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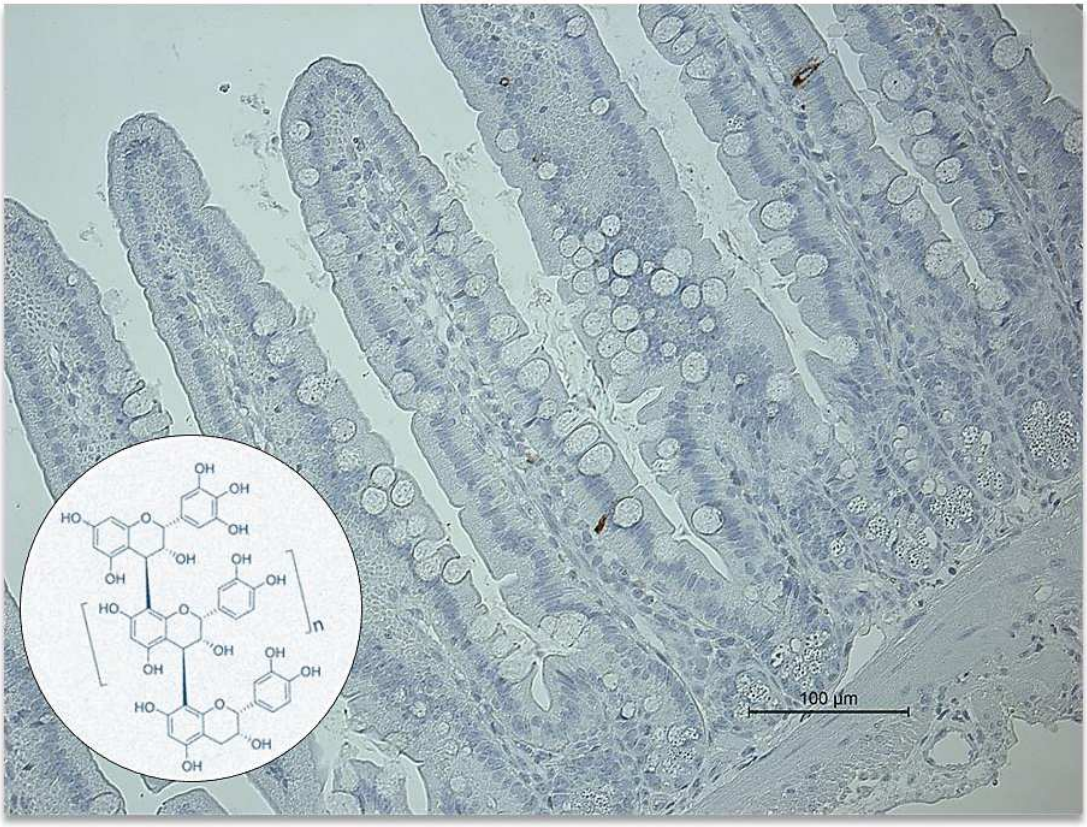

PhD Thesis

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Immuno-modulatory Properties of Proanthocyanidins and Implications during Helminth-induced Inflammation in the Gut

Impact of Proanthocyanidins in Cell-based Models and on Immune Responses in Mice and Pigs

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen [31-05-2021]

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Front Page Figure: Intestinal tissue from *Heligmosomoides polygyrus* infected mouse and proanthocyanidin molecule (*Source*: Author)

Preface

The work presented in this PhD thesis was conducted between June 2018 and May 2021. The majority of the experimental studies took place at the Section of Parasitology and Aquatic Pathobiology, Department of Veterinary and Animal Sciences, University of Copenhagen (Denmark), while chemical extractions and purifications were performed during a 3-month stay abroad at the Department of Chemistry, University of Turku (Finland).
Associate Professor Andrew R. Williams has been the main supervisor, and Professor Stig M. Thamsborg has been co-supervisor. Furthermore, Professor Juha-Pekka Salminen was advising the work conducted at the University of Turku, where I also received technical guidance from Milla Leppä. All studies discussed herein were conducted with the generous help and support from my colleagues of the PIGH (Parasites, Immunology and Gut Health) and VETPAR (Veterinary Parasitology) groups. Furthermore, technical assistance and advice was also received from Dennis Nielsen and Nilay Büdeyri Gökgöz (Department of Food, Copenhagen University), Charlotte Lauridsen (Department of Animal Science, Aarhus University), and Louis Bornancin (Department of Biotechnology and Biomedicine, Technical University of Denmark).
The funding of this PhD project was provided by the Independent Research Fund Denmark.

This PhD thesis entails an introduction and main background knowledge on the subject, a methodology section, followed by a discussion of main findings and reflection on study limitations, leading to a conclusion, and perspectives for future research. Finally, the thesis contains the following published papers and manuscripts:

I. Andersen-Civil, A. I. S., Arora, P. & Williams, A. R. Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins. *Frontiers in Immunology* vol. 12 (2021).

II. Andersen-Civil, A. I. S., Leppä, M. M., Thamsborg, S. M., Salminen, J., & Williams, A. R. Structure-Function analysis of Purified Proanthocyanidins Reveals a Role for Polymer Size in Suppressing Inflammatory Responses.

III. Andersen-Civil, A. I. S., Arora, P., Zhu, L., Myhill, L. J., Castro-Mejia, J. L., Leppä, M. M., Zeller, W. E., Thamsborg, S. M., Salminen, J., Nielsen, D. S., Desjardins, Y., & Williams, A.W. Dietary Proanthocyanidins and Enteric Nematodes Interact to Modulate Gut Microbiota-derived Metabolites and Promote Type 1 Intestinal Immune Responses in Mice.

IV. Andersen-Civil, A. I. S., Myhill, L. J., Gökgöz, N. B., Engström, M., Mejer, H., Salminen, J., Lauridsen, C., Nielsen, D. S., Thamsborg, S. M., & Williams, A. R. Dietary Proanthocyanidins Exert Localized Immunomodulatory effects in Porcine Pulmonary and Gastrointestinal Tissues during *Ascaris suum*-induced Type 2 Inflammation.

Acknowledgements

First and foremost, I want to thank Associate professor Andrew R. Williams for assigning me this great opportunity, and for challenging me with tasks, which I learned a lot from. Thank you for always being supportive, positive and patient, I am truly grateful to have had you as my supervisor throughout the past 3 years. Likewise, to my co-supervisor Professor Stig M. Thamsborg, thank you for your continuous encouragement, for always having your door open, and for adding new perspectives and ideas on the conducted work with your great experience. To both of you, I admire your tremendous knowledge and research within the field of parasitology, immunology and bioactive diets. Thank you for teaching me invaluable practical and theoretical skills within these fields, and for guiding me through this PhD – it certainly would not have been possible without you.

To my office roommate, Charlotte Smith Bonde, although we have both been in our own distinct "PhD bubble", I have really enjoyed our talks and laughs during late office hours, car rides to the farms, and during our stay in Viborg. Thank you for always being helpful and for all the good times we spent together in farmer overalls!

I would like to give a special thanks to Mette Schjelde for your help in the lab, as well as with the mouse and pig studies, you made the long working hours seem shorter! In this regard, I want to extend my deepest gratitude to my colleagues Ling Zhu, Laura Myhill, Pankaj Arora, Angela Valente, Helena Mejer, Lise-Lotte Christiansen and Stine Thorsø Nielsen – thank you all for the many shared hours in the lab and in the stables, for all the interesting scientific meetings and the fun dinner parties. As corona made its unfortunate entry to our department at a critical time point during my main pig study, I want to say a big thank you to everyone at the Section for the moral support I felt from all of you. In this regard, I would like to express my sincere appreciation to Penille Jensen, Karen Schou Møller, and Mita Sengupta for stepping in with such short notice during the necropsy days, I am really thankful that you made it possible to conclude that study as planned. Thank you also to the personnel at the department of Experimental Medicine, especially Tine and Bettina – thank you for your care taking and support with the animal studies.

Next, I want to thank Professor Juha-Pekka Salminen for the helpful meetings and for broadening my knowledge within the field of proanthocyanidins from a chemist's perspective. Thank you to everyone in the Natural Chemistry Research Group for making me feel welcome and part of the team for 3 memorable and snowy months in Finland. A special thanks goes to Milla Leppä for teaching me exciting skills within plant chemistry and purification methods, and for all the good memories from celebrating the Finnish Independence day, meeting at the conference in Austria, as well as horseback riding during the NCRG team-building day.

In relation to some preliminary studies conducted at the department of Veterinary Clinical Microbiology, I would like to thank Professor John Elmerdahl Olsen for your advice and

contribution on the *C. rodentium* studies, and Tony Poul Bønnelycke and Dan Friis Ryttov for your guidance in the lab – it was nice to be back!

Overall, these past years have led me on a memorable journey, where I have met many inspiring people, and it has been truly enriching academically and personally – thank you to everyone who has been part of it.

Finally, but not least, I would like to thank my loving family and my dear friends for always being there and for reminding me of what is important in life outside of the PhD-life. Patrick, my steady rock, thank you for everything you do, and for being who you are.

Table of contents

Summary

Proanthocyanidins (PAC) are specialized plant metabolites, which are associated with the health benefits of a plant-based diet due to anti-oxidant and anti-inflammatory properties. However, the complexity of their diverse chemical structures challenges the study of these promising molecules, and their potential application during infectious diseases remains to be comprehensively determined. This research project investigated the structure-activity interplay and cellular mechanisms leading to the immuno-modulatory effects of purified PAC *in vitro.* Furthermore, this project aimed at exploring the effect of PAC on helminth-induced type 2 immune response *in vivo*, and thus investigating the trilateral interactions between PAC, parasites and the immune system.

Paper I reviews the known immune-modulatory effects of PAC and possible modes of action during enteric diseases, and elaborates in further detail on the subject discussed in this PhD thesis. The aim of **Paper II** was to investigate the consequence of defined PAC structure in inducing bioactivity in cell-based models. Highly purified PAC samples were thus isolated from PAC-rich natural sources, such as grape pomace (GP) (*Vitis vinifera*) and alpine currant (AC) (*Ribes alpinum*) with novel chemical techniques. Purification was performed by a series of extractions with acetone, followed by Sephadex LH-20 fractionation and semi-preparative liquid chromatography (LC). A total of 19 Sephadex fractions and 152 samples derived by semi-preparative LC were consequently analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS), to assess chemical characteristics. PAC samples (especially those derived from GP) significantly reduced the secretion of the pro-inflammatory cytokine interleukin 6 (IL-6) in lipopolysaccharide (LPS)-activated macrophages by up to 60 %. Moreover, a structural characteristic, known as the mean degree of polymerization (mDP), significantly influenced IL-6 secretion and immune-related gene expression levels in LPS-activated macrophages. Likewise, mDP-dependent alterations in gene expression were also demonstrated *in vivo* with mice dosed orally with either low or moderate mDP PAC derived from GP. Finally, mechanistic studies demonstrated a potential role of PAC in regulating the autophagy pathway in LPS-activated macrophages, by upregulating the expression of genes related to lysosome function, and by inhibiting the fusion of autophagolysosomes.

In **Papers III** and **IV**, the ability of PAC in modulating type 2 immune response in animal models of helminth infection was investigated. Intestinal helminthiasis is a worldwide problem affecting around 1 billion people, causing detrimental inflammation in the gastro-intestinal tract among other severe comorbidities. Likewise, helminth infections in animals, especially livestock, is a global concern, and is responsible for important economic losses, including cost of control. Thus, to assess the potential anti-inflammatory properties of dietary PAC, feeding studies in mice infected with *Heligmosomoides polygyrus* or *Trichuris muris,* and pigs infected with *Ascaris suum* were conducted.

Unexpectedly, our main findings in mice demonstrated that PAC tended to induce a Th1 polarized immune response, indicating a higher susceptibility to helminth infection. Thus, dietary PAC

resulted in *H. polygyrus* infected mice with fewer mast cells in the intestinal tissues, while PAC-dosed *T. muris* infected mice had increased expression levels of interferon-related genes, enhanced IgG2a serum levels and harbored increased adult worm burdens. Both models demonstrated that PAC significantly enhanced cell hyperplasia in the mesenteric lymph nodes (MLN), suggesting a strong immune-stimulatory effect of PAC during infection. Thus, PAC did not promote a beneficial immune response in helminth-infected mice.

On the other hand, PAC induced subtle, but generally favorable immune-modulatory effects in *A. suum* infected pigs. These were mainly characterized by localized changes in intestinal tissues, especially transcriptional pathways related to anti-oxidant status. Furthermore, two weeks of PAC supplementation resulted in significantly lower C-reactive protein (CRP) serum levels, however this effect subsided during concurrent *A. suum* infection and larval worm burdens remained unaffected by dietary PAC. Investigation of the potential implications of PAC on the gut-lung axis in *A. suum* infected pigs, revealed limited effect of dietary PAC, based on *ex vivo* stimulation of alveolar macrophages. However, the upregulation of genes, such as connective tissue growth factor (*CTGF)* and arachidonate 15-Lipoxygenase (*ALOX15)* in lung tissues, suggests that PAC may enhance healing and antioxidant status in the lungs during infection.
Finally, PAC and infection status were able to significantly modulate gut microbiota composition by altering β-diversity in both mice and pigs, suggesting that the interactions between gut microbiota and PAC and/or PAC metabolites may influence the observed immunomodulatory effects.

In conclusion, our findings demonstrated how PAC isolated from different natural sources with the same chemical techniques provide a large variety of diverse PAC polymers with distinct bioactivity. Moreover, this PhD project offered novel insights into the structure-function interplay of PAC, and we showed that PAC were able to significantly modulate immune responses in different helminth-infected animal models, thus demonstrating that interactions were depending on host and helminth species. Unraveling a role for PAC in modulating type 2 immune response may thus shed light onto new intervention strategies against helminth infections, as well as other diseases linked to dysregulated type 2 or type 1 immune responses.

Sammendrag

Proanthocyanidiner (PAC) eller kondenserede tanniner er specialiserede stoffer fra planter, såkaldte sekundære metabolitter, med antioxidative og antiinflammatoriske virkninger, som er forbundet med de sundhedsfremmende effekter, der associeres med en plante-baseret kost. Stoffernes komplekse kemiske strukturer vanskeliggør systematisk undersøgelse af deres bioaktivitet, herunder deres mulige potentiale ved infektiøse sygdomme, som således endnu ikke er klarlagt.

Formålet med dette forskningsprojekt var at undersøge sammenhængen mellem den molekylære struktur af oprensede PAC og deres bioaktivitet i cellebaserede assays, samt at undersøge hvilke cellulære mekanismer, der betinger deres antiinflammatoriske effekter. Dernæst blev effekten af PAC undersøgt i eksperimentelle modeller med helminth-inficerede mus og grise for at undersøge mulige immunmodulerende effekter af det associerede type 2 immunrespons. Forsøgene undersøgte således den trilaterale sammenhæng mellem PAC, parasitter og immunsystemet.

Artikel I omhandler de immunmodulerende effekter af PAC i forbindelse med bakterielle og parasitære infektioner og uddyber således selve hovedemnet for dette forskningsprojekt. Formålet med **Artikel II** var at undersøge PAC med forskellige kemiske strukturer og deres indvirkning på cytokinsekretionen i cellebaserede forsøg. Ved anvendelse af nyudviklede kemiske metoder blev PAC fraktioner fra almindelig vin (*Vitis vinifera*) og fjeldribs (*Ribes alpinum*) oprenset ved ekstraktion med acetone efterfulgt af Sephadex LH-20 separation og semi-preparative liquid chromatography (LC). Et samlet antal på 19 Sephadex fraktioner og 152 prøver udvundet via semi-preparative LC blev analyseret ved ultra-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) med henblik på en detaljeret karakterisering af hver enkelt prøve. Derefter blev prøverne testet i et cellebaseret assay med lipopolysaccharid (LPS)-aktiverede makrofager. Flere af prøverne reducerede interleukin-6 (IL-6) sekretion i betydeligt omfang, og prøver fra almindelig vin resulterede i de højeste reduktioner i IL-6 på op til 60 %. Prøvernes bioaktivitet var signifikant korreleret med deres grad af polymerisering (mDP), hvilket også var gældende for deres evne til at regulere gen-ekspressionen i LPS-aktiverede makrofager. I overensstemmelse med dette, observeredes markant flere ændringer i gen-ekspression i tarmvævet fra mus doseret med PAC (peroral) med høj mDP sammenlignet med mus doseret med lav mDP. Endeligt undersøgtes mekanismerne bag den antiinflammatoriske effekt, som kan iagttages ved PAC. I en model med LPS-aktiverede makrofager fandtes en potentiel modulerende effekt på cellulær autofagi, karakteriseret ved opregulering af gener med afgørende betydning for funktionaliteten af lysosomer samt hæmning af autofago-lysosom fusion.

I **Artiklerne III og IV** undersøgtes PACs påvirkning af det typiske type 2 immunrespons i helminth-inficerede dyremodeller. Helminther (eller orm) i tarmen udgør et væsentligt globalt sundhedsproblem, som afficerer omkring 1 milliard mennesker og forvolder tarmbetændelse med en række følgesygdomme, herunder reduceret vækst og kognition. På samme vis er tarmhelminther også et globalt sundhedsproblem hos dyr, især produktionsdyr, med store økonomiske

omkostninger. Formålet med fodringsforsøgene med PAC i mus inficerede med *Heligmosomoides polygyrus* og *Trichuris muris* og grise inficerede med *Ascaris suum* var primært at undersøge potentielle antiinflammatoriske effekter. Imod forventning medvirkede PAC til en øget følsomheden overfor helminth-infektioner i mus, karakteriseret ved et type 1 polariseret immunrespons. Mus inficerede med *H. polygyrus* havde således færre mastceller i deres tarmvæv efter oral dosering med PAC. I mus inficerede med *T. muris* gav PAC anledning til en opregulering af interferon-relateret gener, øget IgG2a niveau i blodet samt signifikant flere voksne orm. I begge modeller forårsagede PAC hyperplasi i krøslymfeknuderne, hvilket tyder på en markant immunstimulerende effekt af PAC ved helminthinfektion i mus.

Til sammenligning havde PAC en svagere immunmodulerende påvirkning i *A. suum* inficerede grise, hvor effekten primært var øget antioxidativ aktivitet i tarmene. Tildeling af PAC i foderet i 2 uger resulterede desuden i et signifikant lavere niveau af C-reaktivt protein (CRP) i serum, men denne effekt udeblev efter infektion med *A. suum.* Derudover, var heller ingen effekt af PAC på ormebyrden. For at undersøge en potentiel virkning af PAC på lungerne blev makrofager isoleret ved lungeskyl, og der forekom en begrænset effekt af PAC som følge af *ex vivo* stimulering af disse alveolære makrofager. PAC opregulerede flere gener såsom connective tissue growth factor (*CTGF)* og arachidonate 15-lipoxygenase (*ALOX15)* i lungevævet, hvilket indikerer at PAC fremmer det antioxidative forsvar og heling i lungerne.
PAC og infektion med helminther var hver for sig i stand til at ændre tarmfloraen i betydeligt omfang. Således fandtes signifikante ændringer i β-diversiteten hos både mus og grise, hvilket indikerer at interaktioner mellem tarmfloraen og PAC (eller PAC metabolitter) potentielt kan influere de observerede immunmodulerende effekter.

Afslutningsvis indikerer disse forsøg, at oprensede og velkarakteriserede PAC fra naturlige kilder har vidt forskellig bioaktivitet. Derudover giver projektet indsigt i potentielle mekanismer, hvormed PAC udøver deres antiinflammatoriske effekter. Resultaterne viser, at PAC kan påvirke type 2 immunsvaret ved helminther i flere dyremodeller. Yderligere undersøgelser af PACs påvirkning af type 2 immunresponset kan potentielt bidrage til nye interventionsstrategier mod helminthinfektioner samt forbedre muligheder for forebyggelse af andre sygdomme, der relaterer sig til dysregulerede type 1 eller type 2 immunsvar.

Abbreviations

AC	Alpine currant
APC	Antigen presenting cells
BAL	Broncho-alveolar lavage
BW	Bodyweight
CO	Cocoa
CRP	C-reactive protein
E/S	Excretory/Secretory
GP	Grape pomace
GS	Grape seed
IEL	Intraepithelial lymphocyte
IFN	Interferon gamma
IgA, IgE, etc.	Immunoglobulin A, E etc.
IL	Interleukin
ILC	Innate lymphoid cells
L1-L4	First-fourth stage larvae
LC	Liquid chromatography
LN	Lymph nodes
LPS	Lipopolysaccharide
Mcpt1	Mast cell protease-1
mDP	Mean degree of polymerization
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
M/S	Mass spectrometry
O/N	Overnight
PAC	Proanthocyanidins
PBMC	Peripheral blood mononuclear cell
PC	Procyanidin
PD	Prodelphinidin
p.i.	Post infection
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
SPF	Specific pathogen free
Th0	Naïve T helper precursor
Th1	Type 1 T helper
Th2	Type 2 T helper
Th17	Type 17 T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
UPLC	Ultra-Performance Liquid Chromatography
V-ATPase	Vacuolar-type Adenosine triphosphatase

1. Introduction

Proanthocyanidins, also known as condensed tannins, are one of the many types of dietary anti-oxidants that belong to the group of polyphenols, which may have protective and health-promoting effects in both humans and animals[1–3]. These bioactive properties have made them especially interesting for cancer research, and other research fields have also demonstrated their beneficial potentials towards metabolic, cardiovascular, and neurological diseases[4–6]. The ability of PAC to neutralize free radicals is well established and, besides their anti-oxidant activities, numerous cell-based and animal studies indicate powerful anti-inflammatory properties. However, the modes of action responsible for the various effects of PAC during inflammation are still not fully understood.

The term "Gut health" has received much attention in the recent years, due to emerging evidence connecting intestinal inflammation to other diseases at extra-intestinal sites of the body, e.g. the bidirectional links involving the gut-brain and gut-lung axis have been well-documented[7,8]. Thus, the effect PAC on gut health has also gained increasing interest, including in the setting of intestinal disease[9]. However, many studies have focused on the effect of PAC in models of metabolic and autoimmune diseases, whereas only a small amount of research has focused on the implication of PAC on immunity to enteric infection. Moreover, the possible effects of dietary PAC on type 2 immune response induced by helminth infections remains to be elucidated. Of note, more than a billion people around the globe are currently infected with intestinal helminths, causing detrimental disease and co-morbidities[10,11]. Reducing the prevalence of these parasitic infections remains a challenging goal, due to the lack of effective medication and vaccines, as well as emerging drug resistance, and high risks of reinfection from the environment where transmission persists[12]. However, endemic regions of the world with high parasitic burdens, generally have lower prevalence of autoimmune diseases, which in turn are highly prevalent in developed countries with low parasite burdens[11,13]. This suggests that helminths may offer protection against dysregulated immune function[14,15], and this effect is also commonly known as part of the hygiene theory, as originally suggested by Strachan in 1989[16].

Thus, understanding how helminth-induced host immune responses may be modulated, could offer new insights in novel intervention strategies, not only for parasite infections but also for other immune-related dysfunctions. Whereas immune responses induced by helminths have been heavily investigated, the effect of dietary PAC on mucosal immune function during helminth infections remains unclear.

Herein, we thus explore which chemical PAC structures and cellular mechanisms may be responsible the immunomodulatory effects of PAC, and how dietary PAC may affect mucosal immune function and inflammation in animal models of helminth infection.

1.1. Research Objectives

The overall aim was to investigate the relationship between chemical structure and bioactivity of purified PAC, leading to their modulatory effects on immune cells, and then to elucidate the effects of PAC on helminth-induced inflammation in the gut of monogastric animal models.

Formulation of main hypotheses:

- Distinctive chemical structures of PAC affect cellular immunomodulatory bioactivity
- PAC modulate type 2 immune response during helminth-induced intestinal inflammation

Thus, the primary objectives of this project were as follows:

- Extract, purify and determine the structural characteristics of PAC from selected natural sources
- Conduct *in vitro* screening of purified PAC samples and mechanistic studies to evaluate bioactivity and immuno-modulatory effects
- Assess the effect of PAC on gut inflammation and immune response in helminth-infected mice[i] and pigs
- Investigate the implications of PAC bioactivity on the gut-lung axis in helminth-infected pigs

1.2. Conflicts of Interest

The author of this PhD project has no conflicts of interest to declare. The synopsis provided herein was written with reference to specified research articles and provided manuscripts.
All experimental work was performed at the University of Copenhagen and Turku University.

[i] Mouse infection studies conducted with *Trichuris muris* were performed in close collaboration with Dr. Pankaj Arora who also shares primary authorship of Paper III

BACKGROUND

2. Bioactive compounds and Health

2.1. Polyphenols

Plants have been used for centuries to prevent and cure diseases, and the chemical structures of plant-derived molecules are the foundation of many of the commercial drugs used nowadays[17–19]. It is commonly known that the intake of fruits and vegetables is beneficial to health and decreases the risk of non-communicable diseases, due to their high contents in vitamins, fibers, minerals, plant sterols, flavonoids and other antioxidants[20]. Of note, the World Health Organization estimated that 3.9 million deaths in 2017 could be linked to inadequate consumption of fruits and vegetables[20], which emphasizes the importance of a healthy diet.

Polyphenols represent a large group of diverse anti-oxidants, which have been extensively studied for their potential as therapeutic agents in relation to various diseases, including gastrointestinal diseases. They are specialized plant metabolites, commonly found in plant-based diets, and numerous studies have demonstrated the ability of polyphenols to modulate the course of inflammatory responses in various experimental models[21]. Notably, epidemiological studies in humans have associated the Mediterranean diet, which is generally rich in polyphenols, with longevity[22]. However, lack of standardized purification and analytical methods, and variations of polyphenol content in dietary composites challenge the evidence of the effects on health attributed to polyphenols[23]. Thus, comprehensive human clinical trials have so far not been able to support the notion of unambiguous beneficial properties of polyphenols on human health[24–26]. Nonetheless, research related to polyphenols has intensified over the years, to unravel which specific molecules may be responsible for the effects observed in experimental settings.

As more than eight thousands of compounds with widely varying bioactivities are referred to as polyphenols, they are divided into different groups and subgroups based on chemical structure[27]. Thus, phenolic acids, flavonoids and non-flavanoids represent the three main groups of polyphenols[26]. Flavonoids are the most extensively studied group with thousands of identified compounds[28,29]. Based on the chemical structure of their heterocycle, flavonoids are thus further categorized into flavonols, flavones, flavanones, isoflavones, anthocyanins and flavanols[28], the latter comprising proanthocyanidins, serving as key focus of this thesis.

2.2. Proanthocyanidins

Proanthocyanidins (PAC), also known as the non-hydrolysable condensed tannins, are water-soluble secondary plant metabolites[30]. They are among the most common dietary polyphenols found mainly in plants and fruits, and are known to cause the astringent taste of e.g. grapes and the bitter taste of chocolate by precipitating salivary protein[21,31,32]. The average intake of PAC in American adults has been estimated to 95 mg/day, whereas the intake in European countries may range between 96-175 mg/day[33–35]. However, it remains challenging to estimate the precise amount of PAC in the diet, due to the complexity of their chemical structures[36]. Moreover, although PAC are highly prevalent in our diet, the amount of conducted research within this field is considerably limited when compared to the available research on polyphenols as depicted in **Figure 1**.

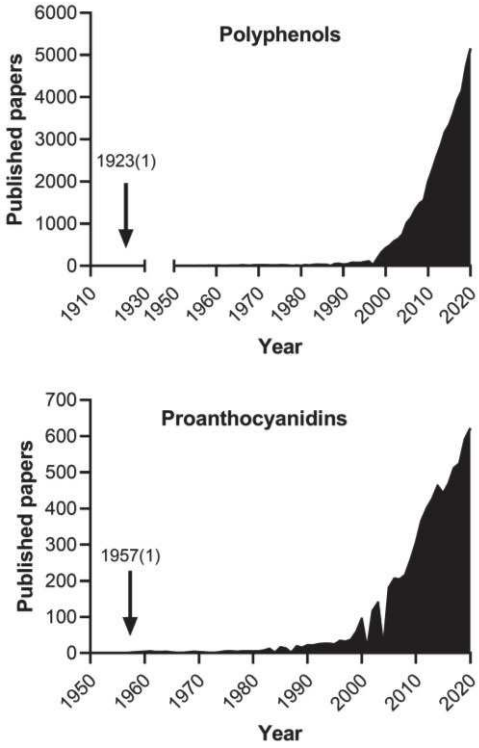

Figure 1. Published papers related to polyphenols and proanthocyanidins. (*Source*: Pubmed search)

Similarly to other bioactive compounds, PAC have been extensively studied for their beneficial effects towards several diseases such as cancer, arthritis, cardiovascular and neurodegenerative diseases[6,37–44]. In the course of inflammation, cellular processes and tissue injury is associated with the production of free radicals such reactive oxidative species (ROS). Thus, excessive amounts of ROS result in oxidized protein and lipid cellular constituents and damage the DNA, leading to oxidative stress[45,46]. Thus, the potential of PAC to scavenge free radicals, such as ROS, has been extensively studied[46,47]. Moreover, their anti-oxidative effects have been shown to be comparable, if not superior, to vitamin C (ascorbic acid) and vitamin E (α-tocopherol)[47–49]. The protective effects of PAC against metabolic diseases have also been described in numerous studies with promising outcomes, whereas the implications of PAC during infectious enteric diseases are less known. However, a few studies have described a potential role for PAC against bacterial, viral and parasitic diseases as discussed in section 5 and in **Paper I**.

Although the modes of action of PAC are not yet fully understood, their beneficial and various functions have been largely attributed to their chemical characteristics[50–52]. Thus, the complex structures of PAC offer them limited absorption from the intestinal tract but they may however exert local bioactive effects, which may lead to a systemic impact by interacting with i.e. the gut microbiota, or residing immune and epithelial cells of the mucosal tissues[53,54].

2.2.1. Chemical Characterization of Proanthocyanidins

The chemical structure of PAC varies greatly based on the natural source from which they are extracted, the extraction method, and the conditions in which the plants, fruits or vegetables have

been cultivated, stored and processed[21,55–59]. Their molecular weight typically varies between 500 and 3000 Da, depending on the number of flavanol monomers that form the building blocks of oligomers and polymers[30]. Flavanols are made of two phenyl rings A and B, and one heterocyclic ring C, with different hydroxylation patterns of rings A and B[60,61], as shown in **Figure 2**. PAC can also differ structurally depending on the stereochemistry of the asymmetric carbons of the heterocycle[62]. The most common dietary PAC are procyanidins (PC) characterized by a 3′,4′-dihydroxy substitution on the B-ring, and PAC molecules composed of both PC and prodelphinidins (PD), which are characterized by a 3′,4′,5′-trihydroxy substitution on the B-ring[62]. On the other hand, propelargonidins with 4′-hydroxy B-rings are only scarcely found in food sources[62].

PAC are also characterized based on their galloylation and polymerization. The percentage of galloylation describes the proportion of galloylated monomers from the over-all amount of monomers that constitutes the molecule, while the number of linked monomers influences the mean degree of polymerization (mDP)[63]. The mDP of PAC vary widely depending on the source and within a single sample, and it generally lies between 3 and 11 but can also exceed 50[62,64]. Furthermore, PAC mDP was shown to be a decisive parameter for the observed outcome in a few *in vitro* studies, with higher mDP often correlating with higher bioactivity[52,65,66].

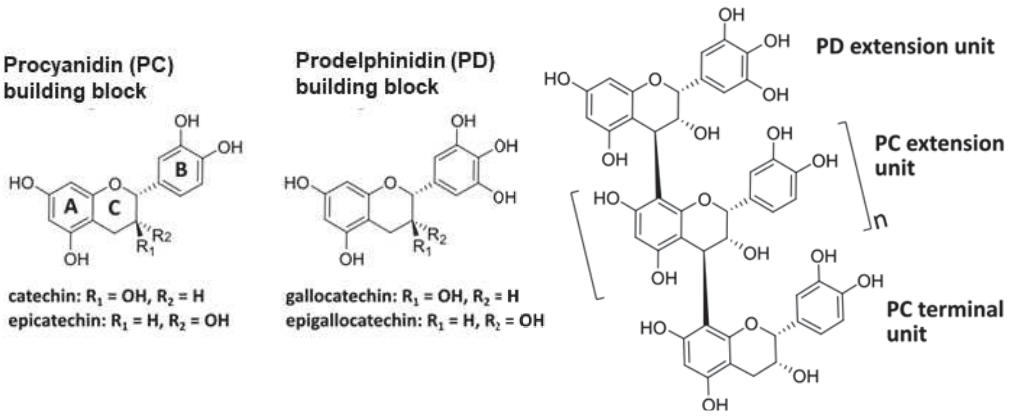

Figure 2. Molecular structure of Proanthocyanidins
(*Source*: Paper II)

3. The Mucosal Immune System and Inflammatory Responses to Pathogens

3.1. Innate Immune Function

Inflammation is a natural response induced by the immune system towards harmful stimuli such as pathogens, and is characterized by the renowned cardinal signs; *calor, rubor, tumor, dolor* and *functio laesa*. It is generally classified into acute and chronic inflammation, depending on whether the source of inflammation is cleared while initiating healing within days (acute), or whether it persists over months/years (chronic) and resolution of inflammation is impaired[67]. Although inflammation is a necessary physiological reaction for survival, chronic inflammation has become one of the major health concerns in the Western world, as the prevalence of e.g. obesity and inflammatory bowel diseases (IBD) is increasing due to lifestyle and environmental factors[68–71]. Thus, the immune system is constantly challenged by intrinsic and extrinsic stimuli, which may be detrimental depending on the level of pathogenicity and host immune function. An adequately functioning immune system is therefore crucial to sustain a healthy life status.

The immune system is a complex network consisting of organs, tissues, cells and molecules, which help the organism in combatting pathologies. The innate immune response is responsible for the early induction of the immune system towards harmful pathogens that manage to circumvent the physical barriers of the body, such as the skin or the protective mucosal barriers of the gut, airways or genital tract[72,73]. It is generally initiated by pathogen-associated molecular patterns (PAMPS) and damage-associated molecular pathogens (DAMP) that are recognized by pattern recognition receptors (PRRs), which are mainly expressed on leukocytes, enterocytes, and various other mucosal cell types[74–77]. PRRs include membrane bound receptors, such as Toll-like receptors (TLR), and receptors located in the cytoplasm, such as NOD-like receptors[9]. Thus, receptor mediated phagocytosis by macrophages and dendritic cells, and induction of apoptosis by natural killer (NK) cells, are some of the main events during innate immune response, which results in the upregulation of inflammatory signaling pathways, such as the NF-κB signaling pathway, and the release of cytokines[78–81]. Of note, macrophages opt different phenotypes depending on the surrounding signals from the microenvironment. Exposure to lipopolysaccharide (LPS) or type 1 cytokines, such as pro-inflammatory interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), will lead to classically activated (M1) macrophages secreting interleukin 6 (IL-6), TNFα, IL-1β, IL-12 and IL-23. On the other hand, naïve monocytes exposed to IL-4, IL-13 or helminth-derived molecules, produce alternatively activated (M2) macrophages promoting Th2 cells, eosinophils and basophils[79]. Intraepithelial lymphocytes (IEL) such as γδ T cells are also crucial for the induction of the immune response, and for maintaining gut barrier function[82].

Finally, another essential group of cells during the innate immune response are the innate lymphoid cells (ILCs). ILCs are mainly found at mucosal surfaces and are responsible for secreting effector cytokines and thus regulating both the innate and adaptive immune system. Depending on the

incoming pathogen, ILCs secrete signature cytokines, which classifies them into ILC1, ILC2, or ILC3s making them part of the Th1, Th2 or Th17 adaptive immune response, respectively[83].

3.2. Adaptive Immune Function

The later stages of inflammation lead to a more specialized immune response, namely the adaptive immune response. The adaptive immune response is generally characterized by B cells, which develop into antibody secreting plasma cells leading to humoral immunity, and T cells, which are responsible for cell-mediated immunity. The role of antibodies is mainly directed towards extracellular pathogens by acting as opsonins, inactivating toxins, enhancing phagocytosis, and activating the complement system and leukocytes[84]. Antibodies, also called immunoglobulins (Ig), are divided into 5 categories; IgA, IgE, IgG, IgM and IgD, which are each associated with specific functions. Of note, IgA levels are considerably high at mucosal surfaces, where they exert protection towards bacterial, parasitic and viral infections[84,85]. Furthermore, IgE is associated with hypersensitivity, allergies, and parasitic infections, and has high affinity to the Fc receptor for IgE (FcεRI), which is expressed on mast cells, basophils, and eosinophils[84].

The key function of T cells is to neutralize pathogens, and is characterized by the interaction of T cell receptors with major histocompatibility complexes (MHC). MHC display antigen fragments, which have been engulfed and processed within the cell[79]. There are two main classes of MHC, i.e. MHC class I, which are found on all nucleated cells, and MHC class 2, which are found on antigen presenting cells (APC), such as macrophages, B cells and dendritic cells. Cells displaying MHC-I-antigen complexes are destroyed by cytotoxic T cells, also known as $CD8^+$ T cells[72]. On the other hand, cells displaying MHC-II-antigen complexes are recognized by T helper cells, also known as $CD4^+$ T cells, which enhances the immune response[86–88]. Dendritic cells are disseminated in the peripheral tissues, and once activated they migrate to the draining lymph nodes and induce the polarization of naïve T cells[89]. Moreover, naïve T helper cells (Th0) primarily differentiate into Th1, Th2, Th17 and regulatory T cells (Tregs) sub-types depending on the inductive stimuli[90,91]. Thus, intracellular pathogens such as bacteria and protozoa induce a Th1 polarization. Th1 cells secrete IFNγ, TNFα, IL-2 and IL-3 and regulate type 1 immune response, which is also characterized by the activation of ILC1, cytotoxic $CD8^+$ T cells, macrophages and the production of IgG2a from B cells[88,92,93]. On the other hand, Th2 cells regulate type 2 immunity by secreting IL4, IL5 and IL13, and high titers of antibodies from plasma cells, especially IgE[88,93]. Interestingly, it is generally accepted that Th1 and Th2 mediated immune responses are inversely proportional, implicating an intricate Th1/Th2 balance as shown in **Figure 3**. For example, infection with Th1-inducing pathogens impairs a proper Th2-immune response and may lead to persisting parasite co-infection[94]. Inversely, treatment with helminths have protective effects against several autoimmune diseases, such as IBD, which are caused by a dysregulated Th1 response[95,96].

Finally, the cytokines TGF-β and IL-6 differentiate naïve T cells into Th17 cells, whereas TGF-β alone expands the population of Tregs. Th17 cells are largely involved in the protection towards extracellular pathogens and in auto-immune disease, and are part of the type 3 immune response,

characterized by ILC3 and the secretion of IL-17 and IL-22 cytokines[91,92,97]. Conversely, Tregs function as immunosuppressive cells secreting IL-10[98].

Taken together, the innate and the adaptive immune response are thus central in inducing an inflammatory response in the course of eliminating harmful pathogens. However, a persevering, exacerbated or insufficient immune response, caused by a dysregulation of the immune system, can be detrimental[88]. Thus, the modulation of immune function is a key aspect in the development of new therapeutics to treat numerous disease, and the interest for immune-modulatory molecules, such as PAC, has therefore intensified greatly over the years.

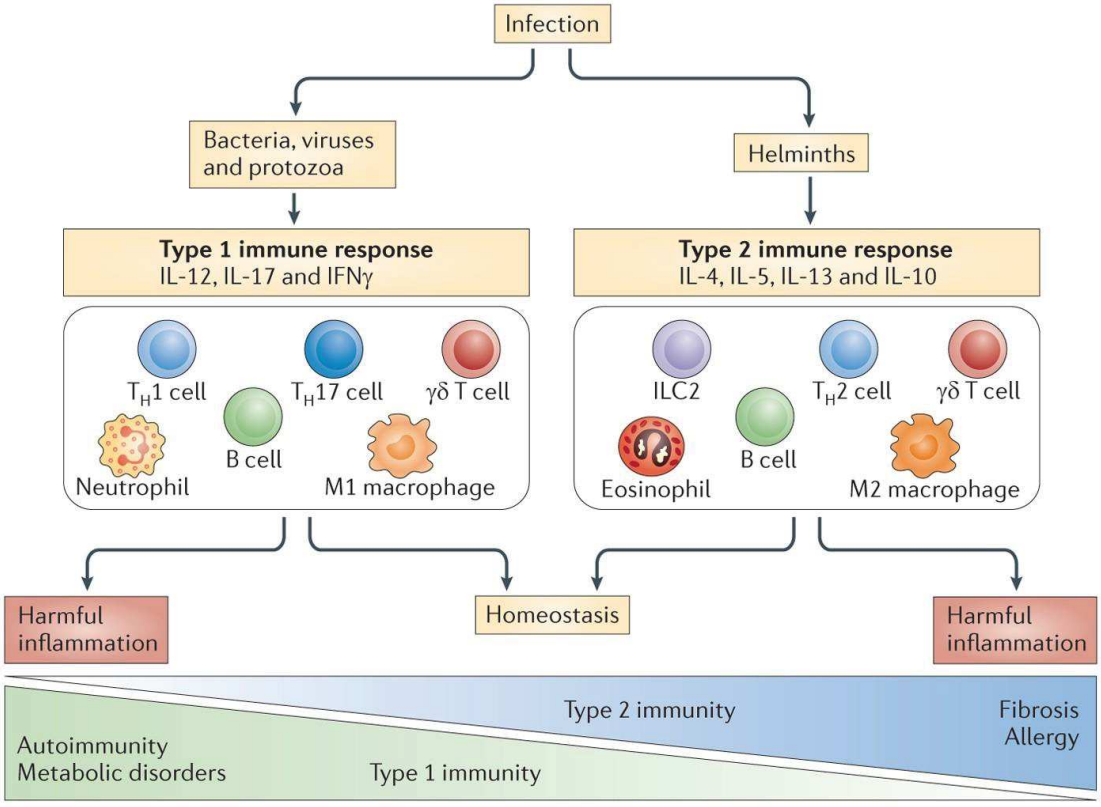

Figure 3 The Th1/Th2 balance (Gause *et al.*, 2013).
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3.3. Intestinal Mucosal Immune Function

The number of research studies focusing on gut health has increased tremendously over the past years, especially as it is becoming clear that gut dysbiosis has tremendous effects on overall health, and may predispose for pathologies outside the gastro-intestinal tract. Thus, an intricate relationship has been described between gut-homeostasis and i.e. the brain, as well as the lungs [41,99,100]. The intestinal epithelium lining is composed of a single columnar cell-layer, which act as a barrier against potential harmful components of the digesta, and forms the mucosal tissues together with the lamina propria and the muscularis mucosae[9,101]. Intestinal cells are constantly and rapidly renewing by proliferation and differentiation from stem cells into enterocytes, goblet

cells, Paneth cells and enteroendocrine cells[102]. The enterocytes are the most predominant cell type in the intestines, and they are absorptive cells that have a brush border with microvilli on their apical surface[103]. In between the enterocytes reside goblet cells, which are in charge of producing mucin to form a protective mucus layer, and they also play an important role as immune function regulators by interacting with APCs[104]. The specialized secretory Paneth cells are only found in the small intestines in mammals at the bottom of the crypts of Lieberkühn, where they secrete antimicrobial proteins, such as lysozymes, cryptdins or defensins[102,105]. The integrity of the epithelial cell layer is essential for maintaining gut health, and is supported by tight-junctions between cells. Tight junctions are comprised of junction adhesion molecules, claudin, occludin, and zonula occludens proteins, which allow adequate permeability to prevent a "leaky gut"[101,106,107]. Intestinal lymphocytes are localized in Peyer's patches (only small intestine) and lymphoid follicles, known as the gut associated lymphoid tissues (GALT), as well as in the intestinal-draining mesenteric lymph nodes (MLN). Antigen presentation to T- and B-cells occurs at these sites e.g. through microfold cells (M cells), whereas effector T cells and plasma cells are located both in the epithelium and in the lamina propria[108–111]. Moreover, GALT plays a fundamental role in regulating and limiting inflammatory responses to the symbiotic gut microbiota and food particles, by promoting a tolerogenic milieu[112]. As mentioned in the section above, ILCs are highly prevalent at mucosal surfaces, and are key elements in mucosal immune function by supporting barrier integrity and exerting protection against pathogenic stimuli[83]. Another type of cells residing at mucosal surfaces, are the epithelial chemosensory tuft cells, which are implicated in the initiation of type 2 immune response by secreting IL-25 upon parasitic infections. However, tuft cells have been found to have several functions in health in diseases, which remain largely unknown[113].

Collectively, gut health is dependent on the appropriate function of many key factors, which implies numerous defense mechanisms toward pathogens, while sustaining adequate feed conversion, mucosal integrity, and absorption of nutrients and water. Thus, a fundamental function of the gut is to eliminate pathogenic organisms while minimizing immune reactivity, i.e. tolerance, to commensal bacteria and food antigens[114,115].

3.4. Inflammatory Response to Intestinal Helminths

In relation to intestinal mucosal immune function, intestinal helminth infections cause detrimental changes at this site in both humans and animals. Remarkably, parasites are capable of evading or even suppressing host immune response for their own survival, which also suggest a protective effect of the immune hypo-responsiveness for the host with less pathology[116,117]. However, due to the considerable size and invasiveness of intestinal helminths, they cause important tissue damage [118]. Thus, upon infection, parasite-derived molecules and direct injury of the intestinal epithelial barrier prompt the initiation of an inflammatory response, with the initial release of IL-33, IL-25, thymic stromal lymphopoietin (TSLP), along with other alarmins. These induce the activation and infiltration of numerous immune cells including dendritic cells, basophils, eosinophils and ILC2 cells, leading to the secretion of signature type 2 cytokines IL-4, IL-5, and IL-13[119–122], which are often involved in both the early and later stages of infection[117,118] as depicted in **Figure 4**. Residing

tuft cells are also believed to play an important role as sentinels and in producing IL-25, as well as by inducing ILC2 in secreting IL-13, which in turn promotes tuft and goblet cell hyperplasia[73,123]. However a clear role of tuft cell hyperplasia in relation to helminth infection remains to be fully investigated[124,125]. Notably, along with epithelial cells, basophils and eosinophils are also known to secrete IL-25, whereas IL-33 is mainly secreted by epithelial cells[126,127]. IL-4 has similar various functions as IL-13, and is involved in the class switching of B cells into IgG and IgE secreting plasma cells, and increased smooth muscle contractibility in the intestines, contributing to worm expulsion[128,129]. Moreover, the production of IL-13 is regarded as a critical cytokine in relation to protection against helminths. Notably, IL-13 is involved in the migration of dendritic cells[130].

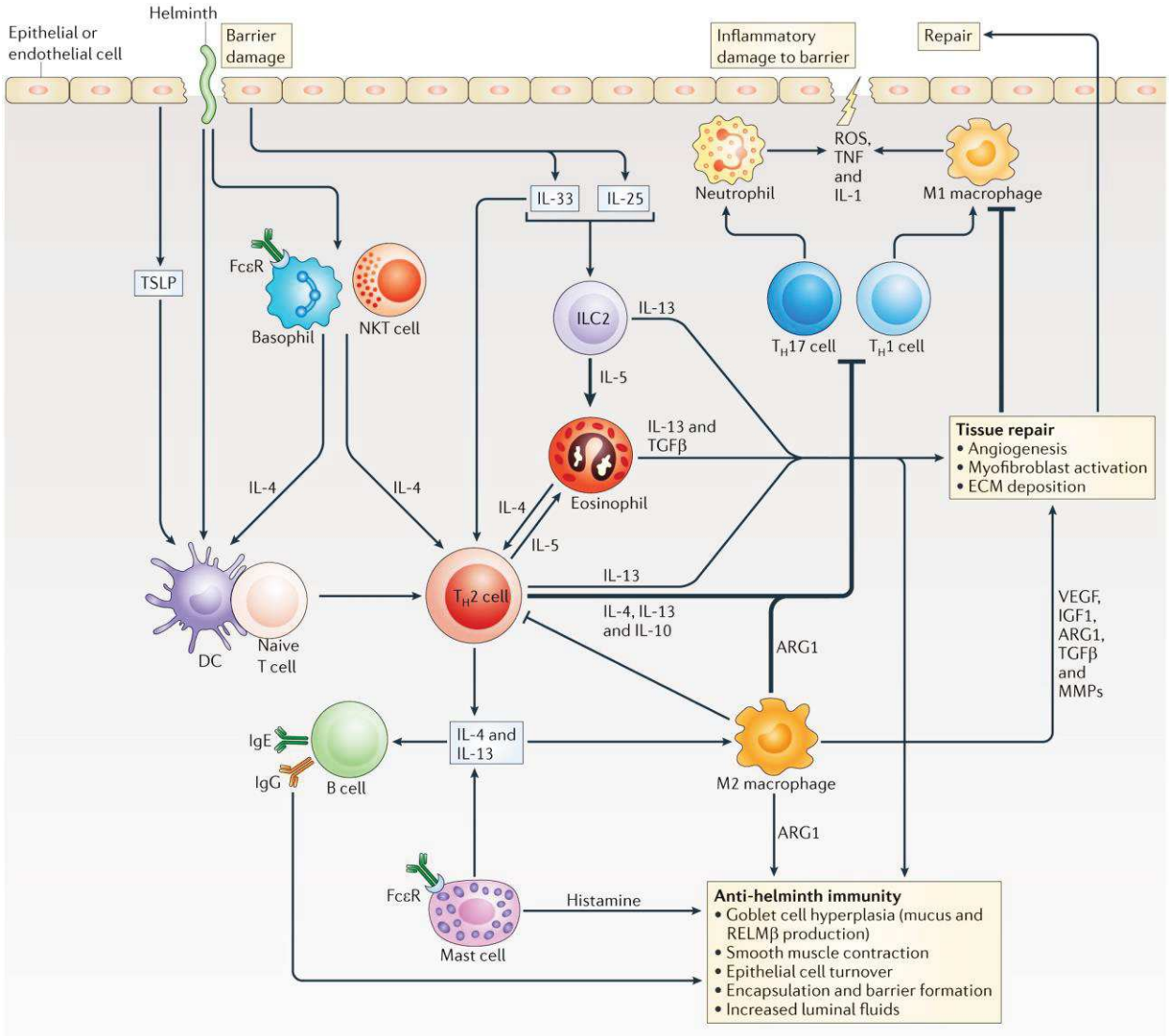

Figure 4 Classical type 2 immune response against intestinal helminths (Gause *et al.*, 2013).
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As indicated earlier, dendritic cells play a crucial role in the induction of the adaptive immune response. Thus, activated intestinal dendritic cells, migrate from the lamina propria to the mesenteric lymph nodes in order to differentiate naïve Th0 cells into Th2 cells[131]. Collectively, the abundant secretion of type 2 cytokines combined with the activation of dendritic cells primes the proliferation of Th2 cells to act as key regulators of innate effector mechanisms when homing to the lamina propria[132]. The production of IL-5 is a key cytokine in the activation and recruitment of eosinophils, which are fundamental for tissue repair[133,134]. Mast cell activity is largely induced by IL-9 secretion, and has been shown to significantly increase paracellular permeability of the gut barrier[135]. Furthermore, goblet cell hyperplasia and thereby increased intestinal mucus ensures a fortified gut barrier contributing to a "weep and sweep" expulsion mechanism[136]. Thus, increased mucus, epithelial hyper-proliferation, and increased peristalsis and luminal fluids, are part of the physiological response that facilitate the expulsion of helminths from the intestines[128,136,137]. However, in the instance of persisting helminth infection, host-immune responses are characterized by a marked upregulation of Tregs, and the secretion of IL-10 and TGF-β, which dampen the protective effects of Th2 cells[138–140]. Thus, in chronic infections the host is unable to expel the parasites, and the immune system "permits" long-term survival of the helminths within the host.

