] reported
314 that the expression of IL1-β gives rise to inflammatory Th2 cells that are specialized to induce
315 allergic inflammatory responses, whereas Th2 primed in the absence of IL1-β are more important

as regulatory cells, that is amplifiers of Th2 cells and antibody response by B cells. Our results showed IL1-β in the top upstream regulator genes controlling infection response.

Transforming growth factor beta (TGF-β) is a multifunctional cytokine known for its regulatory activity and the induction of peripheral tolerance [44]. We found that the gene expression profile of TGF-*β1* was the top significant upstream regulator when comparing the dynamics of infection in resistant and susceptible animals. TGF-*β1* was activated in susceptible and inhibited in resistant animals at 35 dpi. The same was previously reported in other studies in goats [22, 45] and also a study on sheep infected with *H. contortus* [15]. The underlying mechanisms could be a manipulation of the host immune response by *H. contortus*, notably through the induction of the secretion of *IL-10* and *TGF-β1* by goat monocytes to promote an anti-inflammatory environment favorable for worm survival [46]. This hypothesis needs to be investigated.

A gene expression profiling study of the abomasal mucosal and lymph nodes of resistant and susceptible goat in response to *H. contortus* infection at 42 dpi has previously reported that the maintenance of the integrity of the mucosal barrier is one of the priorities of the host response at the late stage of infection [22]. The study presented here studied the dynamics of the gene expression in the goat abomasal mucosa in response to *H. contortus* infection using information from the whole transcriptome of resistant and susceptible kids. A time series activation of Th2 genes was identified for resistant animals compared with the susceptible ones. The later activation of some genes in susceptible animals indicated that the Th2 response was activated earlier in resistant kids compared to susceptible kids. Transcriptional profiling of the abomasal lymph node from Scottish Blackface lambs showed that resistant animals are generating an earlier immune response to *T. circumcincta* infection compared to susceptible animals [19]. This

difference was through pathways relating to the inflammatory response, migration of T lymphocytes and synthesis of reactive oxygen species [19].

IL17 is the leading inflammatory cytokine in the Th17 cell populations [47]. Neither the IL17A nor the IL17F genes have been described in studies analyzing the resistance to GIN in sheep. Nonetheless, IL17 transcripts have been shown to be upregulated in the bovine abomasal mucosa after 24 days of single *O. ostertagi* challenge and 60 days of trickle experimental or natural infection [42]. However, the positions of these interleukin genes have been found to be relatively close to the DRB1 gene in sheep [48], which has been reported to be associated with GIN resistance in sheep [49, 50]. In the present study, *IL17F* was the gene showing the most significant expression difference at day 0 of infection, having an expression three times higher in resistant compared with susceptible kids. Future experiments should investigate the potential of this gene as a pertinent biomarker in a selection program.

The present study showed that *H. contortus* infection in goat induces a marked immune response at the mucosal level in resistant animals, which is characterized by the simultaneous upregulation of Th1 and Th2 genes. Our results suggested differences in the time series activation for Th2 genes, indicating that the immune response is activated earlier in resistant kid goats compared to the susceptible ones. We also found that TGF-*ß1* has a major regulator role during GIN infection in goats.

Availability of data and materials

All data generated during this study are available in the NCBI SRA repository. All other relevant data are included in this published article.

Competing interests

The authors declare no competing interests.

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Table 1. List of target genes for qRT-PCR validation and assay IDs according to the manufacturer

Gene symbol	Gene description	Assay IDs
ACTB	*actin beta*	Ch04810274_s1
CYP4F2	*phylloquinone omega-hydroxylase*	Ch04672252_m1
DUOXA2	*dual oxidase maturation factor 2*	Ch04786286_m1
CCL20	*C-C motif chemokine ligand 20*	Ch04791475_m1
IFI6	*interferon alpha inducible protein 6*	Ch04807049_g1
LST1	*leukocyte specific transcript 1*	Ch04741898_m1
NKX6-3	*NK6 homeobox 3*	Ch04677616_m1
OLFM4	*olfactomedin 4*	Ch04796577_m1
TFF3	*trefoil factor 3*	Ch04767901_m1
TLR4	*toll-like receptor 4*	Ch04654181_m1

568

569 **Table 2.** Number of differentially expressed genes (n) for the different comparisons including

570 log2 fold change and the number of human orthologues (including proportion of genes with

571 human orthologues).

Comparison	n	Log2 fold change	Human orthologues
Inf. R 0 vs 8 dpi	1336	-11.15, 24.17	1017 (76.12%)
Inf. R 0 vs 15 dpi	678	-10.81, 4.57	549 (80.97%)
Inf. R 0 vs 35 dpi	2263	-10.58, 6.66	1881 (83.12%)
Inf. S 0 vs 8 dpi	2221	-10.60, 9.30	1744 (78.52%)
Inf. S 0 vs 15 dpi	1748	-11.84, 8.82	1439 (82.32%)
Inf. S 0 vs 35 dpi	3316	-11.83, 9.23	2811 (84.77%)
R vs S 0 dpi	456	-7.39, 6.00	337 (73.90)
R vs S 8 dpi	679	-4.27, 27.7	490 (72.16%)
R vs S 15 dpi	318	-5.1, 7.82	247 (77.67%)
R vs S 35 dpi	758	-7.34, 8.48	579 (76.39%)

572 Inf. R: Infected resistant, Inf. S: infected susceptible, R vs S: resistant versus susceptible. Dpi:
573 days post infection.

574

Figures

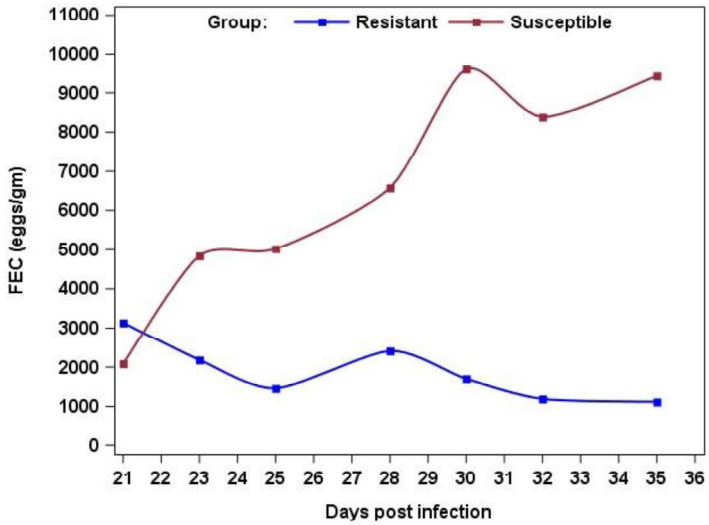


Figure 1. Geometric means of fecal egg count (FEC) comparing resistant and susceptible Creole kids (blue: resistant, red: susceptible) experimentally infected with 10,000 *H. contortus* infective larvae (L3) at day 0 post-infection.

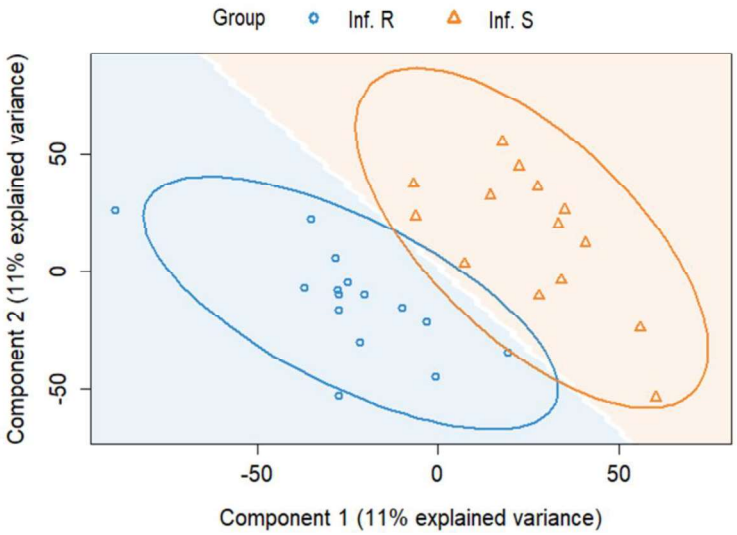
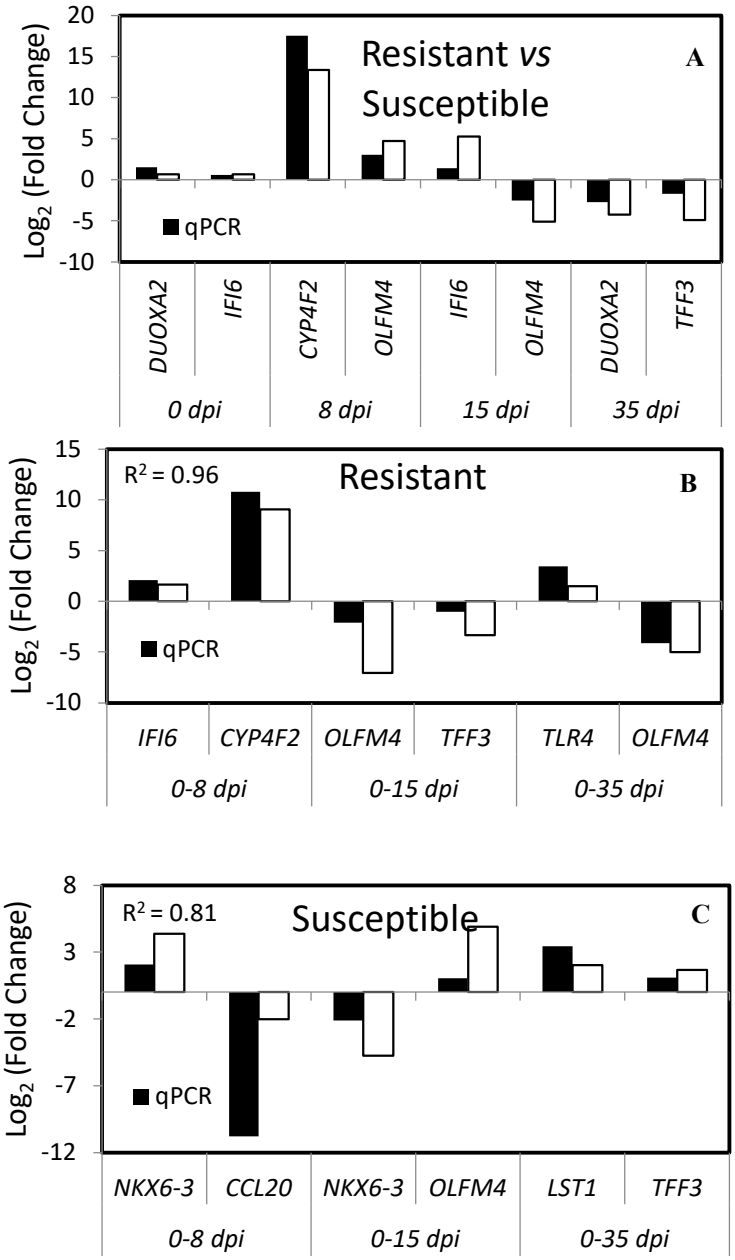


Figure 2. Multilevel PLS-DA (reprenting 22% of the total variance) of the gene expression of infected resistant (Inf. R) and infected susceptible (Inf. S) animals.

583



584

585

586 **Figure 3.** Fold changes of deferentially expressed genes measured by RNA-Seq (white) and
587 qRT-PCR analyses (black) according to the comparisons: resistant versus susceptible at 0, 8, 15
588 and 35 days post-infection (dpi), 0 versus 8, 15 and 35 dpi for resistant and susceptible animals
589 respectively.