In summary, a strong Th2 immune response is elicited by the host in order to eliminate helminth infection and minimize tissue damage. Moreover, immunity towards helminths is generally associated with a concomitant downregulation of Th1 and Th17 immune responses[141]. Numerous studies, primarily mouse models of helminth infection, have significantly contributed to enhance our understanding of how helminths impact on the immune system. However, it has also been shown that distinct helminths and distinct mouse models may affect the Th2 immune response differently. For example, as TSLP is mainly expressed in the large intestines, studies suggests that TSLP is not involved in the expulsion of parasite harbored in the small intestines. Furthermore, aspects such as infection dose, immune status, and host genetics have important implication on the induced host immune response. Thus, although an overall understanding of the fundamental mechanisms are widely accepted, the complex immune function in animal models of helminth infection should be interpreted carefully.

4. Modulation of Immune Responses by Proanthocyanidins

4.1. Immune and Epithelial Cell-based studies

Numerous studies have described the anti-oxidant properties of PAC, whereas the cellular and molecular mechanisms underlying the potential anti-inflammatory effects of PAC based on chemical structure are still not fully understood. However, *in vitro* studies investigating the effect of PAC in immune and epithelial cell lines, have offered some insights into the modes of action of PAC. So far, suggestions on the immune-modulatory effect of PAC include direct interactions with immune and epithelial cells, either by cell surface binding and/or cellular internalization[142–144].

Studies conducted in LPS-activated macrophages have shown that PAC were able to suppress several inflammatory markers, including ROS, TNF-α, IL-6, as well as the expression of inducible nitric oxide synthase (iNOS) and cyclooxynase-2 (COX-2) proteins[145,146]. The consequent suppression of nitric oxide (N O) and prostaglandin E_2 (PGE2) formation may thus be due to the downregulation of specific immune related pathways, such as the p38 mitogen-activated protein kinase (MAPK), p65, and Akt signaling pathways[146,147]. Accordingly, the inhibition of the important immune regulator NF-κB by PAC has been demonstrated in numerous studies[66,146,147]. Similar findings were also described in LPS-activated bovine mammary epithelial cells, with a downregulation of iNOS, IL-6, TNFα, and IL-1β expression in PAC-treated cells[148]. Moreover, a previous study showed that peripheral blood mononuclear cells (PBMC) isolated from PAC-fed pigs secreted lower levels of IL-6, TNFα and IL-1β when stimulated with LPS, compared to PBMCs isolated from control pigs[149]. Another consistent observation is that PAC could suppress NOD-like receptor protein 3 (NLRP3) inflammasomes, caspase-1 and IL-1β secretion in LPS-activated THP-1 macrophages and HUVEC cells[46,147,150].

PAC were shown to down-regulate transcription and secretion of pro-inflammatory cytokines, while up-regulating the secretion of anti-inflammatory cytokines in lymphoid cell lines and PBMC[61,66,151]. Moreover, PAC supplementation in humans, has also shown to induce γδ T cell proliferation in *ex vivo* stimulated PBMC, suggesting a protective effect of PAC on first line cell defenses[152]. The implication of PAC structure was demonstrated in a study showing that the activation of γδ T cells was highly dependent on mDP[65]. Of note, it has also been suggested that PAC may directly bind to LPS, or inhibit the adherence of bacteria to cells, which denotes possible extra-cellular modes of action whereby PAC exert protective effects[153,154].

Interestingly, a potential role of PAC on the autophagy pathway has been suggested as PAC endocytosis and localization to lysosomes was demonstrated in dendritic cells[143]. Autophagy is a complex process involving protein and organelle degradation and with diverse functionalities on immune regulation[155]. Mainly, autophagosomes and lysosomes play a key role in maintaining homeostasis following stress-induced cellular damage[22]. Of note, several phytochemicals, including PAC have been shown to induce autophagy, however the number of studies remains limited[156–159].

In summary, the anti-inflammatory effect of PAC has been investigated in different cell lines, however only limited research has looked at how the varying complex molecular structures of PAC may distinctively influence immune regulation.

4.2. Gut Barrier and Immune Function

Animal models have offered valuable insights in many fields of research including elucidating the diverse effects of nutritional supplements on intestinal immune function[2,160]. Mouse, rat, and pig models of various diseases have thus significantly contributed in unraveling the effect of PAC on gut health.

It is generally accepted that PAC molecules remain stable in the stomach, and protective effects towards gastric inflammation induced by non-steroidal anti-inflammatory drugs (NSAIDs) and ethanol have been demonstrated[149,161,162]. These effects may be attributed to protective protein-binding oligomers coating the gastric mucosa or by decreasing hydrochloric acid (HCl) secretion[64]. As PAC descend toward the large intestine, they are metabolized into metabolites, such as valerolactones and various aromatic acids by the commensal microbiota[9,163]. Notably, valerolactones, which are readily absorbed, have been associated with beneficial local and systemic health effects[163–166]. Absorption of non-metabolized PAC through the gut barrier appears to be limited and related to low mDP molecules, such as naturally occurring monomers, dimers and trimers in the diet[36,161,167]. However, larger PAC molecules may exert local protective effects on the intestinal mucosa, which may also be true for some PAC metabolites[21], and these effects may differ between the sections of the intestinal tract[168].

A direct effect of dietary PAC on the gut barrier was shown by significantly increased goblet cell density and villus/crypt ratio in the terminal ileum in both C57BL/6 and IL-10 deficient mice[169]. Goblet cell hyperplasia, increased mucin secretion, and upregulated IL-4 and IL-13 expression in ileal tissues was also observed in PAC-fed mice with impaired mucosal barrier function[170]. In rats, the effect of PAC on tight junctions was demonstrated by the upregulation of occludin and zonula occludens expression[171]. Similar findings were reported in pig studies, where PAC supplementation was found to increase the expression of occludins, thereby decreasing gut permeability and the incidence of diarrhea in weaned pigs[172–174]. Moreover, PAC were shown to ameliorate dextran sulfate sodium (DSS) induced colitis in a mouse model by down-regulating the expression of pro-inflammatory NF-κB and NLRP3 inflammasome signaling in intestinal tissues [46]. A similar protective effect was demonstrated in a recent study conducted with *ex vivo* stimulated human colon tissues with PAC prior to a DSS challenge[175]. Finally, mouse models of metabolic syndrome i.e. induced by high fat diets, have demonstrated substantial anti-inflammatory properties of PAC in the gut by decreasing inflammatory markers such as TNFα, LPS, IL-6, ROS and iNOS[6,47,176].

Taken together, several studies have described the beneficial effects of PAC in various models of intestinal disease, which point towards a general anti-inflammatory effect of PAC, as well as a the ability of PAC to enhance overall gut barrier and immune function.

4.3. Gut Microbiota

Up to 100 trillion microbes reside in the human intestines and contribute to proper digestion by retrieving important nutrients from the diet[177]. *Firmicutes* and *Bacteroidetes*, represent the main phyla to which the intestinal microbiota of adult humans belong[178]. Although, a high gut microbiota diversity is generally deemed favorable, specific bacteria at phyla, genus and species level have been linked as markers of a healthy gut microbiome, such as *Bifidobacterium* spp. and *Lactobacillus* spp.[179–181]. Thus, several studies have aimed at investigating the ability of PAC to modulate the gut microbiota by enhancing the abundance of specific bacteria.

Interestingly, the effect of PAC on gut microbiota has been well described in mouse models of metabolic syndrome, which have associated PAC supplementation with increased abundance *Akkermansia muciniphila* in high-fat fed mice[176,182,183]. Notably, *A. muciniphila* has been extensively studied for its anti-inflammatory properties in several disease models[184–186]. Although there are important inconsistencies, pig and rodent studies have shown that PAC supplementation could affect the abundance of several bacteria, such as *Bifidobacterium, Bacteroides, Lachnospiraceae, Clostridales, Lactobacillus* and *Ruminococcacceae*[187–192]. Furthermore, PAC significantly enhanced gut microbiota diversity in minipigs, however results are conflicting, as other studies have indicated a decrease in diversity[187,191,193–195]. Thus, further studies are still needed in order to associate PAC supplementation with signature bacteria with relevance to gut health.

The composition of the gut microbiota greatly influences the production of several metabolites such as short chain fatty acids (SCFA), which are known to have beneficial effects in health and disease e.g. by improving gut barrier function and protecting against colitis[196–198]. Moreover, SCFA, such as butyrate are important signaling molecules and a fundamental source of energy in colonocytes[199]. Thus, the influence of PAC on the abundance of SCFA has also been assessed in previous studies and PAC supplementation have been shown to enhance propionate in pigs[188,200]. Thus, current evidence is suggesting that PAC could be useful in ameliorating intestinal dysbiosis by modulating the gut microbiota. However, most studies are mainly linked to metabolic diseases and the reported effects of PAC may therefore be context-dependent.

4.4. Proanthocyanidins and Intestinal Infection in Animal Models

In accordance with the numerous studies demonstrating anti-inflammatory effects of PAC in cell-based studies, emerging research has investigated the potential of PAC against infectious intestinal diseases in animal models. However, the known effects of PAC on viral and fungal infections remain negligible, and thus this thesis will only touch upon effects on bacterial and helminth infections of the intestinal tract, which is also reviewed in further detail in **Paper I**.

4.4.1. Intestinal Bacterial Infections

Closely related to the effect of PAC on gut microbiota as discussed above, the effects of PAC on enteric pathogenic bacteria have been explored in some studies. For example, PAC was able to ameliorate disease models of *Helicobacter pylori* (rodents)*, Campylobacter* (pigs)*, Clostridium perfringens* (poultry) and *Escherichia coli* (pigs)[201–206]. Thus, PAC may enhance the growth of beneficial bacteria in the gut, occasionally defined as PAC-resistant bacteria[207], while exerting anti-bacterial effects on pathogenic bacteria. The mechanisms by which PAC may act as antibacterial molecules may include direct bactericidal effects, agglutination or neutralization of toxins and fimbria, the latter inhibiting bacteria to adhere and invade host cells[208–210]. Furthermore, anti-proliferative and anti-adhesive effects of PAC on *E. coli* were found to be highly correlated with PAC molecular structure, with high mDP exerting higher anti-bacterial effects *in-vitro*[211].

Conversely, PAC supplementation in mice infected with *Citrobacter rodentium*, a model for human intestinal disease, was shown to exacerbate colitis, possibly by reducing gut microbiota diversity[194]. However, treating *C. rodentium* infected mice with hydrolysable tannins had a favorable effect on infection[212], which highlights the importance of distinct structure-function within the large group of polyphenols. Moreover, as the effect of PAC on beneficial and pathogenic bacteria may vary, this suggests that host-pathogen interactions may also influence the modes of action of PAC. Thus, next to assessing the anti-bacterial effects of PAC, the immune-modulating effects of PAC during enteric bacterial infection require further investigation.

4.4.2. Intestinal Helminth Infections

Observational studies have reported that certain animals, especially great apes and ruminants, self-medicate by preferentially ingesting plants containing high levels of PAC, among other secondary plant metabolites, when infected with intestinal parasites[213–216]. Congruently, a few animal studies conducted in goats and sheep reported that PAC supplementation had anthelmintic effects by reducing worm burdens and worm fecundity[217,218]. The anthelmintic properties of a given compound can be evaluated by several methods, classically by assessing faecal egg counts over time, egg hatchability, larval motility/migration, and ultimately, worm burdens. Thus, PAC was shown to have direct anti-parasitic effects *in vitro,* by reducing feeding, migratory and motility abilities, and survival of several helminths, such as *Ascaris suum, Ostertagia ostertagi, Cooperia oncophora,* and *Trichuris suis*[52,219,220]. However, substantial anthelmintic efficacy of purified PAC (or PAC-rich diets) *in vivo* has mainly been demonstrated in ruminants, whereas the anthelmintic efficacy of PAC is less evident in monogastric animals[200,221–223].

Next to the direct effect of PAC on intestinal helminths, a limited amount of studies have indicated that PAC may alter the immune response by enhancing type 2 immune response. Thus, a recent study showed that a polyphenol-rich diet enhanced intestinal eosinophilia and mastocytosis in *Ascaris* infected pigs[200]. Furthermore, *Ascaris suum* infected pigs fed a PAC-rich diet in combination the probiotic bacteria *Lactobacillus rhamnosus* LLG significantly reduced serum IgG2a levels and delayed larvae expulsion[224]. However, as described above, the helminth-host interplay is characterized by a complex orchestration of numerous immunological mechanisms. Thus, further research is still needed to fully elucidate the possible modes of action of PAC during helminth infections. Finally, recent efforts have established a clear impact of helminths in modulating host gut microbiota, which may favor the survival of the parasite but may also in turn be modulated i.e. by phytochemicals in favor of the host[100,225].

Clearly, the interaction between diet and immune status may influence resistance or susceptibility towards infectious disease, and a large body of research within nutritional immunology are continuously joining efforts in unraveling the mechanisms and main molecules of interest. Notably, the potential role of PAC during enteric infectious diseases remains largely undiscovered.

5. Models of Helminth-induced Intestinal Inflammation

5.1. *Heligmosomoides polygyrus*

Heligmosomoides polygyrus, previously named *Nematospiroides dubius,* is a small intestinal parasite found in wild mice[226]. It is believed to originate from wild Californian mice and has been used for research purposes worldwide since the 1950s[227]. *H. polygyrus* is generally accepted as the referral name but it has been suggested that the laboratory strain should be referred to as *H. polygyrus bakeri*, to differentiate it from the wild strain of the parasite, considered to be *H. polygyrus polygyrus*[228]. However, this was disputed in a more recent paper, which implies that a true differentiation between "wild type" and "laboratory strain" remains challenging[229]. For consensus purposes, *H. polygyrus* will refer to the laboratory strain of the parasite herein.

H. polygyrus is a trichostrongylid nematode, and thus a member of the same taxonomic superfamily as the major ruminant parasites *H. contortus* and *O. ostertagi*, which cause important pathology and economic losses in livestock[116,230]. It is also part of the same order, Strongylida, as the human hookworms *Ancylostoma duodenale* and *Necator americanus*, and is one of the commonly used model for chronic infection and immune-regulation in humans[12,116]. Immunity to *H. polygyrus* has been extensively reviewed previously[226]. Thus, *H. polygyrus* induce a classical type 2 immune response with several immuno-modulatory mechanisms on both the innate and the adaptive immune response of infected hosts. However, the outcome *H. polygyrus* infection in mice is highly dependent on the mouse strain, with C57BL/6 mice being fully susceptible, while SJL and BALB/c mice expel worms within 2-3 weeks[231]. Thus, the genetic background strongly influences susceptibility and resistance to (re)infection[226,231]. In a laboratory setting, mice are typically orally gavaged with infective L3 larvae (200 larvae/mouse) in order to induce an infection or to maintain the strain for future experiments[232].

5.1.1. Life cycle

H. polygyrus has a direct life cycle, meaning that no intermediate host is needed to establish the parasitic infection in mice. Infective L3 larvae are ingested by faeco-oral transmission and migrate through the lamina propria and muscularis externa to the subserosal layer of the duodenum. Here, they encyst by day 5 post infection (p.i.) and develop through two larval moults. On day 8 p.i., adult worms emerge from the cysts and into to the lumen of the proximal small intestine where they mate and produce eggs, which are excreted in the faeces by day 10 p.i.[138,232]. The adult worms measure around 8-14 mm in length and have a characteristic reddish color [233]. Furthermore, they are tightly coiled around the villi of the small intestines where they feed on the epithelial cell layer but not on the host digesta or blood[234].

5.2. *Trichuris muris*

There are over 70 *Trichuris* species infecting a variety of hosts. *Trichuris muris* is a whipworm found in mice, which is commonly used as a model for the human *Trichuris trichiura.* Trichuriasis is believed to affect 800 million people and is causing malnutrition, cognitive, iron and growth deficiencies, and anemia during pregnancy[235–237]. Thus, research conducted on *T. muris* along with other soil-transmitted helminths, is of high importance both due to the welfare and disability-related concerns, and also due to the negative economic impact.

Infection models with *T. muris* allow the study of a classical Th2 immune response when mice are infected with a high dose of 300 eggs[238]. However, while most mouse strains are resistant to *T. muris*, a distinctive feature of the *T. muris* mouse model is also the dose-dependent Th1-driven immune response leading to persistent infection. Thus, by infecting mice with a low dose of 20 *T. muris* eggs, mice become chronically infected[239,240]. Furthermore, an immune response induced by trickle infection i.e. mice infected with low doses of *T. muris* eggs at several time points, offers a model that is more closely related to natural infections[241,242]. Thus, mice initially develop at Th1 immune response to the low dose of *T. muris* eggs, and a Th2 polarization occurs over time leading to worm expulsion[137]. Studies conducted with *T. muris* have thus provided important insights to the development of susceptibility and resistance towards parasitic infections[239].

5.2.1 Life cycle

T. muris has a direct life cycle and the anatomical predilection site of the worms is in the caecum[243]. Here, the characteristic morphology of whipworms allows adult worms to embed their elongated thin anterior end – the stichosome - into the gut mucosal lining, while the thickened posterior end in the lumen permits reproduction[244]. Eggs are passed into the faeces, where they embryonate in the environment for about 2 months until the eggs are ingested by a susceptible host[137,245]. Once the eggs are ingested, they hatch in the caecum where the released L1 larvae invade the intestinal epithelial layer[246]. Here, the larvae undergo a series of 4 moults over the course of maturation, moving up the crypt axis in a luminal direction. On day 32 p.i. the larvae have fully developed into adult worms measuring approx. 1.5 cm in length[137].

5.3. *Ascaris suum*

Ascaris suum is found in pigs and causes significant economical losses due to lower feed conversion efficiency, reduced growth, predisposition for secondary infections, and cassation/condemnation of livers[247]. *A. suum* is closely related to *A. lumbricoides*, and the zoonotic potential of *A. suum* has been described in several studies[248,249]. Ascariasis is one the most prevalent helminth infection in humans, with over 1 billion people infected worldwide. *Ascaris lumbricoides* is among the largest intestinal nematodes found in humans, causing 60 000 deaths per year mainly in children. It also causes important complications, such as malnutrition and development deficiencies and is considered a neglected tropical disease (NTD)[10,236,237].

A number of studies have been conducted in order to establish how *A. suum* modulate immune function and molecular mechanisms, although it should be noted that many of these studies were conducted in mice[250,251]. However, pigs are the definitive host of *A. suum*, whereas mice can only harbor the infection until the larvae reach the L3 stage in the lungs, which limits the study of the full life cycle in this model[225]. *A. suum* infections elicit a classical Th2 polarized immune response in pigs, making them an excellent model to study parasite-host interactions[252]. As the larvae migrate through a number of organs, the gut-hepatic-lung migration of *A. suum* infection is characterized by lung and intestinal eosinophilia, increased serum IgE, and downregulation of the Th1 immune response[225,252–254]. Notably, the induction of the immune system leads to the expulsion of the large majority of L4 larvae, which normally occurs between day 14-21 p.i.[253]. However, some pigs become chronically infected with adult worms, which may persist in the small intestines for 1-2 years, and may occasionally cause obstruction of the intestines[255]. Thus, a model for chronically infected pigs can be attained by inoculating pigs with smaller doses of *A. suum* eggs (25 eggs/kg) twice weekly over several weeks[252], whereas single inoculations for experimental purposes typically contain 5000 eggs[200].

5.3.1 Life cycle

A. suum infection occurs via the faecal-oral route. The direct life cycle is characterized by the ingestion of infective ascaris eggs, which hatch and release L3 larvae in the small intestines[256]. The L3 larvae then migrate to the caecum and proximal colon, penetrating the mucosa to reach the liver via the portal blood circulation. Migration through the liver causes the characteristic white liver spots due to liver fibrosis[257,258]. Next, the larvae migrate to the lungs on day 6-8 p.i. and enter the alveolar space, which may cause severe coughing by the host[259]. The larvae are then swallowed and reach the small intestines on day 10 p.i. where they moult into L4 and L5 larvae, and finally reach sexual maturity with eggs being passed into the faeces by approx. day 42 p.i.[259–262]. The adult worms measure approx. 20 cm, and females can produce approx. 200 000 eggs per day, which are excreted in the faeces. Eggs undergo embryonation within 6-7 weeks and can remain viable in the environment for several years [263–265].

METHODS

This chapter entails a brief overview of the main applied methods and experimental set-up for the given studies discussed in this PhD thesis. Further details on specific methods and materials used can be found in each of the relevant **Papers II-IV** that form the basis of this PhD together with **Paper I**.

6. Chemical Purification and Analysis of Proanthocyanidins

6.1. Extraction and Purification

There are numerous well-established methods and techniques, which can be used for the isolation of molecules from natural sources. These methods include extraction and solvent partitioning, as well as fractionation and isolation with a chromatographic system, such as flash chromatography, column chromatography, or preparative high performance chromatography with appropriate solvents and a stationary phase[266]. The isolation of PAC with high polymerization remains a challenge due to the difficulties related to separating polymeric molecules of different sizes from each other. However, a new method recently developed by Leppä et al. (2018), purifies PAC by means of Sephadex LH-20 separation and splits the characteristic PAC polymeric 'hump' into distinct purified samples by semi-preparative liquid chromatography (LC). A combination of these methods resulted in chemically well-characterized PAC samples with higher purity, differing mDPs and PC/PD ratios, and thus allowing a better comprehension of the relation between the structure and bioactivity of PAC.

A comprehensive review on chromatographic separation techniques lies outside of the scope of this thesis, and this chapter will therefore focus on the methods used during a 3-month stay at the Department of Chemistry at Turku University (Finland). The methods included liquid-liquid extraction, Sephadex LH-20 separation, semi-preparative LC and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), and the samples were derived from grape pomace (*Vitis vinifera*) (GP), alpine currant (*Ribes alpinum*) (AC), and cocoa (*Theobroma cacao*) (CO).

6.1.1. Liquid-liquid Extraction

Initial solid phase material derived from GP, AC and CO were macerated in 80 % acetone, and the liquid phase was filtered via a series of extraction through a Büchner funnel. For this study, liquid-liquid extraction was used for "pre-fractionation" of complex mixtures to remove eventual impurities. Liquid-liquid extraction, also known as partitioning, is an important and frequently used separation method in research and chemical analysis[267]. It is a suitable technique for the initial purification of polyphenols although there is no systematic approach regarding selection of solvents which may have differing adequacies[268]. The purpose of the technique is to transfer a solute from one solvent to another, the two solvents being immiscible or partially miscible with each other. Initially, liquid-liquid extraction comprises a step of mixing, or contacting, to facilitate

the extraction without forming emulsions, which may impair the separation efficiency[269]. Here, liquid-liquid extraction was only applied to samples derived from alpine currant with ethyl acetate followed by butanol. The solvents were left for evaporation overnight (O/N) and the butanol phase was further evaporated by rotary evaporation.

6.1.2. Sephadex LH-20 Separation

Sephadex LH-20 fractionation was used for the purification of samples after liquid-liquid extraction was applied. The product name Sephadex is derived from SEparation PHarmacia DEXtran and was commercialized by Pharmacia Fine Chemicals in 1959[270]. It is a cross linked-dextran gel that is obtained by reacting an alkaline solution of dextran with epichlorohydrin, and is used for molecular size chromatography[270,271]. Sephadex gels can thus fractionate and purify molecules of different sizes from complex substances by acting as a molecular sieve[270,272]. Water and smaller molecules readily channel through the gel while macromolecules are being retained[271]. The motion of molecules through the gel is supported by hydrostatic pressure and diffusion[270]. Here, the samples were mixed with Sephadex material by magnetic stirring O/N and poured into a Büchner through a Whatman filter. A series of filtrations through the Sephadex material was performed for each sample, with 5 fractions of 200 ml milliQ water (W1-W5), 5 fractions of 200 ml methanol (M1-M5) and 5 fractions of 200 ml 80 % acetone (A1-A5).

6.1.3. Semi-preparative Liquid Chromatography

An amount of 100-120 mg of each of the 19 Sephadex samples was further fractionated by semi-preparative LC, and collected in 168 2 ml-Eppendorf tubes. The gradients of 0,1 % Formic acid and acetonitrile increased from 8 % to 55 % throughout a 33 min run. TargetLynx software (V4.1 SCN876 SCN 917© 2012 Waters Inc) was used in order to divide the chromatograms into 8 equally sized “slices”, and the 168 tubes were pooled accordingly into 8 samples. Acetonitrile and formic acid was evaporated from the samples by rotavap, rotary evaporation or centrifugal concentration and freeze-dried.

6.1.4. Ultra-Performance Liquid Chromatography

Ultra-Performance Liquid Chromatography (UPLC), also known as Ultra-High Performance Liquid Chromatography (UHPLC), is a powerful chromatographic separation technique, which was used in this study to analyze isolated molecules from complex natural products. The UPLC was launched and trademarked by Waters (Milford, MA, USA) in 2004, and offers a more advanced and superior analysis tool compared to the popular and widely used High-Performance Liquid Chromatography (HPLC). The UPLC offers improved separation and resolution of molecules, short analysis time and uses smaller amounts of solvent(s) as a mobile phase. It can be coupled to various detectors, such as ultraviolet (UV) and/or photodiode array (PDA) detectors, mass spectrometry (MS) or nuclear magnetic resonance (NMR) for structure elucidation[273].

Here we used UPLC-MS/MS for the analysis of a matrix of molecules (Sephadex fractions, and samples derived from semi-preparative LC), which are being separated from each other in time by the UPLC and in mass by MS. The molecules were then characterized by analyzing the output in

form of chromatograms using methods developed by Engström *et al.*[274] , Leppä *et al*[275]. and Malish *et al.*[276].

7. Cell-based studies

In order to assess initial bioactivity and to investigate the modes of actions of PAC samples derived by Sephadex LH-20 separation and semi-preparative LC, a number of experiments were conducted using RAW 264.7 macrophages and mLC3 cells.

7.1. Cell culture

RAW 264.7 murine macrophages (ATCC TIB-71) were cultured under sterile conditions and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. RAW-Difluo^{TM} mLC3 cells (InVivoGen) were cultured with media supplemented with Normocin (100 µg/ml), and Zeocin (200 µg/ml, every 2nd passage). mLC3 cells are autophagy-reporter cells in which autophagosome maturation can be monitored by fluorescence microscopy, due to the expression of chimeric proteins consisting of a red fluorescence protein (RFP), a green fluorescence protein (GFP), and a membrane-bound LC3 protein[277]. The RFP is resistant to acidic environment of the autophagolysosome, while dual red and green fluorescent RFP::GFP::LC3 puncta represent the early stages of the autophagic flux. Low passage numbers (< 20) were used for all experiments. Cells were plated at a concentration of 2.5 x 10^5 cells/ml and were always allowed to adhere for 2 hours before they were stimulated. Cell stimulations were conducted in the following order with Bafilomycin (10 nM), PAC (15 µg/ml), and LPS (500 ng/mL) when appropriate, with 30 min incubation time between each treatment for all conducted experiments. Cytotoxicity in LPS-activated macrophages stimulated with select PAC fractions with differing mDPs was assessed. Cells stimulated with concentrations ranging from 3 µg/ml-100 µg/ml showed no signs of toxicity using a neutral red cytotoxicity assay.

7.2. Screening for Anti-inflammatory Activity

Macrophages were plated out on 96-well plates at a concentration of 2.5 x 10^5 cells/ml. Cells were allowed to adhere for 2 hours before LPS stimulation (500 ng/mL). Where appropriate, cells were pre-incubated with AC or GP PAC (15 µg/ml), or vehicle control (PBS) 30 minutes prior to LPS addition. After 24 hours of incubation, the supernatant was collected and frozen at -20 °C, until IL-6 secretion was assessed by ELISA (R&D Systems). Each PAC sample was tested in triplicates, and at least two independent experiments were performed. For equimolar tests, the molarity of samples was calculated based on PC/PD ratios and mDP.

Data involving gene expression and pathways analysis was obtained from stimulated cells and appropriate controls left for incubation for 6 hours before RNA extraction. RNA extraction was performed using RNAeasy kits (Qiagen), and Quantitect Reverse Transcriptase kits (Qiagen) for cDNA.

7.3. Mechanistic studies

As autophagy was considered a possible mechanism underlying the effects of PAC, numerous mechanistic studies were conducted, including measuring autophagic vacuoles by flow cytometry in RAW 264.7 macrophages with the Autophagy Assay Kit (Abcam ab139484) according to the manufacturer's protocol. Cells were stimulated with either Bafilomycin (10 nM) or GP PAC (15 µg/ml) followed LPS stimulation (500 ng/mL) where appropriate. Furthermore, autophagy was assessed by fluorescence microscopy in mLC3 cells. mLC3 cells were plated out on 6-well plates containing cover slips, and stimulated with either Bafilomycin (10 nM), GP PAC (15 µg/ml) and/or LPS stimulation (500 ng/mL) where appropriate. After appropriate incubation times, the coverslips were recovered and placed onto a slide for microscopy using a Leica DM 5000B Fluorescence Microscope. Images were prepared using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA). Finally, Transmission Electron Microscopy (TEM) was used to visually assess the morphology of RAW 264.7 macrophages stimulated with Bafilomycin (10 nM) and GP PAC (15 µg/ml). The cells were incubated for 24h before fixation and preparation for TEM.

8. Animal studies

8.1. Mouse studies

All mouse studies were performed with 6-week old female C57BL/6JOlaHsd mice (Envigo, the Netherlands). They were allowed 1-week of acclimatization, and were weighed weekly and fed with a purified control diet (13 kJ % fat; ssniff Spezialdiäten GmbH, Germany). Oral gavage was performed on alternate days with either low mDP GP PAC, medium mDP GP PAC (200 mg/kg BW) Sephadex fractions or grape seed extract (GS) (300 mg/kg BW) (Bulk Powders, Denmark) dissolved in distilled water, or water alone. Study lengths ranged between 10-49 days depending on the given experiment. Helminth infections with *H. polygyrus* were conducted by inoculating each mouse with a single dose of 200 larvae by oral gavage[232]. Infection studies with *T. muris* were conducted by inoculating each mouse with 20 eggs every 2 weeks and thereby inducing a trickle infection[242].

8.1.1. Sample collection

Mice were humanely euthanized by cervical dislocation and collected samples included intestinal tissue (*H. polygyrus* study: duodenum, *T. muris* study: caecum) for RNA sequencing, qPCR and histology, MLN for flow cytometry and *ex vivo* stimulations, and blood samples for assessment of antibody levels. Gut digesta (*H. polygyrus* study: caecum, *T. muris* study: faecal) was analyzed by 16S sequencing (Department of Food Science, Copenhagen University). In *T. muris* infected mice, blood samples and caecal content were also collected for the analysis of PAC metabolites. The stainings used for histology samples included PAS-staining for assessment of goblet and Paneth cells, and Mcpt1-staining for the enumeration of Mcpt-1 positive mast cells. Antibody-levels in serum were determined by ELISA, and flow cytometry on MLN was used to assess T cell populations. Worm burdens were enumerated by manual count at necropsy.

8.2. Pig studies

A total of 2 pig studies were conducted in the course of this PhD project. The aim of the initial study, comprising of 6 pigs, was primarily to assess whether pigs would thrive on a diet containing 1 % GS, and to optimize the experimental setup for broncho-alveolar lavage (BAL) and *ex vivo* stimulation of alveolar macrophages. As the pilot study was satisfactory, we next performed a main study with 24 pigs (Duroc/Danish Landrace/Yorkshire; 12 castrated males and 12 females) selected from a Specific Pathogen Free (SPF) Danish farm. We vaccinated a total of 100 pigs (p.o.) against *Lawsonia intracellularis* (ENTERISOL® ILEITIS VET., Boehringer Ingelheim) 4.5 weeks prior to the start of the experiment in order to select 24 pigs with no symptoms a few days before transport to the experimental animal facilities at Copenhagen University.

The pigs were 9-weeks old at arrival and were confirmed free of helminth-infection by faecal egg count and serology. They were randomly distributed into 4 treatment groups that were balanced for sex and initial bodyweight. Each of the four groups was housed in two pens consisting of three pigs each. Throughout the entire experiment, 12 pigs were fed the basal diet and 12 pigs were fed

a 1 % PAC supplemented diet derived from GS extract (Bulk Powders, Denmark). Bodyweights were recorded weekly, as well as blood and faecal samples, which were also taken weekly. Blood was collected by venipuncture of the jugular vein and serum separated and frozen at -80 °C. At day 14 after arrival, half of the pigs in each diet group were inoculated with 5000 embryonated *A. suum* eggs by gastric intubation, as described in a previous study[278].

8.2.1. Sample collection

Pigs were euthanized at day 28 of the experiment (i.e. day 14 p.i.) by captive bolt pistol stunning followed by exsanguination., At necropsy, the entire small intestine was removed and processed for *A. suum* larval counts using a modified agar-gel technique[279]. Worm burdens were assessed by manual enumeration of blinded samples conserved in 70 % ethanol under a stereomicroscope. Sample collection included digesta from the jejunum, proximal and distal colons, lung, liver and mid-jejunal tissues, lung LN, and BAL fluids. Digesta samples were analyzed for gut microbiota composition by 16S sequencing (Department of Food Science, Copenhagen University) and abundance of SCFA (Department of Animal Science, Aarhus University). Tissue samples were used for RNA sequencing, qPCR and histology (H&E staining). Lung LN were analyzed by flow cytometry to assess T cell populations. Finally, BAL was performed in order to retrieve alveolar macrophages for *ex vivo* stimulations. Blood IgM, IgA, IgG1 and CRP levels were measured by ELISA.

9. Ethical statement

All experimentation was conducted in line with the Danish Animal Experimentation Inspectorate (License number 2015-15-0201-00760), and approved by the Experimental Animal Unit, University of Copenhagen according to FELASA guidelines and recommendations.

RESULTS AND DISCUSSION

10. Proanthocyanidins and Gut Immunity

Research in bioactive compounds is largely motivated by the aim of discovering dietary components, which may strengthen the immune system. Furthermore, potent phytonutrients may act as novel medicine, which could be employed as alternative treatments in light of the increasing prevalence of drug resistance, as seen with e.g. antibiotics and anthelmintics[280,281]. As indicated in **Paper I**, unraveling the modes of action by which phytonutrients, such as PAC, modulate the immune system could be highly relevant for the treatment of several diseases. Although growing evidence is demonstrating various effects of PAC in relation to infectious disease, little is known on the structure-activity relationship of PAC. This is in part due to limited ability in isolating purified and well-characterized PAC, but also because the complex molecular structures of PAC may vary from one natural plant/food source to another.

Based on this evidence, **Paper I** provides an overview of recent research conducted on the effect of PAC on gut health. Thus, PAC generally demonstrate various beneficial effects both in healthy subjects, as well as during infectious diseases of the intestinal tract. Moreover, the implications of dietary PAC during bacterial, viral and parasitic intestinal disease were reviewed. The paper also suggests possible modes of action by which PAC may modulate gut immunity to pathogens. The main mechanisms include direct interactions of PAC or PAC metabolites with gut epithelial cells and immune cells, which may trigger an immune response. Furthermore, PAC may also induce local and systemic effects on the immune response by interacting with the gut microbiota, and thereby influence gut homeostasis, which is known to have an important impact on overall health. As the topic discussed in **Paper I** has largely been presented earlier in this thesis, further elaboration is not needed here.

11. Structure-function and Cellular Mechanisms of Proanthocyanidins

11.1. Isolation, Purification and Structural Characterization of Proanthocyanidins

Due to scarce evidence addressing the modes of action of PAC, the aim of **Paper II** was to investigate possible structure-function relationships of structurally distinct PAC molecules. By using novel chemical techniques including Sephadex LH-20 fractionation and semi-preparative LC, we produced highly purified PAC fractions derived from GP, AC and CO. The selection of these three natural source was based on their general PAC structure regarding of PC and PD ratio, which entails that AC is rich in PD, CO is rich in PC, whereas GP contains both PC and PD[282,283].

By initial Sephadex LH-20 fractionation with water, methanol and acetone, we obtained 48 Sephadex fractions (16 from each natural source), which were pooled based on chromatographic similarities. This resulted in a total of 19 Sephadex fractions, which included 8 GP fractions, 6 AC fractions, and 5 CO fractions with sample weights varying from 400-1500 mg. From each of these Sephadex fractions, 100-120 mg were used to produce 152 samples derived from semi-preparative LC. Thus, the samples derived from semi-preparative LC consisted of 64 GP samples, 48 AC samples, and 40 CO samples, with sample weights varying from 2-17 mg. These highly purified samples were analyzed by UPLC-MS/MS, and characterized by their mDP and PC/PD ratio. The CO samples yielded fractions with fairly low mDPs ranging between 1.7-4.0, whereas AC and GP had mDPs reaching up to 13.2 and 32.3, respectively. Furthermore, samples collected with acetone by Sephadex LH-20 separation generally yielded higher mDPs than samples collected with methanol and water. It should be noted that only half of the AC samples were included for the purpose of **Paper II** due to technical discrepancies between two batches in the purification methods involving liquid-liquid extraction with butanol. Finally, the analysis of sample PC/PD ratios revealed that PAC derived from GP and CO were mostly PC-rich (78-100 %), and PAC derived from AC were mostly PD-rich (81-99 %).

11.2. Purified Proanthocyanidins demonstrate Bioactivity in an mDP-dependent manner

All samples derived by Sephadex LH-20 separation (19 fractions) and semi-preparative LC (152 samples) were initially tested *in vitro* on LPS-activated RAW 264.7 macrophages to assess their potential anti-inflammatory properties in suppressing IL-6 secretion. Interestingly, samples derived from semi-preparative LC generally demonstrated higher bioactivity than Sephadex fractions. This suggests that the purification method successfully isolated PAC molecules with a relatively high purity, which allowed us to demonstrate potential relationship in structure-function. Furthermore, these results clearly show that a multitude of PAC molecules have distinct bioactivity within one plant/fruit source, as shown in **Paper II**. Notably, GP samples derived from semi-preparative LC could reduce IL-6 secretions by approx. 60 % in LPS-activated RAW 264.7 macrophages compared to LPS controls. Samples from AC also demonstrated important suppressive activity on IL-6 secretion, however these effects were less clear in cells stimulated with CO. Further analysis demonstrated an increasing bioactivity correlating with increasing mDP,

but the bioactivity plateaued and decreased at the highest levels of mDP. Thus, a statistically significant non-linear correlation between mDP and IL-6 suppression was observed for GP and AC samples. Transcriptomic analysis of macrophages stimulated with GP samples with differing mDPs also clearly demonstrated that medium mDP (9.1) PAC induced higher gene expression levels, compared to low (2.6) and high mDP (12.3) PAC. Notably, all PAC samples modulated similar genes but the fold change was considerably higher in cells stimulated with medium mDP PAC regardless of LPS-activation. As discussed in **Paper II**, this indicated, that there is no fundamental difference in the mechanism by which PAC affect cell activity, however the mDP may influence the ability of the cells to process the molecules.

Interestingly, pathways related to vacuolar-type H^+ATPases (V-ATPases), including the upregulation of *Atp6v0d2*, were highly regulated by medium PAC regardless of LPS activation, suggesting an effect of PAC on lysosomes. Thus, V-ATPases are involved in the acidification of phagolysosomes, facilitating endosome trafficking and neutralization of microorganisms[284–286]. PAC also demonstrated a clear tendency of downregulating of inflammatory markers, such as the cytokine inducible SH2-containing protein (*Cish*) and colony stimulating factor 2 (*Csf2*) in LPS-activated macrophages[287,288]. Moreover, the upregulation of the Jak Stat and Inflammation gene pathways in LPS-activated macrophages, was significantly down-regulated by co-stimulation with medium mDP PAC. Furthermore, *Slamf8*, a negative regulator of inflammatory responses[289], was significantly upregulated in LPS-activated macrophages stimulated with PAC regardless of mDP. However, PAC upregulated some inflammatory markers in resting cells, such as the expression of *IL1a* and numerous chemokine-encoding genes, which suggest that it is recognized as an immunogenic compound. As PAC generally suppressed inflammatory responses, we investigated whether this may be due to the downregulation of *Tlr4*, which may render the cells less responsive to LPS. However, we observed no difference between treatment groups when assessing extracellular and intra-cellular TLR4 expression by flow cytometry.

We next assessed the bioactivity of PAC in C57BL/6 mice, which were orally gavage every 2nd day with 200 mg/kg BW PAC derived from GP Sephadex fractions with either low or medium mDP. After 10 days of treatment, RNA-sequencing of ileum tissues demonstrated a significant increase in modulated genes in the mouse group treated with high mDP PAC compared to mice dosed with low mDP PAC and the control group. Similarly to our *in vitro* data, the modulated genes in mice dosed with medium mDP PAC were characterized by the downregulation of immune-related genes, such as *Asah2* and *Enpp7*, and the upregulation of R-spondin 1, which promotes cell proliferation of crypt cells[290]. Thus, an mDP-dependency of PAC bioactivity was demonstrated in mice.

Taken together, these findings suggest that PAC may exert direct effects on immune cells and mucosal tissues in the gut, and are able to modulate immune-related genes in mice, which is in coherence with the literature. Furthermore, we demonstrated a fundamental role for mDP in the anti-inflammatory bioactivity of PAC molecules, which was characterized by a non-linear

relationship between mDP and IL-6 secretion. Thus, this emphasizes that the chemical structures of PAC may be imperative for the elicited bioactivity.

11.2.1. Proanthocyanidins may Affect the Autophagy Pathway

Based on the gene pathway analysis revealing that PAC significantly upregulated the KEGG Lysosome and transferrin pathway, including the vacuolar gene *Atp6v0d2*, we investigated the potential role of PAC on the autophagy pathway, as described in **Paper II**. Notably, *Atp6v0d2* was highly regulated by medium-sized PAC regardless of LPS activation, and is involved in lysosome acidification and autophagosome-lysosome fusion, which allows appropriate turnover of intracellular organelles and infective agents[291]. Furthermore, the implication of PAC in the regulation of V-ATPase has been suggested in previous studies demonstrating localization of PAC to lysosomes following endocytosis[143,292].

Interestingly, we observed characteristic autophagosome-like structures in PAC stimulated macrophages, which was also demonstrated as double-membraned structures by TEM. Furthermore, the autophagic flux was determined by flow cytometry and fluorescence microscopy, using mLC3 autophagy reporter cells. Here, we observed that similarly to Bafilomycin, a V-ATPase inhibitor, PAC was able to inhibit autophagic flux. Furthermore, PAC stimulation in LPS-activated mLC3 cells induced an accumulation of auto-phagosomes, while inhibiting the formation of autophago-lysosomes. In contrast, mLC3 cells stimulated with LPS only, demonstrated that auto-phagolysosome formation was completed. Thus, these mechanistic studies suggest that PAC may act as an autophagy inhibitor, similarly to Bafilomycin, and that PAC may induce anti-inflammatory effects in LPS-activated macrophages by modulating the autophagy pathway.

11.3. Critical Reflections and Limitations of Cell-based studies

Although research aiming at demonstrating the potential health benefits of PAC as nutraceuticals has been increasing, the various methods applied for the extraction and purification of PAC from natural sources lack standardization. Thus, this research is solely demonstrating the effects observed by PAC isolated by means of Sephadex LH-20 separation and semi-preparative LC, as described elsewhere in the method section. Furthermore, molecules will often vary in structure, including PC/PD ratio and mDP, depending on plant source, storage, and climate, leading to further challenges when comparing experimental outputs. Our findings thus demonstrate the unambiguous complexity in allocating observed effects of PAC to a specific chemical structure. Furthermore, some studies suggest that a combination of phytochemicals lead to more pronounced beneficial health effects, rather than singled molecules[25,151]. However, synergistic effects of PAC samples were not investigated in this study but we clearly demonstrated a variation in bioactivity when comparing PAC samples derived by semi-preparative LC. Although these samples were derived from the same Sephadex fraction, there were significant differences in bioactivity. Thus, this suggests that single molecules may exert higher bioactivity than several molecules combined, which further encourages future research in identifying and characterizing these compounds.

12. Proanthocyanidins Modulate Immune Responses in Helminth-infected Animal Models

12.1. Immunomodulatory Impact of Proanthocyanidins in Helminth-infected Mice

While the anti-inflammatory effects of PAC have been demonstrated in numerous studies, only limited research has been conducted in models of infectious diseases, including helminth-infected mice. The aim of **Paper III** was thus to assess the potential implications of PAC in two mouse models infected with the small intestinal nematode *H. polygyrus,* and the murine whipworm *T. muris*, a parasite of the large intestine. Dietary PAC was administered on alternate days by oral gavage prior to infection and p.i. throughout the entire study durations. As previous studies had demonstrated strong Th1-suppressive effects of PAC, we expected to see an enhanced Th2 immune response in helminth infected mice.

Initially, both models demonstrated that PAC induced significant cell hyperplasia in the MLN of infected mice, which suggests considerable immune-stimulatory effects of PAC. However, surprisingly, PAC tended to increase susceptibility to helminth infections by enhancing the expansion of T-bet$^+$ Th1 cells in both models. Thus, in *H. polygyrus* infected mice, we observed a strong downregulation of Th2-related genes such as *Mcpt1,* which is a marker for mast cells. Coherently, the number of Mcpt1$^+$ cells enumerated in intestinal tissue samples from PAC-dosed *H. polygyrus* infected mice was not different to naïve mice. In comparison, *H. polygyrus* mice fed a control diet had significantly higher numbers of Mcpt1$^+$ compared to naïve mice. Moreover, mice infected with *T. muris* had significantly higher serum levels of IgG2a (Th1-marker), which were significantly enhanced by PAC supplementation. Furthermore, by investigating the expression of immune-related genes in caecal tip tissues by qPCR, we observed an upregulation of IFN-related genes *Irgm1* and *Ifit3b* in *T. muris* infected mice dosed with PAC. No effect of PAC on total worm burdens were observed in either infection models, however PAC supplementation resulted in significantly higher adult worm burdens in *T. muris* infected mice. Thus, a Th1-polarized immune response caused by dietary PAC was indicated in mice infected with *H. polygyrus*, and the effects were further substantiated in *T. muris* infected mice. This was similar to a previous study with a mouse model of *T. muris* fed inulin, a commonly known prebiotic that enhances gut health, which however exacerbated inflammatory responses and prevented worm expulsion[238]. Thus, these studies suggest that PAC supplementation may not be beneficiary to the host during helminth infection, and may in fact increase susceptibility.

As dietary PAC have been shown to enhance gut health, we next investigated the effect of PAC on gut microbiota. Here, we saw that PAC was able to significantly alter β-diversity compared to mice fed a control diet in both naïve and *H. polygyrus* infected mice. Notably, PAC significantly increased the abundance of *Bifidobacterium animalis* in *H. polygyrus* infected mice. *B. animalis ssp.* have been shown to positively contribute to gut health by improving gut barrier function[293]. On the other hand, PAC supplementation increased the abundance of the opportunistic pathogen *Escherichia fergusonii*[294] in *T. muris* infected mice. Thus, the effect of PAC on gut microbiota

appeared to be highly context dependent. However, the impact on gut microbiota was not only limited to PAC, as infection status also significantly altered the abundance of specific bacteria. Notably, *H. polygyrus* significantly enhanced the abundance of *Lactobacillus jonhsonii,* which is in accordance with previous studies[295]. Also, compared to PAC-dosed naïve mice, *H. polygyrus* significantly decreased the abundance of *Turicibacter sanguinis,* which is involved in lipid metabolism[296], and a similar trend was observed in *T. muris* infected mice.