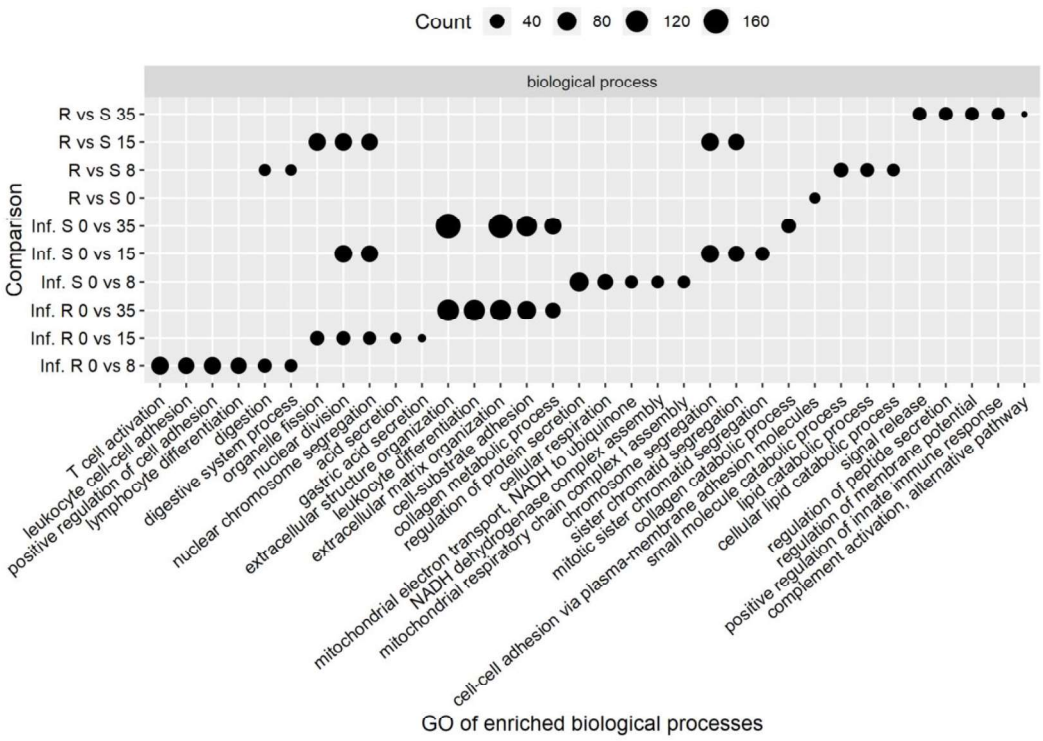


Figure 4. Gene Ontology (GO) of the top 5 biological processes for the three comparisons. Infected resistant (Inf. R), infected susceptible (Inf. S) and resistant versus susceptible (R vs S) kids.

Footnotes:

R vs S: non-infected Resistant compared to Susceptible kids

Inf. S: Infected Susceptible kids (comparison between days post-infection within the susceptible kids)

Inf. R: Infected Resistant kids (comparison between days post-infection within the resistant kids)

0, 8, 15 and 35: days post-infection

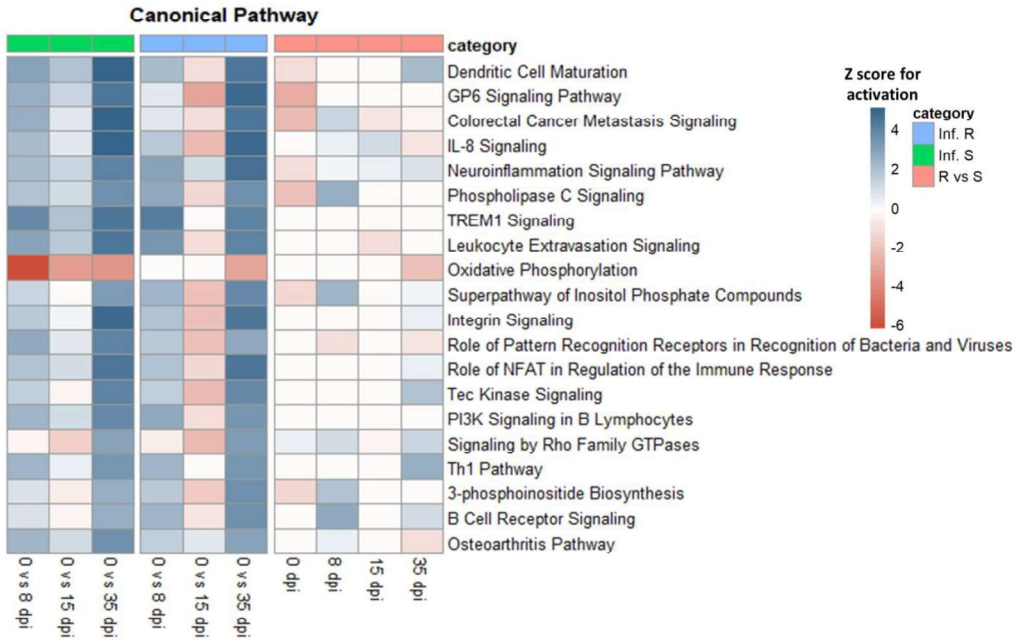
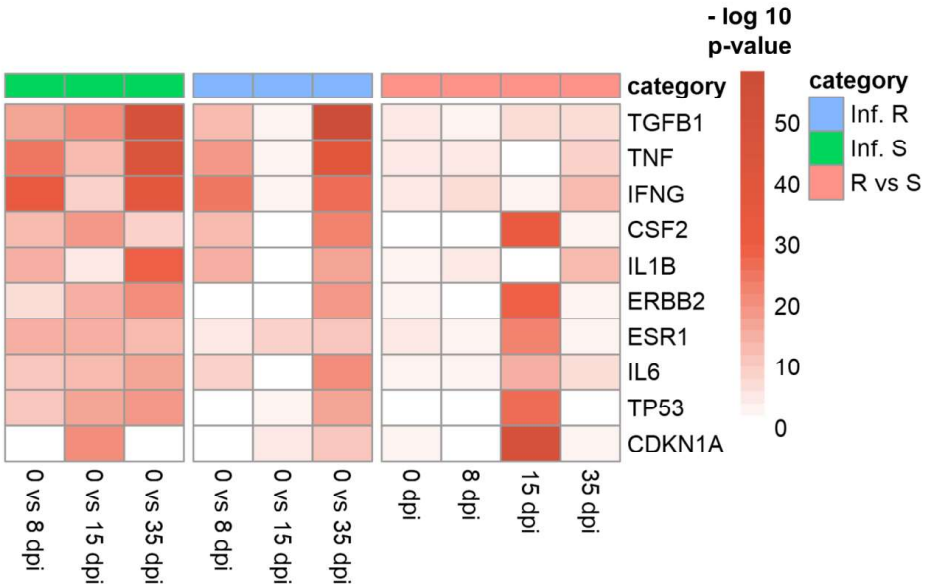


Figure 5. Top 20 canonical pathways of differentially expressed genes for infected susceptible (Inf. S) and infected resistant kids (Inf. R) comparing day 0 with 8, 15 and 35 dpi and resistant versus susceptible kids (R vs S) at 0, 8, 15 and 35 dpi. The color gradient moves from red (down-regulation, z-score for activation= -6) to blue (up-regulation, z-score for activation= 4).

Footnotes:

Z-score for activation: According to Ingenuity systems, the activation z-score is used to infer likely activation states of upstream regulators based on comparison with a model that assigns random regulation directions.



609

610 **Figure 6.** Top 10 upstream regulators of differentially expressed genes for infected susceptible
611 (Inf. S) and infected resistant kids (Inf. R) comparing day 0 with 8, 15 and 35 dpi and resistant
612 versus susceptible kids (R vs S) at 8, 15 and 35 dpi. The color gradient moves from white (no
613 significant difference, $-\text{Log}_{10}$ (p-value) = 0) to red (significant difference, $-\text{Log}_{10}$ (p-value) ≥ 2).

614 *Footnotes: -log10 p-value: -Log10 (p-value)*

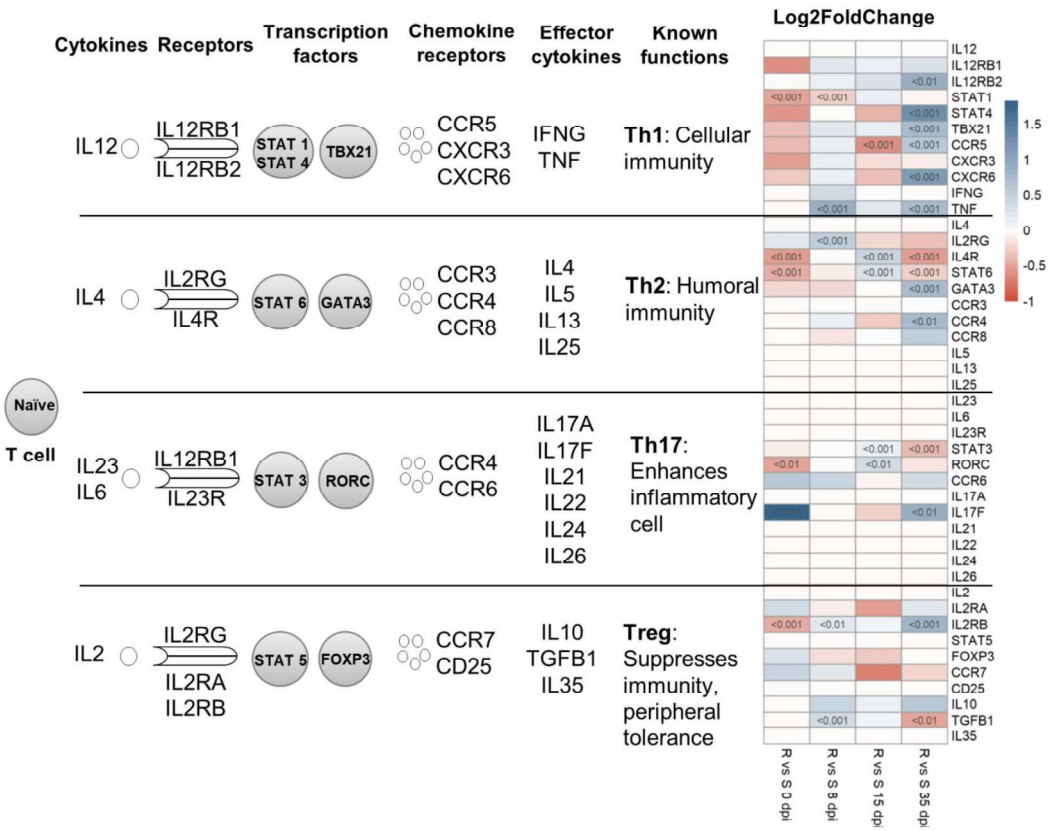


Figure 7. Differential of CD4+ T cell activation and visualization of the gene expression controlling the differences when comparing resistant versus susceptible (R vs S) kids at 0, 8, 15 and 35 dpi. The color gradient moves from red (Log_2Fold Change range from -1 to 0) to blue (Log_2Fold Change range from 0 to 1.5).

Genomic variants from RNA-seq for goats resistant or susceptible to *Haemonchus contortus*

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Abstract

The gastrointestinal nematode is a blood-feeding parasite which is considered one of the top constrain in small ruminant production. Genetic selection for resistant animals is a potential sustainable control strategy. Advances in molecular genetics have led to the identification of several molecular genetic markers associated with genes affecting economic relevant traits. In this study, the variants in the genome of Creole goats resistant or susceptible to *Haemonchus contortus* were discovered from RNA-sequencing. We identified SNPs, insertions and deletions that distinguish between the resistant and susceptible group and we characterized these variants through functional analysis. The T cell receptor signalling pathway was one of the top significant pathways that distinguish the resistant from the susceptible group with 78% of this pathway genes having genomic variants. These genomic variants are expected to provide useful resources especially for molecular breeding for gastrointestinal nematode resistance in goats.