Finally, we looked into the distribution of SCFA and PAC metabolites in the caecal content of naïve and *T. muris* infected mice. Expectedly, dietary PAC resulted in the identification of a number of metabolites, and PAC consistently enhanced the concentration of SCFA in naïve mice. However concomitant *T. muris* infection had a suppressive effect on the abundance of both SCFA and specific valerolactones, which are suggested to have potential beneficial effects[297]. As discussed in **Paper III**, *T. muris* may thus impair the efficiency by which PAC are metabolized due to an altered gut microbiota. Alternatively, PAC metabolites may be more readily absorbed in the intestinal tract of *T. muris* infected mice due to the compromised gut barrier. However, as the abundance of PAC metabolites was also reduced in the serum, the altered gut microbiota may be the primary suppressing factor. Furthermore, the negative effect on SCFA is similar to a previous study demonstrating that concomitant *T. muris* infection and inulin supplementation resulted in a fewer SCFA in caecum[238]. Thus, PAC seemingly enhanced the abundance of beneficial PAC metabolites and SCFA in naïve mice, but had a negative effect on these parameters during *T. muris* infection.

These are the first studies to date describing the effect of dietary PAC on the immune response in helminth infected mice. Collectively, the results show that PAC may enhance the susceptibility towards intestinal helminths, and that both PAC and infection significantly altered the gut microbiota and concentration of specific metabolites. Moreover, the effect of PAC on the immune response may be indirectly linked to the effect of PAC on gut microbiota, SCFA and/or the production of PAC metabolites. Thus, our studies offered new insight in the effect of PAC on acute inflammation in *H. polygyrus* infected mice, and on chronic inflammation induced by trickle infection in *T. muris* infected mice, indicating an unfavorable effect of PAC during helminth infection. Although, the results differed from our initial expectations, they revealed a strong immunomodulatory and context-dependent effect of PAC, which may be of relevance for their possible implications in other Th1 or Th2 immune related diseases.

12.2. Proanthocyanidins exert Localised Alterations on the Immune Response in *Ascaris suum* infected Pigs with Limited effect on the Gut-lung axis

Similarly to the mouse studies, only limited research has investigated the effect of PAC on the immune response towards helminth infections in pigs. Moreover, the implication of dietary PAC on the gut-lung axis has only scarcely been explored. Thus, in **Paper IV**, we present the experimental results of the effect of PAC in *A. suum* infected pigs, which offer a valuable model

to assess the potential immuno-modulating impact of diet on the gut-lung axis, in part due to the extensive migratory properties of *A. suum* larvae.

Infected pigs fed a control diet elicited a classical type 2 immune response, which was characterized by increased serum antibody levels (IgM, IgG1, and IgA), intestinal and lung eosinophilia, and a significantly increased Th2/Th1 ratio in lung LN. Furthermore, the lung LN were comprised of significantly fewer $CD3^+$ T cells, which was similar to what has been observed in mice infected with *A. suum*[250]. These parameters were not significantly altered by dietary PAC, indicating that dietary PAC did not impair nor enhance major markers of Th2 immune response in pigs. These findings were in accordance to a previous study conducted with *A. suum* infected pigs fed PAC derived from cocoa[224]. Furthermore, no impact on worm burdens was observed, which suggests that PAC had no effect on hatching efficiency or larval migration and survival. Although anti-parasitic properties of PAC have been demonstrated *in vitro*, our findings are in agreement with a previous studies[200]. The lacking impact on worm burdens may be explained by the more complex setting *in vivo,* which may interfere with the bioactivity of PAC, i.e. interactions with the gut microbiota, and potential biodegradation of PAC leading to fewer and/or less bioactive metabolites. Moreover, the concentration of active compounds at the site of infection may not have been substantial in order to exert an anthelmintic effect. However, the primary aim of this study was not to investigate the anthelmintic effect of PAC but to assess how PAC may modulate immune function.

The classically induced type 2 immune response was substantiated by investigating regulated genes and gene pathways in the intestinal tissue of infected pigs. Notably, *A. suum* induced the upregulation of IL4 and granulocyte pathways, including the enhanced expression of signature gene *IL4,* as well as *IL9, IL10, IL21,* the eosinophil marker *EPX,* and the TCR related genes *CD28* and *CD80*. Interestingly, transcriptomic profiling of intestinal tissues revealed an effect of PAC in both naïve and infected pigs. Thus, PAC promoted the upregulation of genes such as *GPX* and *TXNRD1*, exerting cytoprotective effects, while downregulating immune pathways related to heat shock proteins in naïve pigs. This was similar to previous studies where PAC supplementation was found to improve antioxidant status in the intestinal tissues of pigs fed a diet containing PAC derived from GP[298,299]. Dietary PAC also induced the upregulation of the oxidative stress pathway in infected pigs, with significant enrichment of *SOD3, GPX3* and *NQO1,* which all are protein-coding genes with known anti-oxidant and detoxifying properties[300]. Thus, these findings suggest that dietary PAC may enhance gut health in both naïve and *A. suum* infected pigs by exerting anti-oxidative effects. Notably, two weeks of PAC supplementation resulted in significantly lower serum levels of the acute-phase C-reactive protein (CRP), suggesting that the PAC-fed pigs had a lower inflammation status than pigs fed a control diet. However, this effect subsided, as there were no differences in CRP levels between treatment groups 14 days p.i. Furthermore, PAC and *A. suum* in isolation appeared to regulate similar immune-related genes and gene pathways by increasing the expression levels of *CD19, LYN* and *FYN,* which are involved in B-cell function and signaling[301], which suggests that PAC also exerted immune-stimulatory effects in the intestines of naïve pigs.

We next assessed how dietary PAC and infection may alter gut microbiota. Initially, we found that *A. suum* significantly altered β-diversity in the small intestine compared to uninfected groups irrespectively of diet, primarily by enhancing the abundance of *Lactobacillus spp.* Furthermore, the abundance of *Lactobacillus spp.* and *Bifidobacterium thermacidophilum* were significantly enhanced in the large intestine by *A. suum.* Interestingly, dietary PAC significantly decreased the abundance of *Bifidobacteria spp*. in the large intestine in both naïve and infected pig. Furthermore, PAC decreased the abundance of *B. thermacidophilum* in infected pigs, which suggests a negative effect of PAC and stands in contrast to a previous study conducted in pigs[189]. However, PAC tended to increase the abundance of *Limosilactobacillus reuteri* in the small intestine compared to control-fed pigs, and this effect was significant in the colon. Notably, *L. reuteri* is considered a beneficial bacterial strain for improved gut health with probiotic and anti-inflammatory properties[180,302,303]. Finally, we also investigated the effect of PAC and infection on the production of SCFA. Interestingly an effect of PAC in the reduction of iso-butyric acid and iso-valeric acid was observed regardless of infection, which is similar to a previous study demonstrating that concomitant PAC supplementation and *A. suum* infection significantly decreased the concentration of iso-butyric acid and iso-valeric acid[200].

Thus, PAC had varying effects on the gut microbiota, however by significantly enhancing the growth of *L. reuteri* in both naïve and infected pigs, PAC demonstrated beneficial effects on gut health. Moreover, by promoting probiotics, PAC may also have protective effects outside the intestines during infection, including the lungs, as previous studies have demonstrated that probiotics may alleviate allergic lung disease induced by *A. suum* allergens[304]. Thus, to investigate the potential implications of PAC on the gut-lung axis, we performed broncho-alveolar lavage at necropsy to retrieve alveolar macrophages from pig lungs, and the isolated cells were stimulated *ex vivo*. Interestingly, relative to control pigs, alveolar macrophages isolated from *A. suum*-infected PAC-fed pigs tended to secrete higher levels of TNFα and IL-1β after *ex vivo* stimulation with either LPS or *T. suis* excretory/secretory (E/S) products. However, a significant increase was only demonstrated for *T. suis* activated macrophages isolated from of *A. suum* infected pigs compared to naïve pigs. Coherently, RNA sequencing analysis of lung tissues revealed limited effect of infection and PAC supplementation. The main outcome of PAC in lung tissues from infected pigs was the upregulation of connective tissue growth factor (*CTGF*), involved in tissue healing[305], and the downregulation of *ALOX15*, an oxidative stress inducer. Notably, a previous study demonstrated that mice lacking *ALOX15* displayed attenuated allergic inflammation when challenged with airway allergens[306]. Thus, dietary PAC (or PAC metabolites) had subtle effects in the lungs, but may promote healing and reduce oxidative stress, which may be beneficial in the context of the predisposition to secondary infections during *A. suum* infections.

Collectively, these findings suggest that PAC had an immune-modulatory effect, which was primarily localized to the site of infection in the small intestines, however with no impact on the polarized type 2 immune response induced by *A. suum*. Furthermore, we demonstrate a clear effect of dietary PAC on gut microbiota composition, by enhancing probiotics such as *L. reuteri*, which may promote enhanced gut health in both naïve and infected pigs. However, a decrease of

Bifidobacteria spp. also indicates some negative effect of PAC, as *Bifidobacteria* spp. are considered "good bacteria"[307]. Moreover, the implications of PAC in modulating the gut microbiota, may also be an indirect mechanisms by which PAC may alter the observed immune-related alterations. Limited effect was observed in the lungs, suggesting that dietary PAC may have restricted implications on the gut-lung axis. However, future studies should investigate in further detail whether PAC potentiate healing in the lungs.

12.3. Critical Reflections and Limitations of Animal studies

The optimal animal model should have high predictive, etiological, construct, and face validity[308]. In other words, the validity of an animal model is evaluated based on how well they resemble the pathogenesis and expected outcomes in humans or other animals. As discussed in previous chapters, the outcome of mouse models of helminth infections greatly varies depending on host genetics, which somewhat limits the interpretation of these results to C57BL/6 mice. On the other hand, pigs are generally less inbred and are largely accepted as appropriate models for humans. Furthermore, as *A. suum* is closely related to *A. lumbricoides,* the pig model used in this study has a certain validity as a model for human ascariasis. However, aspects such as study duration, PAC dose, interference with diet, sample size, inter-individual variation and host genetics greatly influence the translatability of any given outcome of an *in-vivo* study, including the studies described herein. Thus, mice and pigs were dosed with 200-600 mg/kg BW PAC on alternate days or daily, which is considerably higher than what is expected to be consumed by humans. Furthermore, the infection models with a single bolus of *H. polygyrus* or *A. suum* by oral gavage, are not representative of a natural infection dose[226]. Thus, some research groups have shown how trickle infections with these helminths seemingly lead to a better construct validity of the models[252,257,309,310]. Furthermore, as described in the method section, pigs were vaccinated against *L. intracellularis*, prior to the experimental study, which may potentially be a confounding factor for the interpretations of immune-related data analysis.

The analysis of the gut microbiota revealed that the abundance of several probiotic bacteria were affected by either PAC and/or infection. However, some considerations should be made for the interpretation of the gut microbiota data, as large variabilities in i.e. probiotic effects of bacteria are seen both a species and strain level. Also, PAC are known to be metabolized in the gut, and the observed effects may thus be caused by either parent compounds or metabolites, which limits the ability to define the biological effect of a specific PAC molecule. Therefore, continuous improvement of the analysis and purification of PAC is needed to investigate and predict the effects and stability of well-characterized PAC molecules.

The mechanism(s) leading to the unexpected results in the mouse studies leading to higher susceptibility towards helminths are unknown, but a possible explanation could be the influence of the basal diet composition. Specifically, the mice were fed a purified, refined diet, which may potentially result in a microbiota of low diversity and low bacterial abundance. Consequently, dosing mice with PAC may induce the blooming of specific bacterial taxa that result in the stimulation of a Th1-polarized immune response, which could explain the observed cell

hyperplasia observed in the MLN in both mouse models. On the other hand, pigs were fed a whole-grain diet (whole grain ground barley/wheat plus protein concentrate), which may contain traces of phytonutrients, such as flavonoids, as well as higher amounts of fermentable fibre and non-starch polysaccharides. This may suggest that the intestinal gut flora is more diverse from a starting point, with a more tolerogenic immunological environment. Notably, a large difference between a coarse and a refined, pelleted diet in pigs has earlier been shown to result in greatly enhanced susceptibility to helminth infections[311]. Thus, as the gut environment may already be composed of a diverse microbiota, the introduction of a complex plant material such as PAC has a less disruptive effect on the microbiota, which may in turn explain the lower immunomodulatory effect of PAC observed in pigs. In order to investigate this hypothesis, faecal samples from several time points throughout the studies should be analyzed to assess a potential difference in α-diversity between treatment groups. Furthermore, a study with mice fed chow, a non-refined diet, could be conducted in parallel to ascertain whether the effects of PAC are dependent on the diet-induced microbial diversity. Similarly, pigs could be fed a refined, synthetic basal diet, in order to disentangle the effects of host species, parasite, and underlying diet composition and how this impacts on the gut response to PAC during parasitic infection.

Finally, it should be noted that naturally infected hosts may have co-infections with other parasites/pathogens, and the infection exposure may greatly vary between individuals, which is an on-going challenge in the study of immunity towards parasites[241].

13. Conclusion

Based on the studies conducted in this PhD project, the following conclusions can be made:

- The cellular bioactivity of PAC is correlated to their chemical structures, which was demonstrated by mDP-dependency in cytokine secretion and gene regulation. PAC instigated overall suppression of the inflammatory response in LPS-activated macrophages *in vitro*, and the ability to regulate genes in an mDP-dependent manner was also demonstrated *in vivo.*

- PAC may affect the autophagy pathway by upregulating genes related to lysosome function while inhibiting autophagolysosome formation. This suggests a possible mechanism by which PAC may induce anti-inflammatory responses.

- In *H. polygyrus* and *T. muris* infected mice, PAC demonstrated immune-stimulatory properties and tended to drive a Th1-polarized immune response, resulting in enhanced susceptibility.

- PAC supplementation exerted localized changes in the intestinal tract in naïve and *A. suum*-infected pigs by improving antioxidant status with negligible impact on type 2 immune response.

- Limited effect of PAC was observed on the gut-lung axis in *A. suum* infected pigs.

- PAC supplementation altered gut microbiota composition in both naïve and helminth-infected mice and pigs, with varying effect on the abundance of beneficial bacteria.

Taken together, the studies discussed herein demonstrate an effect of chemical structure on bioactivity, and a complex trilateral interplay between dietary PAC, the immune system and helminth infections. While exerting general immune-suppressive functions in LPS-activated macrophages, and enhancing antioxidant status in pigs, PAC rendered mice more susceptible to helminths infection. Thus, PAC demonstrated a broad-spectrum of activities, and strong immune-modulating properties, which were context-dependent. The main and objectives of this PhD project were thus fulfilled and the hypotheses were assessed.

Further research in the area of PAC molecules and their implications in modulating the immune system during enteric infections remains to be conducted, as this area is still largely unexplored. By continuously uncovering the various modes of action of PAC and identifying which specific molecules may be used as potent immune-modulators, they may potentially benefit infectious inflammatory diseases.

14. Future Prospects for Research

To enhance our understanding of the structural properties of PAC and their effect on the immune system, a standardization of chemical purification techniques would substantially improve consistency regarding the interpretation of experimental results conducted in biological sciences. Therefore, continuous efforts should aim at purifying single PAC molecules in large scale with well-characterized structures, although this remains a challenging task.

In this PhD project, we used PAC Sephadex fractions derived from GP, which are rich in PC and have shown promising anti-inflammatory effects in previous studies including our own. However, some studies have suggested that anthelmintic effects of PAC might be correlated with PD-rich molecules[52]. Thus, PD-rich PAC samples derived from AC with high mDP could be investigated for their potential anthelmintic effects in mice.

Although the main focus here was to explore the modulation of helminth-induced inflammation, future research could also investigate in further detail how highly purified PAC molecules may interact with bacterial, fungal or viral infections, as these studies remain scarce. Moreover, the use of genetically modified mice, such as immune-compromised mouse strains or germ-free mice, may also contribute to valuable insights on the effect of PAC on the immune system.

One of the main challenges, which reoccur in most research devoted to nutraceuticals, is the metabolism of the compound of interest before it reaches the target organ to induce a given effect. In this instance, the relatively recently developed concept of nanoparticles may be of high relevance here. Nanoparticles are the result of technical advancements within nano-biotechnology, which may convey controlled delivery of molecules to specific tissues[312]. Notably, a few studies with nanoparticles containing PAC have already be conducted with promising results[313,314] but their implementation in the context of helminth-induced type 2 immune response remains to be explored. Another area of research, is the field of bioenhancers, which are substances that can increase the bioavailability of a given compounds[315,316]. Thus, bioenhancers may also offer an alternate way to potentially improve the efficacy of PAC at the site of inflammation during enteric infections.

15. References

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Figures 3 and 4:

PUBLICATIONS AND MANUSCRIPTS

Paper I

Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins

Andersen-Civil, A. I. S., Arora, P. & Williams, A. R. Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins. *Frontiers in Immunology* vol. 12 (2021).

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frontiers in Immunology

REVIEW
published: 24 February 2021
doi: 10.3389/fimmu.2021.637603

Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins

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The role of dietary components in immune function has acquired considerable attention in recent years. An important focus area is to unravel the role of bioactive dietary compounds in relation to enteric disease and their impact on gut mucosal immunity. Proanthocyanidins (PAC) are among the most common and most consumed dietary polyphenols, and are characterised by their variable molecular structures and diverse bioactivities. In particular, their anti-oxidative effects and ability to modulate gut microbiota have been widely described. However, there is limited evidence on the mechanism of action of PAC on the immune system, nor is it clearly established how PAC may influence susceptibility to enteric infections. Establishing the sites of action of PAC and their metabolites within the gut environment is fundamental to determine the applicability of PAC against enteric pathogens. Some mechanistic studies have shown that PAC have direct modulatory effects on immune cell signalling, isolated pathogens, and gut mucosal barrier integrity. Boosting the recruitment of immune cells and suppressing the amount of pro-inflammatory cytokines are modulating factors regulated by PAC, and can either be beneficial or detrimental in the course of re-establishing gut homeostasis. Herein, we review how PAC may alter distinct immune responses towards enteric bacterial, viral and parasitic infections, and how the modulation of gut microbiota may act as a mediating factor. Furthermore, we discuss how future studies could help unravel the role of PAC in preventing and/or alleviating intestinal inflammation and dysbiosis caused by enteric disease.

Keywords: pathogens, proanthocyanidins, mucosal immunity, microbiota, enteric infection, inflammation

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *03 December 2020*
Accepted: *14 January 2021*
Published: *24 February 2021*

Citation:
Andersen-Civil AIS, Arora P and Williams AR (2021) Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins. Front. Immunol. 12:637603. doi: 10.3389/fimmu.2021.637603

INTRODUCTION

The mammalian gut environment is an intricate ecosystem where multiple components such as epithelial cells, immune cells and the gut microbiota (GM) interact reciprocally with each other and regulate homeostasis (1). The gut plays an important role in digesting and absorbing nutrients, preventing loss of water and electrolytes, tissue injury repair, and communicating with the environment. Moreover, it continually encounters a large amount of exogenous agents derived from the diet and environment, as well as infectious microbes (2). Pathogenic agents such as bacteria, parasites and viruses are a major health and socio-economic burden in both humans and livestock (3, 4). A major task of the gastrointestinal ecosystem is to maintain homeostasis in the presence of harmless dietary components and commensal microbiota, whilst eliciting an adequate protective inflammatory response in response to pathogens (5). Understanding this delicate balance

may be the key to developing novel interventions for control of infectious diseases. This is of critical importance, as new approaches to prevent enteric infections in human and veterinary medicine are urgently required in the face of rising antimicrobial drug resistance (6).

The activities of the gut immune system may be highly responsive to diet, and it is increasingly appreciated that various dietary components can modulate the gut immune environment by directly or indirectly interacting with gut immune cells (7). Consistent with this, various natural products such as fibres, polyphenols, and other plant secondary metabolites have been investigated for their ability to regulate intestinal inflammation and immune function (8, 9). In particular, much recent attention has focused on the proanthocyanidins (PAC), a diverse class of oligomeric and polymeric polyphenols found in a wide range of foods including fruits, nuts and berries (10). PAC have been identified as the fraction most associated with the beneficial effects of fruits and berry extracts on diabetes and obesity (11). Whilst these positive effects of PAC are well-known in context of metabolic diseases, less is known about the role in PAC in regulating gut immunity to intestinal pathogen infection. Moreover, although our understanding of the health benefits of PAC has increased, precisely how PAC modulate the immune system is still mostly unknown. In this review, we examine the immunomodulatory effects of dietary PAC, as well as emerging evidence that PAC may modulate immune function during enteric infection in both humans and animals. We discuss current knowledge on the mechanisms underpinning the effects of PAC on inflammation and immunity, and highlight key studies showing that PAC may play a role in promoting resistance to pathogen infection and/or supressing immunopathology. Finally, we suggest some pertinent areas for future investigation.

GUT PATHOGENS AND THE MUCOSAL IMMUNE SYSTEM

Worldwide, enteric infections are a major cause of mortality and morbidity in humans and production animals. In low and middle income countries, infections of the gastrointestinal tract including bacterial infections (e.g. *Escherichia coli)*, parasitic infections (e.g. hookworm) or viral infections (e.g. rotavirus) cause widespread disease and stunt socio-economic development (12). These infections are less common in high income countries but infections with pathogens such as *Clostridioides difficile* still pose a considerable public health burden (13). Notably, lifestyle factors such as poor diet (high sugar/high fat) and lack of exercise may not only lead to metabolic diseases, such as type 2-diabetes, but may also predispose to opportunistic infections due to dysfunctional mucosal barrier function (14). Enteric infections are also a major constraint in all livestock production systems, causing billions of dollars in lost production annually and compromising animal welfare and food security (15). Importantly, rising resistance to antimicrobial drug treatments in both humans and animals has necessitated the need for novel solutions for promoting healthy gut function and disease resistance (16).

The gut immune system plays a key role in regulating infection, not only by driving protective immune responses that remove pathogens but also co-ordinating wound healing, tissue repair and resolution of inflammatory cytokines to ensure the avoidance of harmful immunopathology. Under homeostatic conditions, the gut mucosal immune system is inherently programmed to be immunologically tolerant towards food antigens and commensal organisms. Loss of this immunological tolerance can result in dysregulated immune responses, which contributes to the development of inflammatory diseases (1, 17).

Anatomically, the gut immune system can be divided into three different components: the intestinal epithelial barrier (IEB), the lamina propria (LP) and the gut-associated lymphoid tissue (GALT), which is further comprised of Peyer's patches (PP), isolated lymphoid follicles, and mesenteric lymph nodes (MLNs) (18). The IEB is comprised of diverse and specialized sets of integrated epithelial cells. At the mucosal surface, enterocytes are tightly joined by well-regulated intercellular junctional complexes, which prevents access by pro-inflammatory agents to the underlying tissue. The epithelium also comprises intra-epithelial lymphocytes (IEL), goblet cells, Paneth cells and enteroendocrine cells among others, which are assembled into a single layer covered by a mucous layer (19). Goblet and Paneth cells secrete mucus, and anti-microbial peptides, respectively, which contribute to the exclusion of allochthonous microbes from the LP tissue. Furthermore, IEL such as γδ T-cells help to maintain barrier integrity by promoting tolerance to commensal microbes, while responding to the presence of pathogens (20).

Within the LP, innate immune cells are uniquely situated to counter infectious microorganisms. These include rapid-responding innate lymphoid cells (ILCs), and professional antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages. The adaptive arm of gut immune system is constituted by B, T and antibody-secreting plasma cells typically arranged within both the LP and the GALT (18). Macrophages and DCs possess several pattern recognition receptors, including toll-like receptors (TLRs), and C-type lectin receptors, that are capable of recognizing pathogen-associated molecular patterns (21). Both the innate and adaptive arms of the gut immune system work in a coordinated manner to maintain immunologic homeostasis and induce protective inflammatory responses (22, 23). Exogenous antigens are sensed by APCs that possess the specialized machinery to sample and process incoming antigens. For the induction of the gut immune response antigen-bearing DCs migrate to the nearby GALT tissue like PP or MLN where DCs communicate with the naïve T-cells and induce their antigen-specific differentiation into effector T cells which can be either $CD4^+$ [T-helper (Th) 1, Th2, Th17, regulatory T cells (Treg)] and/or $CD8^+$ (cytotoxic) T cells (24). ILCs are also involved in initiating immune responses (23). After induction, the effector immune cells either remain in the GALT tissue or migrate to the suitable mucosal sites where they induce

protective inflammatory responses resulting in the elimination of the antigen and the re-establishment of homeostasis.

Depending upon the nature of the pathogen challenge, gut immune responses can be classified into subtypes; type 1 immune responses (comprising mainly ILC1 and Th1 cells) that provide protection against viral and intracellular bacteria, type 2 immune responses (ILC2 and Th2) that help in expelling parasitic helminths, and type 3 immune responses (ILC3 and Th17-Th22) that are required to eliminate fungi and extracellular bacteria (24). Furthermore, an additional type 4 immune response has been suggested to operate at the gut mucosal surfaces that principally block the entry of microorganisms even before they come in contact with the mucosal surfaces. This response is mediated by secretory IgA, which is in turn dependent on the production of TGFβ secretion from Treg cells (25, 26). Regulation of these different pathogen-specific immune programs is crucial. Dysregulation during either chronic infection or autoimmune disease can lead to Th1 and Th17-driven chronic inflammatory diseases like Crohn's disease (27), whereas impaired Th2 immune responses contributes to the progression of food allergies (28) and ulcerative colitis (29). Thus, the effects of immune-modulating dietary interventions (e.g. prebiotics) need to be evaluated not only on how they may boost immune responses to infection, but also how they impact on the resolution of inflammation and regeneration of tissue damage. Ultimately, nutritional manipulation of the gut immune system should aim to promote innate and adaptive responses that allow infected hosts to resist and/or tolerate infections, with a minimum of immunopathology.

PROANTHOCYANIDINS

Proanthocyanidins are a member of the polyphenol class of plant secondary metabolites. Polyphenols represent an important group of naturally occurring anti-oxidants and chemo-preventive compounds, and are found in plants and in food of plant origin, such as fruits, vegetables, cereals, and cocoa. The average intake of polyphenols by European adults has been estimated to ~1 g/day, which is higher than the intake of any other classes of phytochemicals (30, 31). The main challenge that delayed the attention to polyphenols compared to other anti-oxidants, such as carotenoids and selenium, was due to the considerable diversity and complexity of their chemical characteristics (30). Polyphenols have especially been studied to investigate their potential in cancer research due to their anti-oxidative effects, but their mechanisms of action go beyond the modulation of oxidative stress, and are not yet fully understood (30, 32). Epidemiological, *in vitro* and animal studies have shown that polyphenols may have preventive and protective properties towards numerous conditions, such as cardiovascular, degenerative, metabolic, and inflammatory diseases (33, 34).

One of the most widespread polyphenol classes are tannins: water-soluble secondary metabolites found in many different plants. They can be classified into two main groups; the hydrolysable tannins (gallotannins, ellagitannins, and complex tannins) and the non-hydrolysable PAC, or condensed tannins (35). Proanthocyanidins consist of flavan-3-ol monomers, either catechin or epicatechin or their *trans* isomers, arranged into large oligomers or polymers with degrees of polymerization (DP) ranging from 2 to more than 40 (**Figure 1**) (36, 37). They are among the most common dietary polyphenols and are responsible for the bitter taste of chocolate and the astringent sensation in fruits such as grapes, pears and apples. The sensation of astringency is caused by the ability of PAC to bind and precipitate salivary proteins containing high-proline contents (38). The average intake of PAC has been estimated to 95 mg/day in American adults with variations depending on age, sex, wine consumption and ethnicity (39). Of note, due to large variation in PAC DP, no food source can provide high amounts of PAC molecules with one specific chemical conformation (40). Thus, the studied effects of PAC towards a vast variety of diseases often relies on a symbiotic or additive effect of PAC molecules with differing DP.

Gut Immune Regulation by Dietary Proanthocyanidins

Polyphenols (including PAC) display π-electron-rich aromatic nuclei and labile phenolic –OH groups, which confer them a reducing (electron and hydrogen-donating) character. Thus, their antioxidant effects are due to their ability to rapidly reduce reactive oxygen or nitrogen species (ROS/RNS), which are produced in great amounts during the inflammatory phase of chronic diseases (41). In addition, the PAC molecule procyanidin B1 (a dimer of epicatechin) has also shown protective effects towards oxidative stress by inducing the activity of the glutathione S-transferase P1 enzyme (GSTP1) and the nuclear translocation of the transcription factor NF-erythroid 2-related factor (Nrf2), which may in fact be a more important antioxidant mechanism than direct scavenging of ROS (42). Due to their well-known antioxidant effects, and their high concentrations in many health-promoting dietary components such as grapes and berries, PAC have been increasingly studied for their effects on gut health. Numerous studies in mouse and piglet models of intestinal inflammation have shown that dietary PAC can have beneficial effects, including promoting gut barrier integrity and mucosal morphology, and increasing goblet cell density and villi lengths (43, 44). However, the mechanisms by which PAC induce these benefits are largely unknown.

PAC molecules generally remain stable in the stomach (45, 46) which may be due to a protective layer formed by the protein-binding oligomers coating the gastric mucosa or by modulating hydrochloric acid secretion (47). Thus, PAC with a DP of ≥3 remain structurally intact during intestinal transit, and are able to pass to the large intestine where they are metabolized to varying degrees by the GM. This results in the production of numerous bioavailable metabolites such as flavan-3-ol conjugates (e.g. O-methyl-epicatechin-glucuronide) and phenolic acids (e.g. caffeic or coumaric acids). These can be absorbed and result in systemic health benefits, such as positive regulation of cardiovascular disease by binding to low density lipoproteins (48–51).

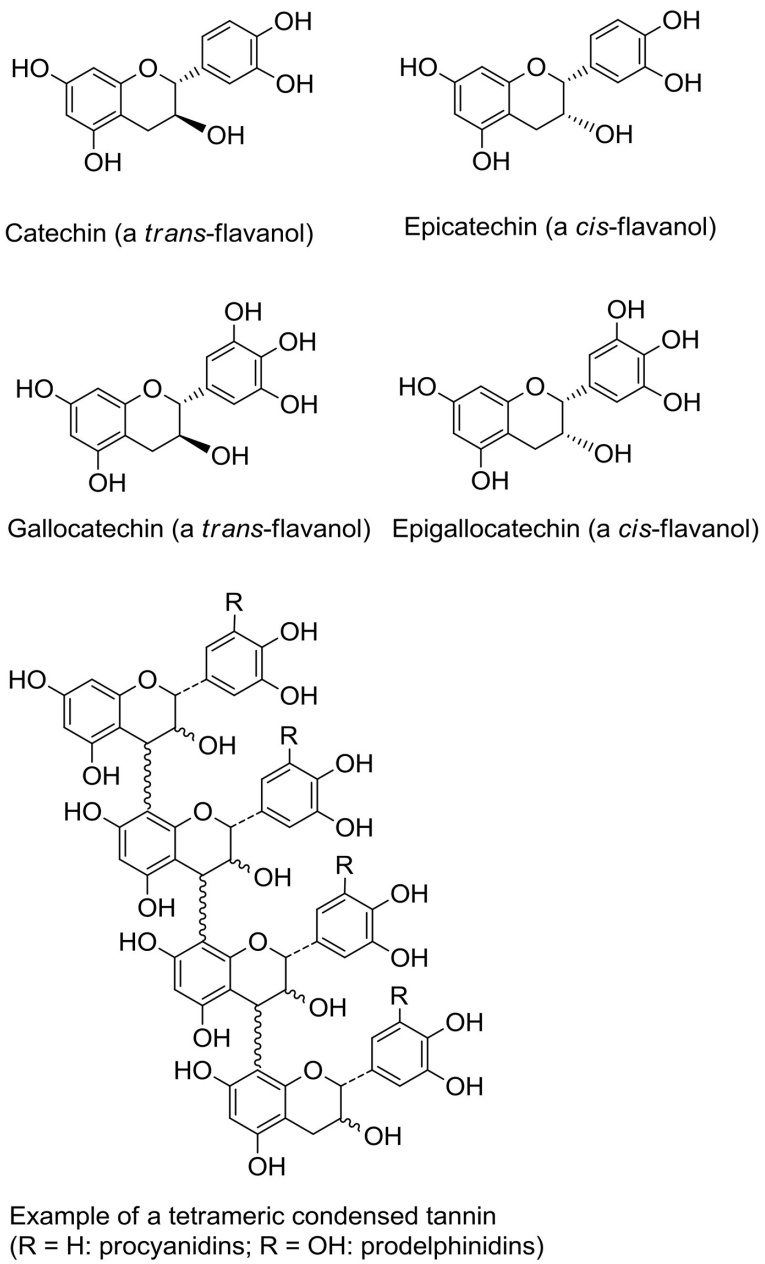

FIGURE 1 | Structures of flavanol monomeric subunits and an example of a tetrameric proanthocyanidin (condensed tannin) oligomer.

A number of recent studies have begun to shed light on how microbial metabolism of PAC may influence gut health. Some caution needs to be applied in ascribing the observed effects solely to PAC, as feeding studies are often conducted with extracts or dietary supplements which contain other polyphenols or fibres which may also impact the GM. Of interest, it has been shown that transfer of GM from mice fed a PAC-rich *Camu camu* extract mice to germ-free mice can protect against metabolic disease during diet-induced obesity (52). Thus, a prebiotic effect of PAC has been speculated to be primarily responsible for their anti-inflammatory and immunomodulatory activity (8, 53). Consistent with this, changes in the GM following administration of pure grape-seed PAC can precede alterations in intestinal immune gene expression (54). However, it is also plausible that PAC interact directly with cells at the mucosal barrier during their intestinal transit, and this may also result in significant immunomodulatory activity (55). Consequently, the effects of PAC on the immune system likely comprise both prebiotic effects and direct modulation of immune cell function (**Figure 2**). We will discuss both these potential mechanisms below.

Regulation of Immune Function by Proanthocyanidins as a Result of Prebiotic Effects

The GM residing in the human intestines is composed of up to 100 trillion microbes (56). Most of the intestinal bacteria belong to different genera of gram-positive Firmicutes, and to some of the many different gram-negative Bacteroidetes, such as *Bacteroides, Prevotella, Parabacteroides*, and *Alistipes*. Other phyla, including *Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia* are also core members of the human GM (57). The number and diversity of bacteria have been shown to vary in the different sections of the gastrointestinal tract. Thus, a low number and few species populate the stomach and the upper part of the small intestine, whereas the number of bacteria progresses from the jejunum to the colon (58). Recently, the concept of "healthy gut" has become very popular given that intestinal dysfunction has been associated with several diseases, both locally as well as systemically (1). Furthermore, perturbed gut immune homeostasis also weakens the gut barrier integrity, increasing susceptibility to opportunistic pathogen infection and allowing gut bacterial translocation to the basal side of the mucosa, resulting in systemic inflammation (5, 59).

A number of studies have shown that dietary PAC can alter the composition of the GM. Piglets given grape-seed derived PAC had improved gut microbial diversity, and increased abundance of OTUs belonging to *Firmicutes*, *Bacteroidetes* spp., and *Clostridiaceae*, and decreasing the abundance of *Lactobacillaceae* (44). Moreover, a consistent feature of PAC-rich diets is a significant increase in the abundance of *Akkermansia muciniphila*, which has been observed in mice, pigs and humans (60–62). *Akkermansia* has become a biomarker for gut health due to its association with mucosal barrier integrity and mucin production. It can also produce metabolites that suppress inflammatory responses directly in intestinal epithelial cells, suggesting that its growth in response to certain dietary components may be responsible for their putative health benefits (63). Various other metabolites with known anti-inflammatory activity, such as short-chain fatty acids (particularly propionate), have also been observed to be increased in the digesta of animals fed PAC-rich diets (61, 64).

How PAC causes these changes in the GM is an open question and an active area of research. Several studies have addressed the metabolism by which PAC molecules are degraded into aromatic acids by the intestinal flora, notably in the large intestines (33). It is clear that, at least *in vitro*, the bacterial metabolism of PAC is dependent on the DP, with polymers being less susceptible to degradation compared to catechin monomers (65). Stimulated digestion of PAC by the GM in *in vitro* systems has demonstrated an active de-polymerization of PAC followed by the appearance of small phenolic metabolites, some corresponding to those observed in the systemic circulation of animals fed PAC *in vivo* (66, 67). Thus, it is clear that PAC can act as a direct prebiotic substrate, similar to dietary fibre. Furthermore, direct anti-bacterial effects of PAC have long been studied, and it is known that PAC induce growth inhibition of some bacteria either by inhibition of enzymes,

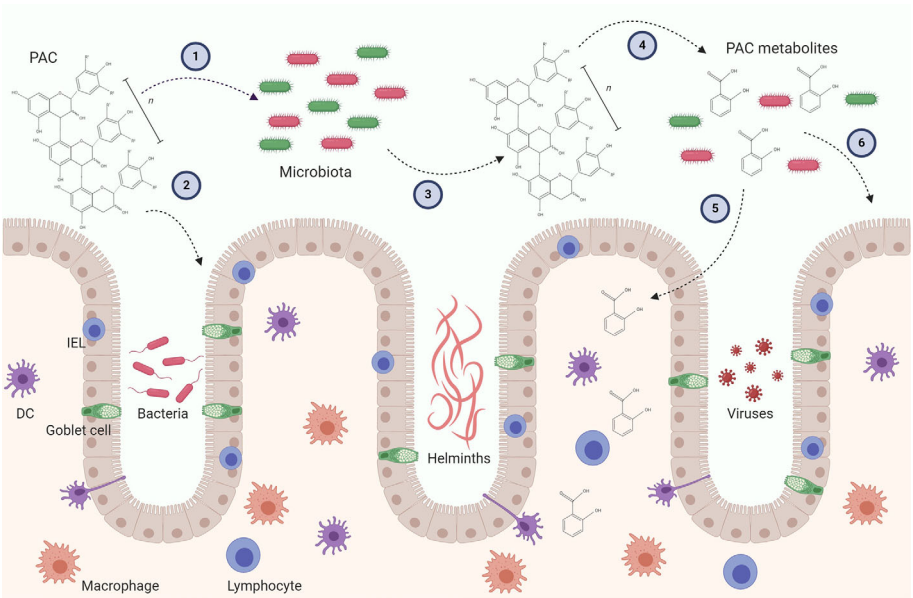

FIGURE 2 | Possible mechanism leading to immunomodulatory activity of proanthocyanidins (PAC) in the gut. PAC molecules enter the gut lumen and may exert direct effect on the gut microbiota by selecting for distinct bacteria (1) and the gut epithelial layer by upregulating i.e. tight junctions (2). In turn, the gut microbiota may alter the chemical structure of the parent PAC molecule and produce metabolites (4). PAC metabolites can be absorbed through the epithelial layer and influence residing immune cells (5). The metabolites may also alter gut microbiota as well as the gut epithelial barrier (6). Furthermore, both parent compounds and PAC metabolites may have direct or indirect effects on gut pathogens, such as bacteria, helminths and viruses. These putative immunomodulatory mechanisms may be caused by isolated PAC molecules and/or PAC-rich extracts. Conclusive experimental evidence *in vivo* is thus far lacking. DC, Dendritic cell; IEL, Intraepithelial lymphocyte.

deprivation of growth substrates, or direct action on the bacterial cell membrane (68). Therefore, selective inhibition of some bacterial taxa, allowing the increased propagation of beneficial bacteria, may also be a mechanism whereby PAC change the composition of the GM. Indeed, selection for *Bacteroides* and *Enterobacteriaceae* is seen in rats fed a PAC-rich diet, and these bacteria were referred to as PAC-resistant bacteria, and their abundance increased in a dose-dependent manner (69). However, it is also clear from *in vitro* studies that direct interactions between PAC and host intestinal cells can stimulate the production of mucins and other mucosal proteins, that may also act as selective nutrient source for bacteria such as *Akkermansia* (70). Consequently, growth of these bacteria may indirectly derive from PAC-mediated effects on host cells rather than a direct interaction between PAC and the bacteria residing within the GM. Whatever the mechanism, it is clear that the consumption of PAC changes the GM and produces soluble metabolites with recognized anti-inflammatory or immunomodulatory activity. This prebiotic capacity of PAC may thus be a major mechanism of their observed health benefits in different models of disease.

Direct Effects of Proanthocyanidins on Immune Cells

In vitro, PAC possess well-characterized anti-inflammatory activity in both intestinal epithelial cells, macrophages and DCs (71, 72). Exposure of macrophages or DCs to pro-inflammatory stimuli such as TLR ligands (e.g. lipopolysaccharide—LPS) in the presence of PAC results in lower production of inflammatory cytokines, ROS, and NFκB translocation (55, 73). Moreover, in epithelial cells, mitochondrial dysfunction induced by inflammation and oxidative stress can be effectively alleviated by PAC (74). Mechanistically, the mode-of-action of PAC has not been fully elucidated. However, PAC do not appear to block the interaction of LPS with TLR4 in DCs, but instead modulate downstream signalling pathways connected to lysosome activity and second-messenger activity (73, 75). Importantly, these effects are consistent with immunological changes observed *in vivo* in models of inflammation, suggesting that at least some anti-inflammatory effects of PAC derive from direct modulation of mucosal immune cells (76–78).

In addition to regulating cellular responses to pro-inflammatory stimuli, PAC are also capable of activating innate immune cells involved in host defence and barrier function. Perhaps the most well described example of this is the *in vitro* response of γδ T-cells to PAC stimulation, whereby these cells undergo proliferation, up-regulate IL-2Rα and display increased stability of transcripts encoding for cellular activation (79, 80). This response appears to be unique to γδ (and not αβ) T-cells, is conserved across multiple species, and has also been observed *in vivo* in humans consuming PAC-rich cranberry juice (81–83). γδ T-cells are found in the intestinal barrier where they respond to conserved pathogen or dietary antigens to fortify mucosal defences and provide signals for the activation of other immune cells such as neutrophils (84). Thus, these cells appear to have evolved a conserved response against PAC, suggesting that intake of these dietary compounds is associated with activation of innate defence mechanisms leading to protection from infection. Consistent with this, stimulation of intestinal organoids with PAC leads to significant up-regulation of antimicrobial defences (e.g. goblet cell and mucin production), again indicating that the mammalian gut has evolved to sense PAC as a signal for strengthening of innate immune defences to prevent infection and/or reduce harmful inflammation (70).

Effects of Proanthocyanidins on Resistance to Enteric Pathogen Infection

A major research effort is currently underway to identify nutritional interventions that can improve resistance to infection in both human and veterinary medicine. Nutritional manipulation of the immune system may enhance protective response to infection but also reduce inflammation and restore homeostasis and tolerogenic immune responses. Much of this research has been directed towards probiotics, and the role that they may play in stimulating the immune system and promoting balanced immune function without overt inflammation (85). The ability of PAC to increase resistance or modulate inflammatory responses to infection is not yet clear. Below, we consider some relevant studies where the effects of PAC on different enteric infections have been studied.

Bacterial Infections

The colonization and invasion of pathogenic bacteria leads to the disruption of gut homeostasis, inflammation and often symptomatic disease. Whilst dietary PAC can protect against pro-inflammatory responses induced by either diet-induced GM dysbiosis or acute challenge with purified endotoxin (86), their role in combating infection with bacterial pathogens is less well described. As mentioned above, PAC have documented anti-bacterial effects which include preventing the entry of *E. coli* into epithelial cells, likely by Fimbriae neutralization or agglutination, and also deactivation of toxins which can cause secretory diarrhoea (70, 87). This may explain the apparent clinical benefits of feeding PAC-rich extracts to piglets infected with enterotoxigenic *E. coli* (88). Moreover, some of the immunomodulatory effects that are apparent during metabolic disease models, (e.g. strengthening of the mucosal barrier by goblet cell differentiation and modulating inflammatory cytokine production), also appear to be relevant in different bacterial infection models. Peptic ulcers are one of the leading gastro-intestinal diseases and can often be attributed by the gram-negative bacteria *Helicobacter pylori*, which affects the duodenum and stomach by inducing inflammation, and increasing the risk of adenocarcinoma (89). A number of studies have investigated how PAC may alleviate infections with *H. pylori*. In a rat model of gastric ulcers, the beneficial modes of actions of PAC were attributed to elevated mucus secretion, increased recruitment of neutrophils and mast cells, as well as a thicker regenerative gastric mucosa (90). In pigs, provision of grapeseed PAC reduced infection with *Campylobacter jejuni*, with the effects ascribed to enhanced mucosal barrier function as a result of reduced ROS, and consequently less disruption of epithelial tight junctions (91).

In addition, the anaerobic and gram-positive bacteria *Clostridium perfringens* is the causative agent of necrotic enteritis in poultry. It primarily affects the jejunum and ileum, and causes important economic losses in the poultry industry (92). A study using concomitant infection of both coccidia and *C. perfringens*, demonstrated a significant decrease in the amount of intestinal lesions and mortality, when feeding PAC as a boosting agent to increase the efficacy of a vaccine (93), with the authors hypothesizing that PAC may be capable of simulating both cytotoxic and T-helper cells during infection.

Whilst these studies have demonstrated that dietary PAC may help to protect against pathogenic bacterial infections, Forgie *et al. (94)* recently reported that mice fed a PAC-rich diet were more susceptible to infection with *Citrobacter rodentium* (a model for *E. coli* infection in humans). These authors speculated that a reduction in GM diversity stemming from PAC consumption deprived the mucosal barrier of protection from commensal bacteria that could prevent *C. rodentium* attachment. In addition, it could be postulated that PAC may inhibit the production of inflammatory cytokines, such as IL-6, which are important for defense against *C. rodentium* (73, 95). Thus, the immunomodulatory effects of PAC could, in some contexts, render hosts more susceptible to infection if a strong inflammatory response is necessary to clear the pathogen. Thus, careful appraisal of the effects of PAC in different host-pathogen systems is required to determine their potential benefits. Interestingly, treatment of mice with extracts from pomegranate that contain hydrolysable tannins (as opposed to PAC, or condensed tannins) reduced *C. rodentium* infection and also appeared to alleviate some of the dysbiotic effects induced by the infection (96). Thus, exploration of structure-function relationships of different polyphenols and how these relate to regulation of responses to bacterial infections are clearly required. Moreover, studies to determine whether there are differences in the ability of PAC to regulate chronic, low-grade infections, or acute infections accompanied by significant epithelial inflammation are highly warranted.

Parasitic Infections

Recent estimates are that more than a billion people are infected by intestinal worms (helminths), making parasite infections one of the most common infections worldwide, and causing substantial morbidity (97). Parasitic infections also cause significant economic losses in farming industries. Helminths are also ubiquitous in livestock where they cause reduced performance and as well as clinical disease (98). In addition, protozoan parasites such as *Giardia* are extremely common in tropical regions, and related parasites such as *Eimeria* are the cause of coccidiosis which is a major cause of clinical diarrhoea in animals such as chickens and calves (99). Unlike bacteria and viruses, immunity to helminth infection is critically dependent on Th2-driven immune responses. IgE production, eosinophilia and production of type-2 cytokines such as IL-13 and IL-33 are all hallmarks of helminth infection (100). In natural infections, mixed Th1/Th2 responses are induced. Modulation of this balance can either induce Th1-driven immunopathology with tissue necrosis, pro-inflammatory responses and chronic infection, or Th2-related protective immunity involving remodeling of the intestinal barrier and mucus secretion that results in parasite expulsion (101).

It has been repeatedly shown that high levels of PAC in the diet may help animals cope with helminth infection. Interestingly, animals have a natural preference for specific plants when infected with parasites, and self-medicate by ingesting secondary plant metabolites, including PAC (102). Worms recovered from sheep or cattle consuming forage pastures rich in PAC (e.g. from the Fabaceae family) are smaller and less fecund than worms from control-fed animals, indicating a beneficial effect of PAC on response to infection (103), and *in vitro* experiments have shown that PAC can directly bind to helminths and reduce their survival (104, 105). More recently, a number of studies have shown that PAC can substantially alter the immune response during helminth infection, suggesting that host resistance to infection can be enhanced. *In vitro*, DCs exposed to PAC have an enhanced ability to drive helminth-induced Th2 responses in naïve T-cells, suggesting that parasite-specific immune function can be enhanced during infection (73). Consistent with this, helminth-infected animals consuming PAC have higher numbers of Th2-associated mucosal eosinophils and mast cells, as well as parasite-specific antibodies and γδ T-cells (64, 106–110). Heightened Th2 polarization may result from the selective down-regulation of pro-inflammatory Th1 responses by PAC (73). Alternatively, direct stimulation of gut epithelial cells, either by parent PAC molecules or GM-derived metabolites, may enhance innate defenses that favor anti-helminth immunity (e.g. goblet cell responses). Beneficial effects of PAC are not confined only to helminth parasites. Poultry infected with *E. tenella* and fed with PAC have significantly decreased mortality rates and increased weight gain, which was linked to amelioration of oxidative stress caused by infection (111).

Collectively, these studies suggest that PAC may offer multifaceted benefits to parasitized hosts. These may include a reduction in parasite fitness, as well as augmentation of immune responses that may suppress harmful inflammation, oxidative stress and pathology, whilst promoting immune responses that fortify and repair the mucosal barrier, thus protecting from secondary bacterial infections that are also a feature of worm infections (112). Further research is needed to understand these effects, and helminth infection models may offer a valuable model for assessing the ability of PAC to modulate naturally induced type-2 mucosal immune responses.

Viral Infections

Enteric viruses are mainly transmitted *via* the fecal–oral route, either by person-to-person contact or by the ingestion of contaminated food or water (113). The most important viruses causing gastroenteritis in humans include norovirus and hepatitis A (114). In addition, sapoviruses, rotaviruses, coronavirus, astroviruses, and hepatitis E virus (HEV), are also known causes for enteric diseases (113).

Research into the potential role of PAC on combating enteric viral infections is in its infancy. Several studies have investigated the ability of PAC to neutralize viruses *in vitro*. PAC derived from persimmon were shown to inactivate 12 pathogenic viruses,

including rotavirus (115). Similar effects with other PAC preparations have been established in other *in vitro* studies, which demonstrated anti-viral effects of PAC against the norovirus surrogates Bacteriophage MS2, murine norovirus (MNV-1), and Feline Calicivirus (FCV-19), as well as Hepatitis A and coxsackievirus (114, 116).