Introduction

Gastrointestinal nematodes (GIN) are one of the most pathogenic parasites in sheep and goats that cause large economic losses. The wide geographic distribution and increasing resistance against anthelmintic control require alternative control strategy [1]. Selection for resistant animals using genetic information is a promising strategy. Genetic selection based on phenotypic traits such as faecal egg count (FEC) has been successfully used [2–4]; however, FEC is an indirect measure of resistance which implements a certain degree of uncertainly. The measurements of FEC is also time consuming and costly as it requires animals to be challenged with parasites. On the other hand, selection using information from the genome could provide a faster and more sustainable tool in breeding for gastrointestinal nematode resistance. Identifying genomic variation is a main step for understanding the relationship between genotype and phenotype. Single nucleotide polymorphisms (SNPs) showed potential as

molecular markers to link genotypes with desired phenotypes in goats, such as milk traits [5–7] , litter size [8,9], growth traits [8,10], fiber quality [11–13] and disease resistance [14].

Recently, Piskol et al. [15] showed that genomic variants detected from RNA sequencing (RNA-seq) data offers a cost-effective and reliable alternative for SNP discovery. They showed that out of the SNPs called from the RNA-seq data, more than 98% were also identified by whole-genome sequencing or whole-exome sequencing approaches. RNA-seq technology was developed primarily for mapping and quantifying transcriptomes to analyze global gene expression in different tissues. Besides allowing the detection of differentially expressed genes, through RNA-seq functional genes are sequenced at high coverage, allowing to full scale variants (SNPs, insertions and deletions) discovery in coding genes. Up to date, this technique has been used as a method to detect SNPs in transcribed regions in an efficient and cost-effective way [13,16–19]. In this study, we performed a genomic variant discovery analysis in the abomasal mucosa transcriptomes of resistance and susceptible Creole goats to *Haemonchus contortus* at four time point of infection and characterized the variants identified.

Materials and Methods

Experimental design

The experimental designs, with detailed laboratory procedures including isolation of total RNA, library preparation and sequencing using the Illumina TruSeq RNA sample prep kit for mRNA analysis, were performed as previously described (paper III). Briefly, eight 10-month old Creole kids (abomasal cannulated) from two lines (n=4 resistant and n=4 susceptible to GIN) were reared indoors at the experimental facility of INRA in Guadeloupe (PTEA, Plateforme Tropicale pour l'expérimentation Animale). Kids were orally infected with a single dose of 10,000 *Haemonchus contortus* third-stage larvae (L3) in two consecutive challenges (5 weeks per each). During the second infection, abomasal mucosa samples were recovered at 0, 8, 15 and 35 days post infection (dpi) and frozen until RNA extraction and sequencing.

Bioinformatics analysis

The quality control check on raw reads in FASTQ format was processed using FASTQC. The remaining reads were aligned to the *Capra hircus* genome (assembly ARS1 from NCBI) using the Burrows-Wheeler Aligner (BWA). Genomic variants including SNPs as well as small insertions and deletions (indels) were detected using mpileup in SAMtools. Variant filtering criterion: A detected variant was kept only if met four criteria: the read depth was more than 10, the quality score was over 20, the minor allele frequency was over 0.05 and the variant present at least in 50% of the group individuals and replicates.

Variant statistics and functional annotation

SNPs, insertions and deletions were compared between samples from the resistant and susceptible group. Common variants were excluded and only different variants between the two groups were kept for the subsequent analysis. Variant information for distribution among genes was calculated within the R program after merging variants with the respective annotated genes. The effect of the variants (SNPs, insertions and deletions) on genes were determined using variant effect predictor (VEP) web interface tool provided by Ensemble online tools (https://www.ensembl.org/info/docs/tools/vep/index.html) within the goat genome reference (Assembly: ARS1) and results were extracted as .txt file for graphical interface using the R program. Genes containing variants were annotated with Gene Ontology (GO) terms under the categories of biological processes, molecular functions, and cellular components using clusterProfiler R package [20]. The Bonferroni-corrected P-value ≤ 0.05 was used as the threshold. Additionally enriched Kegg pathways for genes containing variants were identified using the same package. The Pathview package was used for visualization [21]. The R version 3.5.1 was used.

Results

Sequencing and SNP discoveries

The RNA-seq produced an average of 15.3 million raw reads per sample. Our read alignment results showed that 99.3% sequencing reads (15.2 million) were successfully aligned to the ARS1 goat's reference genome with an average 79% paired sequencing.

Using RNA-seq reads a total of ~2.33 and ~1.82 million raw genomic variant positions expressed in the abomasal mucosa of resistant and susceptible kids were detected at different time points of infection (Table 1). After variant filtering analysis we were able to identify 354,598 and 253,218 SNP, 20,463 and 15,645 insertion and 20,397 and 14,841 deletion records for resistance and susceptible kids, respectively. These variants were then used to produce venn diagrams (Fig 1) to present variants in common and different between resistance and susceptible kids. Comparing genomic variants from the resistant and susceptible group at different time points of infection, 200,053 SNPs, 10,095 deletions and 8,755 insertions were in common (Fig 1). To explore the different genomic variants in resistant and susceptible kids, we excluded the common variants and made the subsequent analysis with non-common variants between the resistant and susceptible group.

Non-common SNPs were annotated with the respective genes. A total of 12,142 and 8,635 genes containing SNPs were identified in the resistant and susceptible group, respectively. In the samples from the susceptible group an average of 5.19 SNPs were identified per gene. Meanwhile, the double number of SNPs (10.53 SNPs per gene) were identified in the data from the resistant group. Among genes containing SNPs, genes with 1 SNP were more common (1759 for resistant and 2629 for susceptible). Genes with 10 SNPs or less accounted for 69.1% and 88.9% of all SNPs identified in the samples from the resistant and susceptible group, respectively. Results for SNPs distributions among genes showed that more genes containing

one or two SNPs were identified in the susceptible group, while more genes containing 3 or more SNPs were identified in the resistant group (Fig 2).

Following the same procedure, non-common insertions and deletions were annotated to genes. A total of 4848 and 3292 genes containing insertions and 4660 and 2610 genes containing deletions were identified in data from the resistant and susceptible group, respectively. Among these genes, genes with one insertion or deletion were more common and accounted for more than 50% of all insertions or deletions identified. Insertion and deletion distributions among genes are shown in Fig 3. Data from the resistant group always contained more insertions and deletions among genes.

All variants (SNPs, insertions and deletions) were combined in two files, one for variants identified in data from resistant animals and one identified in data from susceptible animals. A variant effect prediction analysis was made for both files and the results are shown in Fig 4. The highest variants ratio was around 50% for intron variants from variants in the resistant (56%) and susceptible (47%) group, followed by downstream and upstream gene variants (20% and 8% in the resistant and 25% and 8% in the susceptible group). A total of 3% of missense variants were predicted in the resistant and 4% in the susceptible group (Fig 4).

After the GO enriched analysis, 10,736 and 8,538 genes containing genomic variants in the resistant and susceptible group were assigned with one or more GO terms. The top significant 10 GO terms under the categories of biological processes, molecular functions, and cellular components of these annotated genes are shown in Fig 5. Six to seven of the top 10 terms under each GO category were similar between data from both groups. For the cellular component, the major category that was identified in the data from the resistant and not the susceptible group was mitochondrial matrix. For the molecular function, phosphoric ester hydrolase activity was the most represented term for the variants identified in data from the resistant group which were not present in data from the susceptible group. Under the GO category of biological process, phospholipid metabolic process and macroautophagy were the

most significant GO terms which were identified in the data from the resistant and not the susceptible group.

Top 10 significant Kegg pathways for genes containing genomic variances identified in data from both groups are presented in Fig 6. MAPK signaling pathway, T cell receptor signaling pathway, hepatitis B and longevity regulating pathway were the top significant pathways identified in data from the resistant group only. Activation of T lymphocytes is a key event for an efficient response of the immune system. Therefore, we focused on T cell receptor signaling pathway that was identified in the top kegg pathways for the resistant group. Fig 7 shows the T cell receptor signaling pathway and the number of genomic variants for each gene in this pathway in the data from the resistant group. A total of 100 genes are known to control this pathway in *Capra hircus* species. Out of the 100 genes, we identified one or more genomic variants in 78 genes (Fig 7).

Discussion

In this study, we used data from RNA-seq analysis of Creole goat abomasal mucosa samples at four time point of infection with *Haemonchus contortus* for resistant and susceptible kids to identify putative gene-related genomic variants. To our knowledge, this is the first study to identify genomic variants from transcriptome in goats infected with gastrointestinal nematode. Other studies identified SNPs in goats using RNA-seq analysis for climate adaptation traits [17] and fiber quality [13].

One benefit to variant calling from RNAseq is the focus on genes/transcripts that are actually expressed. However, the information from such data can also be problematic. It was previously shown that some false positive SNPs identified in cDNA arise from alignment of a read to the wrong gene which represents a problem in gene families with highly conserved domains when using short sequence reads [16,22]. This situation has also been observed in regions associated with sequence repeats [23]. Therefore, one great challenge of using Illumina

sequencing for transcriptome analysis is the short read length. We have used the Illumina TruSeq RNA sample prep kit that generated read lengths of 2 X 75 base pair with paired-end reads to increase the base coverage within expressed genes in a sample and as a result improved variant detection sensitivity.

Studies for GIN infection in cattle indicated that GO terms associated with genes that were differently expressed between resistant and susceptible cattle were predominantly related to lipid metabolism and the top function of regulatory networks identified was associated with lipid metabolism [24]. Our results showed that the most significant GO term associated with genes containing variants in resistant goats was phospholipid metabolic process. T cells are a subset of lymphocytes that have a central role in adaptive immune response. T cell receptor is a complex of integral membrane proteins on the surface of T cells, which takes part in the activation of T cells in response to antigen recognition and eventually results in cellular proliferation, differentiation, cytokine production, and/or activation-induced cell death [25]. In the present study, we found that genes containing genomic variants that distinguish resistant goats were associated with T cell receptor signalling pathway in Kegg pathway enriched analysis. Mitogen-activated protein kinase (MAPK) is a highly conserved module that is involved in various cellular functions, including cell proliferation, differentiation and migration. Besides, MAPK has been shown to play a key role in transduction extracellular signals to cellular responses [26]. MAPK signalling pathway was in the top significant pathways identified from genes containing genomic variants of the resistant group. This pathway was previously reported to be regulated by differently expressed genes of resistant and susceptible Yichang White goats and indicated to play significant role in the resistance of this goats to GIN infection [27].

One major problem for goat and sheep genomic analysis was that the functional analysis was not available with goat or sheep as reference species due to the lack of data for *Capra hircus* and *Ovine* in functional analysis programs. Therefore, almost all previous publications in goats and sheep genomic filled used human [27–29] or bovine [30] genome as reference for functional

analysis. Nowadays, the availability of goat genome reference within Ensemble and Kegg pathways made the variants effect prediction and pathways analysis possible and more specific for goats. Our study considers, as one of the first studies, to use the goat genome as reference in the functional analysis.

Conclusion

The present study verifies the possibility to use RNA-seq data as an efficient and cost-effective method to detect genomic variants in transcribed regions. It implements an additional use of such high throughput data and is a great resource to gain further knowledge of animal resources. Genomic variants in genes involved in T cell receptor signaling pathway plays a role in gastrointestinal nematode resistance in goats. This work provides valuable resources for genomic differences between resistance and susceptible goats to nematode infection and serves as a basis towards developing genomic markers for gastrointestinal nematode resistance in goats.

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279 **Table 1. Number of SNPs and indels called from transcriptome data.**

Group	Days after infection	Raw			Filtered			Source file
		SNPs	Insertion	Deletion	SNPs	Insertion	Deletion	
Resistant	0	712902	37798	43176	300268	14956	16841	
	8	747086	38536	43672	365088	17179	20179	
	15	818301	42407	48589	367130	17746	20267	
	35	973405	49260	56391	416626	19667	22492	
	All times	**2107899**	**104250**	**124743**	**354598**	**20463**	**20397**	
Susceptible	0	479135	26102	29129	186577	9572	10689	
	8	680515	35922	40202	324887	15288	17953	
	15	753841	40504	44964	327288	16336	18546	
	35	575608	30821	33198	245840	12025	13837	
	All times	**1635811**	**84872**	**99606**	**253218**	**15645**	**14841**	

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Figures

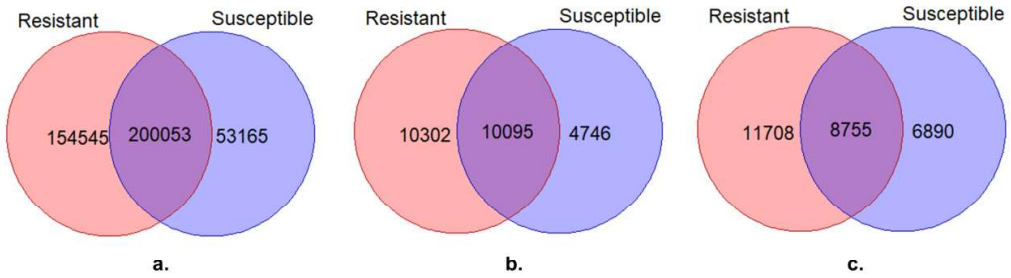


Fig 1. Venn diagram for SNP (a) deletion (b) and insertion (c) variances identified in samples from the resistant and susceptible group.

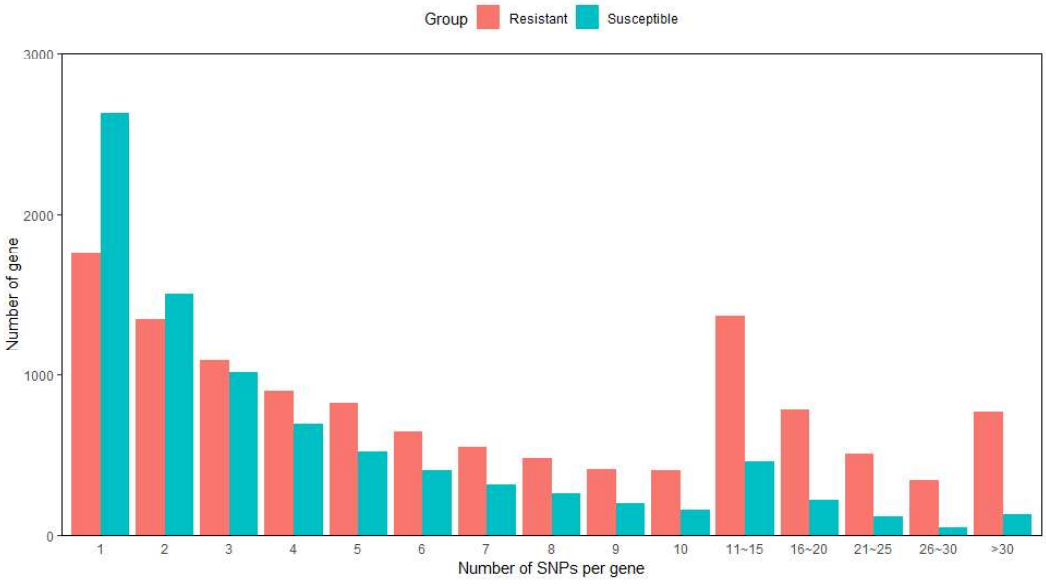
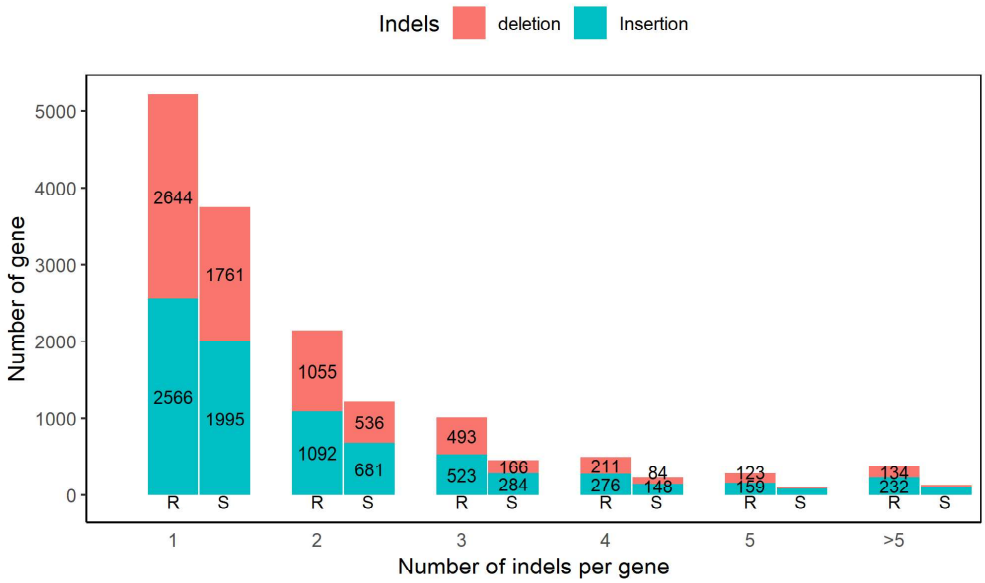


Fig 2. Single nucleotide polymorphism (SNP) distribution among genes.



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291 **Fig 3. Insertions and deletions (indels) identified among genes using the data from**
292 **resistant (R) and susceptible (S) kids.**

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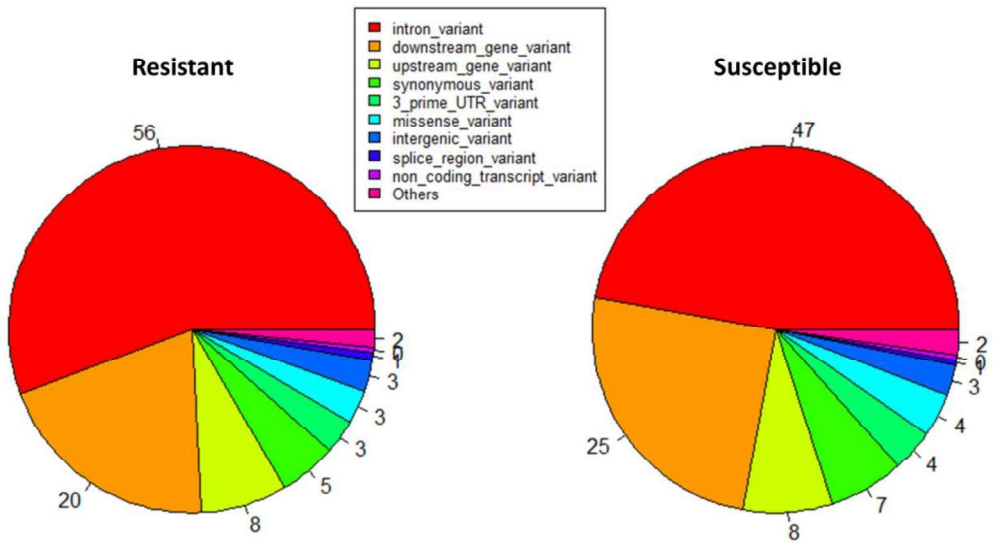
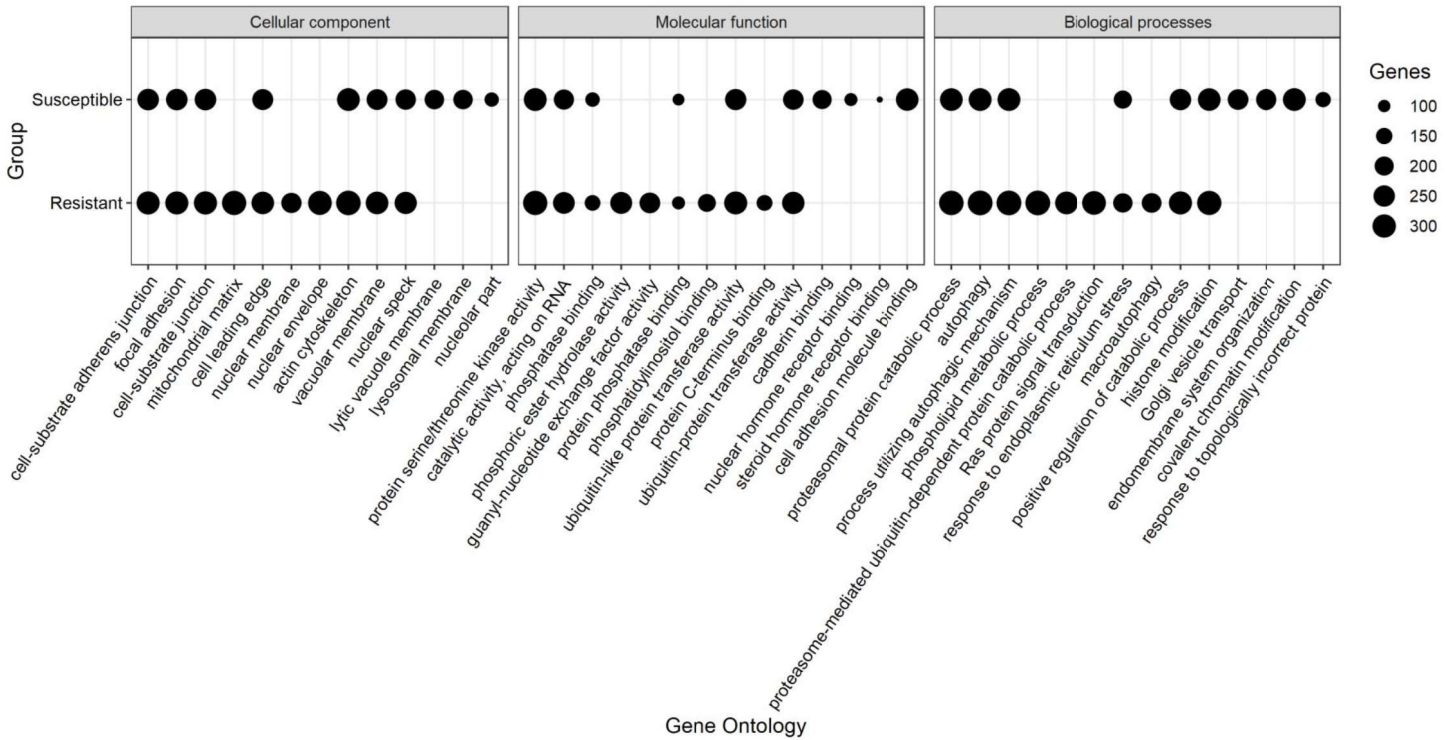


294
295 **Fig 4. Variant effect prediction for the variants identified in the data from the resistant**
296 **and susceptible group.**



297

298 **Fig 5. Gene ontology for genes containing variance for data from the resistant and susceptible group.**

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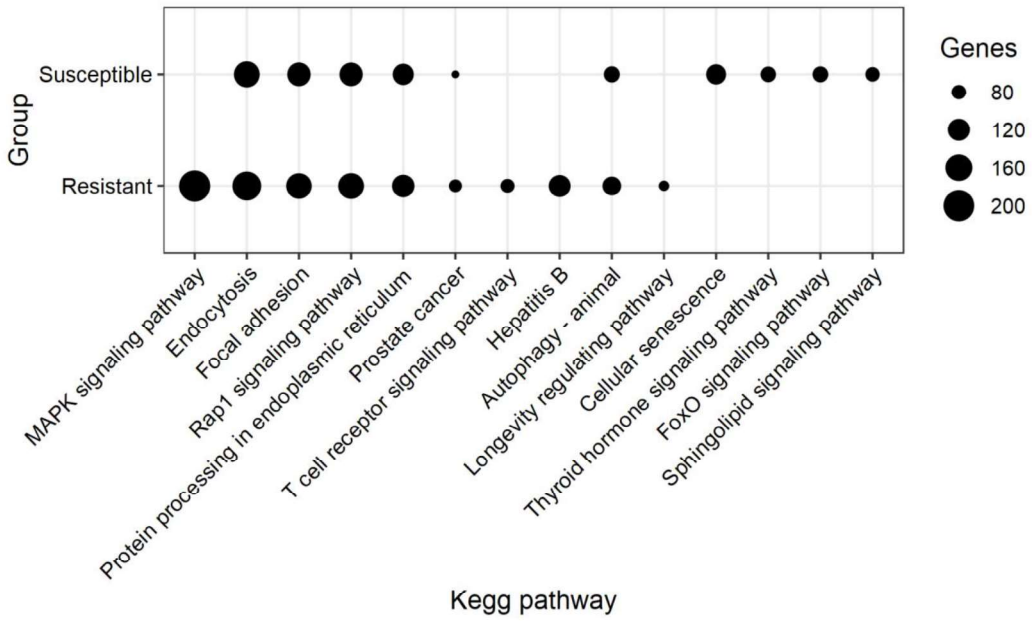