The ability of PAC to modulate immune responses to viral infection have also been investigated *in vitro*. Exposure of human PBMC infected with dengue virus to oligomeric PAC resulted in enhanced type-1 interferon responses, and lower viral titers, suggesting that PAC can directly impact on the intracellular response to viral infection (117). Consistent with this, consumption of apple-derived PAC by mice also resulted in augmented type-1 interferon activity (118). Some limited clinical evidence exists in support of this from a study where prolonged consumption of PAC-rich cranberry juice reduced influenza symptoms (83). These results imply that PAC have the ability to activate innate immune effector mechanisms that target intracellular viruses, similar to the triggering of defense molecules such as mucins in the gut mucosa, suggesting a highly conserved response whereby sensing of PAC leads to a rapid response designed to protect against pathogens. Importantly, PAC also appear to down-regulate excessive inflammatory cytokine production in response to viral infection which, in many cases, is responsible for the pathology and clinical symptoms (119). However, further work is clearly needed with detailed *in vivo* studies to explore if dietary PAC can effectively modulate immunity and inflammation during enteric viral infection.

CONCLUDING REMARKS

A vast number of studies have now documented that, *in vitro*, PAC possess anti-oxidant, anti-bacterial, anti-parasitic, and anti-viral effects. Moreover, the ability of dietary PAC to modulate the GM and protect against obesity and metabolic syndrome is becoming well-established. Whilst there is clear evidence that dietary PAC affect immune function, our understanding of the implications of this is only just starting to be formed. The balance between effective immune responses and harmful immunopathology is a fine one. Dietary supplements that induce immunosuppressive effects may be beneficial for autoimmune disease, but may in turn be detrimental against infectious diseases by potentially giving rise to increased susceptibility of infection. Understanding the mechanisms leading to the anti-inflammatory effects of PAC may therefore lead to better decision-making on how/when to use PAC during aberrant immune responses towards pathogens, while not supressing the vital immune components required for overcoming infections. It has also become clear that development of infection is highly dependent on complex host-pathogen interactions, and future studies should consider the implications of PAC during the early and late stages of acute or chronic enteric disease. This would broaden the possible applications of PAC throughout the course of disease, as well as elicit further investigations into the modes of action of PAC as the gut homeostasis alters during disease progression. The possible benefits of PAC may also be explored prior to infection, to unravel its potential at priming the gut environment towards enhanced immunity, or post-infection where PAC may improve healing of the gut mucosal barrier.

Further understanding the mechanisms of how PAC modulate the activity of immune cells will be crucial to further optimize their use as health-promoting dietary additives. Key questions to be addressed include determining the specific pathways that are modulated in immune cells such as macrophages after exposure to PAC—does their anti-inflammatory activity stem purely from antioxidant effects or do PAC specifically alter key intracellular signalling pathways? How much of the observed immunomodulatory activity of PAC is due to direct effects of parent PAC compounds on mucosal immune cells, compared to the activity of GM-derived metabolites that are formed during breakdown of PAC in the colon? Which specific metabolites can alter the activity of immune cells and gut barrier function? Can PAC directly kill pathogens in the gut, or are beneficial effects purely related to effects on host barrier function and immunity? A detailed understanding of how PAC influence immune responses towards different pathogens that induce type-1 responses (intracellular bacteria), type-2 response (helminths), or type-3 responses (extracellular bacteria) will also be important if PAC-rich dietary components are to be strategically used to combat enteric infections in human or veterinary medicine. Indeed, PAC may prove to be a useful model for answering basic questions about the regulation of mucosal immunity by dietary compounds. However, the lack of standardized methods in the purification techniques of PAC limits the comparison of data between studies. Also, most *in-vivo* studies make use of PAC-rich extracts, which may give rise to misleading conclusions regarding the isolated effect of PAC. Future studies should therefore aim at finding a general consensus as to how to isolate chemically identical PAC molecules. This is crucial, as numerous studies have demonstrated that there are substantial differences in bioactivity of PAC subtypes (based on DP or monomeric subunits) (73, 74). Mechanistic studies could consequently attribute a specific mode of action to distinct PAC molecules. This would aid minimizing the considerable variability observed between research groups working with isolated PAC molecules of various purity compared to plant extracts rich in PAC among other phenolic compounds. Increasingly rigorous methods will need to be applied, including the use of germ-free animals and other experimental approaches to disentangle the role of the GM in the immunomodulatory activity.

In conclusion, PAC seem to be a promising group of natural dietary compounds that can regulate inflammation. Emerging evidence exists that mucosal immunity to pathogen infection can be enhanced by PAC. Specific mechanisms identified include the priming of innate defenses in mucosal epithelial cells and leukocytes as well as promoting antibody and balanced lymphocyte responses through regulation of inflammatory responses during infection. A multidisciplinary approach

involving phytochemistry, microbiology and immunology will be necessary to understand the interactions between PAC and the immune system, and lead to the full exploitation of defined PAC molecules as health-promoting dietary agents.

AUTHOR CONTRIBUTIONS

AA-C, PA, and AW wrote the paper. AW revised the paper. All authors contributed to the article and approved the submitted version.

FUNDING

The authors were supported by the Independent Research Fund Denmark (Grant # 7026-00094B). The funding body had no role in the preparation of the manuscript or decision to publish.

ACKNOWLEDGMENTS

Figures were created with the aid of BioRender.com.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper II

Structure-function Analysis of Purified Proanthocyanidins Reveals a Role for Polymer Size in Suppressing Inflammatory Responses

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Submitted Manuscript under review in Communications Biology

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Abstract

Proanthocyanidins (PAC) are dietary compounds that have been extensively studied for beneficial health effects due to their anti-inflammatory properties. However, the structure-function relationships of PAC and their mode-of-action remain obscure. Here, we isolated a wide range of diverse PAC polymer mixtures of unprecedented purity from plant material. Polymer size was a key factor in determining the ability of PAC to regulate inflammatory cytokine responses in murine macrophages. PAC polymers with a medium (9.1) mean degree of polymerization (mDP) induced substantial transcriptomic changes, whereas PAC with either low (2.6) or high (12.3) mDP were significantly less active. Short-term oral treatment of mice with PAC modulated gene pathways connected to nutrient metabolism and inflammation in ileal tissue in a polymerization-dependent manner. Mechanistically, the bioactive PAC polymers modulated autophagic flux and directly inhibited lipopolysaccharide-induced autophagy. Collectively, our results highlight the importance of defined structural features in the health-promoting effects of PAC-rich foods.

Keywords

Proanthocyanidins; V-ATPase; Autophagy; Inflammation; Polymerization; Macrophage

Introduction

There is currently intense interest in elucidating how dietary components may influence health and disease, in part due to increasing prevalence of chronic inflammation in humans throughout the world [1,2]. Plant bioactive components found in the diet that may modulate inflammation include carotenoids, plant sterols, glucosinolates, and polyphenols [3]. Polyphenols represent one of the major groups of natural antioxidants with more than 8000 polyphenolic compounds identified in various plant species [4]. They are specialized plant metabolites and are commonly found in the human diet, especially in fruits and vegetables [5,6]. Polyphenols act as the active component in many commercialized plant preparations, and have been associated with beneficial effects on health [3,6]. Epidemiological and experimental studies have thus investigated how polyphenols may have protective properties against cardiovascular diseases, cancer, diabetes, neurodegenerative disease and microbial infections, among other diseases [4,7,8].

Proanthocyanidins (PAC), also known as condensed tannins, are among the most common dietary polyphenols [9]. They are characterized by their high molecular weight, and consist of oligomers and polymers of flavan-3-ol monomeric sub-units [6,10]. Flavan-3-ols are formed by two phenyl rings, A and B, and one heterocyclic ring C, with different hydroxylation patterns of rings A and B [11,12]. They may also display gallic acid residues attached by an ester bond to the C-ring hydroxyl, as seen in catechin gallates [13]. The four most common flavan-3-ols are (+)-catechin and its *cis* isomer (-)-epicatechin, and (+)-gallocatechin and its *cis* isomer epigallocatechin [10], which can be joined by inter-flavanol bonds to form oligomers or polymers. When part of an oligomeric or polymeric PAC structure, (epi)catechins are known as procyanidin (PC) units and (epi)gallocathechins are known as prodelphinidin (PD) units (**Figure 1A)**. Depending on the nature of their linked flavan-3-ol subunits, PAC are thus referred to as either procyanidin (PC)-type PAC, which are the most common type of PAC, prodelphinidin (PD)-type PAC, or as PC/PD mixtures [10,12]. PAC are further characterized based on their galloylation and polymerization. The percentage of galloylation describes the proportion of galloylated monomers, while the number of monomeric sub-units dictates the degree of polymerization [13]. The polymer chain lengths of PAC molecules vary greatly depending on the plant sources and thus, mean degree of polymerization (mDP) is a commonly used measure for the average number of monomeric sub-units.

Inflammation is a natural response to harmful stimuli, and results in the secretion of several cell-derived mediators, including cytokines, prostaglandins and reactive oxygen species (ROS) to protect cells and tissues. The detrimental effects of inflammation arise when it perseveres and causes permanent damage of the tissues due to homeostatic imbalance of the immune regulatory functions [5]. Numerous studies have shown that PAC can have beneficial effects on inflammatory and metabolic disorders, such as obesity and inflammatory bowel disease [14,15]. Furthermore, epidemiological studies recorded lower incidences of cancer, atherosclerosis and metabolic syndrome in human populations consuming procyanidin-rich foods, although conclusive evidence is still lacking to support beneficial effects on human health [8,16]. The mechanisms underlying the anti-inflammatory effects of PAC are unclear, but are speculated to include direct interactions between PAC molecules and immune cell

functions and pathways, as well as a potential prebiotic effect on the gut microbiota. There is a general consensus that PAC molecules remain stable in the stomach, and that only monomers are absorbed in the small intestine [17,18]. Thus, the majority of ingested PAC reach the colon, where they may be metabolized by the gut microbiota[19,20]. Moreover, polymeric PAC remain at the lumen-mucosal interface where they may also directly interact with epithelial or immune cells. Several studies have shown that PAC can suppress several inflammatory markers in macrophages and intestinal epithelial cells *in vitro*, such as ROS and tumor necrosis factor-α (TNF-α), as well as the expression of inducible nitric oxide synthase (iNOS) and cyclooxynase-2 proteins, and thereby suppressing nitric oxide (NO) and prostaglandin E_2. This may be due to the downregulation of the p38 mitogen-activated protein kinase (MAPK) and Akt signaling pathways, leading to the inhibition of the transcription factor NF-κB, which is important for the regulation of genes involved with inflammation [21–24]. The direct effects of PAC on other cell lines, including lymphoid cell lines and peripheral blood mononuclear cells, has also been demonstrated by their ability to down-regulate transcription and secretion of pro-inflammatory cytokines and to up-regulate the secretion of anti-inflammatory cytokines [12,22,25,26]. Furthermore, anti-inflammatory properties of PAC have also been described in animal models of various diseases, where dietary PAC have been shown to modulate NF-κB sigaling and inhibit the production of ROS and pro-inflammatory cytokines such as IL-6 and TNFα [14,15,27–29].

Despite these documented effects of PAC on inflammatory activity, mechanistic and structure-function activity studies are limited. Investigation of the bioactivity of PAC is challenging due to the complexity of the PAC molecules and associated difficulties with characterization and purification. The isolation of PAC with high polymerization remains a challenge due to the difficulties related to separating polymeric molecules of different sizes from each other. Thus, structure-function relationship studies with PAC have been limited to relatively crude fractions consisting of mixtures of low or high mDP. Studies utilizing such fractions have suggested that the degree of polymerization of PAC may be an important parameter for the outcome observed *in vitro* and *in vivo*, with higher mDP often correlating with higher biological activity [22]. For example, γδ T-cells are more effectively activated by PAC fractions with an mDP higher than six [30], and inhibition of lipopolysaccharide (LPS)-induced cytokine production in macrophages also appears to correlate with increasing mDP [22]. In addition, the PC/PD ratio is thought to play a role for the activity of PAC, potentially due to the ability of PD-type polymers to form greater numbers of hydrogen bonds with proteins because they have more hydroxyl groups than PCs [31].

Recently, we developed a new method that splits the characteristic PAC polymeric mixtures into precise fractions by Sephadex LH-20 fractionation followed by semi-preparative liquid chromatography (LC) [32]. This resulted in chemically well-characterized PAC samples with higher purity, differing mDPs and PC/PD ratios, and thus allowed a better comprehension of the relationship between the structure and bioactivity of PAC. Here, we investigated how highly purified PAC with differing structural characteristics may affect inflammatory responses. We utilized interleukin 6 (IL-6) production from LPS-activated RAW 264.7 macrophages as a screening tool to assess structure-function relationships of PAC isolated from two plant sources in unprecedented detail. Combined

with *in vitro* and *in vivo* transcriptomics after exposure to different PAC polymers, we identified novel relationships between PAC structure and bioactivity that may have broad implications for the development of novel, PAC-based dietary supplements.

Material and Methods

Samples and materials

The samples and materials used for this study are depicted in **Supplementary Table 1.**

Purification of Proanthocyanidins

Extraction of PAC from plant material was performed in accordance with the protocol from Leppä et al. [32]. In brief, we conducted a series of extraction with acetone, followed by Sephadex LH-20 separation, and semi-preparative LC. For this study, the method was applied for the isolation of PAC from alpine currant (AC) and grape pomace (GP), and an overview of the purification steps is described in **Supplementary Table 2.**

Extraction

The solid phases from AC and GP were stored at 4 °C in 80 % analytical acetone with shaking. The samples were filtered through a Whatman filter paper in a Büchner funnel, and the liquid phase was transferred to a container and placed for evaporation overnight (O/N). Following evaporation, the extracts were filtered again 1-3 times through a Whatman filter paper in a Büchner funnel and the water phase was stored at -20 °C.

The remaining plant material was immersed in 1 L of 80/20 acetone/water (v/v) and stored at 4°C with shaking O/N for further extractions. A total of five extractions from the plant materials were performed and each sample was analyzed by UPLC-MS/MS [33]. The chromatograms of the GP samples depicted high similarities between the samples, which were thus pooled together. Likewise, the samples derived from AC were pooled and liquid-liquid extraction with 300 ml ethyl acetate followed by liquid-liquid extraction with 300 ml butanol was performed. Finally, the solvents were removed from the samples by rotary evaporation.

Sephadex LH-20 fractionation

The samples were mixed with Sephadex LH-20 material by magnetic stirring O/N. Following O/N mixing, the solution was poured into a Büchner funnel and all liquid material was filtered through a Whatman filter paper. A series of filtrations through the Sephadex material was performed for each sample, with 5 fractions of 200 ml milliQ water, 5 fractions of 200 ml methanol and 5 fractions of 200 ml of 80/20 acetone/water (v/v). A final fraction of 400 ml acetone was also collected to wash the Sephadex material for eventual remaining compounds. In order to remove the solvents from the samples prior to freeze-drying, O/N evaporation and rotary evaporation was applied to the samples containing acetone and methanol, respectively. After the Sephadex LH-20 fractionation, a comprehensive analytical characterization was performed by UPLC-MS/MS, and the samples were subsequently freeze-dried and weighed. Eventually, the methanol and acetone fractions were

selectively pooled based on the similarity of their respective chromatograms. This resulted in 3 Sephadex AC fractions and 8 Sephadex GP fractions with weights varying between 400-1500 mg.

Semi-preparative liquid chromatography

The 11 Sephadex fractions were further purified by semi-preparative LC utilizing similar fractionation procedure and instrumentation as described by Leppä et al. [32]. In short, approximately 100-120 mg of each Sephadex fraction was fractionated via gradient elution utilizing 0,1/99,9 formic acid/water (v/v) and acetonitrile as eluents. The gradient elution started with isocratic 5% acetonitrile following a gradient from 8% to 55% acetonitrile at 4–32 mins and from 55% to 80% acetonitrile at 32–35 mins and lastly finishing with column wash (80% acetonitrile) and stabilization. The flow state was set to 12 mL/min and the column used was a 150 × 21.20 mm, Gemini®10 µm,C-18, 110 Å, Axia packed, Phenomenex. The samples were collected from 5.0 to 33.0 mins into 2 mL Eppendorf tubes resulting in total of 168 individual tubes. The chromatographic PAC mixture (at 280 nm) was integrated from 5 to 33 mins via TargetLynx software (V4.1 SCN876 SCN 917© 2012 Waters Inc) and the total integrated are was divided into 8 equal parts (**Supplementary Figure 6**). The 2 mL Eppendorf tubes were then pooled accordingly resulting in total of 8 semi-preparative samples per Sephadex fraction. All 88 semi-preparative samples (24 AC and 64 GP semi-preparative samples) were analyzed via UPLC-MS/MS [33] for their PC/PD composition and mDP. Semi-preparative samples were brought to water phase and freeze-dried. The weights of the samples derived by semi-preparative LC varied between 2-17 mg.

UPLC-MS/MS analyses

UPLC-MS/MS analyses were carried out with similar instrumentation and methodology as described by Engström et al. [33]. The quantitation of the PC and PD units as well as the calculation of mDP were performed with the Engström method [33] as described by Malisch et al. ³⁴. Similar standards were used for the quantitation of PC and PD as well as calibration curve for the calculation of the mDP as described by Leppä et al. [32]. The stability of the mass analyzer response was monitored with frequent injections of 1 µg/mL catechin [61].

Cell culture

RAW 264.7 murine macrophages (ATCC TIB-71) and RAW-Difluo^{TM} mLC3 cells (mLC3, InVivoGen) were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. mLC3 cells are used to report autophagosome maturation and express chimeric proteins consisting of a red fluorescence protein (RFP), a green fluorescence protein (GFP), and a membrane-bound LC3 protein [62]. The RFP is resistant to acidic environment of the autophagolysosome, while dual red and green fluorescent RFP::GFP::LC3 puncta represent the early stages of the autophagic flux. Low passage numbers (<20) were used for all experiments. When culturing mLC3 cells, the media was supplemented with Normocin (100 µg/ml), and Zeocin (200 µg/ml, every 2nd passage). For experimental purposes, neither Normocin or Zeocin was added to the media.

Cytotoxicity testing

Cytotoxicity of the isolated PAC used for the stimulation of murine macrophages was assessed using neutral red assays (Sigma-Aldrich) according to the manufacturer's instructions.

***In vitro* screening of anti-inflammatory activity of proanthocyanidins**

Macrophages were plated out on 96-well plates at a concentration of 2.5 x 10^5 cells/ml. Cells were allowed to adhere for 2 hours before LPS stimulation (500 ng/mL). Where appropriate, cells were pre-incubated with AC or GP PAC or vehicle control (PBS) 30 minutes prior to LPS addition. After 24 hours of incubation, the supernatant was collected and frozen at -20 °C, and IL-6 secretion was assessed by ELISA (R&D Systems). Each semi-prep sample (15 µg/ml) was tested in triplicates, and at least two independent experiments were performed. For equimolar tests, the molarity of samples was calculated based on PC/PD ratios and mDP.

Stimulation with Bafilomycin A1

As described above, murine macrophages were plated out on 24-well plates at a concentration of 2.5 x 10^5 cells/ml. Cells were allowed to adhere for 2 hours before they were treated in the following order with Baf (10 nM), PAC (15 µg/ml), and LPS (500 ng/mL) with 30 min incubation time between each treatment. Stimulated cells and appropriate controls were left for incubation for 6 hours before RNA extraction.

Assessment of autophagy by flow cytometry

Autophagy in RAW 264.7 macrophages was assessed by measuring autophagic vacuoles with the Autophagy Assay Kit (Abcam ab139484) according to the manufacturer's protocol. In brief, cells were plated out in a 96-well plate and stimulated with either Baf (10 nM) or GP PAC (15 µg/ml) followed LPS stimulation (500 ng/mL) where appropriate. After 20h of incubation, the cells were trypsinized, washed by centrifugation and the pellet was re-suspended in indicator free cell culture medium containing 5% FBS. The cells were then incubated with staining solution for 30 minutes at RT in the dark, followed by a series of washing steps, before analysis by flow cytometry.

Preparation of mLC3 cells for fluorescence microscopy

mLC3 cells were plated out on 6-well plates containing cover slips, at a concentration of 2.5 x 10^5 cells/ml. Cells were allowed to adhere for 2 hours before LPS stimulation (500 ng/mL). Where appropriate, cells were pre-incubated with either Baf (10 nM) or GP PAC (15 µg/ml) 30 minutes prior to LPS addition. After 18 hours of incubation, the cells were washed twice with PBS and fixed for 15 min with 4% formaldehyde, followed by another washing step with PBS. Finally, the coverslips were recovered and placed onto a slide for microscopy using a Leica DM 5000B Fluorescence Microscope. Images were prepared using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Image processing consisted only of adjustment of brightness and contrast. In order to ensure adjustments were equally applied to all samples, images were processed in stacks.

Transmission Electron Microscopy

Murine macrophages were plated out on 24-well plates at a concentration of 5 x 10^5 cells/ml. Cells were allowed to adhere for 2 hours before they were treated as appropriate in the following order with Baf (10 nM), and/or GP PAC (15 µg/ml), and/or LPS (500 ng/mL) with 30 min incubation time between each treatment. The cells were incubated for 24h before fixation and preparation for transmission electron microscopy (TEM). Cells were fixed with 3% glutaraldehyde (Merck, 1042390250) in 0.1 M mNa-phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in 0.1 M Na phosphate buffer, dehydrated stepwise in a graded ethanol series, intermediate solution propylene oxide followed by pure epon O/N and next day, embedded in Epon (TAAB, T031). Semi-thin (2 µm) sections were cut with glass knifes (KnifeMaster II, LKB Bromma 7800) on an ultramicrotome (Leica Ultracut, Leica Microsystems, Wetzlar, Germany), stained firstly with 1% toluidine blue (VWR 34187.185) in 0.1% Borex (VWR 27727.231). Ultra-thin (50 nm to 60 nm) sections were sectioned with a diamond knife (Jumdi, 2 mm) on an ultramicrotome (Leica Ultracut), contrasted with 2% uranyl acetate (Polyscience, 21447) and lead citrate(Reynold, 1963), and examined using a Philips CM100 transmission electron microscope operating at 60 kV. Photographs were taken using Olympus Morada 11 megapixel camera and iTEM software (Olympus).

RNA extraction from macrophages and transcriptomic analysis

LPS-activated and non-activated RAW 264.7 macrophages were cultured with appropriate treatments as described above, and harvested for RNA extraction after 6 hours of incubation using RNAeasy kits (Qiagen). cDNA was synthesized from 500 ng of RNA using Quantitect Reverse Transcriptase kits (Qiagen). Transcriptomic analysis was performed using the GeneChip WT PLUS Reagent kit (Thermo Fisher Scientific, CA, USA) and Affymetrix mouse Clariom S HT 24-array plate pipeline (Eurofins AROS, Denmark), with array plate processing carried out on a GeneTitan Instrument (Thermo Fisher Scientific). Transcriptome Analysis Console (TAC) software (Thermo Fisher Scientific) was used to analyze microarray data, and additional pathway analysis performed using Gene Set Enrichment Analysis software (GSEA; Broad Institute, MA, USA), using values of FDR q-value <0.2 and nominal p-value <0.02 to assess significance. qPCR was performed using perfeCTa SYBR green fastmix (Quanta Bioscience) using the following program: 95°C for 2 minutes followed by 40 cycles of 15 seconds at 95°C and 20 seconds at 60°C. Primer sequences are listed in **Supplementary Table 3.**

Treatment of mice with PAC fractions

6-week old female C57BL/6JOlaHsd mice (Envigo, the Netherlands) were distributed into 3 groups of 5 mice. The mice were allowed 1-week of acclimatization, and were fed with a purified control diet (13 kJ% fat; ssniff Spezialdiäten GmbH, Germany) throughout the entire study. Each group was orally gavaged every second day for 10 days with either low mDP GP PAC, medium mDP GP PAC (200 mg/kg BW) sephadex fractions dissolved in distilled water, or water alone. Mice were euthanized by cervical dislocation, and tissue collected from the ileum was preserved in RNAlater for RNA-sequencing and qPCR. Primer sequences are listed in **Supplementary Table 3.**

RNA-sequencing of mouse intestinal tissue

RNA was extracted from ileal tissue as described above. Libraries were prepared and sequenced (paired-end reads of 100 bp) on a BGISEQ-500 sequencer (BGI, Copenhagen, Denmark). Quality control was performed using SOAPnuke v1.5.2 (github.com/BGI-flexlab/SOAPnuke) to remove adaptors, reads with unknown bases more than 10%, and low quality reads. Clean reads were mapped to the mouse genome (*mm10*) using Bowtie2 (v2.2.5). Differentially expressed genes were detected using NOIseq, using a Q-value for significance of >0.8 and a fold change of at least two[63]. Pathway analysis was conducted using GSEA. Predicted genes and those with no gene symbol assigned were excluded from analysis. Principal Component Analysis was performed using ClustVis [64]. For qPCR validation, cDNA synthesis and PCR was performed as above.

Statistical analysis

Data analysis was performed using the statistical softwares GraphPad Prism 7 (GraphPad Inc.) and SPSS (IBM SPSS Statistics 27) . D'Agostino-Pearson and Shapiro-Wilk normality tests were used to assess data sets for normal distribution. Parametric data was analyzed using t-tests or one-way ANOVA analysis and Tukey's multiple comparisons test. If the data did not follow a Gaussian distribution, non-parametric tests (Mann-Whitney tests, or Kruskal-Wallis tests followed by Dunns post-hoc test) were applied. Regression analysis was conducted using the curve-fit option in SPSS, applying both linear and quadratic analyses.

Results

Purification of PAC and analysis of structural features

As a first step in elucidating the structural characteristics that govern the bioactivity of PAC, we selected two plant sources to serve as source material for isolation of purified fractions. In order to assess the relative importance of PC or PD units, we selected plants that contain either PC rich PC/PD mixtures (hereafter PC-type PAC) or PD rich PC/PD mixture (hereafter PD-type PAC). Grape (*Vitis vinifera*) pomace, hereafter GP, is a by-product of wine production that is rich in PC-type PAC. In contrast, alpine currant (*Ribes alpinum*), hereafter AC, is a temperate herb found in Northern Europe that primarily contains PD-type PAC. Thus, material from these two distinct plant sources allowed us to derive pure PAC samples mainly consisting of either PC- or PD-type polymers, which we further sought to fractionate into samples of a precisely defined mDP. PAC were extracted with acetone/water (80/20, v/v) and fractionated by Sephadex LH-20 gel chromatography. Semi-preparative LC was subsequently used to derive 24 and 64 highly-purified samples from AC and GP, respectively [32]. UPLC-MS/MS analysis of each of the isolated PAC samples was used to assess structural features [33]. The samples derived from AC were 81-99% PD-type PAC with mDP values ranging between 3.2 and 12.1. In contrast, samples derived from GP were 78-96% PC-type PAC with mDP values ranging between 2.4 and 13.2 (**Figure 1B and C**). Thus, our technique successfully isolated highly purified PAC covering a wide range of mDP.

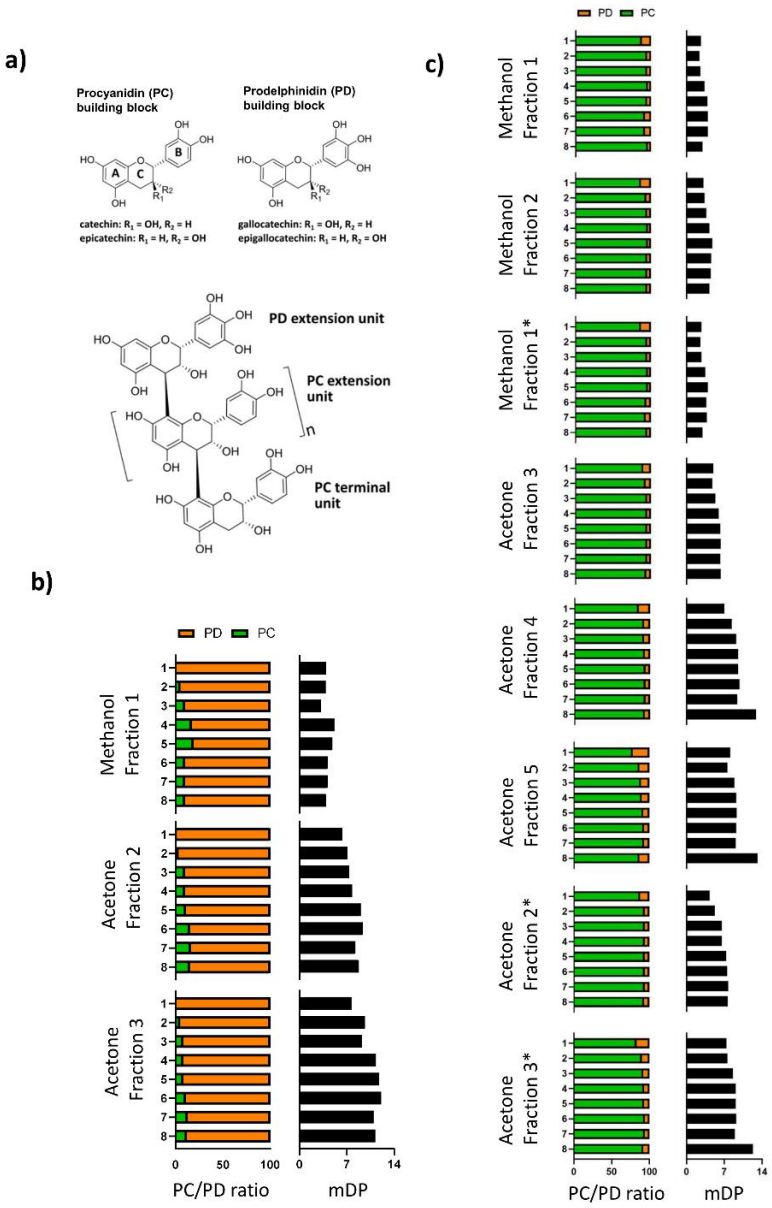

Figure 1 - Molecular characteristics of proanthocyanidins

a) The structures of (epi)catechin and (epi)gallocatechin, which give basis to the most common proanthocyanidin (PAC) structural units, procyanidin (PC) and prodelphinidin (PD) units are displayed as single building blocks. The figure also depicts a model structure of an oligomeric PAC consisting of PD and PC extension units and a PC terminal unit (*n* = number of PC or PD extension units). PAC samples used in this study showed varying chemical characteristics, with samples extracted from **b)** alpine currant (*Ribes alpinum*) being rich in prodelphinidins (PD), whereas samples extracted from **b)** grape (*Vitis vinifera)* pomace were rich in procyanidins (PD). Samples were purified and extracted by Sephadex LH-20 fractionation with methanol followed by acetone. Further purification was conducted by semi-preparative liquid chromatography, resulting in 8 highly purified samples for each of the Sephadex fractions. Each sample was analyzed by UPLC-MS/MS Samples produced from grape pomace were prepared in two batches, thus fractions labeled with "*" represent the second batch of samples. mDP: mean degree of polymerization.

Reduction of IL-6 secretion in LPS-activated macrophages stimulated with PAC

To assess the relative ability of the isolated PAC to modulate inflammatory responses, murine RAW 264.7 macrophages were stimulated with LPS alone, or LPS combined with purified PAC derived from GP or AC. Samples were initially screened at concentrations of 3-50 µg/ml that resulted in no toxicity to the cells (**Supplementary Figure 1**), and was comparable to previous studies, which have demonstrated anti-inflammatory activity of PAC-rich extracts in cells at similar or higher concentrations [21,23]. PAC samples (15 µg/mL) derived by semi-preparative liquid chromatography significantly reduced LPS-induced IL-6 secretion. For GP, samples with low mDP (<4) inhibited IL-6 secretion inefficiently, with around 30% inhibition. Greater inhibition (up to 60%) was observed in samples with higher mDP, with a consistently high inhibition evident in a group of samples with mDPs between 5-10 (**Figure 2A**). However, increasing mDP above 10 did not enhance activity, but resulted in a decrease of IL-6 inhibition. Thus, the relationship between mDP and inhibition of IL-6 secretion followed a quadratic relationship (p <0.001 by regression analysis; **Figure 2A**). This was further demonstrated by grouping samples into three distinct groups with either low, medium or high mDPs which showed clearly that the samples with a medium mDP had a higher (p<0.01) inhibition of IL-6 secretion than those with a low or high mDP (**Figure 2B**). As the different samples were tested at equal w/v amounts, the results suggest that the mDP was an important contributor to the bioactivity. Results obtained from samples with differing mDP tested at equimolar concentrations of 7.8 µM (**Supplementary Figure 2A**), showed similar results, with medium mDP samples being most effective, confirming that the polymerization determined the activity. Overall, these results demonstrate that increasing the mDP of GP PAC enhanced anti-inflammatory activity up to a threshold, where after mDP no longer was associated with high activity.

Evaluation of the AC samples, comprised almost exclusively of prodelphinidin-type PAC, revealed a generally lower suppression of IL-6 secretion than that achieved by GP PAC across a similar range of mDP (**Figure 2C**). Again, the relationship between mDP and inhibition of IL-6 secretion was best described by a quadratic function, with the most active samples falling in the middle of the mDP range of around 6-9 (**Figure 2C**), which was further confirmed by analyzing clusters of low, medium and high mDP (**Figure 2D**). This was also true for AC samples with differing mDP tested at equimolar concentrations of 7.8 µM (**Supplementary Figure 2B**). However, we noted that the magnitude of the IL-6 secretion was generally less than that achieved by GP PAC.

Thus, these data demonstrate a clear relationship between mDP and anti-inflammatory activity, with a positive correlation up until a threshold after which activity declines with increasing mDP.

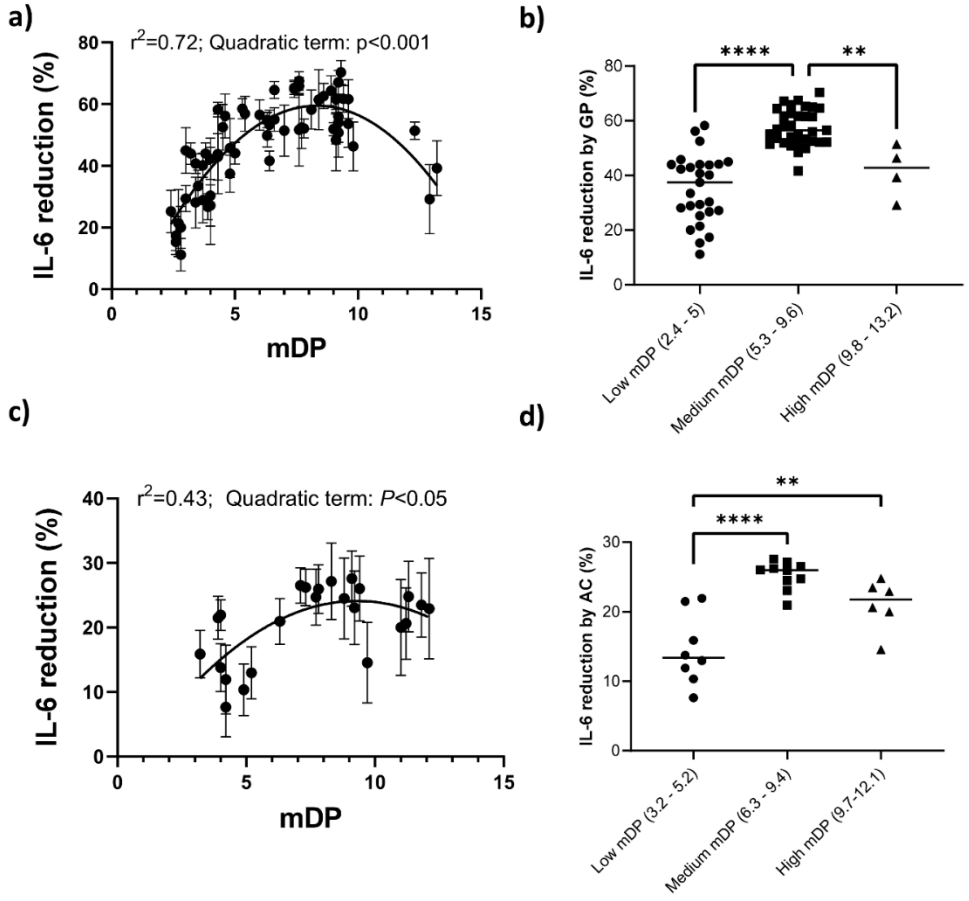

Figure 2 - Suppression of IL-6 secretion in lipopolysaccharide-activated macrophages by proanthocyanidins is influenced by polymerization

a) Proanthocyanidins (PAC) derived from grape pomace (GP) reduced IL-6 secretion in lipopolysaccharide (LPS)-activated macrophages, with the relationship between mDP and IL-6 reduction best described by a quadratic function (Quadratic regression with curve-fitting analysis). Each data point represents mean and S.E.M. of at least three independent experiments. **b)** IL-6 reduction in GP PAC samples stratified into low, medium and high mDP, (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA). **c)** PAC derived from alpine currant (AC) reduced IL-6 secretion in lipopolysaccharide (LPS)-activated macrophages, with the relationship between mDP and IL-6 reduction best described by a quadratic function (Quadratic regression with curve-fitting analysis). Each data point represents mean and S.E.M. of at least three independent experiments **d)** IL-6 reduction in AC PAC samples stratified into low, medium and high mDP, (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA). All samples were tested at 15 µg/mL.

Grape pomace PAC modulate macrophage transcriptional responses in a polymerization-dependent manner

To investigate the mechanistic basis by which PAC regulated macrophage function, three samples derived from GP by semi-preparative LC were selected for transcriptomic experiments. Cells were stimulated with samples with mDP 2.6 (low), 9.1 (medium) and 12.3 (high), respectively, and global transcriptomic profiles were determined 6 hours later. Principal component analysis demonstrated a strong influence of LPS (**Figure 3A**). Moreover, a clear effect of PAC was observed that correlated

closely with mDP. Consistent with the IL-6 inhibition data, the largest transcriptional changes were observed in cells exposed to medium mDP PAC, whereas PAC with high or low mDP were less effective at modulating the cellular responses both in resting cells and those activated with LPS (**Figure 3**). Low mDP PAC were particularly ineffective at inducing transcriptional changes, indicating that an mDP ≥ 3 is necessary for optimal regulation of inflammatory activity. Moreover, cells stimulated with the high mDP PAC were also not as differentiated from their respective controls as the cells stimulated with medium mDP PAC, indicating that increasing mDP beyond a certain threshold appears to decrease bioactivity.

Transcriptional responses in resting macrophages

In resting cells (no LPS), high mDP PAC treatment induced the expression of genes involved with inflammatory responses, such as cytokines from the interleukin 1 family (e.g. *Il1a*, and *Il1b*), as well as a number of inflammatory chemokines (*Cxcl2*, *Ccl22*, *Ccl3*) (**Figure 3B**). This was supported by pathway analysis suggesting that the interferon-signaling and inflammatory pathways were upregulated **(Figure 4A**). Similarly, resting cells stimulated with medium mDP PAC also showed an upregulation of chemokines (*Ccrl2, Cxcr4*) and interleukins. Consistent with this, the most upregulated pathway in resting cells stimulated with medium or high mDP PAC was the Erythrocyte Differentiation Pathway, which includes genes encoding colony stimulating factor (*Csf2* and *Cfs3*), interleukins (*Il1a, Il3, Il6, Il9,* and *Il11*) and transforming growth factors (*Tgfb1, Tgfb2, Tgfb3*). Furthermore, both samples decreased the REACTOME cholesterol biosynthesis pathway (**Figure 4A**), and upregulated the activating transcription factor 3 (*Atf3*). In contrast, treatment with low mDP PAC induced minimal transcriptional changes, with no significantly regulated gene pathways. Thus, in the absence of inflammatory stimuli, PAC with a medium-high mDP induced semi-maturation of resting macrophages, whereas PAC with low mDP lacked this stimulatory capacity.

Transcriptional responses in lipopolysaccharide-activated macrophages

To explore the mechanisms underlying the anti-inflammatory activity during LPS activation, we next investigated transcriptional responses in LPS-stimulated cells. Medium mDP PAC had the most prominent impact on gene expression levels, compared to low and high mDP PAC (**Figure 3C**). However, investigation of the top 15 regulated genes in LPS-activated macrophages showed that 25 % of the genes were regulated by at least two of the three samples (**Figure 3D**). Moreover, we found a large number of gene pathways to be regulated by medium mDP PAC in LPS-stimulated cells, whereas low and high mDP PAC regulated only two and one pathway, respecitveyly (**Figure 4B**). Despite the activation of some inflammatory pathways by PAC in resting cells, in LPS-stimulated cells we observed a strong suppression of multiple inflammatory pathways including JAK-STAT signaling, which was significantly downregulated in LPS-activated cells stimulated with medium mDP PAC (**Figure 4B**).

Another characteristic of the medium mDP PAC was its ability to upregulate pathways connected to lysosome function and transferrin endocytosis. The transferring endocytosis pathway was

upregulated regardless of LPS-activation (Figure **4A-B**). Genes in this pathway encode for numerous vacuolar-type H^+ATPases (V-ATPases) responsible for the acidification of phagolysosomes, allowing for endosome trafficking and neutralization of microorganisms [34–36]. Most of the upregulated V-ATPases belonged to the V1 Domain responsible for ATP hydrolysis, whereas fewer genes belonged to the V0 domain responsible for proton translocation. Of note, *Atp6v0d2* was the most upregulated V-ATPase gene in LPS-activated macrophages stimulated with low, medium, and high mDP PAC, and the expression level was also significantly increased in resting macrophages stimulated with medium mDP PAC **(Figure 3B and 3C).**

Both medium and high mDP PAC significantly downregulated the cytokine inducible SH2-containing protein (*Cish*), which negatively regulates cytokine signaling of the JAK-STAT pathway [37,38], and colony stimulating factor 2 (*Csf2*), which regulates function and differentiation of macrophages. Interestingly, all three samples significantly upregulated *Slamf8*, which has shown to negatively regulate inflammatory response, including *Nox2* activity in macrophages [39].

Collectively, these data show that the anti-inflammatory profile induced by all three samples was qualitatively similar, with a suppression of genes encoding inflammatory cytokine and signaling-related genes, and an up-regulation of genes involved in lysosome function. However, mDP had a substantial impact, with low mDP PAC being ineffective at regulating transcription, and medium mDP PAC clearly being the most efficient at regulating LPS-induced changes in gene expression.

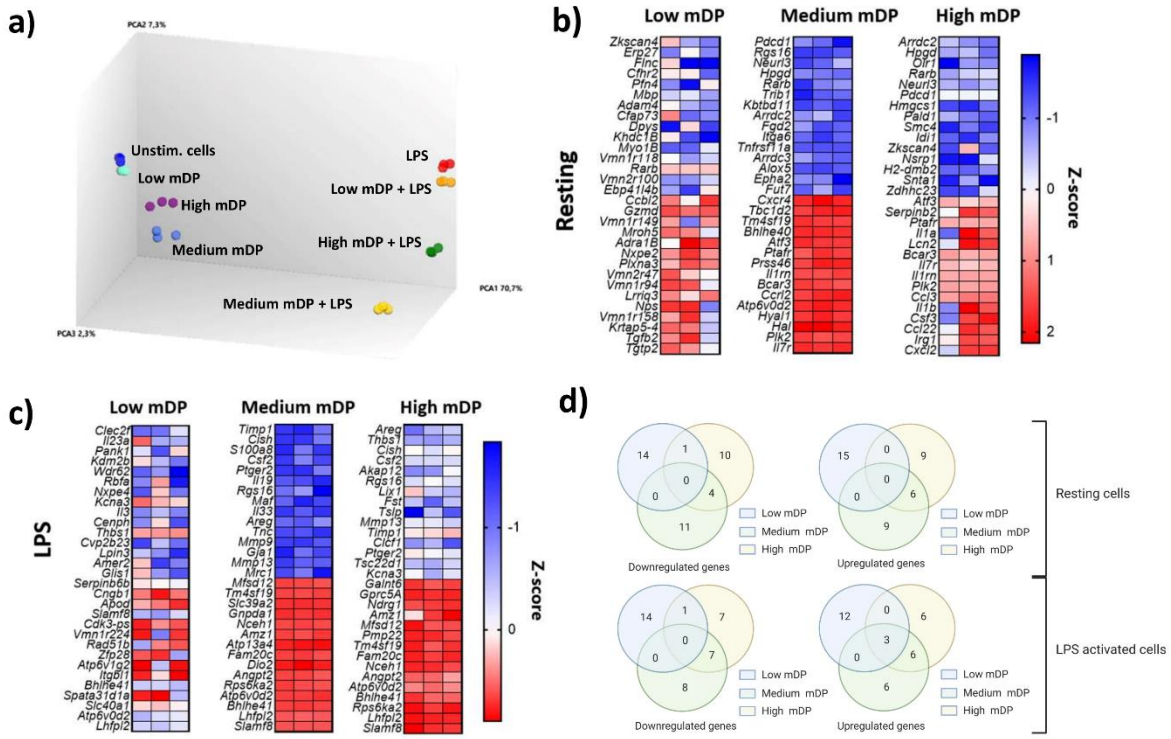

Figure 3 - Proanthocyanidin samples affected the regulation level of similar genes in macrophages in a polymerization-dependent manner

a) Principal Component Analysis plot showing a clear effect of lipopolysaccharide (LPS) and proanthocyanidins (PAC). Medium mDP PAC was the most differentiated treatment from respective controls with and without LPS. The most prominent transcriptional changes were observed in cells exposed to PAC with an mDP of 9.1, whereas PAC with a higher or lower mDP were generally less effective at modulating the cellular responses. **b)** Top 15 up-and down-regulated genes by PAC in resting cells (no LPS treatment). **c)** Top 15 up-and down-regulated genes by PAC in LPS-activated cells). **d)** The top 15 genes regulated by each PAC samples in resting or LPS-activated RAW 264.7 macrophages were investigated. Out of a total of 69 regulated genes, 25% were regulated by at least 2 of the PAC samples with differing mDPs in LPS-activated RAW 264.7 macrophages. Experiments were conducted with triplicate samples.

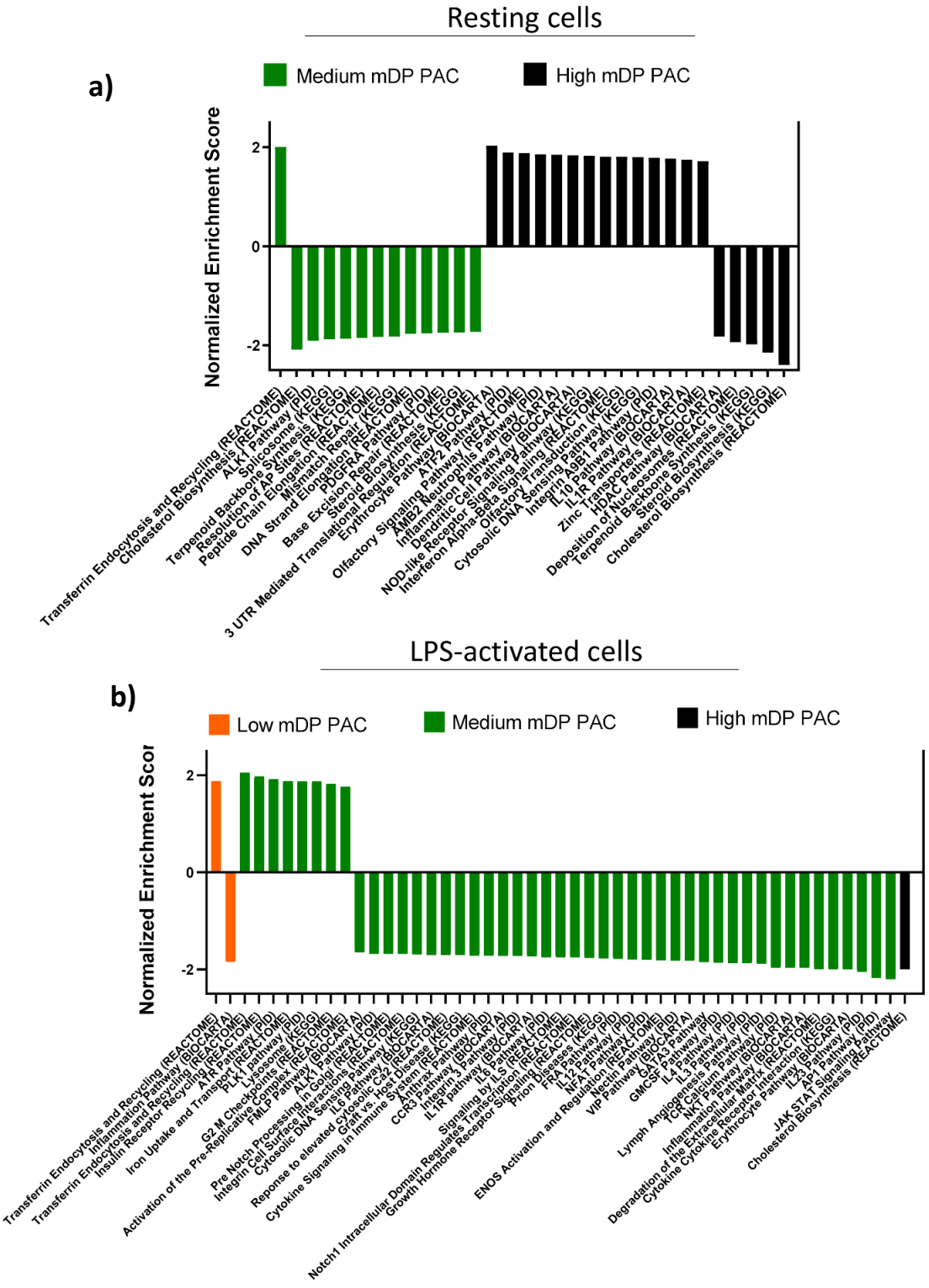

Figure 4 - Regulation of gene pathways in RAW 264.7 macrophages stimulated with proanthocyanidins with differing degrees of polymerization

a) Gene pathways regulated by medium mDP (9.1) and high mDP (12.3) grape pomace PAC in resting RAW 264.7 macrophages . **b)** Gene pathways regulated by low mDP (2.6), medium mDP (9.1), and high mDP (12.3) grape pomace PAC in lipopolysaccharide activated RAW 264.7 macrophages. Experiments were conducted with triplicate samples. Shown are significantly regulated pathways (p value <0.001; q value<0.15) identified by gene-set enrichment analysis.

Grape proanthocyanidins directly induce transcriptional changes *in vivo* in a polymerization-dependent manner

Having established that polymerization was an important factor for direct modulation of cellular responses by PAC, we next assessed whether *in vivo* administration of purified grape-derived PAC Sephadex fractions of differing mDP would also bring about direct transcriptional changes. To this end, mice were dosed repeatedly over a short time-period of ten days to assess responses in the intestine by RNA-sequencing. In mice dosed with low mDP PAC, only 2 significantly altered genes were identified in ileal tissue compared to the control group. In contrast, a total of 27 genes were significantly modulated in tissue from mice dosed with medium mDP PAC that had been shown to be highly active *in vitro* (**Figure 5A**). Principal component analysis also demonstrated a clear effect of medium mDP PAC, whereas low mDP PAC were not substantially differentiated from controls (**Figure 5B)**. Notably, in both groups, the most upregulated gene was *S100g*, encoding the S100 calcium binding protein G, which transports Ca^{2+} from the apical to the basolateral membrane of the enterocytes and sustains the essential intracellular concentration of calcium [40]. Other notable genes upregulated by medium mDP PAC included *Rspo1*, encoding R-spondin-1, a growth factor-like protein that induces crypt cell proliferation and promotes intestinal epithelial healing [41], and which has shown to be protective against intestinal damage and colitis. Downregulated genes were largely related to both inflammation and lipid metabolism, consistent with reports that acute PAC intake modulates bile acid metabolism and lipid homeostasis [42]. Notable genes included the Myd88-responsive anti-microbial peptide gene *Reg3g*, as well as the reactive free radical nitric oxide 2 (*Nos2*) and *Gata4*, a gene known to be involved in bile acid homeostasis [43]. This was supported by gene-set enrichment analysis which demonstrated that gene pathways including lipid metabolism and pro-inflammatory pathways (JAK-STAT signaling) were significantly suppressed (**Figure 5C)**. qPCR analysis confirmed the regulation of these genes in mice dosed with medium mDP PAC, whereas responses in mice dosed with low mDP PAC were generally less differentiated from control mice (**Supplementary Figure 3**). Thus, direct changes in immunological and metabolic-related transcription can be brought about *in vivo* by orally delivered PAC with a defined mDP profile.

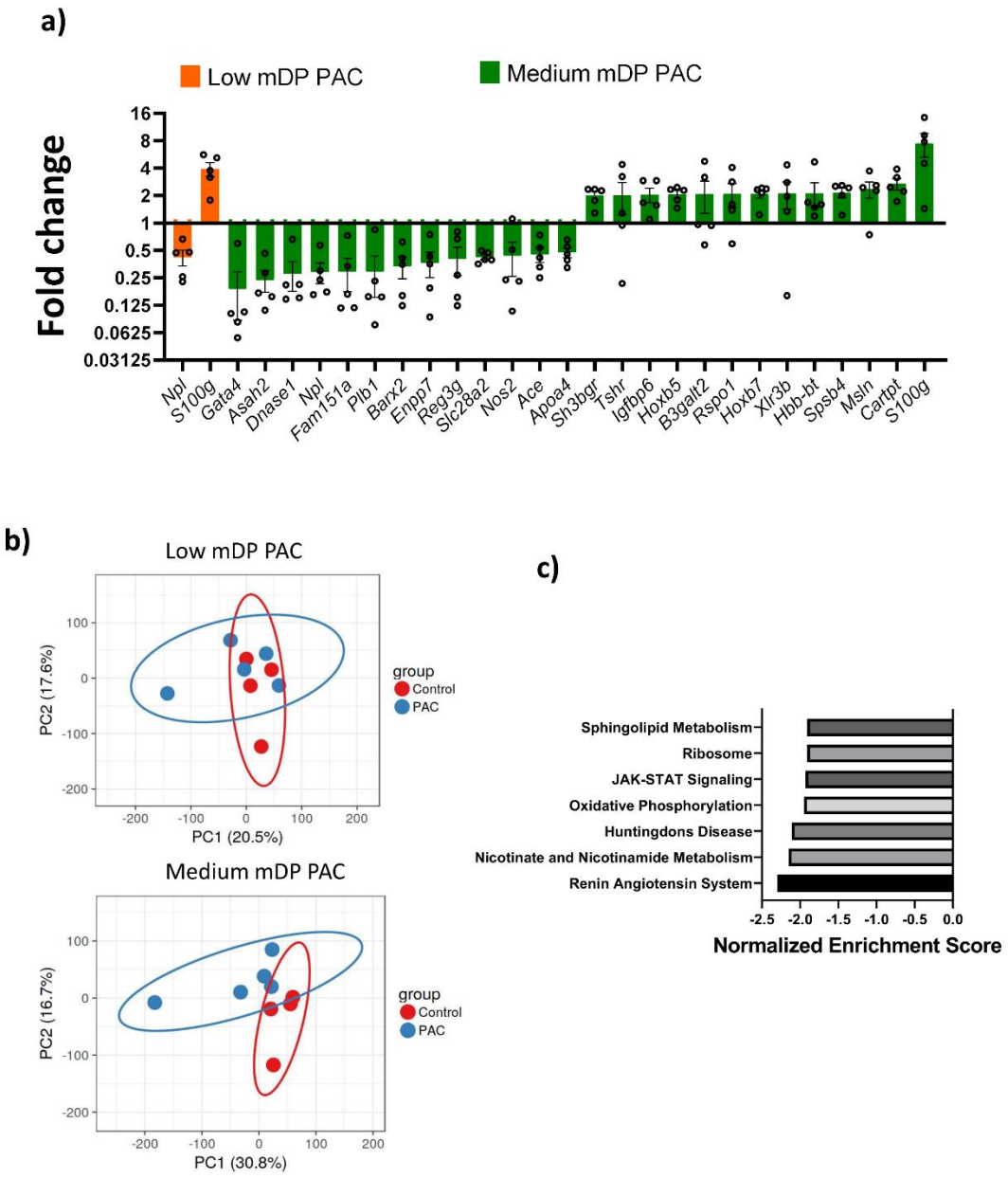

Figure 5 - Regulation of gene expression in mouse ileum tissue by proanthocyanidins

a) Significantly regulated genes (Fold change >2; q-value >0.8 by NOIseq analysis) obtained from RNA sequencing of mouse ileum tissue, in mice dosed with low mDP proanthocyanidins (PAC) or medium mDP PAC. Fold changes are relative to mice dosed with only water. n=5 mice per treatment group. **b)** Principal Component Analysis showed an effect of PAC mDP demarcated in two distinct clusters. *n*= 5 mice per treatment group.

C) Gene pathways identified in the KEGG database that were downregulated (*p* value <0.005; *q* value <0.1) by treatment with medium mDP PAC, identified by gene-set enrichment analysis.

Bafilomycin A1 antagonizes the effect of grape proanthocyanidins *in vitro*

We next sought to establish the mechanistic basis of the activity of the most active PAC sample with medium mDP. We noted that our *in vitro* transcriptomic data showed a suppression of TLR signaling pathways and an up-regulation of genes involved in V-ATPase activity and lysosome function. Therefore, we first assessed the expression of *Tlr4*, *Rab7b* and *Atp6v0d2* in both resting and LPS-activated macrophages by qPCR. Interestingly, we saw a significant increase in the expression of *Rab7b* in resting cells after adding GP PAC, but not in LPS-activated cells. In contrast, *Atp6v0d2* expression was upregulated in both resting and LPS-treated cells, whilst, the expression of *Tlr4* was attenuated by PAC compared to controls (**Figure 6A**). However, we did not observe a decrease in either surface or intracellular expression of TLR4 protein following PAC exposure (**Supplementary Figure 4**). Microscopically, we observed large numbers of vesicle-like structures in PAC-stimulated cells, which were significantly more abundant than in control cells (**Supplementary Figure 5**). Electron microscopy revealed these structures to resemble autophagosomes (**Figure 6B**). This may suggest either a marked increase in phagosome formation, or an accumulation of autophagosomes due to altered lysosomal flux and impaired formation of autophagolysosomes (**Figure 6B**). Together with the increased expression of *Atp6v0d2*, this prompted us to examine the role of V-ATPase activity in the observed effects of PAC. We co-stimulated cells with PAC and Bafilomycin A1 (Baf), a macrolide antibiotic isolated from *Streptomyces gresius*, and a potent V-ATPase inhibitor, to explore whether inhibiting V-ATPase activity would reverse the effects of PAC. Notably, we observed that co-stimulation with Baf prevented the PAC-induced accumulation of the autophagosome-like structures in the cells, suggesting that PAC may inhibit inflammatory responses by modulating V-ATPase activity (**Figure 6B and S5)**.

Interestingly, we observed that Baf treatment alone inhibited IL-6 secretion **(Figure 6C)** and also downregulated *Tlr4* expression in LPS-activated cells **(Figure 6D)**. Surprisingly, whilst Baf is known to inhibit the activity of the V-ATPase, expression of *Atp6v0d2* was markedly increased in Baf-treated cells, similar to that observed with PAC. Thus, Baf and PAC in isolation each induced similar changes in cellular activity, suggesting that they may act through similar mechanisms of action, and that PAC may therefore function as a V-ATPase inhibitor. However, consistent with the observed inhibitory effect of Baf on PAC-induced autophagosome accumulation, during co-incubation we observed that Baf tended to antagonize the effect of PAC. Thus, PAC-induced changes in the expression of *Tlr4* and *Atp6v0d2* were attenuated by co-stimulation with Baf **(Figure 6D)**. Collectively, these data show that PAC induces transcriptional and biochemical changes indicative of a modulatory role in V-ATPase, and hence phagosomal, activity.

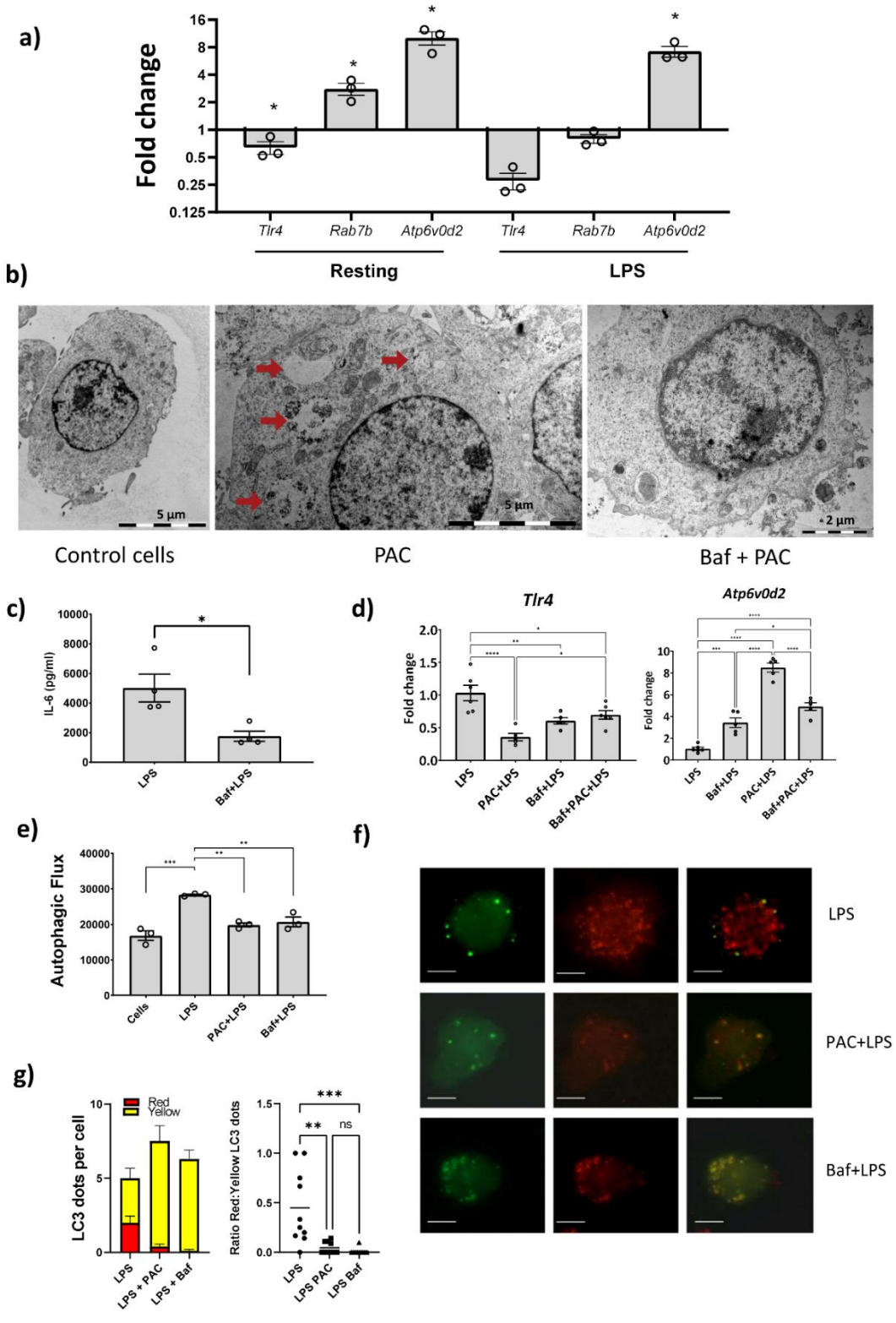

Figure 6 Proanthocyanidins regulate gene expression, autophagosome formation and autophagy in RAW264.7 cells

a) Expression of *Tlr4*, *Rab7b*, and *Atp6v0d2* in RAW264.7 macrophages exposed to proanthocyanidins (PAC) in either resting conditions or following lipopolysaccharide (LPS) stimulation. Fold changes are relative to cells exposed to medium only (resting cells) or LPS only (LPS activated cells). qPCR was conducted with triplicate samples. Error bars represent S.E.M.. (*$p < 0.05$ by paired t-test).

b) Transmission Electron Microscopy (TEM) images of macrophages showing formation of autophagosome-like structures in control cells or in cells incubated with PAC (15 µg/mL), or PAC combined with Bafilomycin A1 (Baf; 10 nM).
c) LPS-activated cells showed a significant decrease in IL-6 secretion when pre-stimulated with Baf (10 nM). Mean and S.E.M. from replicate samples from at least two independent experiments. (*$p < 0.05$ by Mann-Whitney test).
d) Expression of *Tlr4* and *Atp6v0d2* in RAW264.7 cells stimulated with LPS with PAC (15 µg/mL) and/or Baf (10 nM). Mean and S.E.M. from replicate samples from at least two independent experiments. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <0.0001$ by one-way ANOVA and Tukey's tests).
e) Autophagic flux in RAW 264.7 macrophages treated with LPS alone, or LPS combined with either PAC (15 µg/mL) or Baf (10 nM). Mean and S.E.M. of triplicate samples. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA and Tukey's tests).
f) PAC and Baf both reduce the formation of autophagolysosomes in LPS-activated mLC3 cells. LPS-activated cells had marked abundance of red punctate spots, indicating increased autophagolysosome formation. In contrast, punctate spots and co-localization of green and red fluorescence was observed in LPS-activated cells co-stimulated with either PAC or Baf indicating an accumulation of autophagosomes and inhibition of the full autophagy pathway. Scale bar = 5µM.
g) The abundance of red dots, i.e. autophagolysosomes, and yellow dots, i.e. autophagosomes, and the ratio between them, were enumerated in ten individual mLC3 cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Kruskal-Wallis and Dunn's tests).

Proanthocyanidins suppress lipopolysaccharide-induced autophagy

Given the putative association between GP PAC and V-ATPase activity, we next asked whether GP PAC would regulate autophagy, as Baf is a well-known autophagy inhibitor and other natural compounds with anti-inflammatory activity have also been shown to suppress autophagy in macrophages [44]. We found that GP PAC significantly suppressed autophagy in LPS-activated cells, to a similar extent as that seen in Baf-treated cells (**Figure 6E**). These observations were further validated using mLC3 autophagy-reporter cells. LPS-activated cells showed the characteristic appearance of autophagy with a marked abundance of red punctate spots, indicating increased autophagolysosome formation. In contrast, LPS-activated cells co-stimulated with either PAC or Baf showed punctate spots and co-localization of green and red fluorescence, indicating accumulation of autophagosomes and inhibition of the full autophagy pathway (**Figure 6F**). In both PAC-treated and Baf-treated cells, the ratio of red to yellow mLC3 dots was significantly reduced compared to LPS-only treated cells (**Figure 6G**). Overall, these data suggest a novel role for PAC in modulating inflammatory activity in macrophages by suppressing LPS-induced autophagy, in a manner analogous to the V-ATPase inhibitor Baf.

Discussion

The beneficial effects of PAC are numerous, and especially their ability to suppress inflammatory markers has been demonstrated in several mouse models and *in vitro* studies. Direct interaction with immune cells, immune cell-derived mediators, and gut microbiota may cause the prevention and alleviation of gut-related inflammation [27]. Thus, the anti-inflammatory properties of PAC have been attributed to their interaction with protein receptors and transcription factors of the NF-κB and MAPK signaling pathways, and by modulating the arachidonic acid pathway and inhibiting enzymatic activity of eicosanoid generating enzymes [12,22,45,46]. Despite the large body of research investigating

the bioactivities of PAC, their mechanism of action is still not fully understood but their anti-oxidant and anti-inflammatory activities are believed to be dependent to their varying chemical structures [5]. Thus, the bioavailability, stability and activity of polyphenolic compounds may be correlated with the amount of hydroxyl groups, mean polymerization, and/or bond type and position between the monomers [31,47–50]. However, the complex and various molecular structures of PAC challenges the study of their structure-function *in vitro*. Our aim was thus to investigate how differing structural characteristics of highly purified PAC would affect bioactivity, and to explore which cellular mechanisms lead to this activity.

We initially observed a pronounced suppression of IL-6 secretion in LPS-activated murine macrophages stimulated with Sephadex fractions (data not shown) and PAC samples derived from semi-preparative LC. The higher potency of samples derived from semi-preparative LC versus Sephadex LH-20, suggests that Sephadex LH-20 separation followed by semi-preparative LC is a powerful tool to isolate highly purified bioactive PAC with differing mDP. Also, our data showed that the PC-rich GP samples appeared to be more efficient at suppressing inflammatory responses in RAW 264.7 macrophages compared to PD-type AC PAC. Moreover, particularly in PC-type PAC, a non-linear relationship exists between mDP and anti-inflammatory activity, with the most potent inhibition of inflammatory cytokine secretion found in polymers with a medium (7-10) mDP. From a chemical point of view, it is worth considering the stability of compounds in i.e. cell cultures where PD-type PAC might be prone to oxidation, which could explain the lack in bioactivity.

The stimulation of macrophages with GP PAC upregulated the expression of genes related to inflammatory responses, such as *Atf3,* which suggests that PAC are recognized by and have some activating activity in resting macrophages. This is consistent with data showing that immature human dendritic cells adopt a semi-mature state after exposure to PAC [51]. *Atf3* is induced by a variety of stimuli, such as stress, and plays an important role in immune regulation, including downregulation of TLR4, which consequently reduces the expression of several inflammatory genes, such as *Il6, Il12b* and *Tnf* [52,53]. Whilst medium and high mDP PAC tended to induce transcription of similar genes, the small number of genes that were induced by low mDP PAC were markedly different, indicating a qualitative difference in the interaction of different sized PAC polymers with macrophages.

Our data also suggests that GP PAC modulate macrophage transcriptional responses in a polymerization-dependent manner. We demonstrated that PAC with differing mDPs modulate similar genes but with varying impact on expression levels. This suggests, that there is no inherent difference in the mechanism of how different size PAC affect the cell activity, but rather that the mDP may influence the ability of the cells to process the PAC molecules. Thus, we here demonstrated that GP PAC with an mDP of 9.1 had a more prominent impact on gene expression levels, especially in LPS-activated macrophages, than samples with mDPs of 2.6 or 12.3. Overall, our data demonstrated strong immunological properties of medium mDP PAC in presence of LPS in RAW 264.7 macrophages with a downregulation of inflammatory mediators and an upregulation of anti-inflammatory

mediators. The decreased bioactivity seen in low mDP PAC could be caused by limited number of structural units in PAC, thus resulting into e.g. lower affinity towards possible target proteins as shown by Leppä et al. [50]. On the other hand, PAC with high mDP might exceed the saturation of contact points with the cells, and complicate efficient structural interactions and/or endocytosis, and thereby explain the reduced activity seen in cells stimulated with high mDP PAC.

What was also noteworthy was the prominent upregulation of JAK-STAT and inflammation pathways in LPS-activated macrophages, which were consequently down-regulated by co-stimulation with medium mDP PAC. Furthermore, *Slamf8*, which is a negative regulator of inflammatory responses, was significantly upregulated by all three samples in LPS-activated macrophages. A number of genes related to the suppression of inflammatory responses were thus regulated by PAC, including *Cish* and *Csf2*. Interestingly, our data suggests that PAC seemed to elicit an inflammatory response in resting cells by upregulating interleukins as well as chemokines, however, PAC acted as an anti-inflammatory agent in LPS-activated cells.

GP Sephadex LH-20 fractions were tested *in vivo,* and the effects of medium mDP PAC on ileum transcriptomics were substantial in a healthy mouse model compared to low mDP PAC. Here, we gavaged mice every other day for 10 days with 200 mg/kg BW of highly purified GP Sephadex fractions with either low or medium mDP PAC. A significant increase in the number of regulated genes was observed in mice dosed with medium mDP PAC compared to mice dosed with low mDP PAC. Notably, the downregulation of immune-related genes, such as *Asah2* and *Enpp7*, and the upregulation of *Rspo1,* which promotes cell proliferation of crypt cells. Taken together, our *in vivo* data suggests that dietary PAC, when comprised of medium mDP polymers, are able to regulate cellular functions related to nutrient metabolism and immune function in the intestinal mucosa. As mentioned above, PAC remain relatively stable with little to no absorption or metabolism until reaching the colon [54], which supports the assumption that the observed differences in gene expression in the small intestine are a clear effect of mDP. However, it should also be considered that the metabolism of PAC by gut microbiota during *in vivo* studies may strongly affect bioavailability, and further challenges the association of bioactivity to specific molecular structures of PAC.

The question remains as to how and where PAC interacts with immune cells. There are some controversies concerning whether or not larger PAC molecules can be taken up by macrophages like monomers and dimers, or whether they mainly interact with the proteins and lipids on the cell membrane [55,56]. However, we have previously shown internalization of PAC with an mDP of 9.5 in dendritic cells [51]. Based on our microarray data, we hypothesized that PAC may decrease TLR4 receptors on the cell surface due to increased degradation into RAB7B promoted lysosomes [57]. However, our hypothesis was not supported by flow cytometry investigations (data not shown). We observed that the KEGG Lysosome and transferrin pathway was significantly upregulated by medium mDP GP PAC, moreover the vacuolar gene *Atp6v0d2* reached high expression levels. *Atp6v0d22* plays a key role for lysosome acidification and autophagosome-lysosome fusion and thereby ensures the appropriate turnover of intracellular organelles and infective agents [58]. The observed upregulation

of V-ATPase is in coherence with previous studies suggesting localization of PAC to lysosomes following endocytosis in human DCs [51,59]. The upregulation of the KEGG lysosomal pathway, and thereby genes encoding subunits of V-ATPase, in LPS-activated macrophages was also observed in DCs stimulated with PAC isolated from cocoa [59]. Inhibitors of V-ATPase have shown to increase NF-κB activation [60], and thus a higher expression level of V-ATPase may explain the suppression of inflammatory markers observed in our study. Based on our findings with Baf added to LPS-activated cells stimulated with PAC, our data supports the notion that the intracellular activity of PAC is intimately connected to V-ATPase activity. The inhibitory effect of Baf on the expression of *Atp6v0d2* in LPS-activated cells stimulated with PAC, suggests that PAC has an important role in the lysosomal pathway. However, our mechanistic experiments investigating the role of PAC during autophagy, suggested a decrease in autophagy levels. This was also indicated in our studies with mLC3 cells, where we observed fewer autophagolysosomes in LPS-activated cells stimulated with PAC or Baf, compared to cells stimulated with LPS only. Taken together, these findings suggest that PAC in fact inhibits the autophagy pathway in terms of autophagolysosome formation, similarly to Baf. The enhanced expression of *A tp6v0d2* could however be explained by an accumulation of lysosomes or autophagosomes in the cytoplasma, in response to the inhibition of the autophagolysosome formation. Another explanation could be that an inhibition of the ATP6V0D2 protein by PAC and Bafilomycin in isolation, leads to a positive feedback-loop in the expression of the *Atp6v0d2* gene.

A large body of research suggests that a combination of phytochemicals lead to more pronounced beneficial health effects, rather than singled out molecules [8,25]. Synergistic effects of selected PAC samples derived by semi-preparative LC were not investigated in this study, however, we clearly demonstrated a variation in cell stimulation between PAC samples derived from the same Sephadex fraction. These findings strongly suggest an impact of PAC structure on bioactivity, and supports the notion of how polymeric mixtures observed on chromatograms in fact depict numerous PAC molecules with distinctive chemical and biological properties.

Conclusion

We showed that pronounced transcriptional changes induced by PAC occur in a polymerization-dependent manner both *in vitro* and *in vivo*, which suggests that the chemical structure affects the bioactivity of PAC. Thus, there is an indication that medium and high mDP PAC modulate similar pathways but the level of gene expression is dependent on the mDP. Furthermore, we propose that PAC may have an important impact on V-ATPases, lysosome function and the autophagy pathway. Our results highlight the importance of considering structural features of PAC-rich food sources when examining their bioactivity in different models of inflammation. Future research should focus on the intracellular signaling pathways modulated by PAC, as well as how defined structural features may modulate the efficacy of PAC-based supplements as a novel tool for control of health and disease.

Acknowledgements
The authors would like to thank Mette Marie Arnt Schjelde for excellent technical assistance during laboratory work and Anne Koivuniemi for the maintenance of the UPLC instrument. Figure 3D was created with BioRender.com.

Funding
This work was funded by the Independent Research Fund Denmark (Grant # 7026-0094B).

Conflict of interest
The authors declare no conflicts of interests regarding this study.

Ethical statement
All experiments involving animals were conducted in agreement with the Danish legislation and the Danish Animal Experiments Inspectorate with the license number 2015-15-0201-0076.

Data availability statement
Microarray data is available at GEO, under the accession number GSE167063, and raw GSEA data is available in Supplementary File 1. RNAseq data is available at GEO under accession number GSE168138, and raw GSEA data is available in Supplementary File 2. Source data and images underlying the presented graphs is available in Supplementary Files 3 and 4.

Contributions
A.A-C conceived research, designed and performed experiments, carried out data analysis and wrote the manuscript. M.M.L. and J.P.S. planned, supervised and assisted with the chemical analyses. S.M.T. helped supervise the research and interpret he data. A.R.W. conceived and supervised the research, carried out data analysis and wrote the manuscript. All authors participated in discussion of the data and editing the final manuscript.

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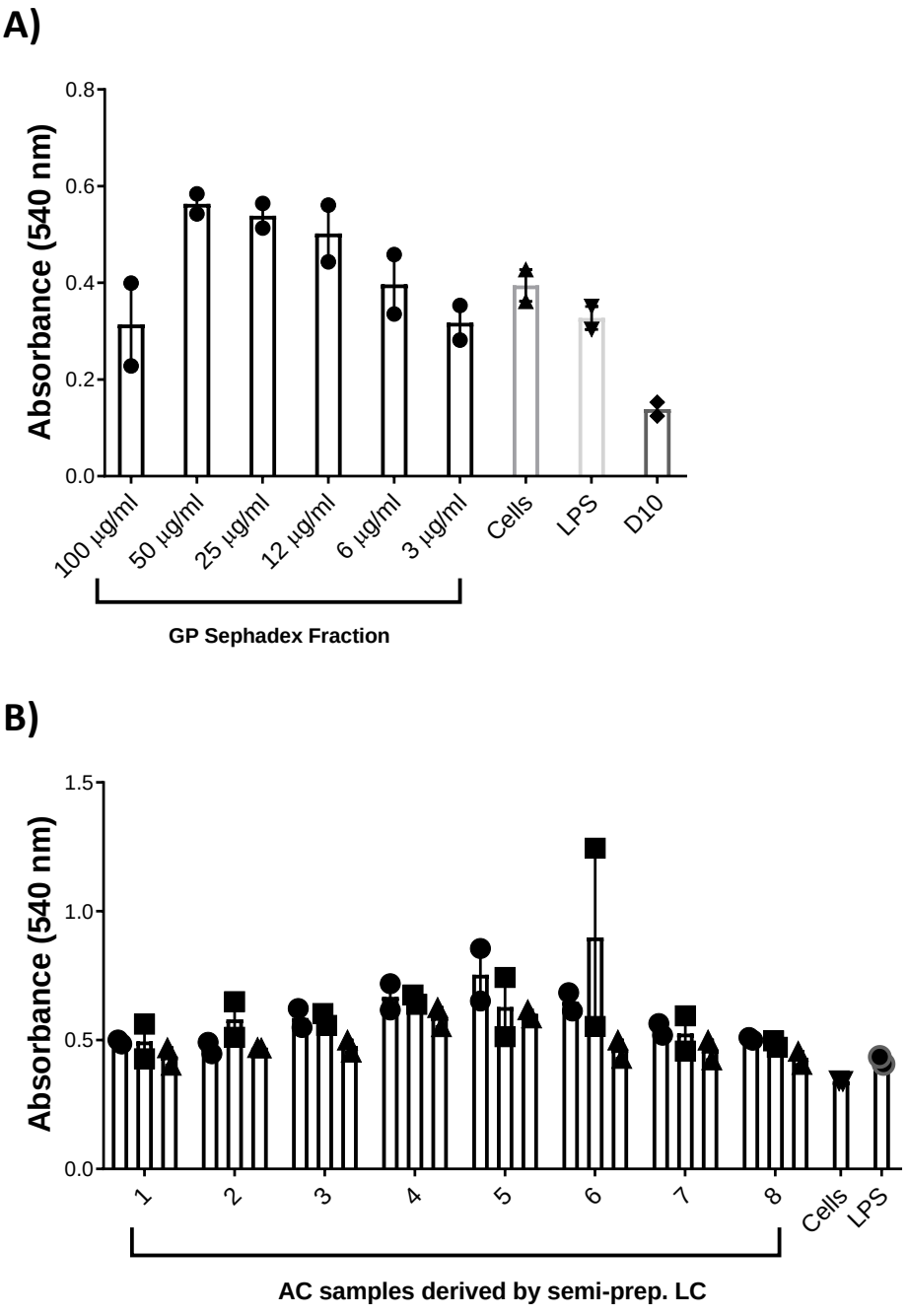
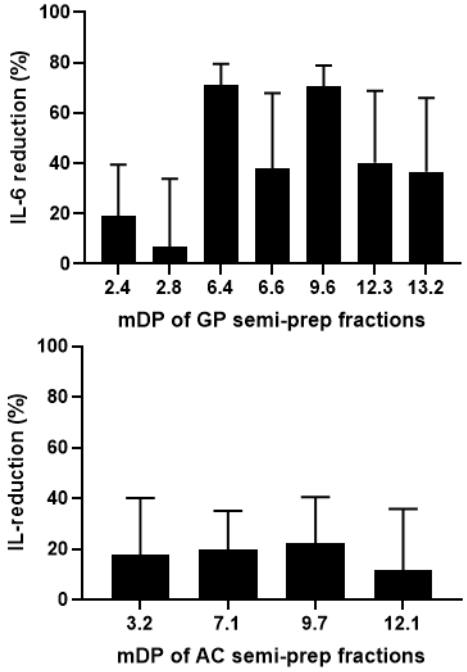
Supplementary Material to Paper II



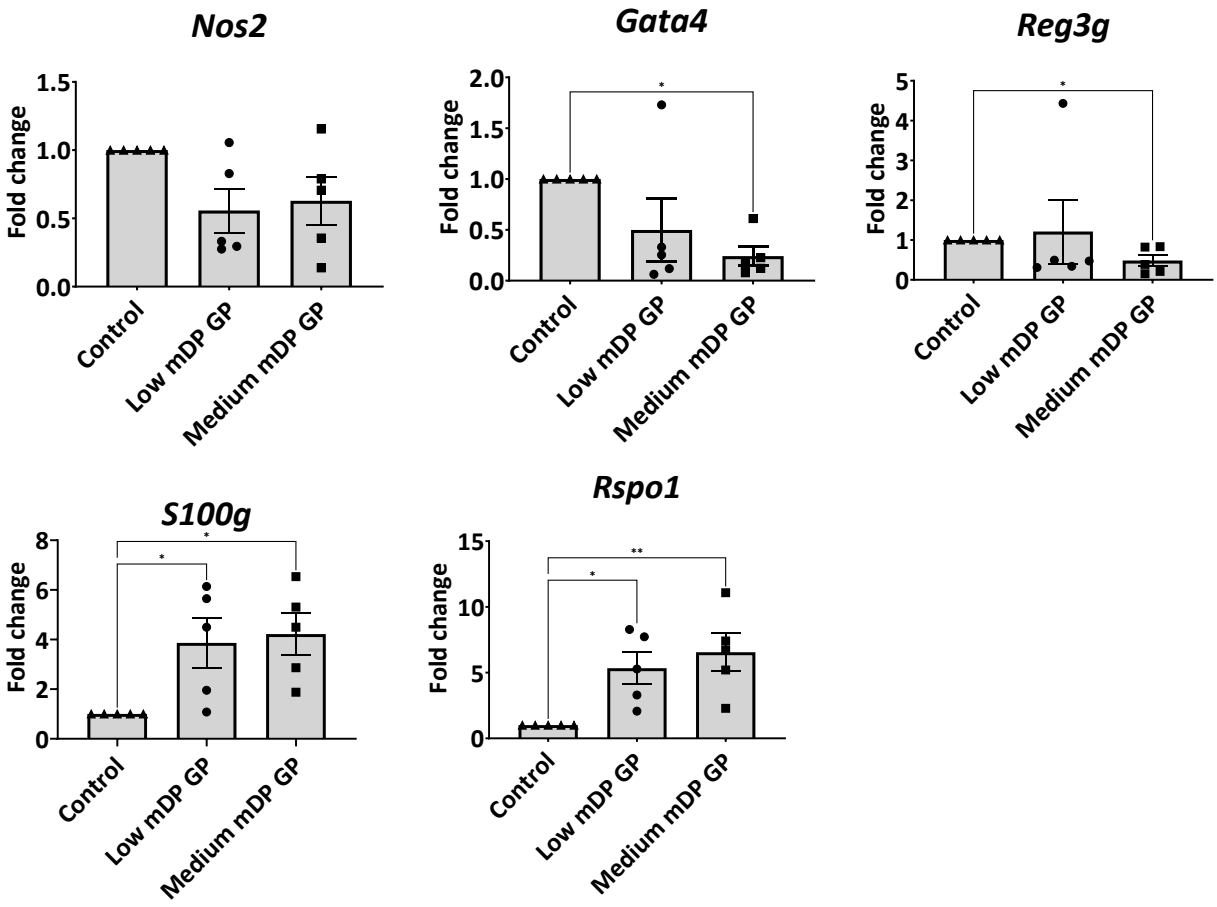
Supplementary Figure 1 Cytotoxicity test by Neutral Red Assay

A) Sephadex fraction were tested in LPS-activated RAW 264.7 macrophages at different concentrations (3-100 µg/ml) in duplicates. No signs of toxicity were reported.

B) Samples derived by semi-preparative liquid chromatography were tested in LPS-activated RAW 264.7 macrophages at a concentration of 15 µg/ml in duplicates. No signs of toxicity were reported as no absorbance values were below the control samples.

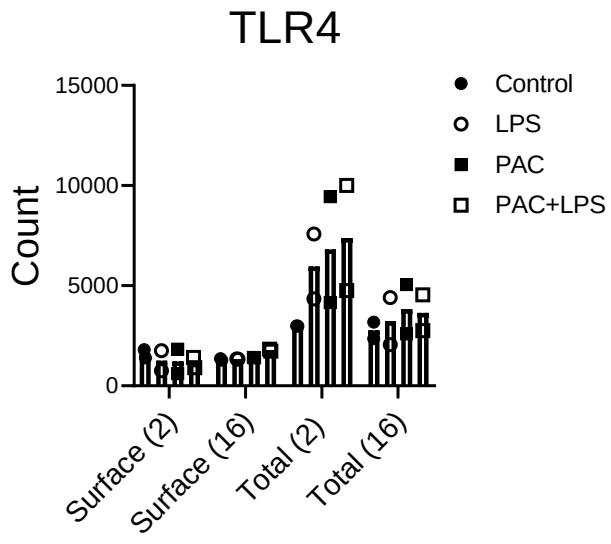


Supplementary Figure 2 - Inhibition of IL-6 secretion in LPS-activated macrophages stimulated with grape pomace or alpine currant PAC at equimolarity of 7.8 µM.
Experiments were conducted at least twice with triplicate samples.



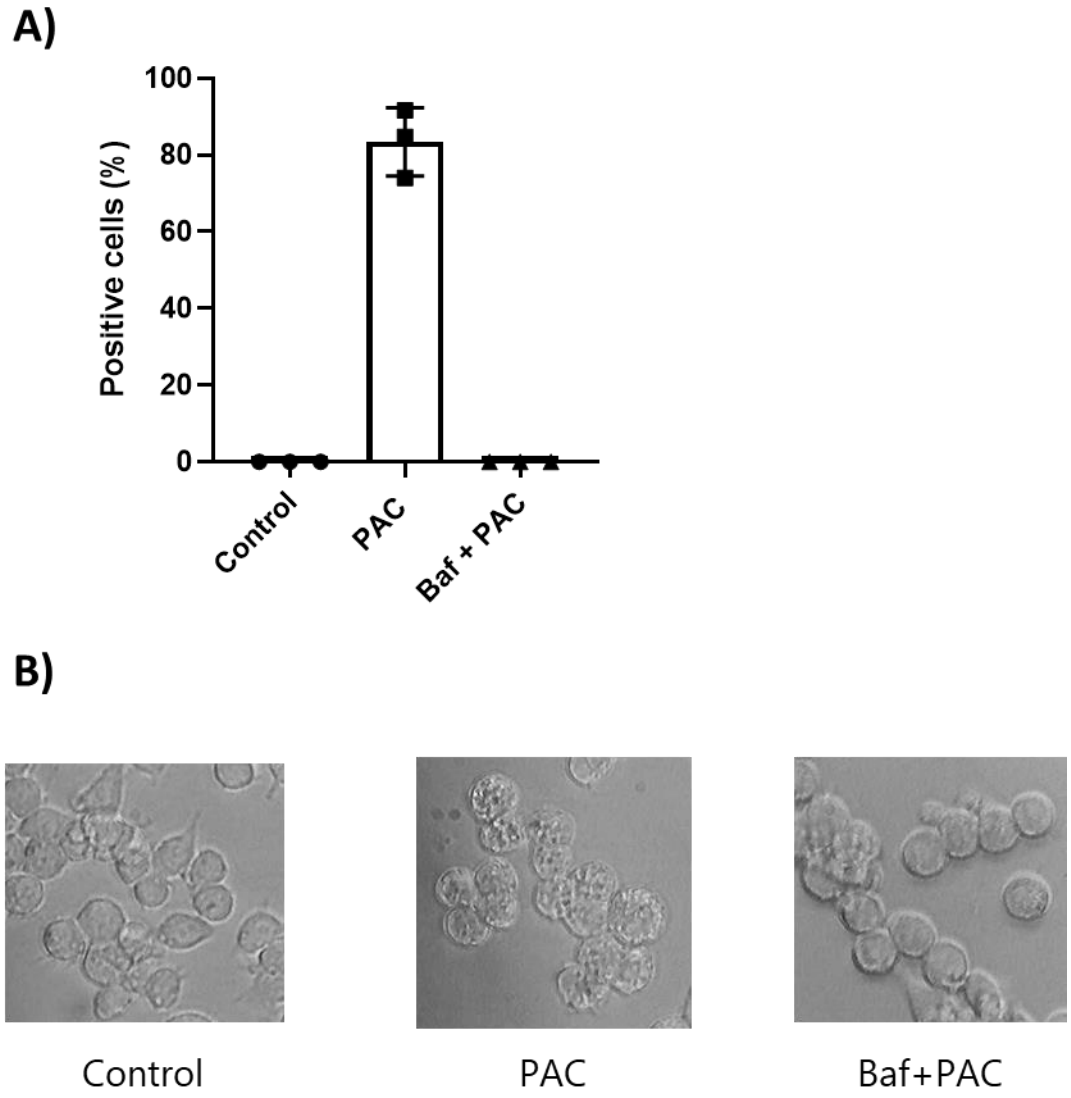
Supplementary Figure 3 - Regulation of gene expression in mouse ileum tissue by PAC

qPCR data depicting the regulation of *Nos2*, *Gata4*, *reg3g*, *S100g* and *Rspo1* in the ileum tissue of mice dosed with either low or medium mDP Sephadex fractions derived from grape pomace (GP). Data is expressed as fold changes relative to mice dosed with water only (n= 5 mice per treatment group). (*p < **0.05, **p < 0.01 by Kruskal-Wallis test).**

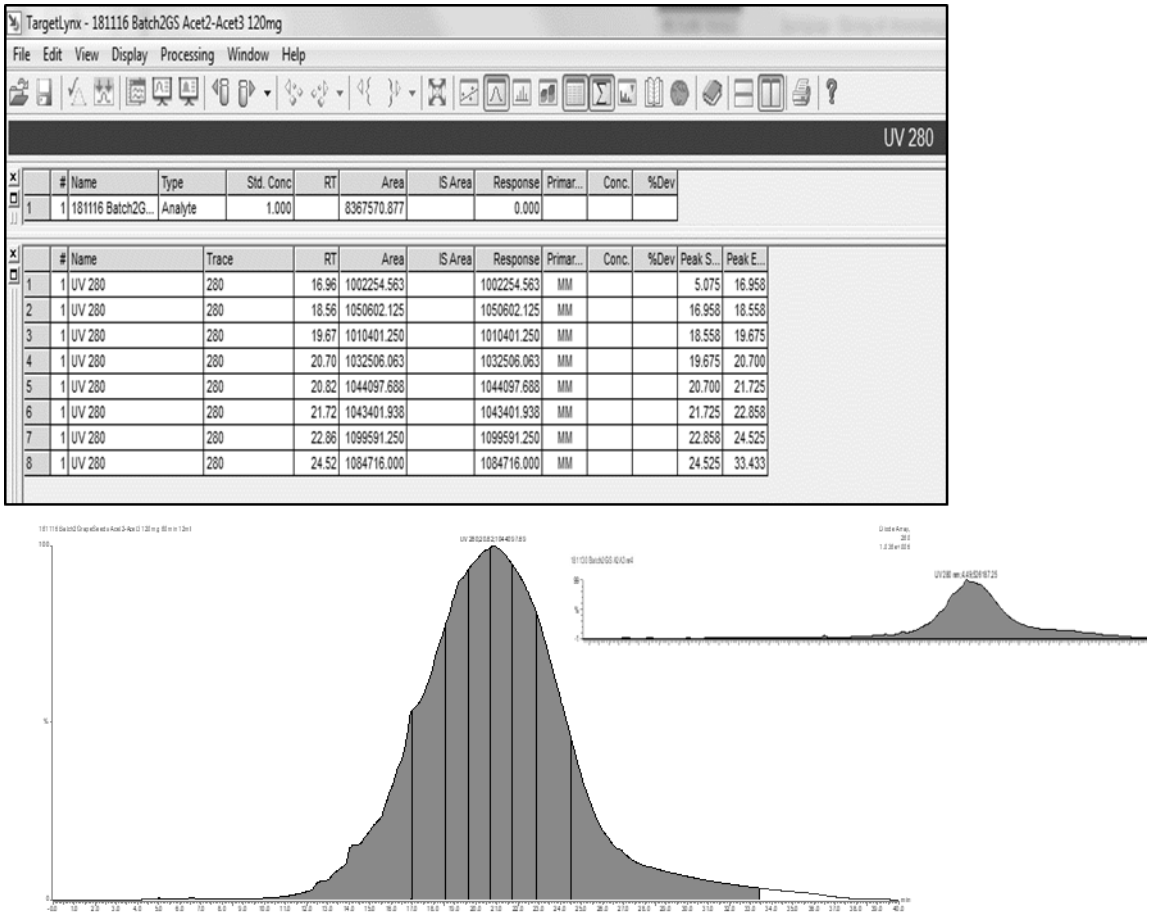


Supplementary Figure 4 Proanthocyanidins do not reduce TLR4 protein expression

No effect of proanthocyanidin was observed on either surface or total expression of TLR4 proteins in LPS-activates RAW 264.7 macrophages stimulated with proanthocyanidins compared to appropriate controls.



Supplementary Figure 5 Proanthocyanidins induce autophagosome-like structures in RAW 264.7 macrophages **A)** RAW 264.7 macrophages stimulated with PAC (15 µg/ml) containing autophagosomes-like structures (= positive cells) were enumerated. Co-stimulation with Bafilomycin (10nM) and unstimulated cells did not display autophagosomes-like structures. **B)** Microscopy pictures of RAW 264.7 cells with appropriate treatments.



#	Name	Type	Std. Conc	RT	Area	IS Area	Response	Primar...	Conc.	%Dev
1	1	181116 Batch2G...	Analyte	1.000		8367570.877		0.000			

#	Name	Trace	RT	Area	IS Area	Response	Primar...	Conc.	%Dev	Peak S...	Peak E...
1	1	UV 280	280	16.96	1002254.563		1002254.563	MM			5.075	16.958
2	1	UV 280	280	18.56	1050602.125		1050602.125	MM			16.958	18.558
3	1	UV 280	280	19.67	1010401.250		1010401.250	MM			18.558	19.675
4	1	UV 280	280	20.70	1032506.063		1032506.063	MM			19.675	20.700
5	1	UV 280	280	20.82	1044097.688		1044097.688	MM			20.700	21.725
6	1	UV 280	280	21.72	1043401.938		1043401.938	MM			21.725	22.858
7	1	UV 280	280	22.86	1099591.250		1099591.250	MM			22.858	24.525
8	1	UV 280	280	24.52	1084716.000		1084716.000	MM			24.525	33.433

Supplementary Figure 6 - Integration of Chromatograms

MassLynx was used in order to determine the Area under the curve (AUC) within the time-span of 5–33 minutes. The Peak Response Area value was divided into 8 equally sized “slices”, corresponding to the 8 areas of the chromatogram. The 168 tubes resulting from the semi-preparative liquid-chromatography were then pooled accordingly into 8 highly purified PAC samples, which were individually analyzed by UPLC-MS/MS.

Supplementary Table 1 - List of material used for the extraction and purification of proanthocyanidins

	Material	References
Samples	Alpine currant (AC) Macerated in 80 % analytical acetone (09-08-2018)	This study
	Grape pomace (GP) Macerated in 80 % analytical acetone (14-09-2018)	Nor-Feed A/S (Hvidovre, Denmark)
Solvents	Acetyl acetate	VWR International S.A.S, France
	Analytical acetone	VWR International S.A.S, France
	Butanol	VWR International S.A.S, France
	Diethyl ether	VWR International S.A.S, France
	Ethyl acetate	VWR International S.A.S, France
	Formic acid 99-100%	WR International, EC
	Formic acid, LC-MS grade	Sigma Aldrich, USA
	Methanol, analytical grade	VWR International S.A.S, France
	Acetonitrile, LC-MS grade	VWR International S.A.S. (USA)
Other	MilliQ water Purified with Millipore Synergy UV system	Merck KGaA, Darmstadt, Germany
	Sephadex LH-20	GE Healthcare

Supplementary Table 2 - Extraction and purification of proanthocyanidins

Overview of the extraction and purification steps used to isolate purified PAC from grape pomace (GP) and alpine currant (AC) by Sephadex LH-20 fractionation followed by semi-preparative liquid chromatography.