300

301 **Fig 6. Kegg pathways for genes containing genomic variances in the data from the**
302 **resistant and susceptible group.**

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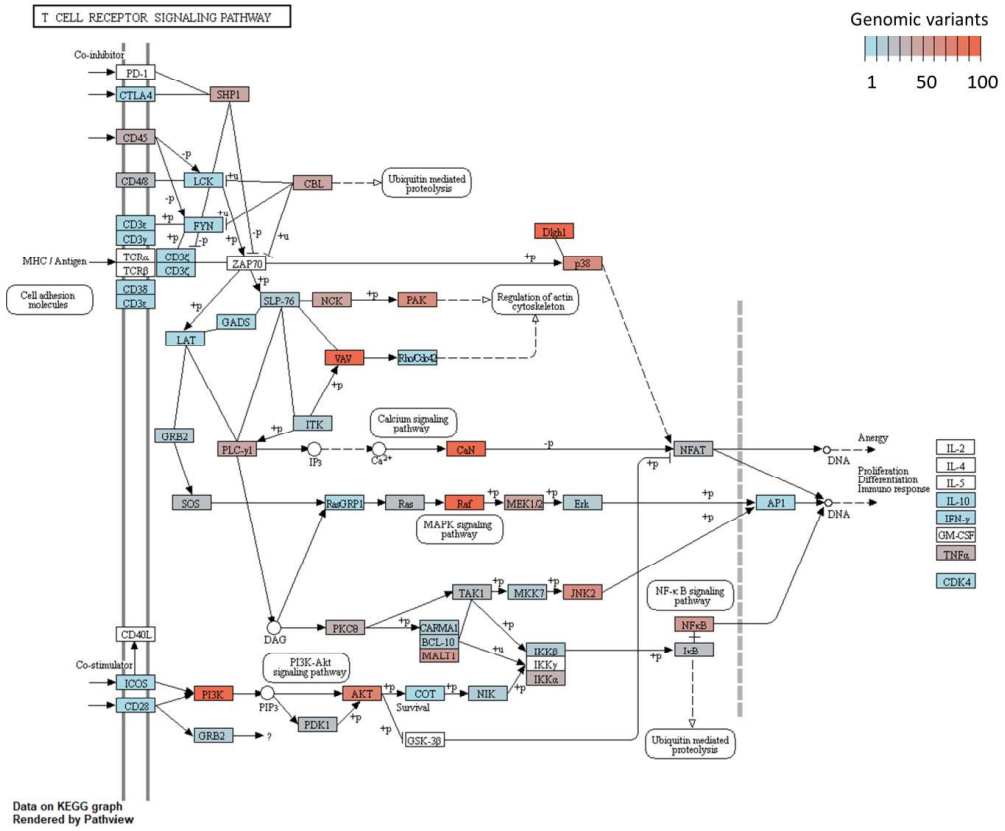

Fig 7. Number of genomic variants for each gene in the T cell receptor signalling pathway as identified in the data from the resistant group.

4 General discussion

Evidence for the genetic variation in host resistance to GIN among small ruminant breeds (Zajac *et al.*, 1990; Baker *et al.*, 1998, 2003; Amarante *et al.*, 1999, 2005; Sayers *et al.*, 2007; Shakya, 2007; Bowdridge *et al.*, 2013) and within the animals of the same breed (Bambou *et al.*, 2013; McRae, Good, *et al.*, 2014; McBean *et al.*, 2016) rise the interest in control strategies based on the host immuno- genetics/genomics. Host resistance has been characterized by rapid genetic progress in small ruminant flocks both under research and commercial conditions (Morris *et al.*, 1997, 2000, 2005; Williams *et al.*, 2010; McRae, McEwan, *et al.*, 2014). Therefore, breeding for host resistance is considered a decisive method of GIN control. A good knowledge of the mechanisms underlying protective immunity in small ruminant is a prerequisite for the development of immune- genetics/genomics methods to control gastrointestinal helminths. A discussion of the host immune response to GIN, which are associated with resistance or susceptibility, and the genetic regulation mechanisms for immunity are summarized here. Besides, the impact of other factors such as chitinas and oxidative status is highlighted.

4.1 Host immunity against GIN

An infection with GIN larvae induces host response to control the infection. The development of immunity to GIN is complex and highly variable depending on host breed, the GIN specie and the intensity of infection (McRae *et al.*, 2015). Protective immunity to GIN is mediated, at least partly, by parasite-specific antibodies response (McRae *et al.*, 2015). Small ruminant antibody response includes IgG1, IgG2, IgM, IgA and IgE isotypes (Schallig, 2000). During the last decades many research groups have studied the possible role of these antibodies in immunity against GIN.

In article 1, we examined and summarized the role of parasite-specific antibody response. We summarized this role according to the three major mechanisms of immunity to GIN that have been described in sheep, prevention of establishment of most incoming infective larvae, suppressed GIN growth and therefore fecundity, and the expulsion of adult worms; or a combination of these mechanisms (McRae *et al.*, 2015). Reduced parasite establishment and survival is associated with IgE activity mainly against incoming third stage larvae (L3) in concert with mast cells as cross-linking of IgE on the mast cell surface leading to mast cell degranulation (Stear *et al.*, 2009; Murphy *et al.*, 2010) with more prominent response in previously infected animals (Huntley *et al.*, 1998). Reduced parasite growth and fecundity is associated with increased local IgA activity against fourth stage larvae (Stear *et al.*, 1995, 2004, 2009). Increased number of inhibited larvae is associated with IgG1 activity against the third stage larvae (Douch, Green and Risdon, 1994; Schallig, van Leeuwen and Hendrikx, 1995) beside IgA activity against the third and fourth stage larvae (Stear *et al.*, 2004, 2009). However, some studies in goats indicated that humoral response is not correlated with GIN resistance in goats (Bambou *et al.*, 2008; de la Chevrotière *et al.*, 2012; McBean *et al.*, 2016).

Eosinophils, mast cells and globule leukocytes (degranulated mast cells) have all been implicated as effector cells mediating resistance to GIN (Schallig, 2000; Arsenopoulos, Symeonidou and Papadopoulos, 2017). Eosinophils are a type of white blood cell and assumed to have a major role in the innate immune response. They have been reported to have a significant role in protection to GIN infections at least against *H. contortus* (Schallig, 2000; Arsenopoulos, Symeonidou and Papadopoulos, 2017). Eosinophilia have been correlated with protection against *H. contortus* in sheep (Balic, Cunningham and Meeusen, 2006; Robinson *et al.*, 2010; Shakya *et al.*, 2011). However, a relationship was neither found between the number of adult *T. circumcincta* and tissue eosinophilia (Henderson and Stear, 2006), nor between FEC of *T. circumcincta* and circulating eosinophil counts (Beraldi *et al.*, 2008). This is probably due to the fact that *T. circumcincta* causes little damage to the mucosal epithelium (Venturina, Gossner and Hopkins, 2013). In goats, blood eosinophil was also reported to increase significantly after infection with *H. contortus* (Bambou *et al.*, 2008) and to have negative correlation with FEC (de la Chevrotière *et al.*, 2012). The hyperplasia of mucosal mast cells is one of the most marked features of a GIN infection (Schallig, 2000; Arsenopoulos, Symeonidou and Papadopoulos, 2017). Mucosal mastocytosis, including globule leucocytes, was associated with GIN, which suggest that type I immediate hyper-sensitivity reactions are important in worm expulsion (Miller, 1984). In this context, significant increases of mast cell in the gastric lymph and globule leukocytes

were observed in infected and reinfected 'immune' sheep (Stear *et al.*, 1995; Huntley *et al.*, 1998). Similarly in goats, globule leukocyte had negative correlations with number of worm (Paolini *et al.*, 2003) and immature worm burden (Bambou *et al.*, 2013) after infection with *H. contortus*. Higher numbers of abomasal mucosal eosinophils, mast cells and neutrophils have been observed in infected compared to uninfected lambs, with higher level in resistant than susceptible breeds (Shakya *et al.*, 2011).

4.2 Regulation of host immune mechanisms

Although antibodies and mast cells have been reported to play the major role in the host control of parasite infection, these factors are regulated by the cytokine environment generated by activated T cells (Venturina, Gossner and Hopkins, 2013). Identifying the type and mechanism of T cell activation involved in the immunological regulation of infection is critical in understanding the host control of GIN infection.

4.2.1 Major Histocompatibility Complex (MHC I and II)

Presentation of antigens via Major Histocompatibility Complex (MHC) class I and class II molecules for recognition by specific T-cell receptors is central to T-cell activation (Vyas, Van Der Veen and Ploegh, 2008). MHC class I presents intracellular peptides at the cell surface of CD8+ T cells when intracellular pathogens such as viruses induce cellular expression of viral proteins. Some of these viral proteins are tagged for degradation, with the resulting peptide fragments entering the endoplasmic reticulum and binding to MHC class I molecules (Neefjes *et al.*, 2011). A MHC class II on the other hand presents peptides from extracellular pathogens at the cell surface of CD4+ T cells which help to trigger an appropriate immune response including localized inflammation or lead to a full-force antibody immune response due to activation of B cells (Vyas, Van Der Veen and Ploegh, 2008; Neefjes *et al.*, 2011).

One candidate region for genes involved in parasite resistance or susceptibility is the MHC. MHC class II regions have been associated with GIN resistance in different breeds of sheep (Schwaiger *et al.*, 1995; Outteridge *et al.*, 1996; Paterson, Wilson and Pemberton, 1998; Charon *et al.*, 2002; Sayers, Good, Hanrahan, Ryan, Angles, *et al.*, 2005; Stear, Innocent and Buitkamp, 2005). In this context, using transcriptional profiling of nematode-resistant and susceptible sheep lines, up-regulation of MHC class II genes was observed in resistant animals (Keane *et al.*, 2007). In a mouse model infected

with *Strongyloides venezuelensis*, MHC class II but not class I molecules were required to induce a predominantly immune response and to achieve efficient control of infection (Rodrigues *et al.*, 2009).

Our results from article 2 (Aboshady *et al.*, 2019) indicated that the top biological functions for the DEG identified from the comparison of lymph node tissue from resistant and susceptible goats were related to antigen processing and presentation of peptide antigen via MHC class I. The 'antigen processing and presentation of peptide antigen via MHC class I' was also reported as one of the major functional annotation cluster of genes differentially expressed in abomasal lymph nodes in sheep breeds known to differ in GIN resistance (Ahmed *et al.*, 2015). The implication of the MHC class I molecules in the mechanisms underlying genetic resistance to *H. contortus* was reported through an association between reduction in FEC and a homozygotes allele for the MHC class I (*OMHC1-188*) in sheep (Castillo *et al.*, 2011). A MHC class I antigen in close linkage disequilibrium with the DRB1 class II antigen, was associated with a 10-fold reduction in FEC following natural predominantly *Ostertagia circumcincta* infection in lambs (Stear *et al.*, 1996). The linkage disequilibrium between MHC class I and II antigen means that it is difficult to say which one is the causative for the FEC reduction.

Our results in goats and other results from previous studies in sheep (Castillo *et al.*, 2011; Ahmed *et al.*, 2015) suggest that MHC class I plays a role in resistance to GIN infection. This result is not expected from the previous known functions for MHC, that class I present in response to intracellular pathogens and class II present in response to extracellular pathogens (Neefjes *et al.*, 2011). Beside that MHC class II but not class I molecules are required for predominantly immune response and control of GIN infection in mice (Rodrigues *et al.*, 2009).