	Alpine currant	Grape pomace
1. Extraction by filtration	Macerated in 80 % analytical acetone (09-08-2018)	Macerated in 80 % analytical acetone (14-08-2018)
	5 extractions through Büchner funnel	
	Evaporation of acetone from samples in fume hood	
	UPLC analysis	
	Pooling of extractions 1-5	Pooling of extractions 1-5
	Liquid-liquid extraction with ethyl acetate and butanol	-
	-	-
2. Sephadex fractionation	Sephadex fractionation 6 water fractions 5 methanol fractions 6 acetone fraction	Sephadex fractionation 6 water fractions 5 methanol fractions 6 acetone fraction
	O/N evaporation of acetone fractions Rotary evaporation of methanol fractions	
	Freeze-drying and weighing of samples	
	Pooling of samples based on similarity of chromatogram → 6 alpine currant sephadex samples	Pooling of samples based on similarity of chromatogram → 8 grape pomace sephadex samples
3. Semi-preparative liquid chromatography	Injection of ~120 mg of each sephadex fractions	
	Collection of samples in 168 Eppendorf tubes	
	Pooling of each semi-prep run into 8 equal sub-fractions → 48 alpine currant semi-prep fractions	Pooling of each semi-prep run into 8 equal sub-fractions → 64 grape pomace semi-prep fractions
	Evaporation of solvents by rotavap, rotary evaporation or centrifugal concentration and freeze-drying	
	A total of 112 semi-prep samples containing highly purified PAC were generated with sample weights ranging between 2-17 mg	

Supplementary Table 3 - Primer sequences used in experiments

Primer name	Primer sequence (5'-> 3')
Used for *in-vitro* studies	
TLR4 forward primer	*ACTGGCCTTTCAGGAACTTT*
TLR4 reverse primer	*ACATCCTAGGGCTGTCTTTCTT*
ATP6V0D2 forward primer	*GGGCCAGTGTTCAGTTGCTA*
ATP6V0D2 reverse primer	*TCCTGCTGAGTTAGGAGGCT*
RAB7B forward primer	*GGAAGTGGCCTCTCACCAAA*
RAB7B reverse primer	*CCTCACACAGGTGGGAGTTC*
GAPDH forward primer	*TATGTCGTGGAGTCTACTGGT*
GAPDH reverse primer	*GAGTTGTCATATTTCTCGTGG*
Used for *in-vivo* studies	
NOS2 forward primer	*GGTGAAGGGACTGAGCTGTT*
NOS2 reverse primer	*TGCACTTCTGCTCCAAATCCA*
GATA4 forward primer	*TTCTGGGAAACTGGAGCTGG*
GATA4 reverse primer	*TGCTTTCTGCCTGCTACACA*
REG3G forward primer	*CACCATCCTAGGGATCTGCAA*
REG3G reverse primer	*ATGGGGCATCTTTCTTGGCA*
S100G forward primer	*GGAGCTGGATAAGAATGGCGA*
S100G reverse primer	*AGAGCGTGCGTTCAATCAGT*
RSPO1 forward primer	*TGTACTTACACAAGGGCCGC*
RSPO1 reverser primer	*GGGACCACTCGCTCATTTCA*
GAPDH forward primer	*TATGTCGTGGAGTCTACTGGT*
GAPDH reverse primer	*GAGTTGTCATATTTCTCGTGG*

Paper III

Dietary Proanthocyanidins and Enteric Nematodes Interact to Modulate Gut Microbiota-derived Metabolites and Promote Type 1 Intestinal Immune Responses in Mice

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Manuscript

Dietary Proanthocyanidins and Enteric Nematodes Interact to Modulate Gut Microbiota-derived Metabolites and Promote Type 1 Intestinal Immune Responses in Mice

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Abstract

Proanthocyanidins (PAC) are phytonutrients commonly found in plant-based diets which have demonstrated immunomodulatory and anti-inflammatory properties at mucosal sites, such as the intestinal tract. PAC and other bioactive dietary components may therefore play a central role in circumventing enteric disease and ensuring appropriate immune response towards harmful stimuli. However, whether PAC can modulate immune responses to enteric pathogens has not yet been elucidated in detail. Here, we investigated the effect of PAC intake on immune responses during parasitic helminth infection in mice. Mice fed compositionally defined diets were orally administered purified PAC during infection with the small intestinal roundworm *Heligmosomoides polygyrus,* or the caecal whipworm *Trichuris muris.* In both infection models, PAC significantly modulated the transcriptomic response to infection, with a notable skewing of the local immune environment towards a type-1 response. Analysis of mesenteric lymph nodes (MLN) showed a heightened cellular response to both parasites, particularly within the T-bet$^+$ T-helper cell population. Furthermore, PAC in *T. muris*-infected mice induced higher levels of parasite-specific IgG2a in serum, and increased the production of inflammatory cytokines in *ex vivo* stimulated MLN cells. Consistent with this, PAC-dosed mice harbored higher numbers of *T. muris* adult worms. In uninfected mice, PAC intake increased the abundance of *Turicibacter sanguninis* within the gut microbiota (GM), increased faecal short chain fatty acids and enriched phenolic metabolites such as valerolactones in the caecum – however, concurrent *T. muris* infection suppressed these parameters during PAC consumption. Furthermore, the combination of PAC and *T. muris* resulted in an expansion of opportunistic

Escherichia fergusonii. Thus, inclusion of PAC in refined mouse diets in the absence of infection promoted gut health, but had a seemingly negative influence on the host response to helminth infection. Collectively, our results suggest a novel inter-relationship between a phytonutrient and enteric infection, which may offer new insights into how diet and infection interact to modulate intestinal inflammation.

Keywords

Proanthocyanidins; *Trichuris muris*; *Heligmosomoides polygyrus*; Intestinal inflammation

Introduction

The immune system plays a crucial role in health status of humans and animals, and is a key component in the search of new treatments and in the comprehension of disease progression and recovery. Several fields of research are exploring how the immune system can be modulated to prevent and treat various diseases. In recent years, interest in the application of phytonutrients as health-promoting dietary substances has been greatly increasing[1]. Proanthocyanidins (PAC) are molecules of plant origin and members of the group of polyphenols, which are present in varying concentrations among commonly consumed foods, particularly grapes, berries and nuts[2]. Several studies have reported the efficacy of PAC to exhibit immunomodulatory properties, of which a common denominator is the downregulation of aberrant T-helper (Th)-1 and Th17 immuno-pathologies, along with promoting systemic anti-inflammatory responses[3–7]. Although these aforementioned studies have increased our knowledge of the possible beneficial immune benefits of PAC, the precise immune-modulatory mechanisms of PAC are still largely unknown. Next to their anti-oxidant potential, they also have beneficial modes of action on gut homeostasis by sustaining a healthy gut microbiota (GM) and improving gut barrier function[8–10].

The intestinal tract is continually challenged by potential harmful stimuli, while balancing the proportion of commensal and opportunistic bacteria[11,12]. Of note, intestinal parasites cause detrimental disease and co-morbidities in humans, with helminthiasis being one of the most common infections worldwide. Thus, about 1.5 billion people globally are infected by different gastrointestinal-dwelling helminths[13,14]. Parasitic infections elicit Th2-biased immune responses, which the parasites can attempt to evade or suppress for their own survival[15]. Under natural conditions, individuals are infected repeatedly with low-dose helminth infections throughout their lifetime, thus allowing the parasite to establish chronic, non-resolving infections[16]. In murine models, expression of type-2 effector mechanisms results in worm expulsion, whereas type-1 responses promote chronic infection. *Heligmosomoides polygyrus* is a parasite of the murine small intestine, and is related to the human hookworms *Ancylostoma duodenale* and *Necator americanus*[15,17]. Primary infection induces type-2 cytokine gene expression in the mesenteric lymph nodes (MLN) and Peyer's patches, where IL-4 is a crucial cytokine yielding protection against *H. polygyrus* by promoting adult

worm expulsion and inhibiting egg production[18,19]. *H. polygyrus* infection also activates colonic Foxp3$^+$ T cells, inducing a regulatory state, which limits the extent of intestinal inflammation[20]. The mouse whipworm, *Trichuris muris*, is a well-established model for human *Trichuris trichiura*. Under laboratory settings, experimental infections can be adjusted to induce different outcomes of *T. muris* infection in C57BL/6 mice. In mice given trickle infections with low numbers of infective eggs, worm persistence and establishment of chronic infection results, with CD4$^+$ Th1 and IFN-γ production being a main feature of the immune response[21,22]. Chronic *T. muris* infections have also shown to cause gut dysbiosis with a marked reduction in microbial diversity[23,24]. In contrast, a single high dose acute infection is characterized by accelerated worm expulsion and the development of CD4$^+$ Th2-driven protective immunity[21,22].

The impact of dietary PAC on the progression of chronic inflammatory diseases, such as obesity, is increasingly well understood, with various studies demonstrating a beneficial role for PAC in chronic inflammation. These effects may derive from both prebiotic effects as well as direct modulation of antioxidant responses in gut epithelial and immune cells[25–29]. In contrast, the effects of PAC on immune function when exposed to a pathogen during infection are less clear. In large-animal models, PAC-rich diets have shown modulatory effects on the immune response to helminth infections, including a boosting of infection-induced γδ T-cells, eosinophils, and antibodies[30,31]. However, PAC-enriched diets have also been shown to exacerbate *Citrobacter rodentium* infection in mice, which may be due to PAC-induced changes in the GM or mucosal immune function[32]. Thus, the effects of PAC on enteric infection are not clear, and may vary depending on factors such as host species or basal GM. As of yet, no studies have investigated the influence of PAC on resistance to helminth infection in controlled mouse models utilizing compositionally-defined diets. This may represent a useful model to examine the ability of PAC to modulate Th1/Th2 polarization in natural models of enteric parasitic infection, which may also be of relevance to other immune-related dysfunctions. Thus, we explored here if the addition of purified PAC to purified, open-source murine diets can alter the immune response towards *H. polygyrus* and *T. muris* infection. Notably, we show that in both infection models, PAC induce a clear modulation of the immune response characterized by a type-1 polarized response. This was especially pronounced during *T. muris* infection, where PAC enhanced adult worm burdens concomitant with the upregulation of numerous genes and gene pathways related to interferon signaling. Thus, our results stand in contrast to the previously described Th1-suppressing effects of PAC and their anticipated anti-inflammatory properties, and suggest that in some contexts, the addition of PAC to refined diets promotes a beneficial intestinal milieu for helminth infection.

Material and Methods

Proanthocyanidins

PAC derived from grape (*Vitus vinifera)* were used for both studies. For the *H. polygyrus* study, PAC were derived from grape pomace and obtained by series of extraction and sephadex separation as described elsewhere[33]. PAC used for the *T. muris* study were a commercial preparation derived from

grape seed (Bulk Powders, Denmark). Both preparations consisted exclusively of procyanidin-type PAC oligomers and polymers (mDP ≥ 4) of at least 90% purity.

Mouse experiments and parasites

6-week old C57/BL6 female mice were used in all experiments, and all mice were fed a purified control diet (E15051-04, ssniff, Germany) throughout the entire study period. All mice were given 1 week acclimatization, were monitored daily and weighed once a week. The mice were subjected to a 12 h light/dark cycle (6:00 a.m. to 6:00 p.m.) with *ad libitum* access to water and food, and randomly assigned to treatment groups. For *H. polygyrus* experiments, mice were orally gavaged on alternate days for 4 weeks with either PAC (200 mg/kg BW, PAC or *H. polygyrus* + PAC groups) or water (control and *H. polygyrus* groups) (**Figure S2A)**. Mice were infected with *H. polygyrus* (200 third-stage larvae/mouse in 200 µl water) on day 14 and were humanely euthanized on day 28 (i.e. 14 days p.i.). PAC was dissolved in sterile water and given by oral gavage in the volume of 200 µl per mouse. For *T. muris* experiments, all mice were orally gavaged on alternate days for 7 weeks with either PAC (300 mg/kg body weight, PAC and *T. muris* + PAC groups) or water only (control and *T. muris* groups) (**Figure S2B)**. After 2 weeks of PAC treatment, the appropriate treatment groups were infected with 20 eggs of *T. muris* (strain E) every 2 weeks (day 0, 14 and 21) to establish naturally occurring chronic infection[34]. At the end of the experimental period (day 35 post first infection), all mice were humanely euthanized by cervical dislocation.

H. polygyrus and *T. muris* were propogated, and excretory/secretory (E/S) antigens produced, as described previously[22,35].

Sample collection

Fecal samples and blood were collected at necropsy. Separated serum was stored at -20 °C until use. After sacrifice, the MLN were dissected and stored on ice in 10 % fetal calf serum (FCS)-supplemented RPMI 1640 media (complete media) until further processing for flow cytometry. Caecal contents were harvested and cooled immediately on ice, and stored at – 80 °C until used for extraction. Tissue samples from the duodenum of *H. polygyrus* infected mice, and the caecal tip of *T. muris* infected mice, were collected and stored in RNA later for RNA sequencing. Full-thickness duodenum samples were collected from *H. polygyrus* infected mice for histology. Histology samples were longitudinally opened, gently rinsed with PBS and stored in 4 % paraformaldehyde until further processing. Worm burdens were assessed by manual enumeration under a stereomicroscope.

Histology

Samples stored in 4% paraformaldehyde. were embedded in paraffin blocks, sectioned, and mounted on glass slides prior to Periodic-acid Schiff (PAS) or Hematoxylin and eosin (H&E) staining. Furthermore, Mcpt1-positive mast cells were in additional paraffin-embedded sections, which were de-waxed, and Ag retrieval was performed with citrate buffer. Tissue sections were incubated with rat anti-mouse primary monoclonal mast cell protease-1 (MCPT-1) Ab (1:100, clone RF6.1; Thermo

Fisher Scientific), followed by secondary staining with biotinylated rabbit anti-rat IgG (Abcam). Mast, goblet and Paneth cells were manually enumerated by blinded microscopy.

Isolation of mesenteric lymph node cells

MLN were carefully dissected and trimmed of fat. Single cell suspensions were prepared by passing through a 70 μM cell strainer. Afterwards, the cell suspension was centrifuged at 450***g*** for 5 minutes at room temperature. Cell counts and viability were assessed manually using a haematocytometer and trypan blue staining.

Flow cytometry, e*x vivo* cell stimulation and cytokine analysis

All cell suspensions were incubated with Fc-block (anti-CD16/CD32, BD Biosciences, cat no. 553142) followed by staining with antibodies against surface and intranuclear markers. Cells were kept at 4°C throughout the staining procedure. Cell surface markers were stained for 20 min using an antibody cocktail containing; FITC-conjugated hamster anti-mouse TCRβ (clone H57-597; BD Biosciences, cat no. 553171), and PerCP-Cy5.5-conjugated rat anti-CD4 (RM4-5; BD Biosciences cat no.550954). For intranuclear (GATA-3 and T-bet) staining, FoxP3/Transcription Factor staining buffer (eBiosciences, 00-5523-00) was used according to manufacturer's instructions. Fixed/ permeabilized cells were incubated for 30 min on ice with: Alexa Fluor® 647-conjugated mouse anti-mouse T-bet (4B10; BD Biosciences cat no. 561264), PE-conjugated rat anti-mouse GATA3 (TWAJ; Thermo Fisher Scientific cat no. 12-9966-42), FITC-conjugated rat anti-mouse FoxP3 (FJK-16s; Thermo Fisher Scientific cat no. 11-5773-82). Cells were analyzed on an BD Accuri C6 flow cytometer (BD Biosciences). All data were acquired and analyzed using Accuri CFlow Plus software (Accuri® Cytometers Inc.).

For cytokine analysis, MLN cells were plated in triplicate into 96-well cell culture plates at the density of 5.0 x 10^6 cells/mL in complete media, and stimulated with *T. muris* E/S antigens or PBS. Cells were incubated at 37 °C/5 % CO_2 and after 24 hours cell-free supernatants were harvested and stored at -20°C for subsequent analyses. Secreted cytokines were measured using a BD Th1/Th2/ Th17 cytometric bead array (CBA) kit (BD Biosciences cat no. 560485) according to manufacturer's instructions. Samples were processed on a BD Accuri C6 flow cytometer (BD Biosciences), with data acquired using Accuri CFlow Plus software (Accuri® Cytometers Inc., MI, USA).

Enzyme-linked immunosorbent assay

T. muris E/S-specific antibodies were measured from diluted (1:50) serum using a previously described ELISA protocol [22]. The antibodies employed for ELISA were: biotin-conjugated rat anti-mouse IgG2a (clone R19-5, BD Biosciences, Denmark cat no. 550332) and anti-mouse IgG conjugated to horseradish peroxidase (HRP; Bio-Rad, Germany cat no. 1706516). Absorbance was measured at an optical density of 450 nm with a Multiskan FC plate reader (Waltham, MA, USA). *H. polygyrus* E/S-specific IgG1 was detected using goat anti-mouse IgG1-HRP conjugate (Invitrogen, cat no. A10551).

RNA extraction, RNA sequencing, and qPCR

Tissue was mechanically homogenized in QIAzol lysis buffer using a gentleMACS™ dissociator (Miltenyi Biotec, Germany) and total RNA was isolated using miRNeasy® Mini Kit (Qiagen, CA, USA) as per manufacturer's instructions. Total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA)**.** Paired-end (100 bp) RNA-sequencing was carried out using the DNBSEQ sequencing platform (BGI, Copenhagen, Denmark). Clean reads were mapped to the mouse genome (*mm10*) using Bowtie2 (v2.2.5). Differentially expressed genes were detected using DEseq2[36]. Volcano plots were constructed using VolcaNoseR[37]. cDNA was synthesized from 500 ng of RNA using Quantitect Reverse Transcriptase kits (Qiagen) and qPCR was performed using perfeCTa SYBR green fastmix (Quanta Bioscience) using the following program: 95°C for 2 minutes followed by 40 cycles of 15 seconds at 95°C and 20 seconds at 60°C. Primer sequences are listed in **Table S1.**

Short chain fatty acids and proanthocyanidin metabolites analysis

SCFA were measured using GC-MS analysis, whilst PAC metabolites were analyzed using UHPLC-MS/MS using previous described methods[22,38].

Microbiota analysis

DNA was extracted from 100mg caecal content (*H. polygyrus* study*)* and fecal samples (*T. muris* study*)* using the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Poland) according to the manufacturer's instructions with the addition of mutanolysin and lysozyme in order to enhance bacterial cell wall degradation. Amplification of the V3 region of the 16S rRNA gene and sequencing was conducted as previously described[39]. The raw data containing paired-end reads were merged and trimmed as previously described, and differences in β-diversity between groups assessed using distance-based redundancy analysis[40].

Statistical analysis and data availability

Statistical analyses were performed in Prism 8.0.2 (GraphPad Software). While using Prism, two-way ANOVA was used for multiple comparisons, as well as t tests when appropriate, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Analysis of data which did not follow a Gaussian distribution was analysed with non-parametric tests in Prism or in SPSS (IBM SPSS Statistics 27). RNA sequence data from caecum and duodenum are deposited at the NCBI Gene Expression Omnibus (GEO: GSE174756).

Results

Proanthocyanidins alter expression of immune-related genes and reduce mastocytosis during small intestinal nematode infection

To examine the effects of PAC on the small intestinal response to helminth infection, mice were fed compositionally defined diets and were orally dosed every 2nd day with 200 mg/kg PAC derived from grape pomace dissolved in water, or water only for 4 weeks. Half-through the experiment, mice were

then infected with *H. polygyrus* (day 0) and euthanized 14 days post-infection (p.i.). RNA sequencing of duodenum tissues from *H. polygyrus*-infected mice showed a strong upregulation of many genes involved in type-2 immunity, relative to uninfected control mice (**Figure 1A**). These included genes involved in mast cell responses (*Mcpt1*, *Ms4a2*) and innate defense molecules (*Defa32*, *Ang4*). Downregulated genes were mainly involved in nutrient uptake and metabolism, consistent with the marked changes in gut function that accompany *H. polygyrus* infections[41]. We next compared duodenal transcriptomic responses of *H. polygyrus*-infected mice receiving either PAC, or water alone. This revealed a suppression of several genes related to immune function by PAC. Notably, we observed a downregulation of B-cell related genes, such as *Fcer2a* and *Cd19*, and mast cell related genes, such as *Mcpt1*and *Ms4a1* (**Figure 1B**). Interestingly, the bactericidal C-type lectin *Reg3b* was also significantly downregulated by PAC in infected mice. The down-regulation of aforementioned genes by PAC was confirmed by qPCR (**Figure 1C**). We also substantiated that there was a significant increase of Mcpt1-positive cells in the intestinal mucosa when comparing infected mice to control mice, but this increase was not observed in infected mice dosed with PAC (**Figure 1D**). The infection induced goblet and Paneth cell hyperplasia in the duodenum, and, interestingly, Paneth cell numbers in infected mice were significantly increased by PAC, indicating a possible stimulatory effect of PAC on the innate mucosal antimicrobial response (**Figure 1E-F**). However, we observed no significant effect of PAC on worm burdens, or *H. polygyrus*-specific IgG_1 levels in serum at day 14 p.i. (**Figure 1G-H**). Thus, in this model PAC did not significantly enhance parasite-specific immunity, but rather tended to restrain the type-2 tissue response induced by *H. polygyrus*.

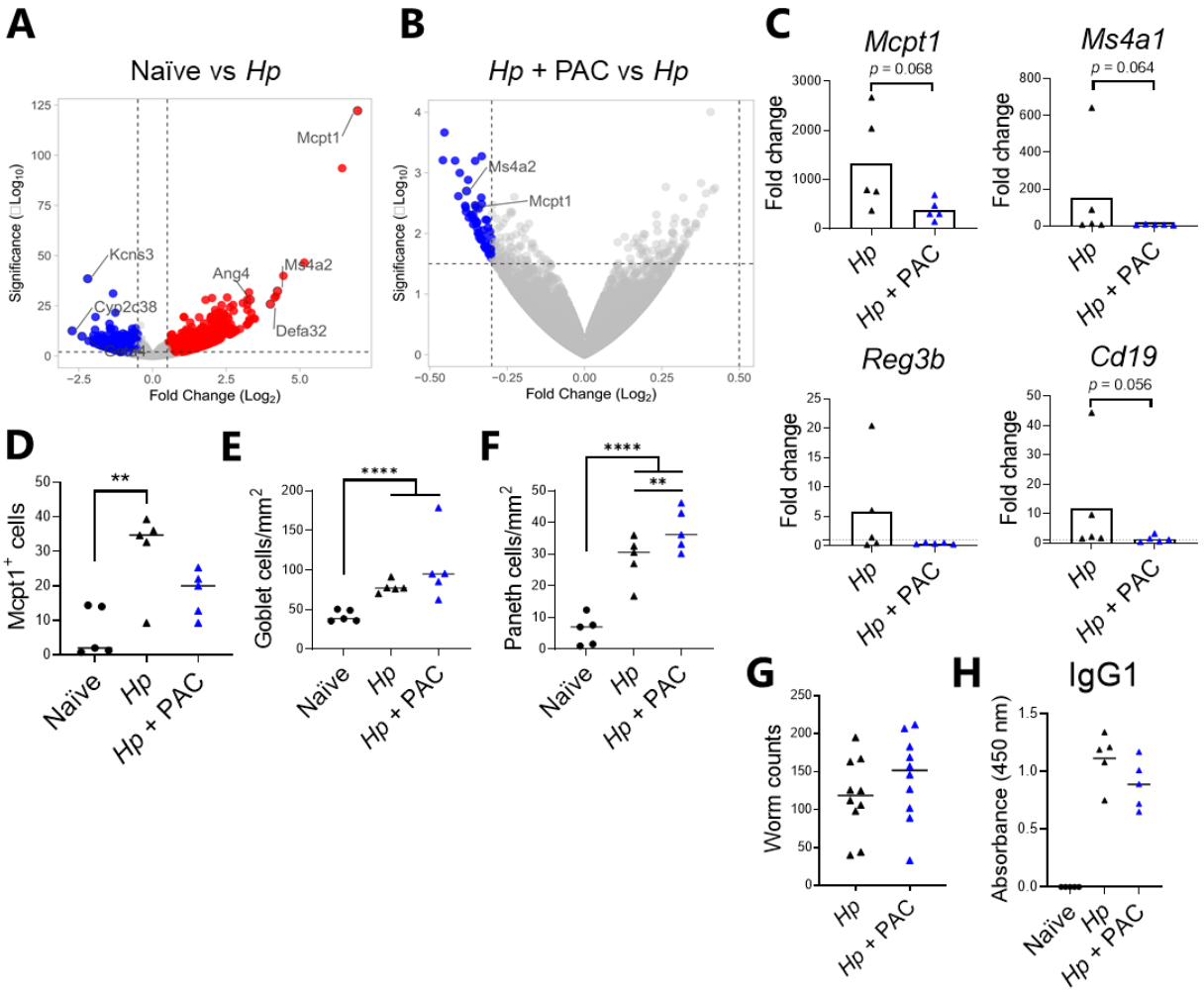

Figure 1 Proanthocyanidins downregulate immune-related genes and affect residing immune cell hyperplasia in duodenum tissues of *Heligmosomoides polygyrus* infected mice with no effect on worm burdens

A) Differentially expressed genes in the duodenum of *Heligmosomoides polygyrus* (*Hp*) infected mice relative to naïve mice, identified by RNA-sequencing (*n*= 5 mice per treatment group).

B) Differentially expressed genes in the duodenum of PAC-dosed *Hp* infected mice, relative to *Hp* infection only (*n*= 5 mice per treatment group).

C) Expression of *Mcpt1*, *Cd19*, *Ms4a1*, and *Reg3b* measured by qPCR (*n*= 5 mice per treatment group). **(Mann-Whitney or unpaired t tests).**

Enumeration of Mcpt1-expressing mast cells (**D**), goblet cells (**E**) and Paneth cells (**F**) in the duodenum (*n*= 5 mice per treatment group). **(*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, by one-way ANOVA or Kruskal-Wallis test).**

G) Worm burdens in *Hp* infected mice (2 independent experiments, each n=5)

H) *Hp* specific serum IgG1 levels (*n*= 5 mice per treatment group). **(Unpaired t test).** Black points = water, blue points = PAC.

Proanthocyanidins modulate caecal transcriptomic responses and increase susceptibility to whipworm infection

We next assessed the impact of PAC on a chronic, trickle infection of the caecal-dwelling whipworm *T. muris.* In contrast to *H. polygyrus* infection, low doses of *T. muris* eggs induce both type-2 and type-1 immune effector mechanisms, with the Th1-bias preventing expulsion, allowing worm

persistence and the development of colitis-like pathology[21]. Consistent with this, RNA-sequencing analysis of caecal tissues showed a clear upregulation of genes involved in pro-inflammatory responses including *Ifng*, *Nos2* and *Gzmb* as well as type-2 genes such as *Mcpt1* following three doses of 20 *T. muris* eggs over a period of 35 days (**Figure 2A**). Furthermore, gene pathway analysis demonstrated the upregulation of numerous pathways related to inflammation, cellular immunity and interferon signaling (**Figure 2B**). We then treated uninfected or infected mice with purified PAC from grape seed extract (300 mg/kg) 14 days prior to, and throughout, the infection period. Interestingly, PAC intake decreased body weight gains relative to mice consuming only purified diets (**Figure S1**). Caecal tissue was harvested from mice at day 35 p.i. (49 days after PAC treatment commenced). In uninfected mice, RNA-sequencing analysis showed that PAC intake upregulated numerous genes related to extracellular matrix remodeling (e.g. *Cd34, Lum, Csrp1*), as well as gene pathways related to cellular proliferation and integrin function, consistent with known functions of PAC in stimulating epithelial cell growth[42]. (**Figure 2C and D**). In infected mice administered PAC, we observed only minor gene expression changes relative to infected control mice. However, it was notable that most of the upregulated genes were related to interferon signaling (**Figure 2E**), which was confirmed by gene-set enrichment analysis (**Figure 2F**) and qPCR analysis of *Ifit3b* and *Irgm2* (**Figure 2G**). Consistent with this, we also found that PAC increased serum levels of *T. muris*-specific IgG2a, a marker of Th1 responses and parasite chronicity (**Figure 2H**). Moreover, adult worm burdens (but not larval worm burdens) were significantly higher in PAC-treated mice (**Figure 2I**). Collectively, these data suggest that PAC intake during whipworm infection promotes Th1 polarization and regulates host susceptibility to adult worms.

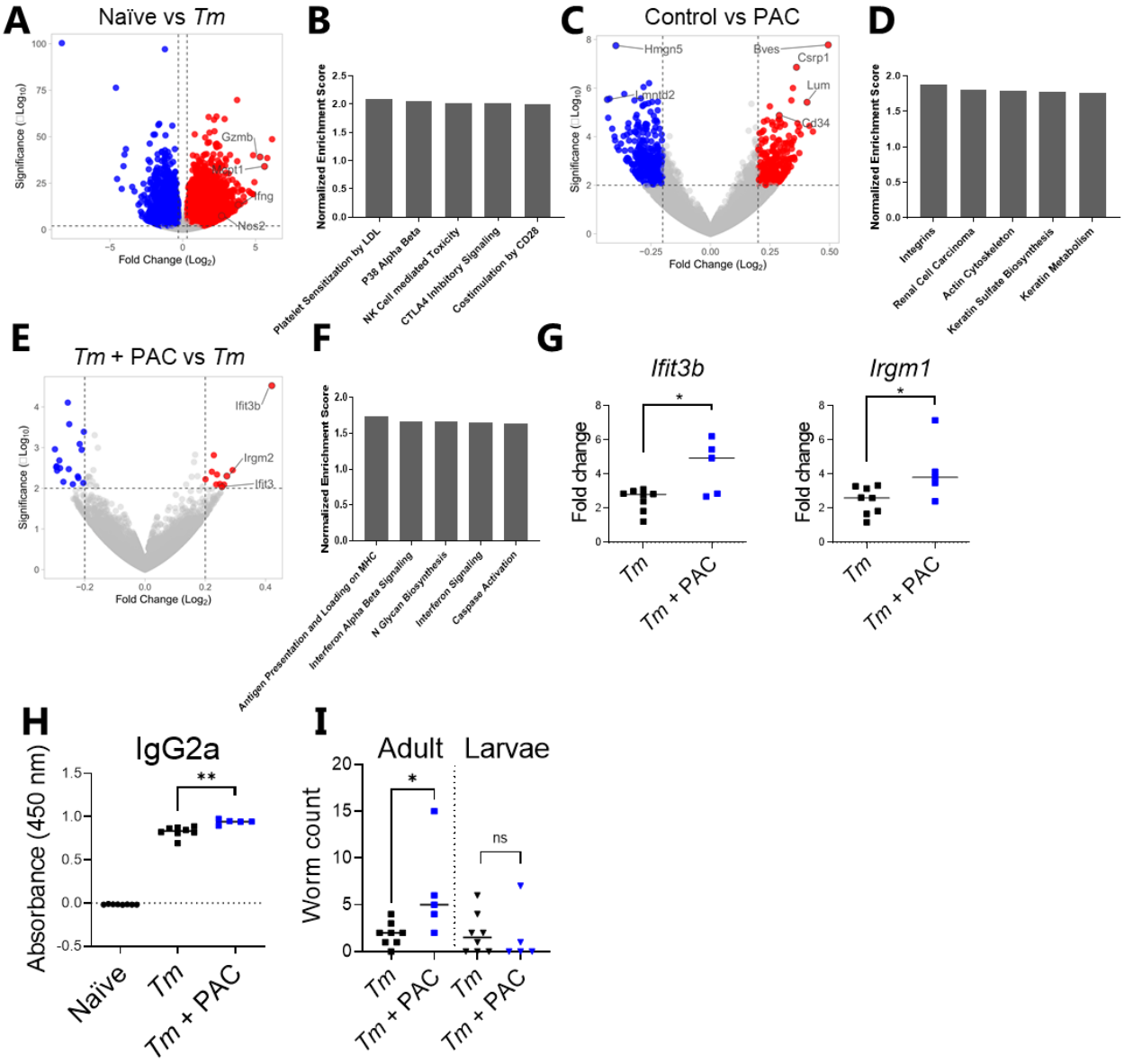

Figure 2 Proanthocyanidins upregulate of interferon-related gene expression in *Trichuris muris* infected mice

A) Differentially expressed genes in caecal tissues of *Trichuris muris* (*Tm*) infected mice, relative to naïve mice (*n*= 8 mice per group, from 2 independent experiments).

B) Significantly upregulated gene pathways in caecal tissues from *Tm* infected mice compared to naïve mice (*n*= 8 per group, from 2 independent experiments). **(*p* < 0.01; q < 0.1 by gene-set enrichment analysis).**

C) Differentially expressed genes in caecal tissues of naïve mice administered proanthocyanidins (PAC), relative to naïve control mice (*n*= 8 mice per group from 2 independent experiments)

D) Significantly upregulated gene pathways in caecal tissues from naïve mice administered PAC, compared to control mice (*n*= 8 per group, from 2 independent experiments). **(*p* < 0.01; q < 0.1 by gene-set enrichment analysis).**

E) Differentially expressed genes in caecal tissues of *Tm* infected mice administered PAC, relative to infected, control mice (*n*= 8 mice in *Tm* group, *n*= 5 mice in *Tm* + PAC group, from 2 independent experiments).

F) Significantly upregulated gene pathways in caecal tissues from *T. muris*-infected mice administered PAC, compared to *Tm* infected control mice. (*n*= 8 mice *Tm* group, *n*= 5 mice in *Tm* + PAC group). **(*p* < 0.01; q < 0.1 by gene-set enrichment analysis).**

G) Regulation of *Ifit3b* and *Irgm1* expression measured by qPCR (*n*= 8 mice in *Tm* group, *n*= 5 mice in *Tm* + PAC group). **(*p < 0.05 by unpaired t-test).**
H) *Tm* specific serum IgG2a levels (*n*= 8 mice in naïve and *Tm* groups, *n*= 5 mice in *Tm* + PAC group). **(**p < 0.01 by unpaired t-test).**
I) Adult and larval worm burdens in *Tm* infected mice (*n*= 8 mice in *Tm* group, *n*= 5 mice in *Tm* + PAC group). **(*p < 0.05 by un-paired t tests).**
Black points = water, blue points = PAC.

Proanthocyanidins modulate *ex vivo* cytokine secretion from mesenteric lymph node cells following *Trichuris muris* infection

To further explore the effects of PAC on *T. muris*-specific immune responses, MLN cells from infected mice were harvested and stimulated *ex vivo* with *T. muris* antigens. Interestingly, cells isolated from infected mice dosed with PAC secreted numerically higher levels of the type 1 cytokines IL-2, TNFα, and IFNγ and significantly higher amounts of the type-1/17 cytokine IL-6 (**Figure 3A-D**). These findings are consistent with the caecal transcriptomic data which suggest a significant impact of PAC, with an indication of a type-1 polarized response elicited by infection.

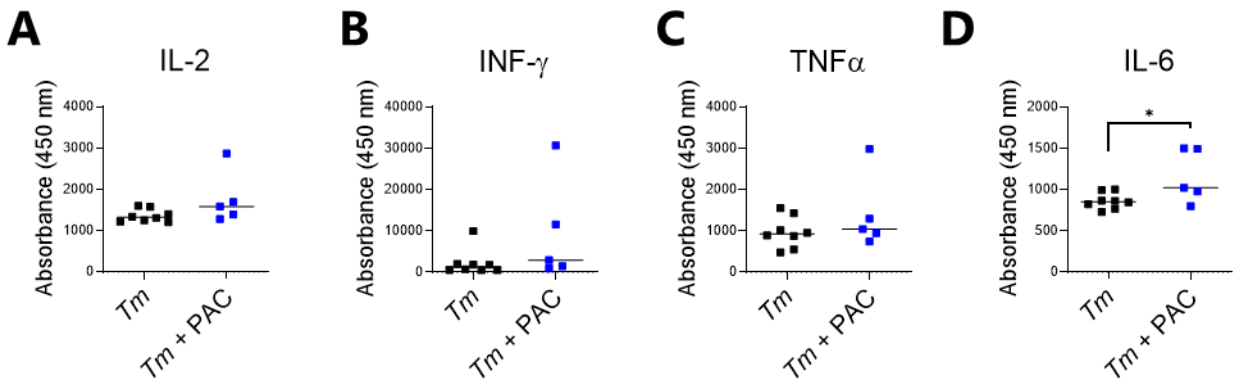

Figure 3 *Ex vivo* stimulation of mesenteric lymph node cells isolated from *Trichuris muris* infected mice and the effect of dietary proanthocyanidins
Effect of proanthocyanidins (PAC) on secretion of **A)** IL-2, **B)** INF-γ, **C)** TNFα **D)** IL-6 by lymphocytes isolated from mesenteric lymph nodes (MLN) in *Trichuris muris (Tm)* infected mice. MLN cells were stimulated with excretory/secretory (E/S) products from *Tm* (*n*= 8 mice *Tm* group, *n*= 5 mice in *Tm* + PAC group). **(*p < 0.05 by un-paired t tests).**
Black points = water, blue points = PAC.

Proanthocyanidins alter T cell populations in mesenteric lymph nodes in helminth-infected mice

The balance of intestinal T-cells induced during helminth infection may play a key role in determining the outcome of infection[43]. To explore if the transcriptomic changes observed in tissue were accompanied by cellular changes in the lymph nodes, MLN were isolated from *H. polygyrus*-infected mice at day 14 p.i. and *T. muris*-infected mice at day 35 p.i. to assess whether these were altered by infection and/or PAC supplementation. Interestingly, PAC intake significantly increased the total

number of cells in the MLN during infection with either *T. muris* or *H. polygyrus* , relative to control mice (**Figure 4A and B**). During *H. polygyrus* infection, equal proportions of Th1 (T-bet$^+$), Th2 (GATA3$^+$) and Treg (Foxp3$^+$) CD4$^+$ T-cells were induced by PAC treatment, resulting in a significantly enhanced cellular response to the parasite (**Figure 4C-F**). A similar tendency was observed in *T. muris* infected mice, however here there was a clearer Th1 polarization resulting from PAC treatment (**Figure 4G-J**). Whilst PAC tended to increase the absolute numbers of Th2 and Treg cells in infected mice, only Th1 cell numbers were significantly increased, suggesting that Th1 cells were selectively enhanced by PAC (**Figure 4K**). Taken together, these data indicate that PAC had a stimulatory effect on lymphocyte proliferation in the MLN, and a propensity towards Th1 polarization that was most pronounced during *T. muris* infection.

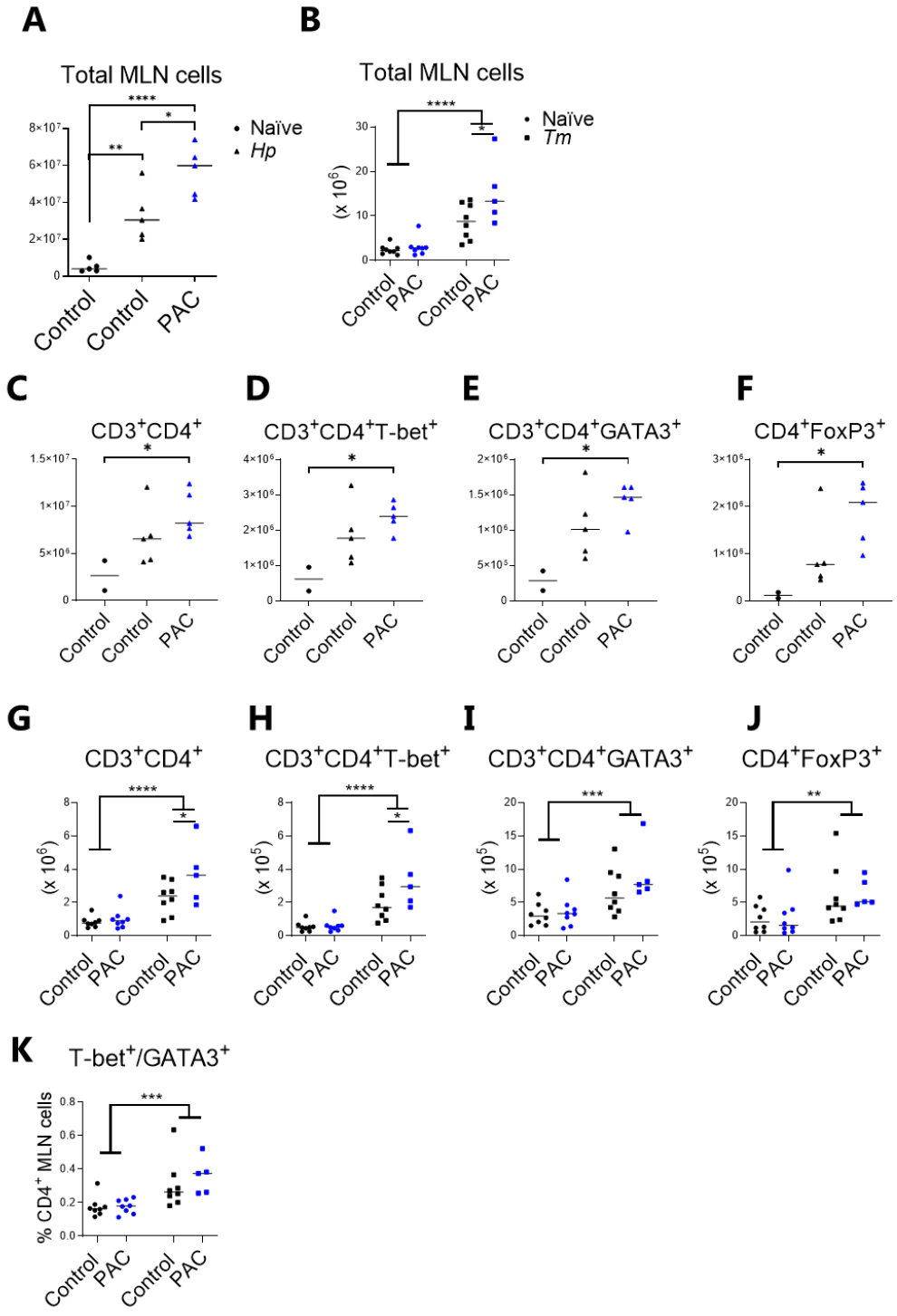

Figure 4 Proanthocyanidins alter T cell populations in the mesenteric lymph nodes in helminth-infected mice

Impact of proanthocyanidins (PAC) on the total number of cells in the mesenteric lymph nodes (MLN) in **A)** *Heligmosomoides polygyrus (Hp) (n*= 5 mice per group) and **B)** *Trichuris muris (Tm)* infected mice (*n*= 8 mice in naïve and *Tm* groups, *n*= 5 mice in *Tm* + PAC group) compared to naïve mice (***p* < 0.05, ****p* < 0.01, *****p* < 0.001, ******p* < 0.0001 by ANOVA).**

Effect of PAC on the distribution of **C)** $CD3^+CD4^+$, **D)** $CD3^+CD4^+$T-bet^+, **E)** $CD3^+CD4^+GATA3^+$ and **F)** $CD3^+CD4^+FoxP3^+$ cells in the MLN of *Hp* infected mice and naïve mice *(n*= 5 mice per group). **(*p* < 0.05 by one-way ANOVA).**

Effect of PAC on the distribution of **G)** $CD3^+CD4^+$and **H)** $CD3^+CD4^+$T-bet^+ **I)** $CD3^+CD4^+GATA3^+$ and **J)** $CD3^+CD4^+FoxP3^+$ cells in the MLN of *Tm* infected and naïve mice (n= 8 mice in naïve and *Tm* groups, n= 5 mice in *Tm* + PAC group). **(*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by two-way ANOVA). K)** $CD3^+CD4^+$T-bet^+/$CD3^+CD4^+GATA3^+$ ratios in *Tm* infected mice (n= 8 mice in naïve and *Tm* groups, n= 5 mice in *Tm* + PAC group).**(***p < 0.001 by two-way ANOVA).**
Black points = water, blue points = PAC.

Proanthocyanidins and helminths interact to change the gut microbiota composition

Given the significant influence of PAC on helminth-induced immune responses, we next assessed how this related to changes in GM composition. Analysis of weighted β-diversity in mice administered PAC with or without *H. polygyrus* infection revealed four distinct clusters corresponding to the treatment groups. *H. polygyrus* infection and PAC supplementation significantly altered the composition of the caecal GM at day 14 p.i. (both $p < 0.05$ relative to control group by distance-based redundancy analysis**; Figure 5A**). Consistent with previous studies, the major taxonomical change induced by *H. polygyrus* was an increase in Firmicutes, particularly *Lactobacillus* species[44], such as *L. johnsonii* (**Figure 5B**). PAC intake led to a significant increase in the abundance of a vOTU corresponding to *Turicibacter sanguinis*, a bacterium associated with lipid metabolism and triglyceride levels[45] – pathways known to be modulated by PAC[46]. PAC also reduced the abundance of *L. animalis*, but only in the uninfected mice (**Figure 5B**). Within infected mice, there was also a significant difference in β-diversity between the PAC-supplemented and control mice ($p < 0.05$ by distance redundancy analysis; **Figure 5A**), suggesting that PAC modulated the response to infection, consistent with the transcriptomic analysis. The major taxonomical difference between these groups was an increase in *Bifidobacterium animalis* (**Figure 5B**). Thus, PAC intake induced distinct changes in GM composition that were dependent on infection status, indicating a profound interaction between diet and helminth infection.

We next assessed faecal GM changes in *T. muris* infected mice, to explore whether the clear changes in helminth burden and immune status as a result of PAC correlated with specific GM parameters. *T. muris* infection had the strongest effect on *β*-diversity, with infected mice being clearly diverged from uninfected mice, regardless of PAC intake (**Figure 5C**). Infection was associated with increases in vOTUs corresponding to *L. animalis* and *B. animalis*, however the expansion of *Bifidobacteria* was attenuated in PAC-supplemented mice (**Figure 5D**). In this experiment, the effect of PAC on *B*-diversity was not as pronounced as observed in the *H. polygrus* experiments, which may relate to the differences in samples analyzed (caecum vs. faeces) or the length of study (28 vs 49 days). Despite this, we again noted a trend for enrichment of *T. sanguinis* in PAC-dosed mice, together with a trend for a reduction in *L. animalis*. Most notably, we found that the combination of PAC and *T. muris* infection led to the expansion of the potentially pathogenic *Escherichia fergusonii* which was absent in all other treatment groups (**Figure 5D**). Thus, consistent with the parasitological and immunological data, administration of PAC to *T. muris*-infected mice tended to result in an increase in parameters associated with dysbiosis.

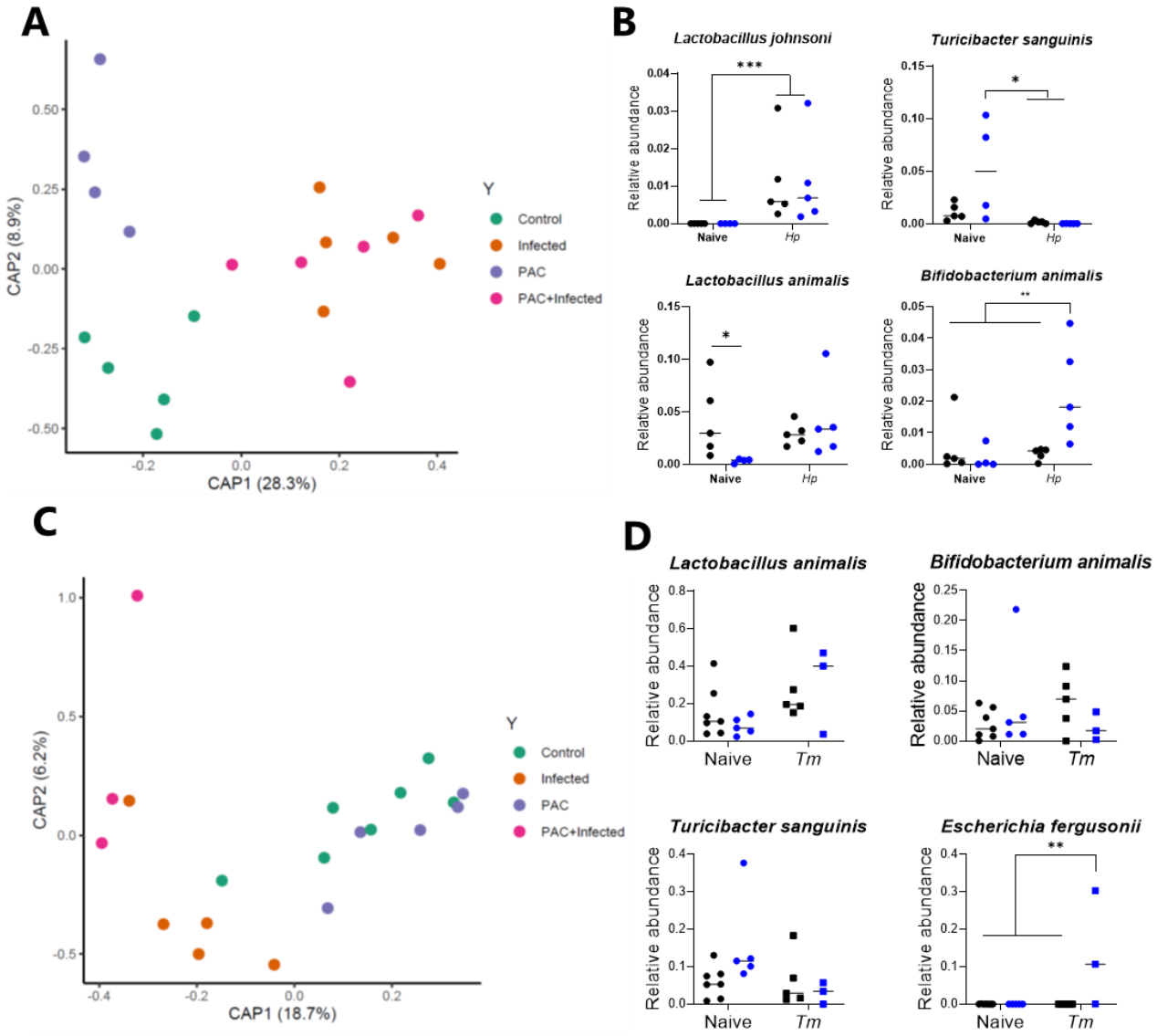

Figure 5 Impact of helminth infections and proanthocyanidins on gut microbiota

A) Distance-based redundancy analysis plot of β-diversity in naïve and *Heligmosomoides polygyrus (Hp)* infected mice dosed with either proanthocyanidins (PAC) or water *(n*= 4-5 mice per group).

B) Relative abundance of vOTUs corresponding to *Lactobacillus johnsonii, Turicibacter sanguinis, L. animalis* and *Bifidobacterium animalis* in naïve and *Hp* infected mice dosed with either PAC or water *(n*= 5 mice per group) **(*p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA).**

C) Distance-based redundancy analysis plot of β-diversity in naïve and *Trichuris muris* infected mice dosed with either PAC or water.

B) Relative abundance of vOTUs corresponding to *L. animalis, B. animalis, T. sanguinis*, and *Escherichia fergusonii* in naïve and *T. muris (Tm)* infected mice gavaged with either PAC or water (*n*= 8 mice in naïve and *Tm* groups, *n*= 5 mice in *Tm* + PAC group) **(**p < 0.01 by two-way ANOVA).**

Black points = water, blue points = PAC.