4.2.2 T cell receptors

The T cell receptor (TCR) is a complex of integral membrane proteins on the surface of T cells, which recognizes the antigens presented by MHC and plays a central role in the adaptive immune response (Vyas, Van Der Veen and Ploegh, 2008; Huse, 2009). Recently, TCR signaling has been linked to gene regulation through downstream pathways which modify gene expression (Huse, 2009).

Results from article 4 showed that the TCR signaling pathway was one of the top significant pathways identified for genes containing genomic variants from resistant animals. By examine genes involved in TCR signaling pathway we found that 78% of these genes have one or more genomic variants that exist

in resistant and not in susceptible animals. TCR signaling pathway was not identified previously as top significant pathway in studies comparing gene expression between resistant and susceptible animals. Despite that TCR signaling pathway was one of the immune pathways identified for immune genes containing SNPs in sheep chromosome 3 (Oar3) which were associated with GIN resistance (Periasamy *et al.*, 2014). In this study, they used a large number of animals (n = 713) which represent 22 breeds across Asia, Europe and South America. The results in this study align with our findings of the role of TCR signaling in the adaptive immune response against GIN infection and that genomic variants in genes involved in it affect animal immune response.

4.2.3 T helper (Th) cells and cytokines

On encountering a foreign antigen, MHC class I or II carrier molecules display the antigens to their cognate T cell receptor, which activates the naïve T cell and initiates the adaptive immune response. Consequentially, this results in release of cytokines, leading to both T cell differentiation and the proliferation of further T cells (McRae *et al.*, 2015).

The adaptive immune response against GIN has been studied extensively in rodent models (Miller, 1984; Sher *et al.*, 1990; Urban *et al.*, 1992, 1996; Finkelman *et al.*, 1997). As a consequence, our knowledge in host immune response comes mainly from these models. Traditionally, it has been accepted from studies on murine models that immunity is dependent on CD4+ T cell (Th0) activation which develops in two mainly distinct pathways, T helper type 1 (Th1) and type 2 (Th2) cell response based on the cytokines that they secrete (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). Th1 cells produce a number of cytokines principally interleukin 2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor β (TNF-β) resulting in a cell mediated immune response (Schallig, 2000). Meanwhile, Th2 cells produce another number of cytokines such as IL-4, IL-5, IL-9, IL-13, IL-25 and IL-33 among others, which induce differentiation and maturation of intraepithelial mast cells, eosinophilia and goblet cell development (Mosmann and Coffman, 1989; Artis and Grencis, 2008; Li *et al.*, 2012). IL-4 and IL-5 induce an inflammatory response that is characterized by IgE production in case of IL-4 (Finkelman *et al.*, 1990) or eosinophilia in case of IL-5 (Coffman *et al.*, 1989; Sher *et al.*, 1990). Besides, IL-4 with IL-3 and IL-9 serve as a co-factor in the development of intestinal mucosal mast cells (Hültner *et al.*, 1990; Urban *et al.*, 1992). Meanwhile, IL-13 activates goblet cells leading to increases the secretion of mucus and prevents contact of parasites with the epithelial surface. Additionally, IL-13 and IL-4 activate macrophages that produce metabolic products to attack and stress

larval stage of GIN within the intestinal mucosa (Artis and Grencis, 2008). A typical Th2 response is characterized by increased immunoglobulin secretion by plasmocytes, in particular IgG1, IgA and IgE, and proliferation of eosinophils and mast cells.

Research using murine models has underlined the role of Th2 response and high levels of cytokines IL-4 and IL-13 with resistance of the host immune system against GIN infection, while a Th1 response with high levels of IFN-γ have been linked with susceptibility (Urban *et al.*, 1992; Maizels and Yazdanbakhsh, 2003; Anthony *et al.*, 2007). In general, Th1 response is activated during intracellular parasite infections, where IFN-γ is the predominant immune activator, while Th2 response is activated during extracellular parasite infections where IL-4 plays a prominent role in elevating humoral immune mechanisms (Urban et al., 1996).

Results from the systematic review in this thesis (article 1) showed that immunoglobulin response and therefore Th2 response differ between sheep breeds. There is strong suggestion that goats develop a different set of strategies to regulate GIN infections and to establish immunity, compared to sheep. Moreover, some goats appear to lack a functional IgA and eosinophil response against natural GIN infection. In this context, results from article 3 showed that the Th1 pathway was one of the top pathways identified in most of the comparison performed. Looking at differential of CD4+ T cell, signals for Th1 and Th2 activation were found in resistant animals when comparing them with susceptible animals. Results suggested that activation for Th2 genes is earlier in resistant goats compared to the susceptible ones. Altogether these results indicate that the Th2 response against GIN infection is less effective in goats than sheep and probably does not play the main role in the mechanism underlying genetic resistance in goats. While a Th1/Th2 balance could be more important than a Th2 response alone.

In ruminants, the view that a Th1 response is associated with susceptibility and a Th2 response with resistance, as well as their balance, consider an issue of conflict. For example, IFN-γ inhibited host protective responses to *Strongyloides papillosus* infection in cattle resulting in increased larvae survival (Nakamura *et al.*, 2002). A Th1 response was observed in susceptible sheep infected with *H. contortus* through an increased expression of TNF-α and IFN-γ (Zaros *et al.*, 2014). In addition, Th1 response was linked to susceptibility and Th2 response to resistance in reviewing genetic resistance of sheep to *T. circumcincta* (Venturina, Gossner and Hopkins, 2013).

On the contrary, another study supported a relationship between IFN-γ and resistance to GIN infection in Texel sheep (Sayers, Good, Hanrahan, Ryan and Sweeney, 2005). TNF-α and IFN-γ expression was increased after *H. contortus*

infection in sheep both in the abomasal mucosa and the draining lymph nodes (Pernthaner *et al.*, 2005, 2006; Robinson *et al.*, 2011). In the same context, infection with *Ostertagia ostertagi* in cattle resulted in decreased levels of IL-2 transcription and increased levels of IL-4 and IL-10 transcription. These observations are consistent with Th1 depression and Th2 activation; however these did not protect the calves against the *O. ostertagi* infection.

Another hypothesis support existing balance/ratio between Th1 and Th2 to express resistance genotype. A study showed an increased expression of IFN-γ and IL-12 despite a predominant Th2 response in immunized sheep during *H. contortus* infection (Meeusen, Balic and Bowles, 2005). Schalling (2000) suggested that the more important factor for the final outcome of the immune response is not the quantity of each cytokine but the ratio of the different cytokines. Our results from article 3 support this hypothesis.

4.2.4 Th17 responses and Regulatory T cells (Tregs)

Another distinct T cell category is Th17 cells which promote inflammation response through production of IL-17 and IL-21 cytokines (Venturina, Gossner and Hopkins, 2013). Inducing T cells to differentiate to Th17 instead of other T cell strains requires IL-23 stimulation following IL-6 and TGF-ß1 stimulation (Kimura and Kishimoto, 2010; Jin and Dong, 2013). IL-17 family members and IL-21 cytokines are known for their important in cleaning pathogens and inducing tissue inflammation at early infection (Korn *et al.*, 2009; McRae *et al.*, 2015). IL-17A and IL-17F mediate their immunological function by inducing pro-inflammatory cytokine, anti-pathogenic peptide and chemokine secretion by responder cells. The release of these pro-inflammatory molecules triggers the recruitment of innate immune cells to the site of infection and eliminate the pathogen (Jin and Dong, 2013).

Human patients with a genetic mutation in the STAT3 gene have defective IL-17A/F production and suffer from high susceptibility to infections from different pathogens (Milner *et al.*, 2008). Our results showed that the expression levels for genes controlling the Th17 response had a positive fold change for STAT3 and RORC genes in resistant compared with susceptible kids at 15 days post infection (dpi), and for IL17F at 35 dpi (article 3). In sheep research, Th17-associated genes have been associated with resistance to GIN at an early stage of infection (MacKinnon *et al.*, 2009). In contrary, increased expression of IL-6, IL-23A and IL-21 have been associated with susceptibility to GIN at 12 weeks after trickle infection (Gossner *et al.*, 2012). These results indicate the role of Th17 response in resistant to GIN at early stage of infection.

The host can control the immune response against parasite infection by the development of T regulatory cells (Tregs) (Venturina, Gossner and Hopkins, 2013). Tregs are known to have two main functions. Firstly, they have the ability to suppress the immune response with IL-10 and TGF-β cytokines after prolonged immune activation to manage inflammation and limit tissue damages (Tang and Bluestone, 2008). Secondly, Tregs are critical for the clinical outcome of GIN infection (Venturina, Gossner and Hopkins, 2013; Arsenopoulos, Symeonidou and Papadopoulos, 2017).

TGF-β is a multifunctional cytokine produced by all white blood cells lineages and best known for its regulatory activity and induction of peripheral tolerance (Nakao *et al.*, 1997). In our studies, we found that the 'TGF-β signaling pathway' in the top significant pathway for the different expressed genes in the comparison of mucosa samples between resistant and susceptible kids at 42 dpi (article 2). Moreover, *TGF-β1* was one of the first upstream regulator gene that was differently expressed in mucosa tissue of resistant versus susceptible and infected versus non-infected kids, with a prediction to be inhibited in resistant kids at 42 dpi (article 2). By studying the expression of *TGFβ1* at different time of infection (article 3), we found it to be significantly higher in resistant compared with susceptible kids at early time of infection (8 dpi) and then down regulated in resistant animals at late infection (35 dpi), which is in agreement with findings in article 2. In this context, TGF-β receptor 1 was highly expressed in lymph nodes of a susceptible sheep breed compared with a resistant sheep breed at 27 dpi with *H. contortus* (MacKinnon *et al.*, 2009). Recently it was found that modulate cytokines profile to increases the secretion of IL-10 and TGF-ß1 in goat monocytes contributes to induce an anti-inflammatory environment (Wang *et al.*, 2017). This confirms the role of Tregs in maintenance of immunological tolerance.

4.3 Other factors related to host control of infection

4.3.1 Chitinase and chitinase-like proteins

Chitinases are a group of digestive enzymes that break down glycosidic bonds in chitin, which is present in the exoskeletal elements of GIN and arthropods (Fuhrman and Piessens, 1985). A mice model showed that chitinases (C) and chitinase-like proteins (CLP) production is an important feature of Th2 immune responses during nematode infection (Nair *et al.*, 2005). In a recent review of the role of C/CLP in immune response, Lee et al. (Lee *et al.*, 2011) reported that C/CLP are produced by the host in the case of mammals as a

defense against infection. They can inhibit chitin-induced innate immune and injury responses. Simultaneously, enhance adaptive immune responses, thereby ensuring the development of selective antigen-specific immunity. C/CLP are further induced during the type 2 immune response, and have the ability to contribute in the production of TGF-β1 and also probably to healing and fibrosis (Lee *et al.*, 2011).

The chitinase-3 like 1 (Chi3L1) transcript was found to be upregulated early (day 5 post infection) in both the abomasum and gastric lymph nodes in response to a *T. circumcincta* challenge of previously infected sheep. But it was upregulated late (day 21 post infection) in the abomasum of naïve sheep (Knight *et al.*, 2007). Expression of the chitinase-3 like 2 (Chi3L2) has been observed in the abomasal lymph node of resistant and susceptible Blackface lambs infected with *T. circumcincta* in comparison to uninfected animals (Gossner *et al.*, 2013). Expression of the same gene (Chi3L2) has also been reported in the abomasum of 18 and 21 week old steers exposed to *O. ostertagi* (McRae, McEwan, *et al.*, 2014). These could indicate that C/CTP play a role in immune response in both susceptible and resistant animals.

Our results for transcriptomic changes of goat abomasal mucosa in response to *H. contortus* infection (article 3) did not show any signature for C/CTP mechanisms. However, looking at the gene expression level we found that expression of Chi3L2 was significantly higher in resistant and susceptible kids at 8 and 15 dpi in comparison to day 0. While the expression was still high in resistant kids at 35 dpi in comparison to day 0, it decreases in susceptible kids. This leads to a significant difference in the expression of Chi3L2 between the resistant and susceptible groups, being 32-fold higher in resistant group. This supports the previous finding, that C/CPT plays a role in immune response in both susceptible and resistant animals, with a new sign for difference in maintaining high level in resistant animals.

4.3.2 Oxidative status

Another significant factor that was reported in parasite control is the generation of host oxidants (Ingham *et al.*, 2008; Patel *et al.*, 2009; Arsenopoulos, Symeonidou and Papadopoulos, 2017). Oxidants that have been associated with GIN resistance include phagocytic oxidase (PHOX) (Dzik *et al.*, 2006), dual oxidase (DUOX) (Ingham *et al.*, 2008; Menzies *et al.*, 2010; Lees *et al.*, 2011) and inducible nitric oxide synthase (NOS2A) (Rajan *et al.*, 1996). Reactive oxygen and nitrogen species (RONS), generated by these factors, have possible roles in facilitating GIN expulsion through direct damages of parasitic tissues or lethality (Colasanti *et al.*, 2002; Lees *et al.*, 2011).

An increase in the reactive oxygen producer dual oxidase 1 (DUOX1) transcript was particularly marked high in resistant sheep following 3 days of *T. colubriformis* challenge in previously infected animals (Ingham *et al.*, 2008). The DUOX2 expression was found to be important in the sheep mucosal inflammatory responses to GIN infection as it raised from 3 d.p.i (Menzies *et al.*, 2010). In this context, an early response to *H. contortus* experimental infection in resistant sheep was marked by an increase in expression of host oxidant producing genes: the dual oxidase group (DUOX2/DUOXA2) during day 1 to day 7 compared to day 0 of infection (Lees *et al.*, 2011). During days 1 to 7 post-challenge, a cluster of four cytokines, IFN-ɣ, IL4, IL5 and TNF-α, showed strong positive correlation to a second cluster containing mast cells, eosinophils and globular leukocytes as well as the expression of DUOX2, DUOXA2 and GPX2 (Lees *et al.*, 2011). It is interesting that this study noted a positive association between IFN-ɣ (Th1 cytokine) and IL4 expression (Th2 cytokine). Again, this result raises the role of both Th1 and Th2 in host resistance to GIN infection.

On the other hand, host reactive oxygen and nitrogen intermediates display high reactivity and low specificity. Therefore, they can damage host tissues, leading to dysfunction of the immune response which explains the requirement for effective host antioxidant defenses for the development of immunity against GIN infection (Arsenopoulos, Symeonidou and Papadopoulos, 2017). It was demonstrated that the host antioxidant response to infection is specific to the time of challenge at the time when oxidants expulsion effect was finished (first 7 days of the infection) and resistance in sheep was established (Lees *et al.*, 2011). This involving an increase in the expression of the glutathione peroxidase family genes (glutathione peroxidase 3, glutathione reductase and glutamyl cysteine deoxygenase gene) at 28 dpi.

Looking at the genes expression from article 3, there were no differences in DUOX1, DUOX2, DUOXA2 expression between resistant and susceptible animals at 8 or 15 dpi. These genes showed down regulation in resistant compared to susceptible animals at 35 dpi. The same genes were differently expressed between abomasal mucosa of infected and non-infected animals at 42 dpi (article 2), showing down regulation in infected compared to non-infected animals. At the same time, both experiments (article 2 and 3) did not verify differences in antioxidant genes expression. Despite no differences in antioxidant gene expressions, oxidants play a role in response to GIN infection in both resistant and susceptible animals with difference in expression prolongation.

4.4 Breeding for resistance to GIN

Breeding for GIN resistance depending on genetic variation has been the subject of many review research articles (Schallig, 2000; Davies, 2006; Stear *et al.*, 2009; Bishop, 2015; McRae *et al.*, 2015; Zvinorova *et al.*, 2016; Arsenopoulos, Symeonidou and Papadopoulos, 2017). Selection for resistance has traditionally been based on quantitative measurements of phenotypic traits as we discussed in the general introduction. One of these quantitative phenotypic traits that has potential to be used in breeding for GIN resistance is the immunoglobulin level. In article 1 we discussed the role of each immunoglobulin for the resistance to GIN infection. CarLA saliva IgA antibody test is currently being marketed (CARLA® SALIVA TEST) as a powerful new tool for measuring parasite immunity in sheep (https://www.agresearch.co.nz/doing-business/products-and-services/carla-saliva-test/). Salivary IgA measurements have been used to calculate an Estimated Breeding Value (EBV) for Lleyn sheep in an ongoing project and results from selection using these EBVs is in the way (https://www.isage.eu/wp-content/uploads/No.6_ORC_NSA-_Assessing-parasite-resistance-on-three-sheep-breeds-in-the-UK_FINAL.pdf).

On the other hand, the identification of molecular markers is potentially a more reliable approach in breeding for GIN resistance (Venturina, Gossner and Hopkins, 2013; Zvinorova *et al.*, 2016). In article 2 and 3, we examine the transcriptome variation between resistant and susceptible Creole kids in response to *H. contortus* infection from abomasal mucosa and lymph node tissue at late infection (article 2) and from abomasal mucosa at 8, 15 and 35 dpi (article 3). The purpose was to compare the genes expressions between resistant and susceptible kids in response to infection and to identify the different mechanisms involved in the control of infection. This is considered a first step to identify possible genes to be used as potential molecular markers in breeding for resistance. Article 2 showed that MHC class I and *TGFβ1* genes have a major role in controlling GIN infection and infection consequences, which make them possible molecular markers. In this context, the implication of the MHC Class I molecules in the mechanisms underlying genetic resistance to *H. contortus* was reported through an association between reduction in FEC and a homozygotes allele for the MHC class I (*OMHC1-188*) in sheep (Castillo *et al.*, 2011). The same role for *TGFβ1* was previously reported in other study in goats (Bhuiyan *et al.*, 2017) and also a study on sheep infected with *H. contortus* (MacKinnon *et al.*, 2009). Article 3 confirms the previous finding for the relevance of the *TGFβ1* gene besides suggestions for other genes for possible use as molecular marker like *IL2R, TNF, IFN-γ, IL4R, STAT6, GATA3, STAT3*, or *RORC*. Interestingly QTL were reported near the *RORC* gene,

transcription factors controlling Th17 maturation and function, on sheep chromosome 1 (OAR1) (Ellis *et al.*, 2009; Gutiérrez-Gil *et al.*, 2009; Marshall *et al.*, 2009). *IL2RB, IFN-γ,* and *TNFA* were also reported as proximate genes to different QTL found on sheep chromosome 3 and 20 which have been associated with FEC (Benavides, Sonstegard and Van Tassell, 2016).

Results from differential gene expression studies during infection assist in understanding the differences in mechanism between resistant and susceptible animals and the genes involved in these differences. Nevertheless, for the purpose of practical selection, variants causing the difference in expression should be identified as this will offer better opportunities that information on gene expression, which might rely on infection experiments. Meaningful and easy accessible molecular markers will be more useful as practical tools for breeding purposes. In article 3 we found that *IL17F* had the most significant difference in expression between resistant and susceptible kids at day 0 of infection (uninfected), having an expression three times higher in resistant compared with susceptible kids. This gene would have therefore a potential to be used as biomarker in a selection program. *IL17A* and *IL17F* were reviewed as proximate genes located in genomic regions that were found on sheep chromosome 20 and were associated with parasite resistance in sheep (Benavides, Sonstegard and Van Tassell, 2016).

In article 4, we discovered genomic variants in the abomasal mucosa transcriptomes of Creole goats classified as resistant or susceptible to *H. contortus* and characterized the variants identified. This could help in the previous raised issue concerning the causative variants to be used in breeding programs. Results from this article showed that 78% of genes involved in T cell receptor signalling pathway have one or more genomic variants that exist in resistant but not in susceptible animals. These genomic variants could be the key for the difference in activated T cells between resistant and susceptible animals and therefore have a potential to be used in breeding for resistance against GIN. This is the first study to examine the genomic variants between resistant and susceptible animals to GIN using information at the transcriptome level. However, there is still need to confirm these variants and to examine if they exist at DNA level or if they are post-transcription variants.

5 Future perspectives

Our results indicated the important of early response to infection in resistance to infection. Hence, studying the differences between resistance and susceptible animals at very early infection could provide better understanding for the resistance mechanisms.

Currently, animal selection and the search for biomarkers depend on low FEC which increase host resistance to parasites. However, resilient animals are not targeted by this approach. Hence, there is a need to identify resilient animals, discovering genes or genetic markers associated with resilient and mechanisms involved to include it in selection programmes.

Furthermore, studies showed that nutrition could be used as control strategy for GIN infection. Studies to determine nutrigenomic effect on resistance to GIN infection should be performed. Studying metagenomics during infection could also provide better information on infection mechanisms and hence better development for control strategy. Selection for resistance and/or resilience to GIN is complicated and polymorphic trait. This highlights the need for non-genetics/genetics methods to complement each other to prevent and control infection.

6 Conclusions

One promising method to control GIN and reduce its negative impact is selecting animals with a high immunoglobulin response. We highlighted factors that differ across studies and affect the immune response to GIN infection. Of these factors, age of the animals, the infection experience and the type of infection should be taken into account when designing future studies. Beside the need to standardize/normalize the measurements of immunoglobulin concentrations to be comparable between studies.

Our results suggested that resistance in Creole goats mainly controlled through reduction in worm fecundity and not worm burden with a major regulator role for MHC class I and TGF-β genes. At late infection, the priority of the host response is to maintain the integrity of the mucosal barrier

Goats infected with *H. contortus* induced simultaneous upregulation of Th1 and Th2 immune response at the mucosal level of resistant animals. Our results indicated an earlier activation in Th2 immune response in resistant goats compared to the susceptible ones. Some genes like *IL2R, TNF, IFN-γ, IL4R, STAT6, GATA3, STAT3*, or *RORC* have potential to be used in breeding for GIN resistance.

Results verified the possibility to use RNA-seq data as an efficient method and great resource to detect genomic variants at functional genes level. Genomic variants in genes involved in T cell receptor signalling pathway plays a role in GIN resistance in goats.

This work provides valuable resources for genomic differences and molecular mechanisms of the host response to GIN infection in small ruminant. This serves as a basis towards developing genomic markers for GIN resistance.

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Popular science summary

Small ruminants are widespread all over the world. They are source of different goods and benefits ranging from food with precious animal proteins (meat and milk) to manure, fibre and skins, draught power in the highlands, food security and important non-market services like insurance, cultural and ceremonial purposes. The world's sheep and goat populations have increased steadily over the past decades, especially in developing countries. One of the main constraints on small ruminant production is the management of animal health. Infection with gastrointestinal nematode parasites has the greatest impact upon animal health and productivity. Anthelmintic treatments had been the main control strategy during the last decade leading to rise of anthelmintic resistance worldwide. Genetic selection for resistant animals is a promising sustainable strategy to control GIN infection.

This work aimed to understand the mechanisms involved in host resistance to GIN and explore possible phenotypic and genomic markers for resistance that could be used to develop appropriate small ruminant breeding strategies. We could show important factors for the design of future studies when summarizing the literature. Furthermore, we did identify relevant biologic pathways for the response to parasite infection in goats. Some of the information will add information to develop a potential selection of resistant goats in the future.

Firstly, we re-analyze and summarize the literature findings on immunoglobulin response to GIN. Immunoglobulins showed good potential to be used as phenotypic markers for GIN resistance. IgE level and mast cell for example could be used to breed for reducing parasite establishment and survival. IgA level has the potential to be used in breeding for reducing parasite growth and fecundity. And other immune parameters are potential biomarkers for the number of inhibited larvae. We highlight factors that should be taken into account to make future research comparable such as age of the animals, the infection experience and the type of infection (natural, single or trickle).