Concomitant *Trichuris muris* infection and proanthocyanidin supplementation affect the production of short chain fatty acid and proanthocyanidin metabolites

Given the observed changes in the GM composition, we next asked how infection and PAC supplementation may affect the production of GM-derived metabolites. To this end, the faeces of uninfected and *T. muris*-infected mice administered PAC were analyzed for short chain fatty acid (SCFA) concentrations. We observed significant interactions ($p < 0.05$) between diet and infection for acetic acid, propionic acid, and total SCFA. These interactions reflected a generally higher level of SCFA in either uninfected PAC-dosed mice or *T. muris*-infected control mice, but a lower level in the combinatorial group of PAC-dosed *T. muris*-infected mice (**Figure 6A-C**). Thus, whilst both treatments in isolation promoted SCFA production, instead of an additive effect we detected an antagonistic trend. Butyric acid followed the same trend but was not significant (**Figure 6D**).

A number of studies have shown that small intestinal absorption of PAC is minimal, and they are available for metabolism by the resident GM in the large intestine. Accordingly, PAC-derived metabolites such as valerolactones have been proposed to play a role in their putative health benefits, such as improved vascular function[47,48]. Therefore, we examined whether phenolic metabolites produced from the breakdown of PAC were altered during *T. muris* infection, by examining the abundance of a panel of PAC-derived metabolites in the caecum of uninfected and infected mice dosed with PAC. As expected, PAC supplementation resulted in substantial increases of these metabolites in the caecum, compared to control mice. Most of the metabolites were similar in both groups of mice that received PAC supplementation, regardless of infection (**Figure 6E**). However, we identified a cluster of related metabolites that were reduced in abundance in infected mice. These were mostly valerolactones and valeric acid derivatives including 3,4-dihydroxyphenyl-y-valerolactone, dihydroxyphenyl-y-valerolactone sulfate, dihydroxyphenylvaleric acid sulfate, and hydroxyphenylpropionic acid (**Figure 6F**). Moreover the serum concentration of dihydroxyphenyl-y-valerolactone sulfate and 4-hydroxy-5-(dihydroxyphenyl)valeric acid sulfate were also significantly reduced in infected mice dosed with PAC (**Figure 6G**). Hydroxyphenylpropionic acid and 3,4-dihydroxyphenyl-y-valerolactone were not detected in the serum in any of the treatment groups (data not shown). Thus, both *T. muris* and PAC supplementation in isolation appeared to have positive effects on the production of microbial-derived metabolites in the gut, but concurrent infection and PAC intake attenuated both SCFA levels and the production and absorption of valerolactones, suggestive of an antagonistic interaction that may have a negative effect on tissue homeostasis and intestinal health.

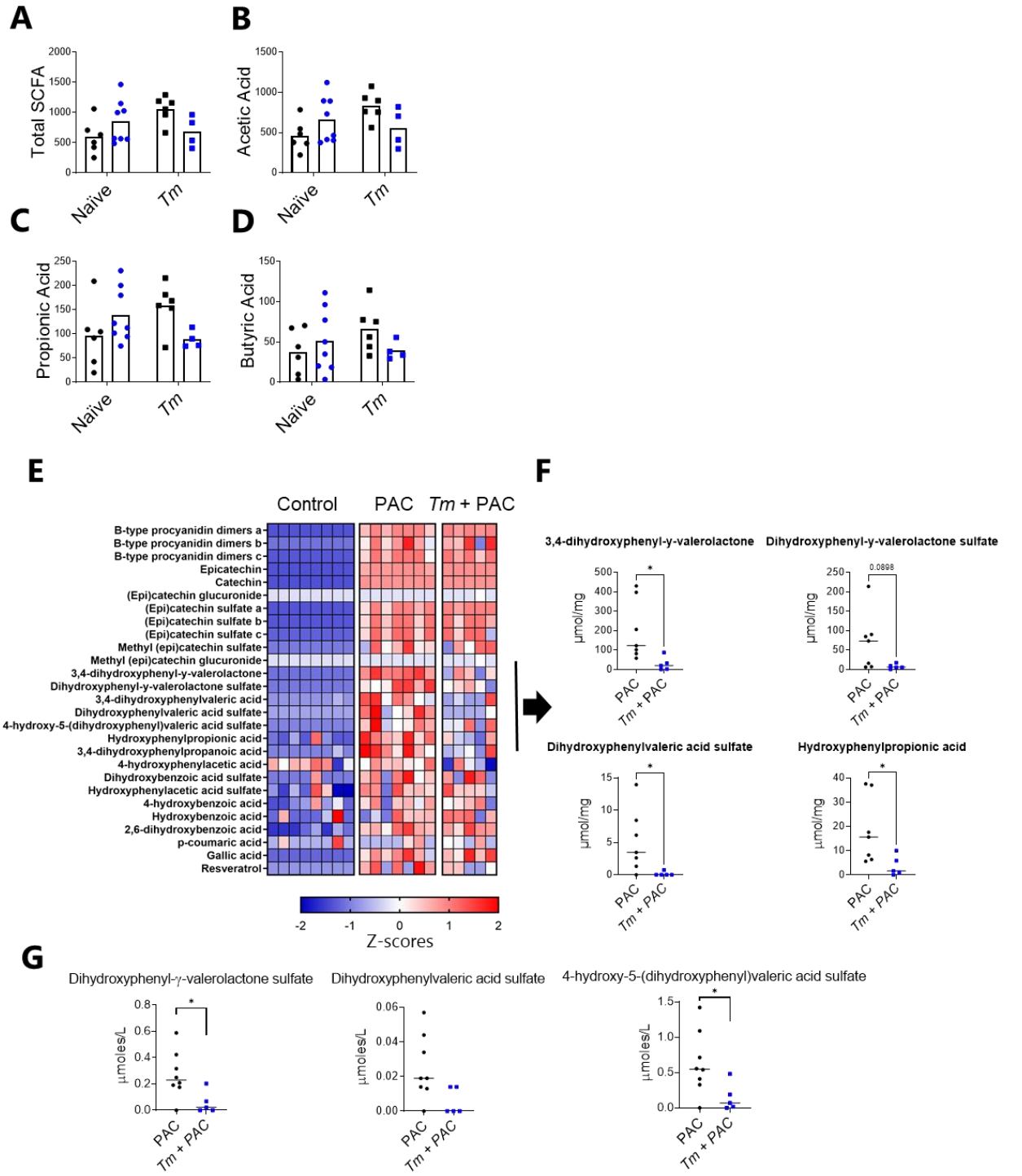

Figure 6 Short chain fatty acids and identification of proanthocyanidin metabolites in *Trichuris muris* infected mice

Effect of proanthocyanidins (PAC) and *Trichuris muris (Tm)* infection on the concentrations of **A)** total short chain fatty acids (SCFA), **B)** acetic acids, **C)** propionic acid and **D)** butyric acid in faecal samples. A significant interaction between diet and infection was reported for acetic acid, propionic acid and total SCFA by two-way ANOVA (n = 6 mice in naïve group, n = 8 mice in PAC group, n = 6 in *Tm* groups, and n = 4 mice in *Tm* + PAC group).

E) Identification of PAC metabolites in the caecum of PAC-dosed naïve and *Tm* infected mice relative to naïve mice dosed with water (n = 8 in controls n= *7* mice in PAC group, n= 5 mice in *Tm* + PAC group).

F) Caecal concentration of 3,4-dihydroxyphenyl-y-valerolactones, dihydroxyphenyl-y-valerolactone sulfate, dihydroxyphenylvaleric acid sulfate, and hydroxyphenylpropionic acid in PAC-dosed naïve and *Tm* infected mice (n= 7 mice in PAC group, n= 5 mice in *Tm* + PAC group). **(*p < 0.05 by unpaired t-tests).**
G) Serum concentration of dihydroxyphenyl-y-valerolactone sulfate, and dihydroxyphenylvaleric acid sulfate and 4-hydroxy-5-(dihydroxyphenyl)valeric acid sulfate in PAC-dosed naïve and *Tm* infected mice (n= 8 mice in PAC group, n= 5 mice in *Tm* + PAC group). **(*p < 0.05 by unpaired t test).**
Black points = water, blue points = PAC.

Discussion

In the current work, we investigated the interplay between PAC and helminth infection in mice, and their effect on immune responses and the gut environment. Whilst the immunomodulatory effects of parasites have been extensively demonstrated in previous studies, less is known on the modulatory implication of bioactive diets during helminth infections[49,50]. Similarly, whilst the impact of helminth infection on the GM has also been investigated in numerous studies, diet-parasite interactions and their effects on gut health are largely unexplored[24,51,52]. Several studies have demonstrated strong anti-inflammatory properties of PAC, which have also been shown to be beneficial for gut health by supporting gut barrier function[6,53]. Moreover, PAC may alter gut microbiota by enhancing the growth of beneficial bacteria, such as *Bacteroides* species[54–56]. Thus, by altering the bacterial flora of the intestinal tract, PAC may indirectly stimulate gut-associated myeloid tissues, and thereby modulate T- or B-cell mediated immune responses[57,58].

Based on these known anti-inflammatory and immunomodulatory properties, we hypothesized that PAC may reduce helminth-induced inflammation by either enhancing the diversity and composition of the GM, or by directly stimulating mucosal immune cells. Both infection models clearly showed a higher number of MLN cells in infected mice dosed with PAC, which suggests a strong effect of PAC on immune reactivity and lymphocyte proliferation. However, we surprisingly found an indication of a type-1 polarized immune response in PAC-treated *H. polygyrus* infected, which was further validated in our *T. muris* model. Indeed, investigation of MLN T cell populations revealed an effect of PAC in *T. muris* infected mice, with significantly increased T-bet$^+$ Th1 cells, enhanced serum IgG2a levels, and induced higher burdens of adult worms. These findings may suggest a priming effect of PAC in the intestinal mucosa. A type-1 polarized immune response in the gut may thus dampen or inhibit the natural type-2 immune response elicited by helminth-infection. An inhibitory effect on Th2 immune response was also indicated in PAC-supplemented *H. polygyrus* infected mice, where the abundance of Mcpt1-stained cells, a marker for mast cells, did not significantly differ from un-infected mice. Interestingly, a recent study conducted with inulin (a prebiotic oligosaccharide with known anti-inflammatory effects) demonstrated similar effects, with higher worm burdens and Th1 driven immune response in *T. muris* infected mice, indicating an important context-dependent immunological effect of inulin[22].

To assess the impact of infection and PAC supplementation on the gut environment, we investigated several factors, such as GM composition, SCFA and PAC metabolites. As expected, the GM was strongly altered by both infection and PAC, which however most prominently in *H. polygyrus*

infected mice. Of note, *H. polygyrus* significantly enhanced the growth of *Lactobacillus johnsonii,* which has been associated with improved gut health by reducing inflammation in several animal models[59–62]. Also, *H. polygyrus* has previously been shown to increase the abundance of *Lactobacilli spp.* in the small intestine[44], and produces antimicrobial C-type lectins[63]. Similarly, PAC-supplemented *H. polygyrus* infected mice had a significantly higher abundance of *Bifidobacterium animalis*, which has also been shown to improve intestinal barrier function[64,65]. Fewer changes were observed in *T. muris* infected mice, however β-diversity was significantly changed in both infection models. This, suggest that the interactions between PAC and the GM, may have beneficial effects on the gut milieu but may also be context-dependent. Accordingly, PAC tended to increase SCFA in un-infected mice. However, we observed that whilst *T. muris* infected mice had significantly higher levels of SCFA, concomitant PAC supplementation tended to decrease this effect, which may be a result of a reduced abundance of Bifidobacteria due to PAC intake. Interestingly, a similar negative effect of inulin on SCFA during *T. muris* infection, was reported a recent study[22]. Thus, the level of produced SCFA by the GM is highly context dependent, and may be indirectly affected by helminths and diet.

As PAC are known to be metabolized in the large intestines, we sought to identify which PAC metabolites were present in the caecum. As expected, a number of PAC metabolites were identified in PAC-treated mice. However, significantly fewer metabolites were found in *T. muris* infected mice dosed with PAC, including valerolactones. This may be closely related to the changes in the GM composition induced by helminth infection, which may alter PAC metabolism efficiency. Another suggestion could be that PAC metabolites are more easily absorbed in the gut, due to the increased permeability and disruption of the gut mucosal barrier caused by the helminth infections. However, the serum concentration of valerolactones was also significantly lower in infected mice, suggesting that an enhanced degradation of these metabolites by the GM seems most plausible.

Taken together, these studies offer novel insights on the effect of PAC on acute and chronic intestinal inflammation induced by helminths. Overall, we observed a stronger immune-modulatory effect of PAC in *T. muris* infected mice, which may be due to a higher activity of PAC in the caecum compared to the small intestine. However, as opposed to our initial hypothesis, both studies indicate that PAC-dosed mice may become more susceptible to helminth infection. Of note, this adverse effect of PAC was similar to a previous study conducted with PAC-treated mice, which exacerbated *C. rodentium* infection rather than inducing an anti-inflammatory response[32]. For now, the precise mechanisms underlying these observations remain unclear. However, our results clearly indicate that whilst PAC and other phytonutrients have demonstrated beneficial effects on gut health and inflammation, in the context of enteric parasite infection in mice a potentially antagonistic relationship exists. Furthermore, these effects may be restricted to the structural characteristic of the PAC, which were primarily PC-rich, whereas i.e. anthelmintic effects have been observed with PD-rich PAC[66].

Thus, these studies offer interesting novel perceptions in the various implications of PAC on immune response, and ability of modulating Th1/Th2 balance during helminth-induced inflammation. Further studies should aim at unravelling the immunological mechanisms underlying the complex relationship between diet, helminth infection and immune response.

Acknowledgements
The authors would like to thank Mette Marie Arnt Schjelde and Penille Jensen for excellent laboratory assistance and support for the conduction of animal studies. We are grateful to Professor Rick Maizels (University of Glasgow) and Dr Sebastian Rausch (Freie University, Berlin), for provision and advice on *H. polygyrus* infections.

Funding
This work was funded by the Independent Research Fund Denmark (Grant # 7026-0094B).

Conflict of interest
The authors declare no conflicts of interests regarding this study.

Ethical statement
All experiments involving animals were conducted in agreement with the Danish legislation and the Danish Animal Experiments Inspectorate with the license number 2015-15-0201-0076.

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Supplementary Material to Paper III

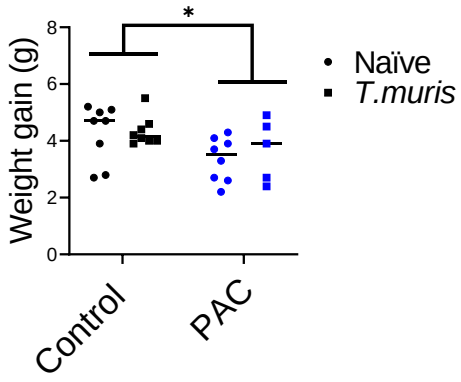

Figure S1 Body weight gain in *Trichuris muris* infected mice

(*n*= 8 mice in control and *Trichuris muris (Tm)* groups, *n*= 5 mice in *Tm* + PAC group).

(*p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA).

Black points = water, blue points = PAC.

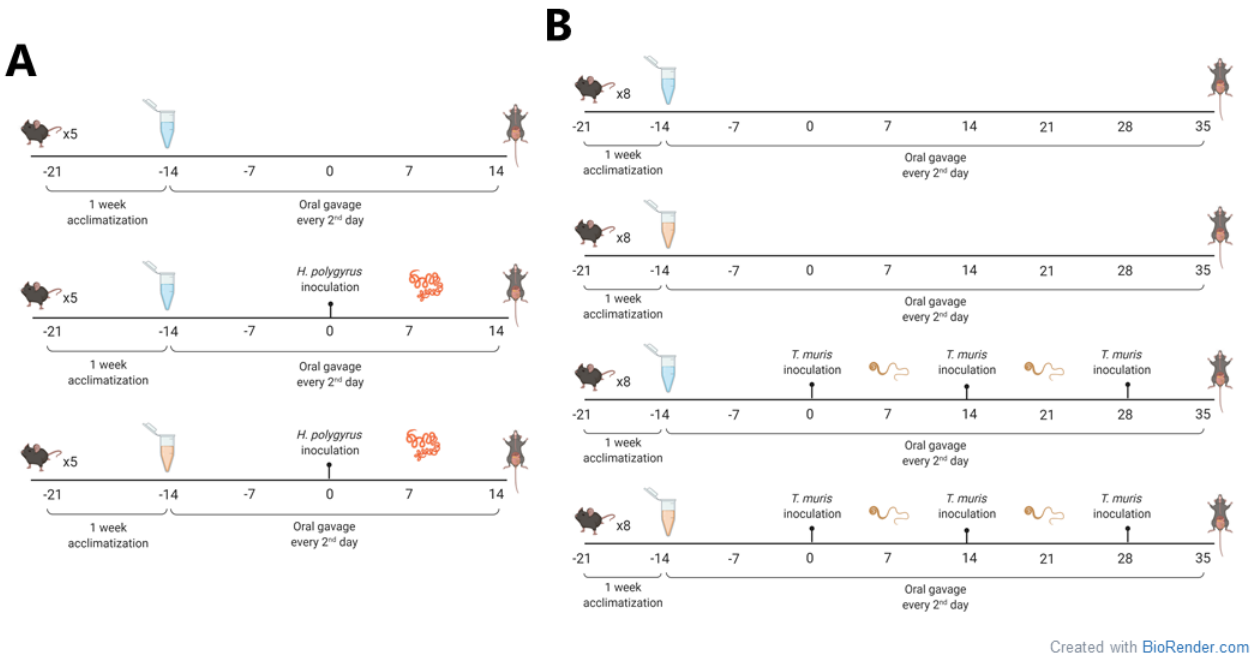

Figure S2 Experimental set-up of mouse studies

A) *Heligmosomoides polygyrus* study. **B)** *Trichuris muris* study. Figures were made with Biorender.com

Table S1 Primers used for qPCR

Primer name	Primer sequence (5'-> 3')
Used for *H. polygyrus* study	
Mcpt1 forward primer	*CTCTGCATATGTGCCCTGGATTA*
Mcpt1 everse primer	*GGGGGCAGACTGGGGATAGT*
Reg3b forward primer	*CAACAGCCTGCTCCGTCAT*
Reg3b reverse primer	*ATGCGTGCGGAGGGTATATT*
Cd19 forward primer	*GAGAGATGTGGGTTTGGGGG*
Cd19 reverse primer	*TGCTGACCTTGCAATGACCT*
Ms4a1 forward primer	*GGGACTTGAGTCAAACCCAGC*
Ms4a1 reverse primer	*TCAGCACATTGCTCACAAAGTA*
GAPDH forward primer	*TATGTCGTGGAGTCTACTGGT*
GAPDH reverse primer	*GAGTTGTCATATTTCTCGTGG*
Used for *T. muris* study	
*Irgm1*forward primer	*CCATGCGTATGCTGCTGTTT*
*Irgm1*reverse primer	*GATCTGCGGAGGGAAGATGG*
Ifit3b forward primer	*TTCCCAGCAGCACAGAAACA*
Ifit3b reverse primer	*GTTGCACACCCTGTCTTCCA*
GAPDH forward primer	*TATGTCGTGGAGTCTACTGGT*
GAPDH reverse primer	*GAGTTGTCATATTTCTCGTGG*

Paper IV

Dietary Proanthocyanidins Exert Localized Immunomodulatory Effects in Porcine Pulmonary and Gastrointestinal Tissues during *Ascaris suum*-induced Type 2 Inflammation

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Manuscript

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Abstract

Bioactive dietary components may considerably influence intestinal health and resistance to enteric disease. Proanthocyanidins (PAC) are dietary polyphenols with putative health-promoting activity that have been increasingly studied for their anti-inflammatory and immunomodulatory effects. However, whether dietary PAC can regulate type-2 immune function and inflammation at mucosal surfaces remains unclear. Here, we investigated if diets supplemented with purified PAC modulated pulmonary and intestinal mucosal immune responses during infection with the helminth parasite *Ascaris suum* in pigs. *A. suum* infection induced a type 2-biased immune response in lung and intestinal tissues, characterized by pulmonary granulocytosis, increased Th2/Th1 T cell ratios in tracheal-bronchial lymph nodes, intestinal eosinophilia, and modulation of genes involved in mucosal barrier function and immunity. We observed that PAC had only minor effects on pulmonary immune responses, regardless of concurrent *A. suum* infection. However, RNA-sequencing of intestinal tissues revealed that PAC significantly induced transcriptional responses related to immune function, antioxidant responses, and cellular stress activity, both in uninfected and *A. suum*-infected animals. *A. suum* infection and dietary PAC both induced distinct changes in gut microbiota composition, primarily in the jejunum and colon, respectively. Notably, PAC substantially increased *Limosilactobacillus reuteri* abundance in the colon of both naïve and *A. suum*-infected animals. Thus, dietary PAC may have distinct beneficial effects on intestinal health during infection with mucosal pathogens, whilst having limited activity to modulate naturally-induced type-2 pulmonary inflammation. Our results shed further light on the mechanisms underlying the health-promoting properties of PAC-rich foods, and may aid in the design of novel dietary supplements to regulate mucosal inflammatory responses in the gastrointestinal tract.

Keywords

Proanthocyanidins; *Ascaris suum;* Immunomodulation; Gut-lung axis

Introduction

Effective immune function is essential for maintenance of health and tissue homeostasis. The role of diet in regulating immunity and inflammation at mucosal barrier surfaces has been well-established, and immunomodulatory dietary components have therefore gained tremendous attention in scientific research in recent years. Polyphenols, terpenoids, and carotenoids are examples of three central groups of phytonutrients, which have been extensively studied for their beneficial impact on health and disease[1–4]. Proanthocyanidins (PAC) are a type of polyphenol, commonly found in a plant-based diet, which have characteristic chemical structures with known anti-oxidant and anti-inflammatory properties[5–7]. Numerous studies have demonstrated that PAC play an important role in the regulation of immune function and may offer therapeutic potential towards inflammatory intestinal diseases[6]. Furthermore, PAC was shown to regulate allergen-induced type-2 inflammation in the lungs of mice by decreasing the expression of IL-4, IL-5 and IL-13[8]. However, the ability of PAC to modulate pathogen-induced type-2 mucosal immune responses, such as those induced by helminth parasites, has not been examined in detail. Such studies may shed light on the interactions between diet and immunity to helminth infection, and other type-2 driven pathologies, such as asthma and ulcerative colitis.

PAC are able to modulate various physiological parameters when consumed as part of the diet, and have been shown to have beneficial healing effects in human patients with ulcerative colitis by increasing mucus production[9]. In rats, PAC alleviate methotrexate-induced enterocolitis, and decreases inflammation in TNBS-induced (ulcerative) colitis by inhibiting NF-kB signaling pathways[5,10]. PAC supplementation in pigs has been shown to downregulate pro-inflammatory cytokines in intestinal tissues, as well as increase serum superoxide dismutase (SOD) levels[11,12]. PAC exert strong antioxidant and anti-inflammatory effects in cellular models[7]. Another consistent outcome of dietary PAC supplementation is changes in gut microbiota (GM) composition, which has been observed in both murine and porcine models, with some evidence also from human studies[11,13–16]. PAC have been shown to increase the abundance of lactobacilli and *Bifidobacterium* species, which are commonly associated with a "healthy gut" environment, as well as increasing levels of faecal short chain fatty acids (SCFA) such as propionic acid[14,17,18]. Thus, the immunomodulatory effects of PAC may be caused by direct interactions with immune cells and/or indirect modulation of immune responses as a result of PAC-induced alteration of the GM[19]. These bioactive effects on intestinal immune cells or the GM may have significant implications for inflammation in the gut, as well as at distant sites, such as the lungs, with increasing evidence suggesting a gut-lung axis and clear connection between gut inflammation, the microbiome, and lung homeostasis[20].

Helminth infections are widespread in humans and animals worldwide and cause substantial morbidity[21,22]. The characteristics of immune responses during helminth infection include a strongly Th2 polarized immune response, characterized by eosinophilia and the induction of dendritic cells triggering an increased secretion of IL-13, IL-10 and IL-4 by T-cells [23]. The porcine roundworm *Ascaris suum* is widely prevalent in pig farms globally and closely related to *A. lumbricoides*, which is the most common helminth in humans[21,24,25]. After infection, *A. suum* larvae have a complex migratory path, which includes migration through the liver and lungs before returning to the small intestine[25–27]. At each of these anatomical sites, the migratory larvae cause strong type-2 biased inflammatory reactions. Studies in both the natural porcine host and murine models have shown that larvae elicit significant levels of type-2 (e.g. IL-5), but also type-1/17 (IL-6 and TNFα) cytokines as they migrate in the liver, lungs and gut[28 29]. Furthermore, *A. suum* infection was shown to increase the susceptibility of bacterial lung infections in both mice and pigs[30–32].

The characteristic life cycle of *A. suum*, with larvae relocating between the intestines, the liver and the lungs, offers numerous sites of interaction with the immune system. In this respect, *A. suum* infections in pigs have proved to be a valuable model for assessing the effects of different dietary components on type-2 immune function in both the gut and the respiratory tract. For example, studies in this model have shown that dietary retinoic acid can enhance *A. suum*-induced pulmonary eosinophilia, whilst treatment with probiotics, such as *Lactobacillus rhamnosus* GG, can suppress the prototypical type-2 response in lymph nodes draining the lungs during infection[17,33]. However, studies on the effects of concomitant PAC-supplementation and helminth infection are scant[17,34]. Here, we thus explored the effect of dietary PAC on host immune function in *A. suum*-infected pigs. We examined the impact of PAC on systemic immune parameters, inflammatory and immune reactions at the mucosal barrier of both the lung and the intestinal tract, and infection- and dietary-induced changes in the gut microbiota, with the aim of understanding how PAC intake may modulate a concurrent naturally-induced type-2 mucosal response in multiple tissue sites.

Material and Methods

Proanthocyanidins and diets

The PAC used for this study were from a standardized grape seed extract (Bulk Powders, Denmark). The extract consisted of at least 90% PAC as determined by NMR spectroscopy. The PAC were composed of 99% procyanidin oligomers and polymers with a mean degree of polymerization of 4.2. The basal diet (NAG, Denmark) was based on ground wheat and barley and was formulated to provide 16.2% crude protein (**Table S1**). Pigs received either the basal diet or the basal diet supplemented with 1% PAC. Feed intake was adjusted for body weight throughout the experiment and was calculated to provide the PAC-supplemented pigs with approximately 300 mg PAC/kg BW. All pigs were weighed weekly and they were monitored and fed twice daily at 8:00 in the morning and 15:00 in the afternoon, with access to water *ad libitum*.

Pig experiment

9-weeks old pigs were selected from a Specific Pathogen Free (SPF) Danish farm with no history of helminth infection. On arrival, pigs were confirmed free of helminth-infection by fecal egg count[81] and negative by serology for *A. suum*. Pigs were vaccinated (p.o.) against *Lawsonia intracellularis* 4.5 weeks prior to the start of the experiment (ENTERISOL® ILEITIS VET., Boehringer Ingelheim). A total 24 pigs (Duroc/Danish Landrace/Yorkshire; 12 castrated males and 12 females) were randomly distributed into four treatment groups that were balanced for sex and initial bodyweight. Bodyweights were recorded weekly (**Figure S2**). Each of the four groups were housed in two pens consisting of three pigs each. From day 1 of the experiment, 12 pigs were fed the basal diet and 12 the PAC-supplemented diet. At day 14, half the pigs in each group were inoculated with 5000 embryonated *A. suum* eggs by gastric intubation (**Figure S3**). Pigs were euthanized at day 28 of the experiment (i.e. day 14 p.i.) by captive bolt pistol stunning followed by exsanguination. Throughout the study, weekly blood and fecal samples were taken. Blood was collected by venipuncture of the jugular vein and serum separated and frozen at -80 °C. At necropsy, the entire small intestine was removed and processed for *A. suum* larval counts using a modified agar-gel technique [82]. Worm burdens were assessed by manual enumeration using a dissection microscope of blinded samples conserved in 70% ethanol. Digesta samples were collected from the jejunum, proximal and distal colons and cooled on ice before transfer to – 80 °C storage. Small pieces ($1cm^3$) of lung (right cranial lobe) and mid-jejunal tissue were preserved in RNAlater for transcriptional analysis. A further piece of jejunal tissue was also collected for histology using BiopSafe® Biopsy Sample System (Merit Medical). Histology slides were stained with hematoxylin & eosin, and eosinophils were enumerated by blinded microscopy.

Broncho-Alveolar Lavage

BAL was performed at necropsy post-mortem by introducing 500 ml PBS into the lungs to recover BAL cells from both lung lobes. The BAL fluid was filtered through 2-layer fine gauze sheets into clean 50 ml centrifuge tubes, and stored at room temperature (RT) until further processing. The recovered cell suspensions underwent a series of washing with HBSS and centrifugation. To remove red blood cell (RBC) contamination we used an RBC lysis buffer, which was added to the cell suspension for 5 minutes at room temperature. Finally, the cells were resuspended in 5 ml RPMI-1640 media supplemented with 10% foetal bovine serum and penicillin/streptomycin. Cells were enumerated and either used for flow cytometry (see below) or plated out on 48-well plates at a concentration of 1.2×10^5 cells/well and incubated overnight (37 °C, 5% CO_2). The next day, cells were stimulated with either *T. suis* (50 µg/ml*), A. suum* (50 µg/ml) E/S products, or LPS (500 ng/ml) for 24 hours. Following stimulation, the supernatant was collected and stored at – 20 °C before further analysis by ELISA.

Gut microbiome analysis - DNA extraction and 16S rRNA gene amplicon sequencing

DNA from small and large intestine samples was extracted using Bead-Beat Micro AX Gravity Kit (A&A Biotechnology, Gdynia, Poland) as per manufacturer's instructions. The DNA purity and

concentration were determined by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and Varioskan Flash (Thermo Fisher Scientific, USA), respectively.
A 16S rRNA gene amplicon library was constructed by amplifying the 16S rRNA gene with unique molecular identifier (UMI) containing multiple forward and reverse primers (**Table S2**) according to protocol by Krych et al. (publication in preparation). PCR conditions for the amplification were as follows: 95°C for 5 min, 2 cycles of 95°C for 20 s, 48°C for 30 s, 65°C for 10 s, 72°C for 45 s, and a final extension at 72°C for 4 min. A second PCR step was then performed to barcode PCR amplicons with the following conditions: 95°C for 2 min followed by 33 cycles of 95°C for 20 s, 55°C for 20 s, 72°C for 40 s, and a final extension at 72°C for 4 min. After each PCR reaction, PCR amplicons were cleaned up using SpeedBeads^{TM} magnetic carboxylate (obtained from Sigma Aldrich). The size of barcoded PCR products (approximately 1500 bp) was checked by 1.5% agarose gel electrophoresis.
A sequencing library from pooled barcoded PCR products were prepared by following the ligation sequencing kit SQK-LSK110 (Oxford Nanopore Technologies, Oxford, UK) manual. Next, prepared library was sequenced by Oxford Nanopore GridIONX5 sequencing platform as described in manufacturer's protocol (https://nanoporetech.com/products/gridion). Sequencing was run until there was no longer active pores.

Data analysis workflow for 16S rRNA gene sequencing

Nanopore sequencing software GridION version 21.02.5 (https://nanoporetech.com) was used for data collection. Base calling and demultiplexing of sequencing data were performed by ONT's Guppy version 4.5.2 (https://nanoporetech.com). Nanofilt version 2.7.1[83] was then used for filtering and trimming of demultiplexed sequences. Briefly, data were filtered on a minimum 1000 and maximum 1600 reads with a minimum average read quality score of 8. After filtering, 15 nucleotides were trimmed from start and end of reads. Taxonomy assignment was achieved by using parallel_assign_taxonomy_uclust.py script of Quantitative Insights into Microbial Ecology (Qiime) 1 version 1.8.0[84]. Greengenes database version 13.8[85] was used as a reference database. The reads classifications did not include UMI correction due to low coverage of UMI clusters.
Qiime 2 version 2020.6.0[86] was used to set rarefraction depth to 5000 reads per sample. Sample reads below 5000 were removed from the analysis; a total of 42 samples were included for microbiome analysis (n=19 for small intestine samples and n= 23 for large intestine samples). Normalized data were then processed in RStudio version 1.3.1073[87] using R version 4.0.2[88] and R packages phyloseq[89], vegan[90], tidyverse[91], ggpubr[92], reshape2[93] and viridis[94].
Short-chain fatty acids including DL-lactic acid were analyzed in colonic digesta samples according to a method by Canibe et al. 2007[95].

Flow-cytometry

Tracheal-bronchial lymph nodes were collected from the bifurcature and stored on ice in FBS supplemented RPMI until further processing. Single-cell suspensions were prepared by passing lymph nodes through a 70 µm cell-strainer. Cells were washed and stained with the following antibodies: mouse anti-pig CD3-FITC (clone BB23-8E6-8C8; BD Biosciences); mouse anti-pig CD4-

PE-Cy7 (clone 74-12-4; BD Biosciences); mouse anti-human T-bet-APC (clone 4B10; BioLegend); mouse anti-human GATA3-PE (clone TWAJ; Invitrogen). BAL cells were collected as described above, and stained with mouse anti-pig granulocytes-Alexa Fluor647 (clone 2B2; Bio-Rad) and mouse anti-pig CD203a-FITC (clone PM18-7; Bio-Rad). Granulocytes were defined as $2B2^{+}CD203a^{-}$. For all stainings isotype controls were included and gates were set using FMO controls. Data was acquired on an Accuri C6 flow cytometer (BD Biosciences) and analyzed using C6 software.

Enzyme-linked immunosorbent assay

IL-1β and TNFα concentrations in alveolar macrophage supernatant and CRP levels in serum were analyzed using commercial ELISA kits (Duosets; R and D systems) according to the manufacturer's instructions. Levels of IgM, IgA and IgG_1 in serum specific for *A. suum* antigen were measured as previously described [96].

RNA-sequencing

RNA was extracted from lung and intestinal tissue following homogenization (gentleMACS, Miltenyi Biotech) using miRNAeasy kits (Qiagen) according to the manufacturer's instructions. RNA was subsequently used for library preparation and 150bp paired-end Illumina NovaSeq6000 sequencing (Novogene, Cambridge, UK). Sequence data was subsequently mapped to the Sus Scrofa (ss11.1) genome and read counts generated which were used to determine DEG using DEseq2. Pathway analysis was conducted using gene-set enrichment analysis (Broad Institute, MA, USA). RNA sequence data from lung and intestinal tissues are deposited at the NCBI Gene Expression Omnibus (Acession numbers: GSE174042 and GSE168840).

Statistical analysis

All statistical analysis were performed using GraphPad Prism 8, IBM SPSS Statistics 27 or R packages. Data were analyzed using a mixed-model analysis, with diet and infection status as fixed factors and pen and pig as random factors, or with two-way ANOVA and t-tests, as indicated. Where appropriate time was included as an additional fixed factor to account for repeated measures. One pig (in the *A. suum* + PAC group) was excluded from analysis as it displayed post-mortem pathology indicative of ileitis and displayed aberrant values on several immunological assays. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to tests for assumptions of normality in analyses, and square-root transformations were used to approximate normal distributions when appropriate. For gut microbiota α-diversity analysis, pairwise Wilcoxon Rank Sum Test from R stats package[88] was used to obtain Benjamin–Horchberg corrected p-values. Statistical analysis for distance-based redundancy analysis (db-RDA) was done by using permutational ANOVA in the R package vegan.

Results

Proanthocyanidins exert minor effects on systemic antibody and inflammatory biomarkers

Acute infections with *A. suum* induce potent immune reactions in the lungs and intestine before expulsion of the majority of the invading larvae starting from around day 18 post-infection (p.i.) [26,28,35]. In this study, pigs were fed either a control diet or a diet supplemented with 1% PAC for 14 days, before half the pigs in each group were infected with 5000 embryonated *A. suum* eggs. Mean larval burdens at day 14 p.i. were not altered by PAC supplementation (2914 larvae ± 928 larvae (mean ± SD, *n = 6)* in control-fed pigs and 3155 ± 1057 larvae (mean ± SD, *n = 5*) in PAC fed pigs). In order to examine whether PAC influenced the development of the immune response to *A. suum*, we first examined serological markers of infection in the different treatment groups. *A. suum* infection resulted in a significant increase in serum IgM, IgA, IgG1 specific for *A. suum* antigenic extracts compared to un-infected groups (**Figure 1A-C**). PAC supplementation increased the levels of all three antibody classes in infected pigs, however the effect of diet was not statistically significant. To assess the effect of both *A. suum* and dietary PAC on systemic inflammation, we quantified C-reactive protein (CRP) levels in serum. CRP levels on day 0 p.i. (i.e. after 14 days of PAC supplementation) ere significantly lower in PAC fed pigs compared to controls (**Figure 1D**). However, CRP levels measured on day 14 p.i. (i.e. after 28 days of PAC supplementation) were no longer affected by diet, and infection had no impact on CRP levels (**Figure 1E**). Thus, whilst dietary PAC appeared to exert transient anti-inflammatory properties in uninfected pigs, PAC had little capacity to alter systemic antibody production induced by infection.

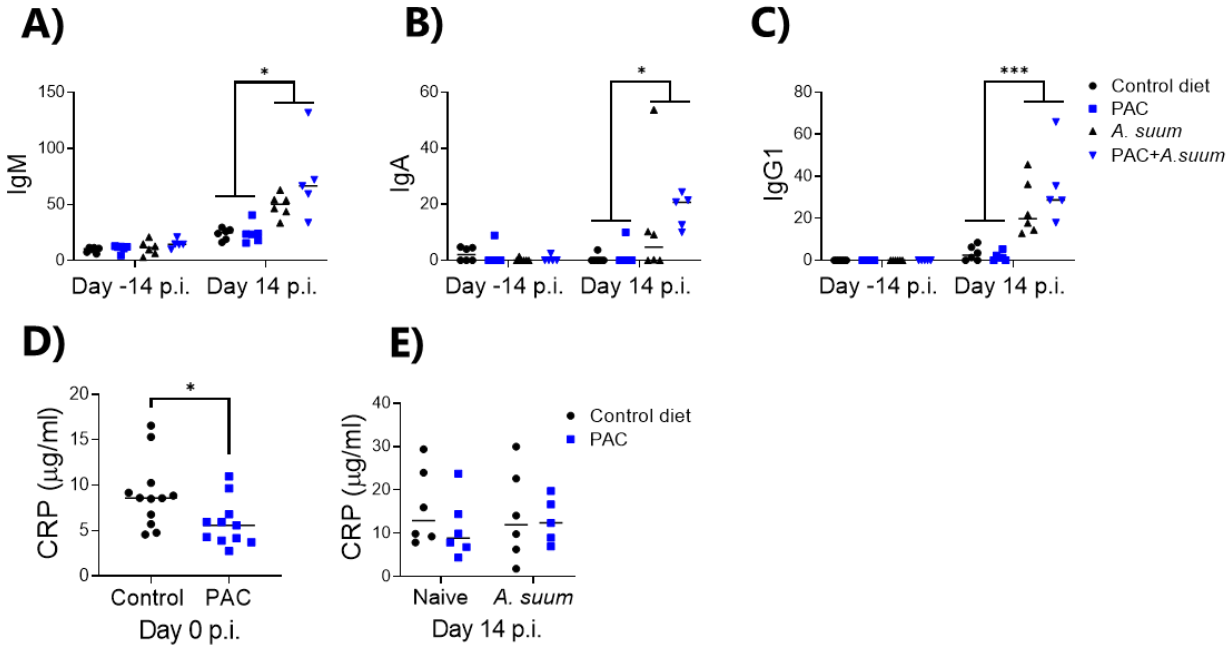

Figure 1 Dietary proanthocyanidins exert limited effects on systemic antibody levels and inflammatory biomarkers Serum levels of **A)** IgM, **B)** IgG1 and **C)** IgA specific for *Ascaris suum* antigen on day -14 pre-infection (i.e. day of arrival) and day 14 post-infection (p.i.) with *A. suum*. **D)** C-reactive protein (CRP) levels at day 0 p.i. (i.e. after 14 days of proanthocyanidin (PAC) supplementation) and **E)** day 14 p.i. (**Mixed model analysis or t test**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 6$ pigs per group, except $n = 5$ pigs in PAC+*A. suum* group).

Impact of *Ascaris suum* infection and dietary proanthocyanidins on Th1, Th2 and granulocytic responses in pulmonary and gut tissues

A. suum infection induced significant cellular changes in the broncho-alveolar lavage (BAL) fluid and tracheal-bronchial lymph nodes (LN). In the LN, the proportion of $CD3^+$ T cells was significantly decreased when comparing infected pigs to controls, similar to what has been observed in mice[28] (**Figure 2A**). The proportions of $CD3^+CD4^+$, $CD3^+CD4^+$T-bet^+ (Th1) and $CD3^+CD4^+GATA3^+$ (Th2) T-cells were not significantly different across treatment groups, however Th2/Th1 ratios clearly demonstrated a strong Th2-polarized immune response as a result of *A. suum* infection (**Figure 2B-E**). Moreover, infection markedly induced granulocytosis in BAL fluid, and eosinophilia in jejunal tissues. PAC did not alter granulocyte numbers in BAL, and whilst intestinal eosinophils were numerically higher in infected pigs fed PAC compared to those fed the control diet this difference was not significant (**Figure 2F and G**). Taken together, these findings indicate that *A. suum* induced a strong type-2 biased cellular response in the lungs and intestinal tissues, which was not significantly altered by concurrent PAC consumption.

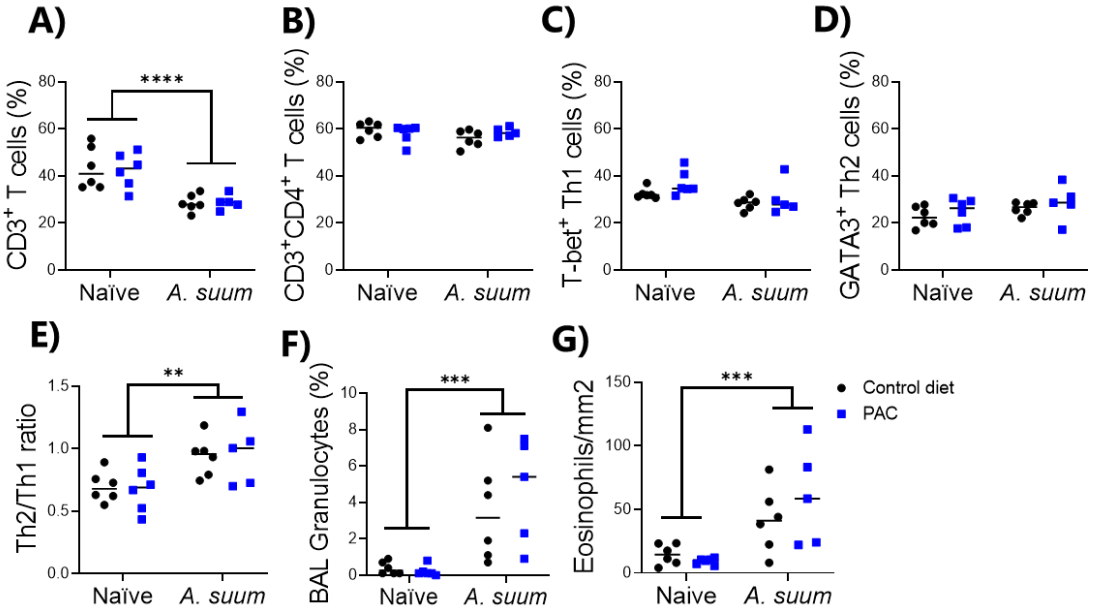

Figure 2 *Ascaris suum* induces Th2-biased cellular responses in the lungs and intestine
A-E) Proportions of $CD3^+$ cells, $CD4^+$ T cells, T-bet^+ Th1 cells , $GATA3^+$ Th2 cells , and Th1/Th2 ratios in lung lymph nodes (LN) on day 14 post-infection (p.i.). **F)** Lung granulocytosis in broncho-alveolar lavage (BAL) fluid at day 14 p.i. **G)** Eosinophils were assessed by microscopy of histology samples of mid-jejunum tissues. PAC: proanthocyanidins (**Mixed model analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$**, n = 6 pigs per group, except n = 5 pigs in PAC+*A. suum* group).

Concomitant *Ascaris suum* infection and dietary proanthocyanidins tend to enhance cytokine secretion in alveolar macrophages stimulated *ex vivo*

To further assess the effects of infection and dietary PAC on the profile of lung immune cells, alveolar macrophages were isolated from BAL of all pigs in each treatment group and stimulated *ex vivo*. Cells were first stimulated with lipopolysaccharide (LPS) to assess how diet and infection may influence secretion of the pro-inflammatory cytokines TNFα and IL-1β. Infection status did not significantly influence LPS-induced secretion of these cytokines. Whilst levels of LPS-induced TNFα and IL-1β were lower in uninfected pigs fed PAC, the secretion of these cytokines tended to be higher in infected pigs fed PAC (**Figure 3A-B**). To explore if *ex vivo* inflammatory responses to parasite antigens were modulated by PAC or infection, macrophages were first stimulated with *A. suum* excretory/secretory (E/S) products, however no cytokine secretion was observed (data not shown). We therefore also stimulated cells with E/S from another porcine parasite, *Trichuris suis*, which we have previously shown to be a stronger activator of innate cytokine production[36]. Interestingly, alveolar macrophages isolated from infected pigs secreted significantly higher levels of TNFα when activated with *T. suis* E/S products (**Figure 3C**). Similarly to LPS-stimulated cells, macrophages isolated from infected pigs fed PAC secreted higher levels of TNFα and IL-1β compared to macrophages isolated from any other treatment groups, albeit not significantly (**Figure 3C-D**). These findings suggest that *A. suum* infection primed macrophages to be more responsive to stimulation from heterologous parasite

antigens (but not LPS), whilst PAC has only minor systemic immuno-stimulatory effects on cytokine production during infection.

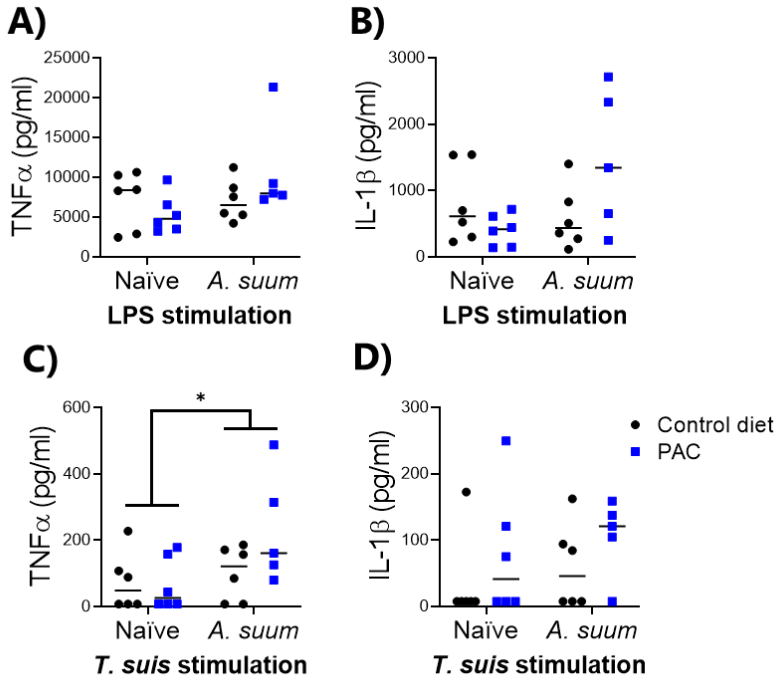

Figure 3 TNFα and IL-1β secretion following *ex vivo* stimulation of alveolar macrophages
Alveolar macrophages were collected by broncho-alveolar lavage (BAL) from *Ascaris suum* –infected and naive pigs fed either the control diet or proanthocyanidin-(PAC)-supplemented diet The macrophages were stimulated *ex vivo* with lipopolysaccharide (LPS) to assess the impact on **A)** TNFα secretion or **B)** IL-1β secretion. Similarly, the macrophages were also stimulated with *Trichuris suis* antigens to assess the impact on **C)** TNFα secretion or **D)** IL-1β secretion. (**Mixed model analysis, *$p < 0.05$**, $n = 6$ pigs per group, except $n = 5$ pigs in PAC+*A. suum* group).

Transcriptional profiling of gut and lung tissues reveals modulatory effects of proanthocyanidins during *Ascaris suum* infection

Jejunum transcriptional responses

To explore in more detail if PAC may influence the immunological response to *A. suum* infection, we conducted RNA-sequencing of jejunal and lung tissues. Both *A. suum* and PAC treatment strongly modulated gene expression in the intestine as compared to controls (**Figure 4 and 5**). Principal component analysis showed a clear clustering of biological replicates according to infection status (**Figure 4A**). *A. suum* infection significantly downregulated the expression of genes such as the aldehyde dehydrogenase-encoding *ALDH1B1*, and the sodium-channel encoding *SCN8A*. Interestingly, three of the top-ten downregulated genes as a result of infection were related to circadian rhythm (*PER3, PER2, NOCT*) (**Figure 4B-C**). Moreover, *A. suum* infection significantly upregulated the expression of interleukins *IL4, IL9, IL10, IL21*, the eosinophil marker *EPX*, as well as TCR related genes *CD28* and *CD80* in intestinal tissue. Furthermore, we noted strong upregulation of genes involved in aryl hydrocarbon receptor (AHR)-signaling including *ARNTL* as well as smooth

muscle contraction (*P2RX1*), which may relate to the increased intestinal motility observed during the immune reaction to *A. suum* larvae[37,38] (**Figure 4B-C**). Analysis of gene pathways modulated by infection revealed that pathways related to peroxisome function, as well as the metabolism of fatty acids and glycerolipids were significantly supressed, suggesting a profound modulation of nutrient metabolism due to larval colonization of the intestine (**Figure 4D**). Unsurprisingly, the main up-regulated gene pathways were related to immune function, such as the IL-2, IL-4, and T-cell receptor related pathways, as well as granulocyte and B-cell signaling (**Figure 4D**). Thus, consistent with the pulmonary and intestinal eosinophilia, *A. suum* induced a type-2 inflammatory reaction concomitant with physiological responses related to the changed mucosal environment induced by larval antigens.