Secondly, we performed two experiments to study molecular mechanisms and genomic variants between resistant and susceptible Creole goats in response to *Haemonchus contortus* infection. In the first experiment, we compared transcriptome profiling of abomasal mucosa and lymph node tissues between non-infected, resistant and susceptible infected Creole goats. This breed showed resistance to GIN infection through reducing worm fecundity and not worm burden. Results indicated that at late infection stage the host response priority is to maintain the integrity of the mucosa. *TGFβ1* and MHC class I genes had a probable role in resistance to GIN infection. In the second experiment, we examined the host response at different time points of infection through studying the dynamic transcriptomic changes of the abomasal mucosa of resistant and susceptible infected Creole goats. Innate (Th1) and adaptive (Th2) immune response was activated in response to infection. Results indicated earlier immune response in resistant animals compared with susceptible ones. The mechanisms underline resistance were controlled trough many genes. *IL2RG, IL4R, STAT6, GATA3, CCR4, STAT3, RORC, TGFβ1* and *IL17F* genes showed an important role in determining animal response to GIN infection, which give them potential to be used in breeding scheme for resistance.

Finally, we used RNA-sequencing data from the second experiment to discover the genomic variants in resistant and susceptible animals. We were able to identify single nucleotide polymorphisms, insertions and deletions in the resistant and in the susceptible groups and compare them. The distinguished variants between resistant and susceptible animals were characterized through functional analysis. One of the top significant pathways that was identified for genes containing genomic variants was T cell receptor signaling pathway. 78% of genes in this pathway had genomic variants in resistant and not in susceptible animals. This study considered one of the first discoveries for genomic variants between resistant and susceptible animals at functional genes level which have potential to be used in breeding for GIN resistance.

Popular science summary in French

Populärvetenskaplig sammanfattning

Popular science summary in Swedish.

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Individual training plan (ITP)

Training (30 ECTS minimum)		
Mandatory courses	**Where/when**	**ECTS**
EGS-ABG research school "emerging technologies"	Wageningen university, The Netherlands February 13-17th, 2017	1.5
Philosophy of Science	SLU, Sweden March 6th – April 6th, 2017	3.0
How to write and publish a scientific paper	SLU, Sweden April 20th –May 23rd, 2017	3.0
Research ethics for PhD students	SLU, Sweden Sep. 12- 27th 2017	2.0
EGS-ABG research school "Animal 4D"	AgroParisTech, France May 28th – 1st June 2018	2.0
Advanced scientific courses (≥ 18 ECTS)		
From Genome Sequence to Genomic Selection	SLU, Sweden April 4-8th, 2016	5.0
Introduction to programming In R	SLU, Sweden May 9 -13rd, 2016	2.0
Genetic Epidemiology of infectious diseases in Livestock	SLU, Sweden May 15-19 th, 2017	3.0
Introduction to Bioinformatics using NGS data	SciLifeLab, Uppsala Nov. 27 th – Dec. 1 st 2017	2.0
Statistics III: Regression analysis	SLU, Sweden Jan 16 - Feb 16, 2018	4.0

Analysis of livestock metagenomics datasets	Centre INRA Antilles-Guyane, Guadeloupe, France. May 13-17th, 2019	1.5
How to write your first grant application	SLU, Sweden January 15-17th, 2017	1.0
Total credits (≥ 30 ECTS)		**30**
Dissemination of knowledge		
International conferences	**Where/when**	
Transcriptome variation in response to gastrointestinal nematode infection in goats. Oral presentation at 69th Annual Meeting of the European Federation of Animal Science (EAAP).	Dubrovnik, Croatia. August 2018.	
Dynamic transcriptomic changes of goat abomasal mucosa during an experimental *Haemonchus contortus* infection in resistant and susceptible genotypes. Poster presentation at 37th International Society for Animal Genetics (ISAG) Conference.	Lleida, Spain. July 2019.	
Oral presentation at 21st seminar of Animal Genetics Division.	Paris, France. May 2018.	

Appendix 1. Summary of molecular genetic markers associated with GIN resistance in small ruminants.

Species	Markers	Parasite(s)	Breed	Chromosome (associated trait)	References
Goats	sM (3, MHC)	*T. colubriformis*	Australian Angora and Cashmere	23 (FEC, EOS)	(Bolormaa *et al.*, 2010)
	M (101, 29 chr.)	Mixed	Creole	22, 26 (FEC), 7, 8, 14 (EOS), 5, 9, 21 (PCV) 6 (BW), 1, 3, 10, 26 (IgE)	(De La Chevrotière *et al.*, 2012)
	SNP (50k)	*H. contortus*	Creole	4, 6, 11, 17 (FEC)	(Silva *et al.*, 2018)
Sheep					
	sM (OLA antigens)	Mixed	Romney	20 (FEC, Peps)	(Douch and Outteridge, 1989)
	sM (OLA, 9 haplorypes)	*H. contortus*	Romanov	20 (FEC)	(Luffau *et al.*, 1990)
	sM (DRB1 alleles)	Mixed predominant *T. circumcincta*	Scottish Blackface	20 (FEC)	(Schwaiger *et al.*, 1995)
	sM (10 lymphocyte antigens)	Mixed predominant *T. circumcincta*	Scottish Blackface	20 (FEC)	(Stear *et al.*, 1996)
	sM (2, MHC)	Mixed predominant *T. circumcincta*	Scottish Blackface	20 (FEC)	(Buitkamp *et al.*, 1996)
	sM (5, MHC)	Strongyle	Soay	20 (FEC)	(Paterson, Wilson and Pemberton, 1998)

	sM (14, IFNɣ)	Mixed	Romney	3 (FEC)	(Paterson *et al.*, 2001)
	sM (3, IFNɣ)	*T. circumcincta*	Soay	3 (FEC)	(Coltman *et al.*, 2001)
	sM (IgE gene)	*T. colubriformis*	Merino	18 (FEC, confirmation filled for another flock for *T. colubriformis* or *H. contortus*)	(Clarke *et al.*, 2001)
	sM (18 on OAR1)	*T. colubriformis* & strongyle	Romney × Coopworth	1 (FEC)	(Diez-Tascón *et al.*, 2002)
	sM (7, OAR5)	Mixed predominant *H. contortus*	Corriedale and Polwarth	5 (FEC)	(Benavides *et al.*, 2002)
	sM (6, OAR20)	*H. contortus*	Rhönschaf	20 (Haematocrit, IgL, FEC)	(Janßen *et al.*, 2002)
	sM (DRB1 alleles)	Mixed	Polish Heath	20 (FEC)	(Charon *et al.*, 2002)
	M (133, 26 autosornas)	*T. colubriformis*	Peppin Merino	1*, 3*, 6, 11*, 12*, 24* (FEC)	(Beh *et al.*, 2002)
	sM (6, OAR20)	*H. contortus*	Merinoland	20 (FEC, IgL)	(Janßen *et al.*, 2004)
	M (165, 26 autosornas)	*T. columbriformis*	Churra	1, 6, 14, 20 (FEC) 1, 9 (IgA)	(Arranz *et al.*, 2004)
	sM (IFNɣ, 4 haplotypes)	*Trichostrongyle* & *Nematodirus*	Texel and Suffolk	3 (FEC in Texel)	(Sayers, Good, Hanrahan, Ryan and Sweeney, 2005)
	sM (DRB1 alleles)	*Trichostrongyle spp.*	Suffolk and Texel	20 (FEC in Suffolk)	(Sayers, Good, Hanrahan, Ryan, Angles, *et al.*, 2005)
	M (203 on 9 chr)	*Trichostrongyle spp.*	Romney × Coopworth	2, 8, 11 (N. adult), 23 (IgG, IgE)	(Crawford *et al.*, 2006)
	M (139 on 8 chr)	Mixed (*Strongyles, Nematodirus*)	Scottish Blackface	2, 3, 14, 20 (FEC) 3, 20 (IgA)	(Davies *et al.*, 2006)

	M (251,whole genome)	*Strongyle*	Soay	1, 6, 12 (FEC)	(Beraldi *et al.*, 2007)
	sM (3 on OAR5 & 4 on OAR20)	Mixed predominant *H. contortus*	Corriedale and Polwarth	5 (FEC)	(Benavides *et al.*, 2009)
	M (27 on OAR1 & 28 on OAR3)	*H. contortus*	Indonesian Thin Tail × Merino	1, 3 (FEC)	(Ellis *et al.*, 2009)
	M (181,whole genome)	*T. circumcincta*	Spanish Churra	1 (IgA), 1, 6, 10, 14 (FEC)	(Gutiérrez-Gil *et al.*, 2009)
	M (140 to 177, whole genome)	*H. contortus*	Merino	1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 15, 16, 18, 20, 21, 22, 24, 25, 26, X (FEC)	(Marshall *et al.*, 2009)
	M (55 on 8chr)	*T. colubriformis*	Romney-Merino Backcross	3 (FEC, EOS), 21 (EOS), 22 (FEC)	(Dominik *et al.*, 2010)
	sM (12 on OAR3 and OAR14)	*Nematodirus* and *Strongyles*	Suffolk and Texel	3 (FEC), 14 (FEC)	(Matika *et al.*, 2011)
	M (172, 25chr.)	*H. contortus*	Red Maasai × Dorper Backcross	3, 6, 14 (FEC), 22 (FEC, PCV)	(Silva *et al.*, 2011)
	SNP (50k)	*T. colubriformis* then *H. contortus*	Mixed breed	1, 2, 4, 5, 9, 13, 15, 20, 26 (FEC T. colubriformis) 1, 17, 18 (FEC H. contortus)	(Kemper *et al.*, 2011)
	M (172, 25 autosomes)	*H. contortus*	Red Masaai, × Dorper	2 (FEC, PCV, WC), 4 (FEC), 10 (FEC), 12 (WC), 18 (WC), 22 (WC), 23 (FEC, WC), 25 (WC), 26 (ADG, LWT, PCV, FEC, WC, AFWL, EPW)	(Marshall *et al.*, 2012)

	M (160) & SNP (50k)	*H. contortus*	Romane × Martinik Blackbelly Backcross	1 (PCV), 2 (PCV, SexR), 3 (FEC, WB) 4 (Len, pH), 5 (FEC, IgG, WB, PCV) 7 (FEC, Len), 9 (FEC), 10 (Peps) 12 (FEC, PCV, pH), 13 (FEC, IgG) 14 (Peps), 15 (FEC), 16 (FEC, WB) 17 (pH, PCV), 18 (Len), 19 (Len), 20 (FEC), 21 (FEC, Peps), 23 (FEC, WB), 25 (SexR, PCV)	(Sallé *et al.*, 2012)
	SNP (n=192)	*Strongyle*	Soay	No clear association with FEC/ specific antibodies and antinuclear antibodies	(Brown *et al.*, 2013)
	SNP (50k)	Mixed predominant *T. circumcincta*)	Scottish Blackface	6 (FEC, BW*), 14 (FEC), 21 (BW) 1* (FEC), 2* (FEC), 4* (FEC, IgA), 20* (FEC)	(Riggio *et al.*, 2013)
	SNP (41 on 38 genes)	Mixed	22 breeds	3 (FEC, PCV, LWT), 13 (PCV)	(Periasamy *et al.*, 2014)
	SNP (50k)	Mixed (*Nematodirus and Strongyles*)	Scottish Blackface, Sarda-Lacaune, Martinik Blackbelly-Romane Backcross	4, 12, 14, 19, 20 (FEC)	(Riggio *et al.*, 2014)
	SNP (50k)	Mixed predominant *H. contortus*	Red Maasai-Dorper Backcross	2*, 6*, 11*, 12*, 15* (FEC) 5*, 7*, 15*, 17*, 26* (PCV) 7*, 8*, 14*, 15*, 17* (LWT)	(Benavides *et al.*, 2015)
	SNP (50k)	*T. circumcincta*	Spanish Churra	6, 8, 22 (LA) 8, 10, 11, 12, 14, 15, 25 (GWAS)	(Atlija *et al.*, 2016)

sM, specific marker; M, microsatellite; SNP, single nucleotide polymorphism; chr., chromosome; FEC, fecal egg count; PCV, packed-cell volume; WB, worm burden; SexR, sex ratio in adult worm population; Len, female worm length; pH, abomasal pH; Peps, pepsinogen; WC, worm count; AFWL, adult female worm length; EPW, eggs per worm; LWT, live weight; ADG, average daily gain; LA, linkage analysis; GWAS, genome wide association selection.

* Suggestive marker.