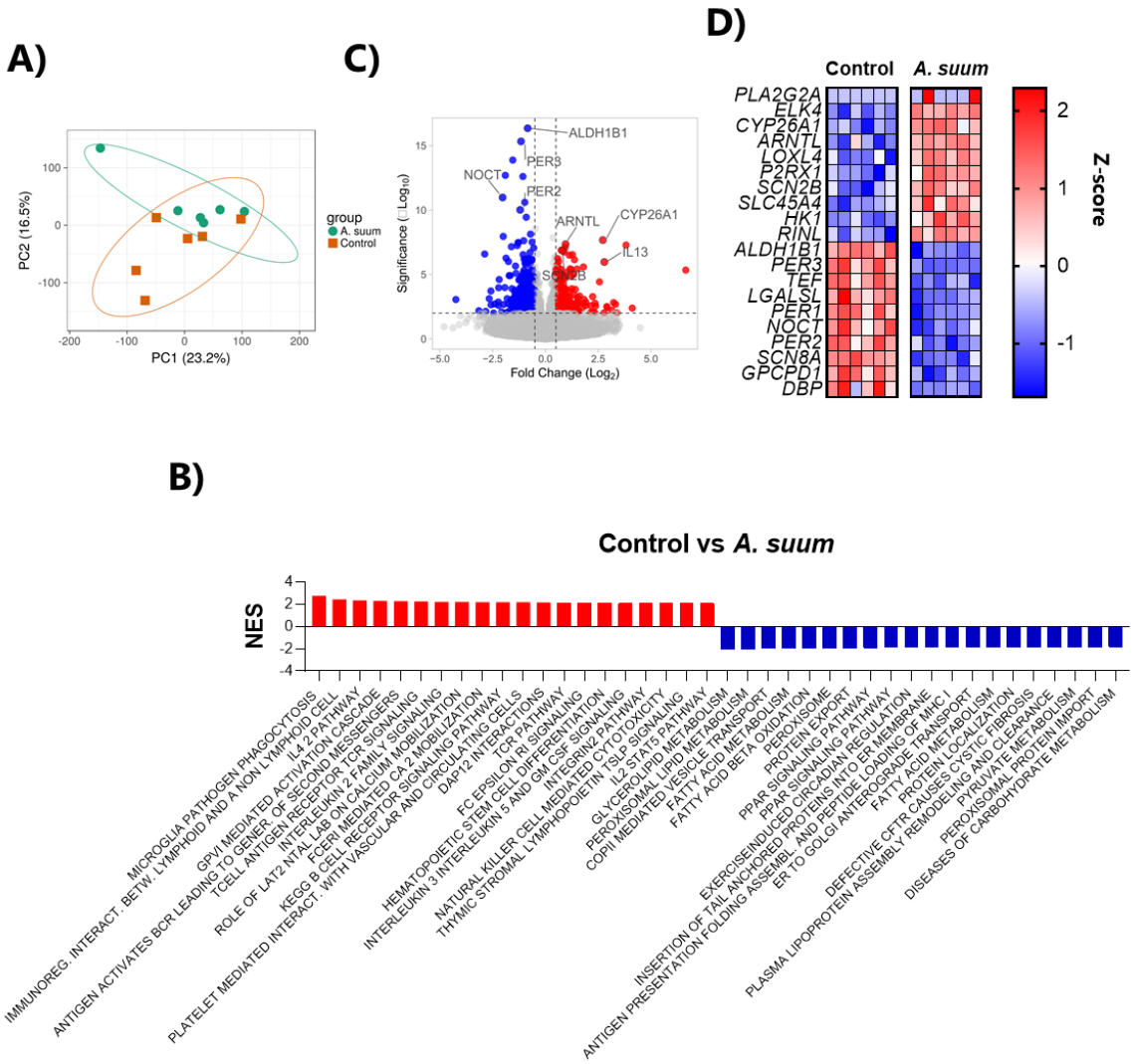

Figure 4 Modulation of gene expression and transcriptional pathways in intestinal tissue by *Ascaris suum* infection
A) Distinct clustering of infected and control groups was demonstrated by principal component analysis. **B)** Significantly up- and down-regulated pathways ($p < 0.01$; $Q < 0.1$) identified by gene-set enrichment analysis as a result of *Ascaris suum* infection. **C)** Volcano plot showing differentially expressed genes resulting from *A. suum* infection **D)** Top ten up- and down-regulated genes identified as a result of *A. suum* infection. ($n = 6$ pigs per group). NES: normalized enrichment score.

In uninfected pigs, dietary PAC resulted in a distinct clustering of treatment groups based on diet as assessed by principal component analysis (**Figure 5A**). We noted significantly upregulated gene pathways related to metabolic processes, such as translation elongation and ribosome function. Interestingly, we noted that a number of pathways that were related to immune function (and that were also induced by *A. suum*), were upregulated by PAC. These included pathways related to granulocyte function and B-cell signaling, indicative of an immune-stimulatory effect of PAC (**Figure 5B**). Indeed, both treatments in isolation significantly increased the expression levels of *CD19, LYN* and *FYN*, which are relevant for B-cell function and signaling, as well as *NFKB1* and *NFKBIE*, which are involved in NF-kB activation. Furthermore, we observed a significant upregulation of pathways related to both detoxification of reactive oxygen species (ROS) and Selenoamino acid metabolism, characterized by increases in *GPX1*, and *PRDX6* expression, suggestive of enhanced antioxidant responses in PAC-fed pigs. Notably, we also observed a strong downregulation of pathways related to heat shock responses, which are normally induced by cellular stressors and offer protection against tissue injury[39–41] . Downregulated genes included the glucose transporter *SLC2A7*, which has previously been shown to be inhibited by polyphenols in cellular models, as well as *MT-2B*, encoding a metallothionein protein known to be associated with intestinal inflammation and oxidative stress in mice[42,43]. Interestingly, we also noted downregulation of *EGFR*, encoding the epidermal growth factor receptor (**Figure 5C**). Significantly upregulated genes included *TXNRD1*, encoding thioredoxin reductase 1, a protein involved in suppression of ROS, as well as genes involved in cellular endocytic processes (*RAB7A*) and extracellular matrix remodeling (*COL6A5*) (**Figure 5D**). Collectively, these data suggest that dietary PAC function as a cytoprotective agent in the intestinal mucosa, by inducing antioxidant responses and regulating responses to cellular stressors.

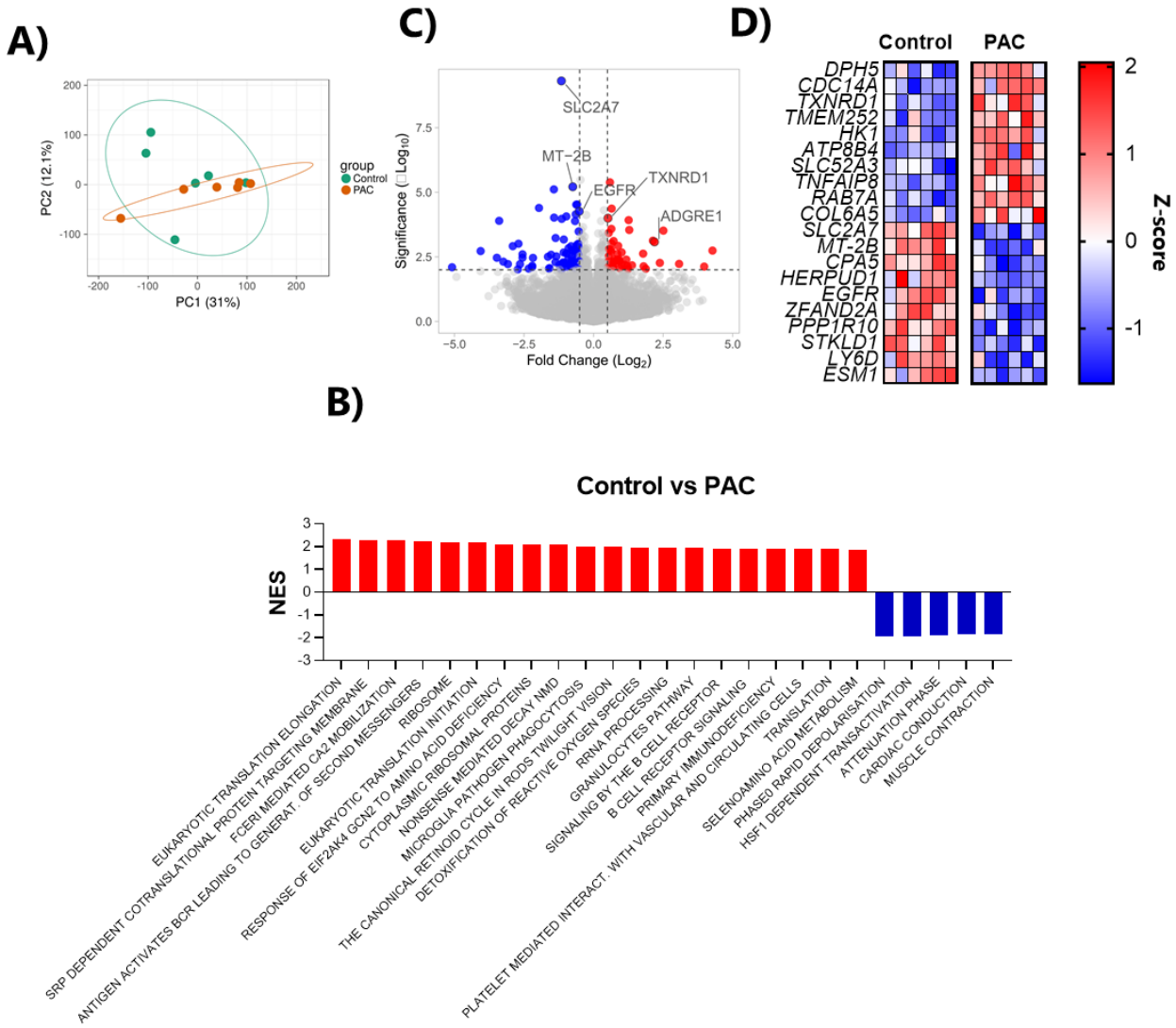

Figure 5 Modulation of gene expression and transcriptional pathways in intestinal tissue by dietary proanthocyanidins

A) Distinct clustering of the two dietary groups was demonstrated by principal component analysis in naïve pigs. **B)** Significantly up- and down-regulated pathways ($p < 0.01$; $Q < 0.1$) identified by gene-set enrichment analysis as a result of dietary proanthocyanidin (PAC) supplementation. **C)** Volcano plot showing differentially expressed genes resulting from dietary PAC supplementation **D)** Top ten up- and down-regulated genes identified as a result of dietary PAC supplementation in naïve pigs. (n = 6 pigs per group). NES: normalized enrichment score.

Given that PAC appeared to induce transcriptional pathways with functions in immunity and inflammation, we next asked whether concurrent PAC consumption could modulate the intestinal transcriptomic response to *A. suum* infection. This did indeed seem to be the case as we observed that within infected pigs, there was once again a clear clustering according to diet based on principal component analysis (**Figure 6A**). Inspection of genes increased in infected, PAC-fed pigs, relative to infected pigs fed the control diet, revealed that expression of genes involved in intestinal nutrient

metabolism were increased, such as *ORAI2* and *AGTR1*, which both play a role in calcium uptake[44,45]. Consistent with the suppression of *EGRF* expression in uninfected pigs fed PAC, the expression of a number of genes related to EGF signaling, including *BTC* and *AREG*, were downregulated. *AREG* encodes amphiregulin, a cytokine involved in type-2 inflammation induced by a number of different helminth species[46] (**Figure 6B)**. In agreement with the data showing an enrichment of antioxidant pathways in uninfected pigs fed PAC, we noted that PAC supplementation during infection also resulted in the upregulation of the oxidative stress pathway, which included significant enrichment of *SOD3*, *GPX3* and *NQO1* - genes encoding proteins with known anti-oxidant properties (**Figure 6C**). Down-regulated pathways in infected pigs fed PAC were mainly related to metabolic activity such as cholesterol metabolism, but these were not significant following FDR adjustment. Collectively, these data show that PAC exert a significant influence on the intestinal transcriptional environment during enteric helminth infection mainly by promoting the transcription of genes involved in regulating oxidative stress and nutrient metabolism.

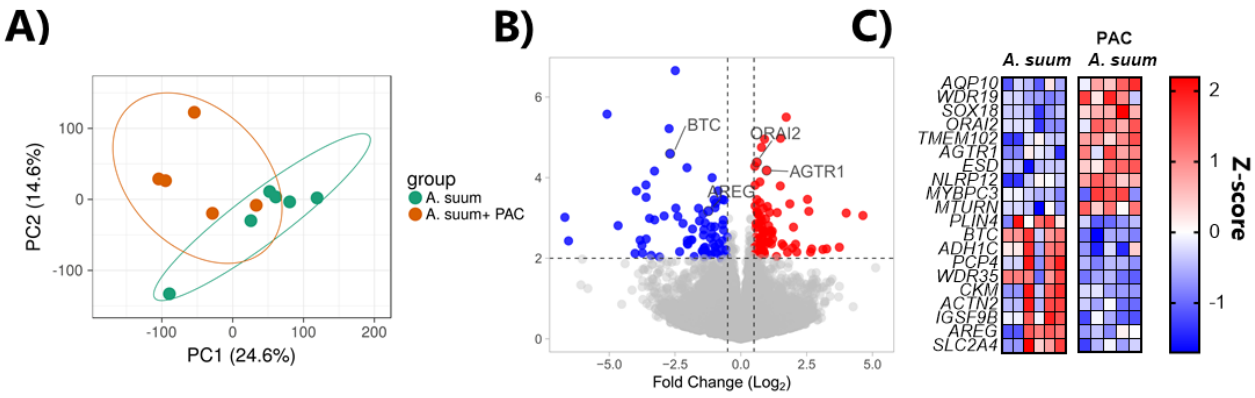

Figure 6 Modulation of gene expression and transcriptional pathways in intestinal tissue by dietary proanthocyanidin supplementation in *Ascaris suum*-infected pigs

A) Distinct clustering of the dietary groups within *Ascaris suum*-infected pigs as a result of dietary proanthocyanidins (PAC) was demonstrated by principal component analysis. **B)** Volcano plot showing differentially expressed genes resulting from dietary PAC supplementation in *A. suum*-infected pigs. **C)** Top ten up- and down-regulated genes identified as a result of dietary PAC supplementation in *A. suum*-infected pigs. (n = 6 pigs in *A. suum* group, n = 5 pigs in PAC+*A. suum* group).

Lung transcriptional responses

Next, transcriptional profiling of the lungs by RNA-sequencing was performed to investigate the effect of larval migration in the lungs and the potential impact of dietary PAC on gut-lung interplay. In comparison to the intestine, the modulation of gene expression in the lungs was only modestly modulated by both *A. suum* infection and/or PAC supplementation. Interestingly, as was the case in the jejunum, *A. suum* infection regulated the expression of numerous genes related to circadian rhythm. Notably, *PER1*, *PER2*, *PER3*, *NR1D1*, *NR1D2* and *DBP* were suppressed, whereas *NPAS2*, *ARNTL* were significantly upregulated (**Figure 7A**). A number of studies have touched upon the importance and complex interplay between circadian rhythm, immune regulation and parasite-host

interactions[47]. Of note, *ARNTL* was also significantly upregulated by PAC (**Figure 7B**). In coherence with the above described granulocytosis in the lungs in BAL fluid, *A. suum* infection upregulated the expression of *CCR3,* which is essential for eosinophil recruitment[48,49]. Infected pigs fed PAC had significantly higher expression levels of genes related to innate immune function (*CD209* and *OAS2*), and connective tissue growth factor *(CTGF)* in lung tissues compared to infected pig fed a control diet (**Figure 7C**). CTGF is involved in wound repair and tissue healing[50], suggesting a protective effect of PAC during *A. suum* infection. Intriguingly, the expression of the oxidative stress inducer *ALOX15* was significantly increased by *A. suum* infection, but was significantly down-regulated in infected pigs fed a PAC diet, which supports previously described reports of PAC acting as a lipoxygenase inhibitor[51–53] (**Figure 7A and C**). Thus, *A. suum* infection induced marked transcriptional responses in the lungs but somewhat less than compared to intestinal tissues, which may indicate that lung homeostasis is somewhat restored by day 14 p.i. when the migrating larvae have returned to the intestine. Furthermore, dietary PAC induced smaller transcriptional changes in the lung compared to the intestine but may ameliorate wound healing and antioxidant status during infection.

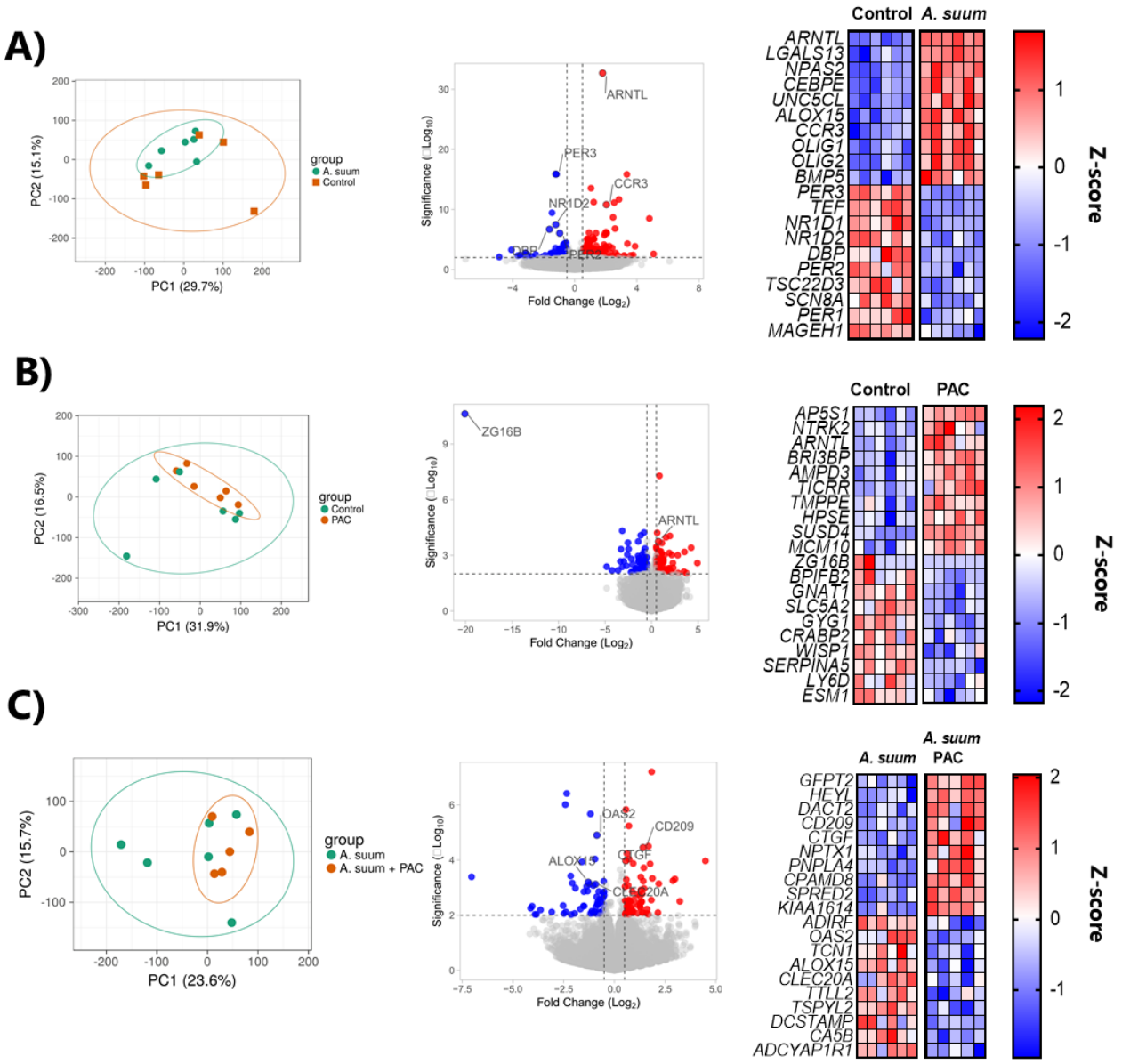

Figure 7 Modulation of gene expression in lung tissue by *Ascaris suum* infection and dietary proanthocyanidins
Effects on lung gene expression as shown by principal component analysis, volcano plot of differentially expressed genes and top ten up- and down-regulated genes identified as a result of **A)** *Ascaris suum* infection in pigs fed the control diet, **B)** dietary proanthocyanidins (PAC) in naïve pigs and **C)** dietary PAC in *A. suum*-infected pigs. (*n* = 6 pigs per group, except *n* = 5 pigs in PAC+*A. suum* group).

Ascaris suum infection and proanthocyanidins alter gut microbiota composition with limited effect on short chain fatty acids

Previous studies have indicated that immunomodulatory and anti-inflammatory effects of PAC may derive from changes in the GM and associated metabolite production[13,54]. Furthermore, *A. suum* and other helminths can markedly change host GM composition[55,56]. Therefore, to explore whether the observed transcriptomic changes induced by diet and infection were accompanied by GM changes,

we used 16S rRNA gene-based sequencing to characterize both the small and large intestinal GM composition.

We initially analyzed the GM composition in the jejunum, at the main site of *Ascaris* infection. Neither *A. suum* nor dietary PAC altered α-diversity (data not shown). Changes in β-diversity were apparent primarily as a result of *A. suum* infection ($p < 0.05$ by distance-based redundancy analysis; **Figure 8A**), with differential abundance analysis on genus level indicating an enrichment in *Lactobacillus* spp. in infected pigs (**Figure 8B**). Moreover, *A. suum* infection decreased the abundance of *Facklamia* spp. ($p = 0.053$ by mixed model analysis; **Figure 8C**). In contrast, PAC did not have a significant effect on the small intestinal GM, with no changes in β-diversity between PAC-fed pigs and control pigs ($p > 0.05$ by distance-based redundancy analysis; **Figure 8A**). However, we did note that, within *A. suum*-infected pigs, those animals fed PAC tended to have a higher abundance of a vOTU corresponding to *Limosilactobacillus reuteri* (**Figure 8D**). *L. reuteri* has been associated with beneficial probiotic and anti-inflammatory effects, and plays a role in the prevention of microbial translocation and inhibits colonization of pathogenic bacteria[57–60].

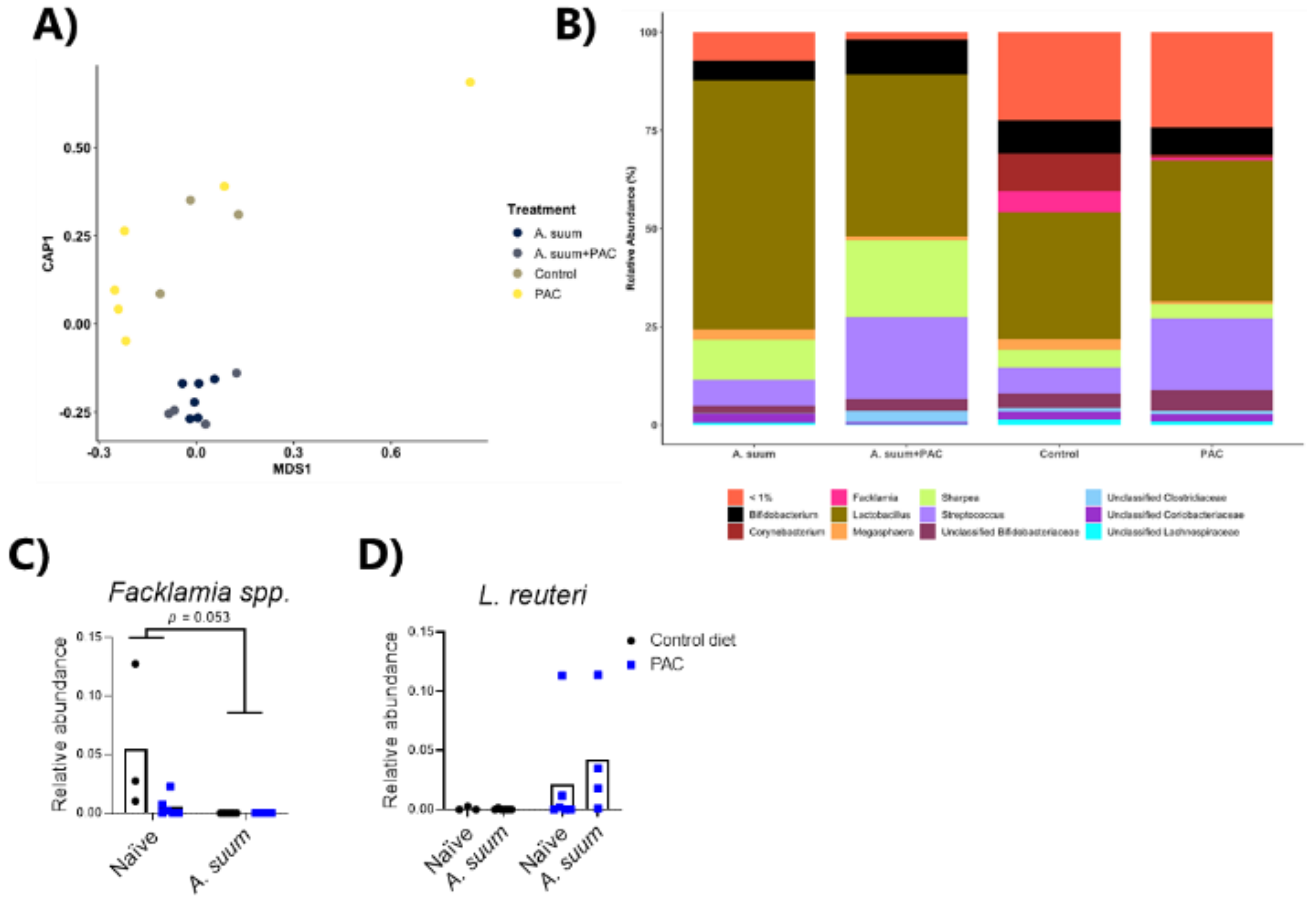

Figure 8 Changes in gut microbiota composition in the small intestine due to dietary proanthocyanidins and *Ascaris suum* infection

A) Changes in β-diversity in the small intestine as identified by redundancy analysis where a significant effect of *Ascaris suum* compared to all other treatment groups was identified. Furthermore, there was an effect of proanthocyanidins (PAC) during infection compared to infection only. No effect on β-diversity was reported when comparing PAC to control-fed naïve pigs **(Pair-wise comparison)**. **B)** Relative abundance at genus level in naive or *A. suum*-infected pigs fed a control

diet or PAC-supplemented diet. Relative abundance of **C)** *Facklamia* spp. and **D)** *Limosilactobacillus reuteri* in naive or *A. suum*-infected pigs fed a control diet or PAC-supplemented diet, as identified by differential abundance analysis and mixed-model analysis**.** (*n* = 3 pigs in control group, *n* = 6 pigs in *A. suum* group, *n* = 6 pigs in PAC group, and *n* = 5 pigs in PAC+*A. suum* group). Columns represent mean values.

In the colon, we found that PAC had the largest effect on the GM composition, consistent with the notion that PAC are extensively metabolized by and modulating the large intestine microbiome[16,34,61–64] ($p < 0.05$ for β-diversity comparison between PAC and control group by distance-based redundancy analysis; **Figure 9A- B**). PAC tended to decrease the abundance of *Bifidobacterium* in both naïve and infected pigs (**Figure 9C**). Notably, the abundance of a vOTU closely related to *Bifidobacterium thermacidophilum* was significantly increased by *A. suum*, but concomitant PAC supplementation significantly suppressed this effect (**Figure 9D**). The reduction of *Bifidobacterium* in pigs fed PAC contrasts to a previous study in pigs showing that PAC increased the growth of this taxa[64]. However, similar to the trend in the small intestine, PAC supplementation resulted in the significant increase of *L. reuteri* abundance in the colon of both naïve and infected pigs (**Figure 9E**). Interestingly, *A. suum* infection increased the abundance of *Lactobacillus* spp. in the colon whilst significantly decreasing the abundance of *Turicibacter* spp. (**Figure 9F and G**). However, β-diversity was not different between *A. suum* and control groups in colon, indicating that the effects of infection on GM composition were mostly limited to the predilection site (the small intestine). Finally, we investigated if the colonic GM changes were accompanied by changes in the concentrations of SCFA in the distal colon. Neither PAC nor *A. suum* infection altered levels of acetic acid, propionic acid, n-butyric acid or D-lactic acid (**Figure S1**). However, we observed that dietary PAC decreased the concentrations of the branched-chain fatty acids iso-valeric acid ($p < 0.05$) and iso-butyric acid ($p = 0.0616$), which may relate to altered protein metabolism or colonic transit time[65], and is consistent with our previous work on pigs fed a polyphenol-enriched diet[34] (**Figure S1**). Taken together, these results indicate distinct effects of *A. suum* infection and PAC on specific bacteria taxa in a site-specific manner.

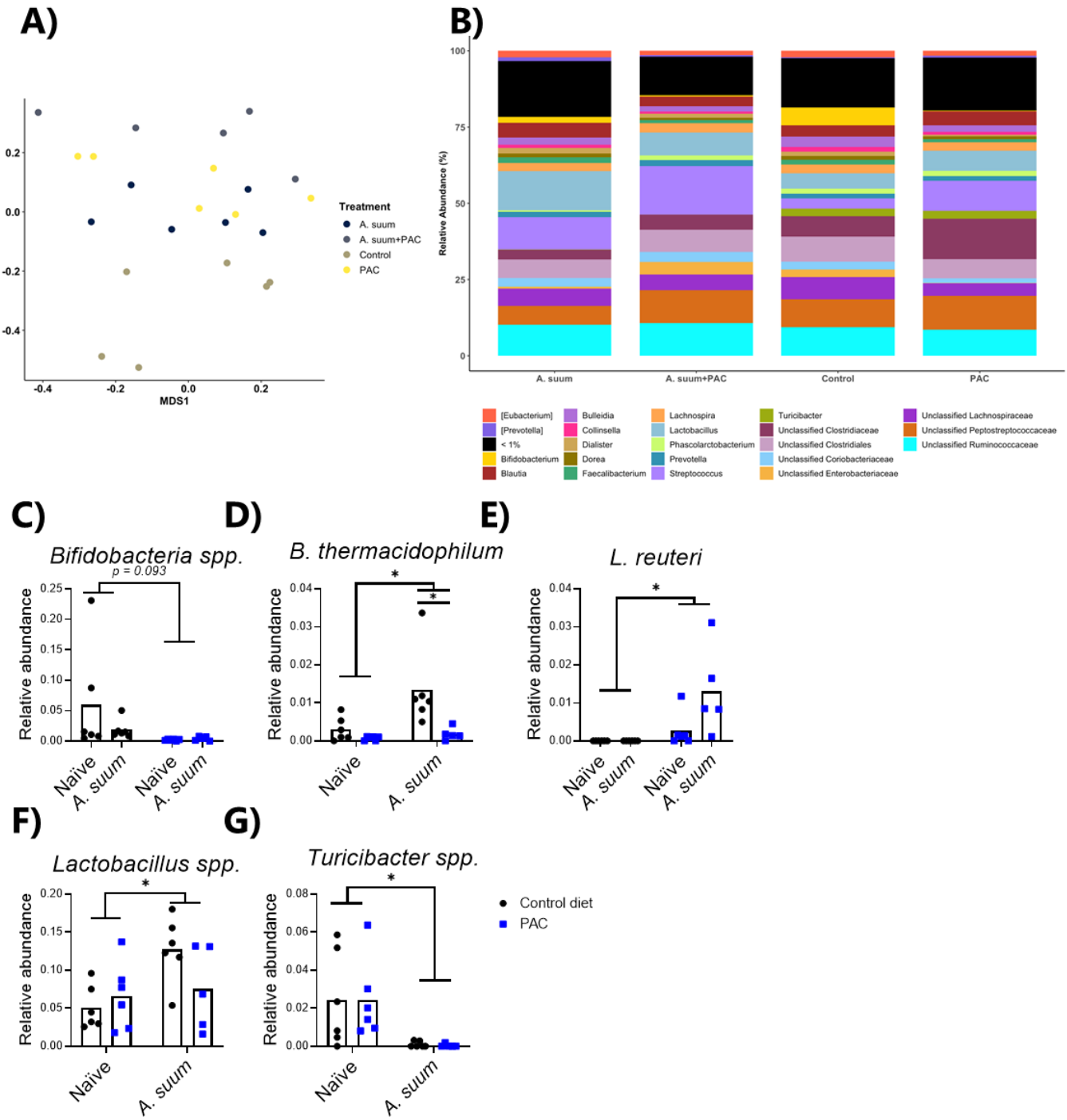

Figure 9 Changes in gut microbiota composition in the proximal colon due to dietary proanthocyanidins and *Ascaris suum* infection

A) Changes in β-diversity in the proximal colon as identified by redundancy analysis, where an effect of proanthocyanidins (PAC) compared to control was identified. Furthermore, there was an effect of PAC-supplemented infected pigs compared to control-fed naïve pigs **(Pair-wise comparison, **p* < 0.05, ***p* < 0.01, ****p* < 0.001). B)** Relative abundance at genus level in naive or *Ascaris suum*-infected pigs fed a control diet or PAC-supplemented diet. Relative abundance of **C)** *Bifidobacteria* spp., **D)** *B. thermacidophilum*, **E)** *Limosilactobacillus reuteri*, **F)** *Lactobacillus* spp. and **G)** *Turicibacter* spp. in naive or *A. suum*-infected pigs fed a control diet or PAC-supplemented diet, as identified by differential abundance analysis and mixed-model analysis (***p < 0.05).** (*n* = 6 pigs per group, except *n* = 5 pigs in PAC+*A. suum* group).Columns represent mean values.

Discussion

The immuno-modulatory effects of PAC have been investigated in numerous studies but their mode of action and impact on immune function is still not fully understood. Furthermore, only limited knowledge has been attained on the effects of PAC on type-2 immune response, which plays a central role during helminth infections and may also be relevant for inflammatory disorders, such as food allergies and ulcerative colitis. Therefore, we used here a model of *A. suum* infection in pigs, which offers a unique opportunity to explore the modulation of parasite-induced inflammation in multiple tissues by dietary components.

Initial assessment of the systemic effects of PAC and *Ascaris* infection were demonstrated by monitoring serum antibody levels and the acute-phase protein CRP, a marker for systemic inflammation [66]. *A. suum* infection resulted in a significant increase in serum antibodies, which were further enhanced by dietary PAC, albeit not significantly. Interestingly, although significantly lower CRP levels were observed after 14 days of PAC supplementation, this effect subsided by the end of the study. Thus, PAC had limited effects on parasite-induced antibody levels, and prolonged PAC supplementation did not appear to persistently alter inflammatory markers in serum.

The gut-lung axis is gaining increasing interest in numerous research fields, and the migratory characteristics of *A. suum* render the investigation of gut-lung interplay greatly relevant in this model. Here, we showed that *A. suum* infection induced granulocytosis in the lungs, and a Th2 polarized immune response was clearly demonstrated by Th2/Th1 T-cell ratios. PAC and *A. suum* in isolation, upregulated a number of similar genes, notably genes related to the circadian rhythm. A recent study showed that *ARNTL,* also known as *BMLA1* and related to circadian rhythm, was fundamental for the time-of-the-day dependent expulsion efficiency of *Trichuris muris* infection in mice[67]. Furthermore, a study conducted in pigs also demonstrated an association between *ARNTL* and adult worm burden[68]. However, in contrast to murine studies, which have demonstrated a role for PAC in suppressing allergic responses in the lungs, we did not find a modulatory effect of PAC on the type-2 cellular response to *A. suum* infection. *Ex vivo* stimulation of lung macrophages by LPS or helminth antigens indicated a tendency of higher cytokine secretion levels in macrophages isolated from infected pigs fed PAC. Moreover, although we observed transcriptional changes in the lungs of infected pigs that are reflective of type-2 inflammation, these did not appear to be markedly altered by concurrent PAC intake. The exception was an indication of regulation of several genes such as *CTGF*, and *ALOX15*, which could suggest that PAC may augment wound-healing and anti-oxidant status in lung tissues during *A. suum* infection. Thus, in our model, dietary PAC had limited capacity to regulate lung immune function during helminth infection, although further studies to elucidate whether PAC may potentiate protection towards secondary airway infection during *A. suum* infection may be relevant.

We next assessed the impact of *A. suum* infection and PAC at the predilection site of infection, the small intestine. *A. suum* infection induced stereotypical intestinal eosinophilia, which was equivalent in both dietary groups. We have previously shown that eosinophilia in the jejunum of *A. suum*-infected pigs could be potentiated by a polyphenol-enriched diet containing 5 % grape pomace[34]. The

composition of this polyphenol-enriched diet may contain several phytonutrients and fibrous components such as lignin, which could contribute to synergistic effects, whereas the PAC diet in the present study was composed only of purified PAC oligomers from grape seed extract. This may explain the discrepancy between these results. Transcriptomic analysis of intestinal tissues revealed that a number of genes and pathways were regulated by both infection and PAC supplementation. As expected, *A. suum* induced the upregulation of type 2 immune related genes and pathways, as well as having an important impact on nutrient metabolism-related genes. Notably, PAC and *A. suum* in isolation were both able to modulate transcriptional pathways related to immune function and antioxidant activity. Interestingly, PAC increased the expression of protein-encoding genes with cytoprotective functions against oxidative stress, suggesting a role in improving gut heath by minimizing cellular stress during inflammation. The antioxidant effect of PAC could be caused by the absorption of PAC-derived metabolites, produced as a result of microbial metabolism. Although PAC metabolites are known to remain relatively stable until they reach the large intestines, PAC molecules with low mDP may also be absorbed in the small intestines[69–71]. Furthermore, PAC and their metabolites may exert direct interactions with the gut mucosa and thus epithelial cells, as described in numerous cell-based studies [72]. Thus, PAC may intervene as scavengers of free radicals due to the numerous hydroxyl groups present in their molecular structures, which can neutralize free radicals via electron delocalization [6,73,74]. Another mechanism of the protective effects of PAC, may be via the induction of cellular antioxidant defenses by modulating Nuclear factor erythroid 2-related factor 2 (Nrf2)-related genes, which play an important role in regulating cellular resistance to oxidants, such as ROS[75–77].

The localized effect of PAC and infection in the intestines was also demonstrated by their impact on the GM. *A. suum* infection caused substantial changes in the GM composition, most notably in the small intestine. This is the first report of alterations in the GM by *Ascaris* in the predilection site of the jejunum, and we found a significantly decreased abundance of *Facklamia* spp. Furthermore, we noted that *A. suum* increased the abundance of lactobacilli in the colon. Consistent with this, an increased abundance of lactobacilli has also been associated with *Heligmosomoides polygyrus* infection in mice[78,79]. This may potentially result from the increased mucus secretion that is a stereotypical feature of helminth infections which may provide a niche environment for lactobacilli to thrive[80]. Interestingly, the abundance of *L. reuteri* was significantly increased by PAC supplementation in both naïve and infected pigs, suggesting a prebiotic effect, which may have functional implications, given the known role of *L. reuteri* in modifying inflammation. However, PAC also significantly decreased the abundance of *Bifidobacterium* spp., including *B. thermacidophilum,* suggesting a complex regulation of the GM. Notably, the suppressive effect of PAC on *Bifidobacterium* spp. stands in contrast to a previous study showing the opposite effect in pigs fed PAC derived from cocoa[64]. These apparently contradictory findings may potentially be explained by the differing molecular structures of PAC derived from different sources, as well as potential interactions with differing basal diets. Given that PAC appeared to change the GM composition, a key question is whether the immunomodulatory effects of PAC in the intestine derive from direct interactions with PAC and mucosal immune cells during intestinal transit, or whether

PAC-derived microbial metabolites are absorbed and exert systemic bioactivity, as has been proposed in previous studies[13,64]. Given that PAC-related transcriptional changes we observed were localized mainly to the gut, and not the lung, this may support a hypothesis that the activities were derived from direct interactions between PAC and cells at the level of the gut mucosa, consistent with a lack of an effect of PAC on SCFA levels. However, further studies are clearly needed to unravel these mechanistic aspects.

In conclusion, pigs infected with *A. suum* offered a robust model to study the effect of PAC on pathogens that induce a strong, type-2 biased mucosal immune response in pulmonary and intestinal tissues. Both *A. suum* infection and PAC in isolation had similar immunomodulatory capacity, notably by modulating gene pathways related to B-cell function. PAC also affected transcriptional pathways related to oxidative stress by significantly increasing the expression levels of protein-encoding genes with cytoprotective properties. However, the canonical markers of type-2 inflammation, such as eosinophilia and Th2 T-helper cells in the lungs, were not modulated by PAC intake. The limited effects of dietary PAC observed in the lungs is in coherence with a previous study demonstrating no effect of PAC on gene expression levels of various immune-related genes in alveolar macrophages and tracheobronchial lymph nodes isolated from *A. suum* infected pigs, which were dosed with PAC derived from cocoa[17]. Thus, in contrast to some murine studies suggesting beneficial effects of dietary PAC on asthma, our results in the porcine model suggest a restricted ability of PAC to influence the development of Th2 responses in the respiratory tract in pigs. However, the significant modulatory effects of PAC on porcine intestinal gene expression suggest a primarily gut-localized effect of PAC. Thus, PAC may play a role in maintaining gut health during enteric infection in pigs and humans, and further studies to address the functional implications of this diet-infection interaction are highly warranted.

Acknowledgements
The authors would like to thank Mette Marie Schjelde for excellent laboratory assistance and Charlotte Smith Bonde, Lise-Lotte Christiansen, Penille Jensen, Stine Nielsen and Pankaj Arora for assistance with the animal study.

Funding
This work was funded by the Independent Research Fund Denmark (Grant # 7026-0094B).

Conflict of interest
The authors declare no conflicts of interests regarding this study.

Ethical statement
All experiments involving animals were conducted in agreement with the Danish legislation and the Danish Animal Experiments Inspectorate with the license number 2015-15-0201-0076.

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Supplementary Material for Paper IV

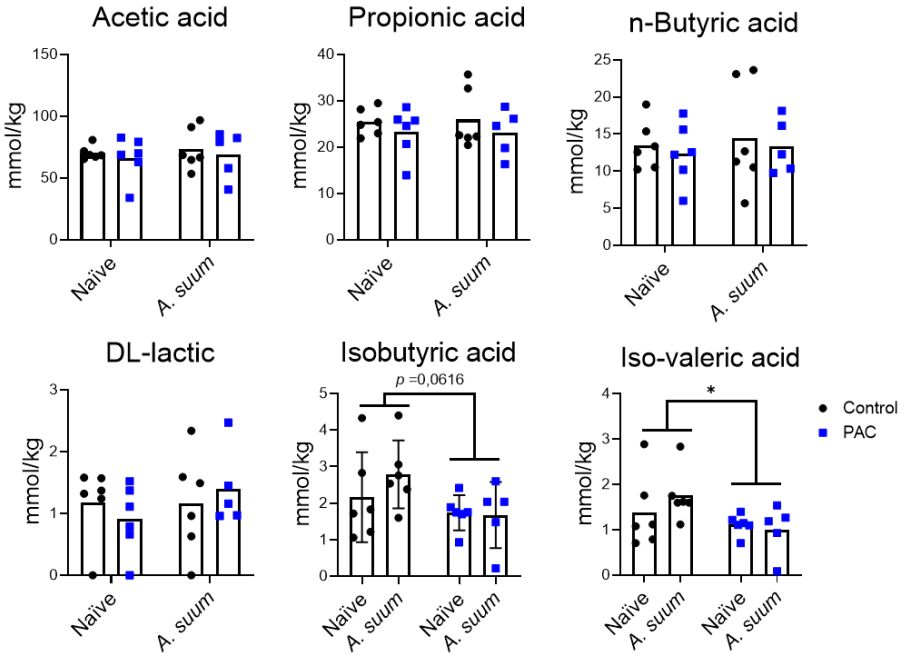

Figure S1 Short chain fatty acids in pig fecal samples

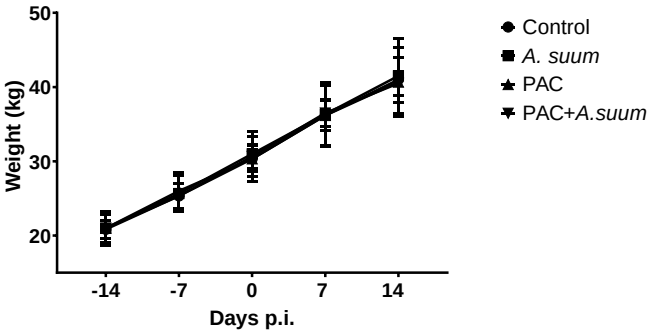

Figure S2 Pig bodyweights

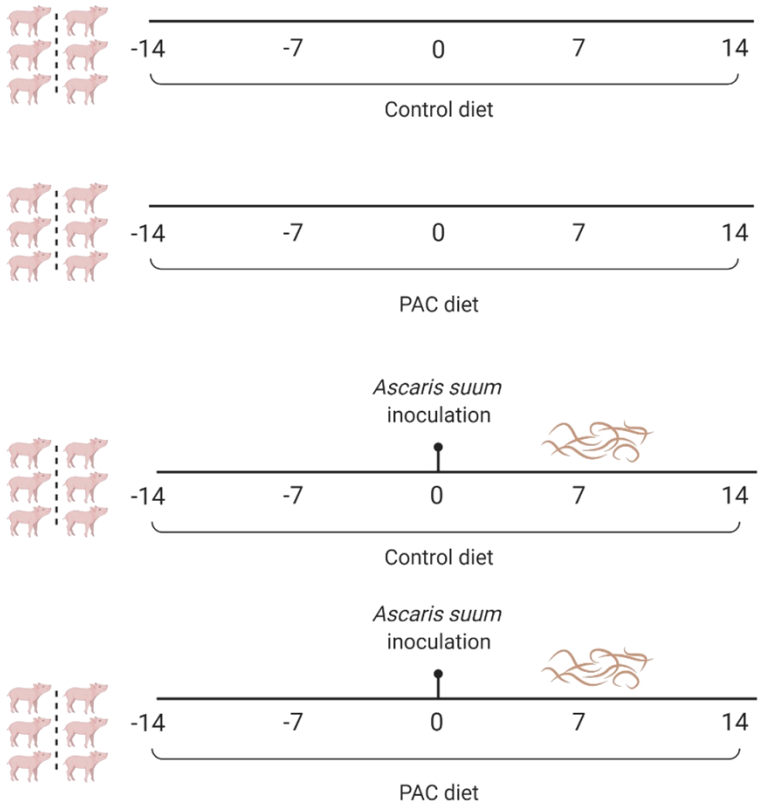

Figure S3 Experimental study design

Figure created with Biorender.com

Table S1 Composition of control diet

Composition of basal feed diet
Analytical constituents: 16,3 % Crude protein 3,8 % Crude fat 3,7 % Lignin 5,2 % Raw ash 0,99 % Lysine 0,29 % Methionine 0,81 % Calcium 0,49 % Phosphor 0,17 % Sodium
Composition: Wheat (57,85%); Soybean meal (20,84%); Barley (15.00%); Molasses (1.50%); Fatty acid distillates from physical refining, palm (1.50%); Calcium carbonate, chalk (1.47%); Monocalcium phosphate (0.70%); Sodium chloride, feed salt (0.37%); L-Lysine monohydrochloride (3.2.3) (0.29%); Premix Vit. Slagt (0.20%); Methionine 40 (3.1.1) (0.17%); L-Threonine (3.3.1) (0.13%);
Additives, added per kg: Nutritional: 4200 i.e Vitamin A (3a672a) 420 i.e. Vitamin D3 (3a671) 84 i.e. E-Vit 3a700 all-rac-alpha-tocopheryl acetate 84 mg Fe, iron II sulphate (3b103) 15 mg Cu, copper II sulphate (3b405) 42 mg Mn, manganese oxide (3b502) 100 mg Zn, zinc oxide (3b603) 0.21 mg I, calcium iodate anhydrate (3b202) 0.30 mg Se, sodium selenite (3b801) Enzymes: 500 FYT 6-phytase (3.1.3.26) (4a18)

Table S2 UMI containing primers used for 16S rRNA gene amplification.

Primers	**Primer Sequence**
UMI_338Fa	5'- GTCTCGTGGGCTCGG- NNNNNNNNNNNNNN - ACWCCTACGGGWGGCAGCAG-3'
UMI_338Fb	5'- GTCTCGTGGGCTCGG- NNNNNNNNNNNNNN - GACTCCTACGGGAGGCWGCAG-3'
UMI_27Fa	5'- GTCTCGTGGGCTCGG- NNNNNNNNNNNNNN - AGAGTTTGATYMTGGCTYAG-3'
UMI_27Fb	5'- GTCTCGTGGGCTCGG- NNNNNNNNNNNNNN - AGGGTTCGATTCTGGCTCAG-3'
UMI_1540R	5'- GTCTCGTGGGCTCGG- NNNNNNNNNNNNNN - TACGGYTACCTTGTTACGACT-3'
UMI_1391R	5'- GTCTCGTGGGCTCGG- NNNNNNNNNNNNNN - GACGGGCGGTGTGTRCA-3'